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Towards microfluidic sperm refinement: impedance-based analysis and sorting of sperm cells†

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The use of high quality semen for artificial insemination in the livestock industry is essential for successful outcome. Insemination using semen with a high number of sperm cells containing morphological defects has a negative impact on fertilization outcome. Therefore, semen with a high number of these abnormal cells is discarded in order to maintain high fertilization potential, resulting in the loss of a large number of morphologically normal sperm cells (up to 70–80% of original sample). A commonly occurring morphological sperm anomaly is the cytoplasmic droplet on the sperm flagella. Currently, no techniques are available to extract morphologically normal sperm cells from rejected samples. Therefore, we aim to develop a microfluidic setup which is able to detect and sort morphologically normal sperm cells label-free and non-invasively. In a proof-of-concept experiment, differential impedance measurements were used to detect the presence of cytoplasmic droplets on sperm flagella, which was quantified by calculating the area under the curve (AUC) of the corresponding impedance peaks. A receiver operating characteristic curve of this electrical analysis method showed the good predictive power of this analysis method (AUC value of 0.85). Furthermore, we developed a label-free cell sorting system using LabVIEW, which is capable of sorting sperm cells based on impedance. In a proof-of-concept experiment, sperm cells and 3 μm beads were sorted label-free and non-invasively using impedance detection and dielectrophoresis sorting. These experiments present our first attempt to perform sperm refinement using microfluidic technology.

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1. Introduction

Artificial insemination (AI) is a well-established technique in the animal industry for livestock production. Selection of sperm samples for AI is based on sperm concentration, cell motility and morphology.¹ All factors have shown impact on the success rate of fertilization and the abundance of offspring.^{1–4} Therefore, insemination stations live up to high standards to supply high quality semen samples to ensure high probability of fertilization after AI. Examples of criteria for semen sample rejection are reduced sperm cell motility (motility < 70%) and/or a high number of morphologically abnormal sperm cells (>20% abnormal cells).^{5,6}

A frequently occurring sperm defect is the presence of a cytoplasmic droplet on the sperm flagellum. This droplet is a part of the cytoplasm of the spermatids, which was not re-

moved from the flagellum at the end of spermiogenesis.⁷ Although the effect of residual cytoplasm retention on human infertility is a controversial subject in the clinic,⁸ several sources show a significant effect of the droplet content on infertility in domestic species.^{8,9} Unfortunately, routine sperm refinement techniques such as sperm density centrifugation and sperm swim-up are not suitable for removal of these sperm cells and therefore, semen samples containing over 15–20% of cells with cytoplasmic droplets are withheld from AI.^{5,6,9}

A potential approach to obtain these healthy and morphologically normal sperm cells from rejected samples is the use of microfluidic technology. Microfluidic systems have been used for the manipulation, analysis and enrichment of viable, motile sperm cells.^{10–14} However, none of these systems is currently capable of performing sperm analysis and selection based on cell morphology (on the single cell level). A possible method to distinguish morphologically normal sperm cells from cytoplasmic droplet-containing cells is impedance cytometry. Numerous reports show the use of impedance cytometry to perform high-throughput analysis of biological species including bacteria, yeast, cancer cells and various blood cell types, investigating cell properties such as cell size,

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membrane integrity and cytoplasm conductivity.^{15–18} Our group's previous work showed potential to perform impedance cytometry on sperm cells, specifically.¹⁹

Besides high-throughput analysis capabilities, the microfluidic system must include an actively-controlled sorting mechanism to separate abnormal sperm cells. Active size sorting of single plastic beads using dielectrophoresis (DEP) based on impedance analysis has been shown before.²⁰ Many have reported the use of DEP as a versatile tool for particle and cell trapping, manipulation and sorting.^{21–23} Furthermore, DEP has been used to manipulate and analyse sperm cells.^{13,24} Therefore, the combination of impedance detection and DEP sorting is an interesting approach towards sorting of sperm cells based on cytoplasmic droplet content.

2. Materials and methods

2.1. Chip fabrication

Microfluidic chips (Fig. S1†) were fabricated using routine photolithography wet etch, sputter and bonding techniques. The fabrication process is illustrated in Fig. S2.† After cleaning two borofloat glass wafers (BF33, 100 mm diameter, 500 and 1100 μm thick), the microelectrodes were fabricated after resist deposition, exposure and developing, BHF wet-etching, deposition of titanium/platinum layers (layer thickness of 30 and 120 nm, respectively) and resist lift-off. Subsequently, inserts for fluidic and electric connections were powder blasted through both wafers (particle size 29 μm). After cleaning the wafers using ultrasound and HNO_3 , a layer of foil (20 μm , PerMX3020, DuPont) was laminated on the 500 μm wafers at 80 $^\circ\text{C}$ and a roller speed of 300 mm min^{-1} . After lamination, the wafers were pre-baked (5 min at 50 $^\circ\text{C}$, 5 min at 65 $^\circ\text{C}$ and 10 min at 85 $^\circ\text{C}$) to improve adhesion of the foil to the glass. Exposure of the layer was performed by illumination through the mask using a 12 mW cm^{-2} UV

source (EVG 620) for 40 s using an 8×5 s interval exposure to prevent gas bubble formation in the polymer layer. Subsequently, a post-exposure bake was performed (5 min at 50 $^\circ\text{C}$, 5 min at 65 $^\circ\text{C}$ and 10 min at 85 $^\circ\text{C}$). The polymer layer was developed with RER-600 (PGMEA, Arch Chemicals, Inc.) using a spin-coater (2000 rpm, 3 runs of 15 s).

After aligning the 500 μm wafers with respect to the 1100 μm wafers in a bond chuck, they were bonded together (30 min, 1 ton piston pressure, 0 V, 10^{-3} mbar, 100 $^\circ\text{C}$) using an anodic bonder (EV-501). Subsequently, the wafer stack was hard-baked (1 h, 1 ton, 150 $^\circ\text{C}$) in a heated press (Carver). After dicing (Dicing Saw Disco DAD 321), the chips were ready to use.

Two different chip designs were used in the reported experiments. For cytoplasmic droplet detection experiments, sperm cells were flown through a 20 μm high and 20 μm wide microfluidic channel (Fig. 1A). Two electrode pairs were used to measure the impedance differentially, in which the width of the electrodes was 10 μm and the distance between the electrode pairs was 20 μm . For cell sorting experiments, the microfluidic chip consisted of a 20 μm high and 100 μm wide channel containing integrated electrodes for impedance detection and DEP focusing and sorting (Fig. 5A).

2.2. Sample and chip preparation

Fresh boar semen was obtained from a local artificial insemination centre ("KI Twenthe", The Netherlands) at a concentration of 20×10^6 cells mL^{-1} . The samples were diluted with Beltsville Thawing Solution (BTS, Solusem, Aim Worldwide) to a concentration of 2×10^6 cells mL^{-1} .

Before each experiment, microfluidic channels were coated with poly(L-lysine)-*graft*-poly(ethylene glycol) (PLL-*g*-PEG, SuSoS) to prevent cell adhesion. PLL-*g*-PEG was rinsed through the channels at a concentration of 100 $\mu\text{g mL}^{-1}$ in DI

Fig. 1 (A) Microfluidic chip consisting of two electrode pairs for differential impedance analysis. (B) Electric circuit model (ECM) of the measurement setup. Without a cell in between the electrodes (input 2), the setup is described by an electrode–electrolyte interface (C_{DL}), electrolyte (R_{el} & C_{el}) and the wire resistance (R_{lead}). A passing spermatozoon adds a cell membrane capacitance (C_{mem}) and cytoplasm resistance (R_i) to the ECM, considering Foster and Schwan's simplified ECM for a single-shelled spheroid in suspension (input 1). (C) Simulated and measured impedance versus frequency. In the inset the simulated impedances between sperm cell and diluent are shown at the resistive plateau (around 1.3 MHz), showing a small impedance difference.

water for at least 15 min at a flow rate of $0.5\text{--}1\ \mu\text{L min}^{-1}$ using a syringe pump (neMESYS, Cetoni GmbH) equipped with a Hamilton gastight syringe (1710N). The BTS solution was rinsed for at least 15 min at a flow rate of $0.5\text{--}1\ \mu\text{L min}^{-1}$ to remove the remaining coating solution. Subsequently, the sperm solution was flushed through the channel at a flow rate of $0.5\text{--}1\ \mu\text{L min}^{-1}$. Upon visualization in the microfluidic channel, the flow rate was changed to $0.02\text{--}0.025\ \mu\text{L min}^{-1}$ before impedance acquisition.

2.3. Impedance detection and analysis

Impedance was recorded using an impedance spectroscopy (HF2IS, Zurich Instruments, Zurich, Switzerland) equipped with a preamplifier (HF2TA), illustrated in Fig. S3†. Two different modes of operation were used in the experiments. For cytoplasmic droplet detection experiments, the impedance was recorded in differential mode (Fig. S3A†) using the chip design illustrated in Fig. 1A. In this mode, an AC signal with an amplitude of 0.5 V and a frequency of 1.3 MHz was generated on output 1 and applied to the differential electrode pair of the device under test (DUT). The two corresponding electrodes of the differential electrode pair were connected to input 1 and input 2 of the impedance spectroscopy *via* two separate current amplifying channels of the current preamplifier (10k amplification factor). The impedance was recorded using a bandwidth of 200 Hz and a sampling frequency of 3598 Hz.

For cell sorting experiments, the impedance was measured in non-differential mode (Fig. S3B†) using the chip design shown in Fig. 5A. In this mode, 4-point measurements were performed. The current was amplified (10k amplification factor) using channel 1 of the preamplifier connected to input 1 of the impedance spectroscopy. The voltage was measured differentially at input 2. In this mode, impedance was recorded using a 1 MHz sinusoidal excitation with an amplitude of 0.5 V. In this mode of operation, impedance was recorded using a bandwidth of 200 Hz and a sampling frequency of 899 Hz unless mentioned otherwise.

The recorded impedance data were imported and processed in Matlab (R2014a, MathWorks). For measurements in the differential mode, the absolute impedance data from input 2 were subtracted from signal 1 to obtain the differential signal (Fig. S5C†). Subsequently, the baseline correction was performed after which the peaks in impedance were detected. In the non-differential mode, drift and offset were removed by baseline correction after which the peaks were detected.

2.4. Cell focusing and sorting by DEP

The sperm cell orientation and location within the microchannels (Fig. 5A) were manipulated by applying DEP using two top-down electrode pairs. Cell focusing was performed by applying a 10 MHz, 3 V sinusoidal excitation on the focusing electrodes (Agilent 33220A, Agilent Technologies, Inc.) unless mentioned otherwise. Similarly, cell sorting was

performed using 15 MHz, 2 V excitation using output 2 of the impedance spectroscopy.

2.5. Video acquisition and optical analysis

Optical data were recorded using a Nikon TE2000-U microscope equipped with a $10\times$ phase contrast objective and a Basler acA780-75 camera at 25 fps. Sperm tracking was performed using the “motion-based multiple object tracking” function of the computer vision system toolbox in Matlab. This function processes every frame one by one and detects objects in comparison to a static background. These objects are tracked over time and assigned to object trajectories. This readily available function in Matlab was adapted to allow storage of objects' time data and location.

2.6. Integrated data acquisition and DEP sorting using LabVIEW

Acquisition of impedance data and actively controlled DEP sorting were performed by using a custom-built LabVIEW program. This program consists of readily available virtual instrument (VI) drivers for all involved equipment (Zurich HF2IS, Basler Camera acA780-75gc, and neMESYS syringe pump) and a control algorithm, which processes measurement data and controls DEP excitation.

When operational, the control program monitors the impedance over time. Upon particle or cell passing, the change in impedance is recorded and matched to a predefined template (Fig. S4†). When the detection criterion, which is based on the quality of the fit and the optimal scaling factor, crosses the threshold value, a positive match is found.²⁵ In the case of a positive match, a Gaussian distribution is fitted to the recorded impedance change after which a derivative is calculated. This derivative is used to estimate the velocity of the particle or cell and the estimated time of arrival (ETA) at the sorting electrodes (t_x). Subsequently, the total change in impedance is matched to the window of interest (WOI). When the impedance change fits within the WOI, the DEP sorting electrodes are activated at interval $t_0\text{--}t_1$ to sort the particle or cell in the top channel. When the impedance change does not match the WOI, the DEP sorting electrodes are inactive at the interval $t_0\text{--}t_1$.

2.7. Simulation

The electrical response of the microfluidic setup (Fig. 1A) was investigated by constructing a numerical model of the circuit (Fig. 1B) in Matlab. This model is well described in the literature²⁶ and is based on Foster and Schwan's simplified electrical circuit model (ECM) for a single-shelled spheroid in suspension. In simulations, a parallel electrode configuration was modelled without field fringing at the electrode edges. Sperm cells were modelled as single-shelled spheroids with a similar cell volume ($1.25 \times 10^{-17}\ \text{m}^3$).²⁷

The absolute impedance response of the microfluidic setup was simulated as a function of frequency (200 Hz–2 MHz). This simulation was compared to a real measurement,

in which the absolute impedance was recorded in an equal frequency range. Furthermore, a sperm cell was simulated in between the electrodes to investigate the expected impedance change. The simulation parameters were: channel height = channel width = 20 μm , electrode width = 10 μm , electrode surface area $A = 2 \times 10^{-12} \text{ m}^2$, sperm cell volume $V_{\text{cell}} = 12.5 \text{ fL}$, cell radius $r = 1.44 \mu\text{m}$, membrane thickness $d_{\text{m}} = 5 \times 10^{-9} \text{ m}$, conductivity of electrolyte $\sigma_{\text{el}} = 1.4 \text{ S m}^{-1}$, conductivity of cell interior $\sigma_{\text{i}} = 0.4 \text{ S m}^{-1}$, conductivity of cell membrane $\sigma_{\text{mem}} = 10^{-8} \text{ S m}^{-1}$, permittivity of electrolyte $\epsilon_{\text{el}} = 80$, permittivity of cell interior $\epsilon_{\text{i}} = 60$, permittivity of cell membrane $\epsilon_{\text{mem}} = 11.3$, double layer capacitance $C_{\text{DL}} = 20 \mu\text{F cm}^{-2}$, and resistance of lead wires $R_{\text{lead}} = 0 \Omega$.

3. Results and discussion

3.1. Simulation

Impedance spectroscopy is a commonly used tool for label-free analysis of adherent cells or cells in suspension. This technique has been used extensively to investigate the dielectric properties of cells in microfluidic systems. Constructing an ECM is a simple way to gain insight into the electrical response of the microfluidic setup (Fig. 1B). The capacitive properties of the microelectrode setup are predominantly determined by the electrode/electrolyte interface (C_{DL}) and the parasitic effects of the microelectrodes, which are mainly determined by the electrolyte capacitance (C_{el}). The resistive response is influenced by the lead wires (R_{lead}) and the conductivity of the electrolyte (R_{el}). When a spermatozoon is introduced between the microelectrodes, the capacitive and resistive properties will be altered by the cell membrane (C_{mem}) and the cell's cytoplasm (R_{cyt}), respectively.

The simulation is based on the equivalent circuitry of Fig. 1B in the absence of a sperm in between the electrodes of a single electrode pair. The corresponding simulation parameters are shown in Fig. 1C. The simulation showed the expected effect of the electrode/electrolyte interface on the absolute impedance. Due to a small electrode surface area and the corresponding small C_{DL} , the impedance continuously decreased over a broad frequency range where C_{DL} is dominant. At a frequency of approximately 1.3 MHz a resistive plateau was formed where R_{el} is dominant. A frequency sweep of a single electrode pair in the absence of a sperm cell showed similar behaviour compared to the simulation, indicating that a measurement frequency of 1.3 MHz is an appropriate choice for sperm impedance analysis in this setup. At this frequency, the simulation showed an impedance increase of 299 Ω , when modelling a sperm cell in between the electrodes using the parameters described in section 2.7.

3.2. Electrical analysis of cell orientation and morphology

Impedance analysis of sperm cells was performed by flowing cells through a 20 μm high, 20 μm wide channel restriction (Fig. 1A) at a flow rate between 0.013 and 0.02 $\mu\text{L min}^{-1}$. In this constriction area, the impedance was recorded differentially using two electrode pairs with an electrode width of 10

μm and an electrode separation of 20 μm using the chip illustrated in Fig. 1A and the measurement setup illustrated in Fig. S3A†. After calculating the difference between the electrical responses of both electrode pairs (Fig. S5C†), baseline correction (Fig. S5C†) and peak detection (within thresholds) were performed (Fig. S5D†). The resulting peak heights showed a wide distribution in impedance, ranging from values between 200 and 1500 Ω (Fig. S6†), which is in agreement with the simulated impedance change of 300 Ω . This broad distribution is caused by the effects of cell location and tilting on the impedance. Especially, cell tilting showed a big effect on the impedance. When the sperm cell is aligned with the electrodes, e.g. the flat side of the sperm cell runs parallel with respect to the electrode surface (high cross-sectional area), a high impedance change is recorded. However, when a sperm cell is rotated 90 degrees over the longitudinal axis and only a small part of the sperm is exposed (small cross-sectional area), the recorded impedance changes are small (around 200 Ω). In the simulation, the sperm cells are modelled as a spheroid with similar volumes, in which its cross-sectional area is much smaller compared to a sperm cell that is aligned with the electrodes. Therefore, the simulated impedance (300 Ω) is more comparable to a sperm cell, which passed the electrodes in a tilted orientation ($\pm 200 \Omega$).

Due to the effects of location and orientation, the absolute impedance change is not a suitable parameter to characterize morphological differences. A different approach is the analysis of the peak shape over time. A sperm cell is shaped as a tri-axial ellipsoid with a head length of 8–9 μm , head width of 4–5 μm , head thickness of <0.5 μm and an average tail length of 40–50 μm).^{28,29} Its total length is longer compared to the channel width and height and the width of the microelectrodes. When a sperm cell is flown through this microchannel, the cell will align itself over its longitudinal axis with respect to the channel wall. Consequently, the distinct parts of the sperm cell (head, midpiece and flagellum) will pass the electrical field between the microelectrodes at different points in time and will affect the recorded impedance, accordingly. As a result, the peak shape may contain information about the cell orientation (head-first or tail-first) and its morphology.

To test this hypothesis, the impedance peak shape of passing sperm cells was investigated (when using an electrode separation of 20 μm). The curves showed a positive peak and a negative peak (Fig. 2), corresponding to a sperm passing through the first and second electrode pairs, respectively. At zero-crossing, the recorded impedance at inputs 1 and 2 is equal, at which point the sperm head is positioned in between the two electrode pairs, approximately. The curves showed a clear effect of the cell orientation on the peak shape. When a sperm cell passed the two electrode pairs head-first (Fig. 2B), the differential impedance change showed, as expected, a positive peak and a negative peak, respectively. However, the negative peak returned to a steady baseline level after clear signal tailing. This tailing effect is caused by the presence of the sperm flagellum in between

Fig. 2 Differential impedance curve of sperm cells in (A) tail-first and (B) head-first orientations, showing the recorded impedance change upon passing of a sperm cell in between the differential electrode pair over time. (C) When a sperm cell contains a cytoplasmic droplet, a distinct feature is observed in the impedance plot. In the insets the microscopic images of the corresponding sperm cells are shown, indicating the orientation at the detection site and the presence of cytoplasmic droplets.

the electrodes. In the tail-first orientation, this tailing effect was observed before the positive peak, *i.e.* when the sperm head arrived at the first electrode pair (Fig. 2B).

Furthermore, information about the droplet content could be extracted from the data. A clear example is shown in Fig. 2C, in which a bump in the signal was observed. This bump in impedance was located in between the negative peak (sperm head) and the residual impedance change (sperm flagellum) and was caused by the presence of a distal cytoplasmic droplet in between the second electrode pair when the sperm head is leaving the sensor volume. Although one would expect a similar response in impedance change when the sperm cell is passing the first electrode pair, the electrical overlap between the two electrode pairs interfered due to which the observed effect was less pronounced. This overlap is evident when simulating the electrical field strength in between the electrodes (Fig. S7†). When a particle is positioned in between the electrodes (Fig. S7†, X -location = 70 μm), the field strength is approximately decreased by a factor of 2 compared to the field strength right in between the electrodes (X -location = 55 and 85 μm). Due to this electrical overlap, the effects of the sperm head and the cytoplasmic droplet could not be discriminated. Therefore, we will focus on the analysis of the second peak to analyse the droplet content.

The cytoplasmic droplet content was investigated by analysing the area under the curve (AUC) of the differential impedance peaks. In total, 18 morphologically normal and 18 droplet-containing sperm cells were selected for analysis which crossed the electrodes in the head-first orientation (Table 1). Using Matlab, the maximum (Fig. 3, point A), mini-

mum (point B) and zero crossing points (point C) were determined. Subsequently, the AUC of the positive and negative peaks were calculated by performing a numerical integration

Fig. 3 Analysis of cytoplasmic droplet content of sperm cells passing the electrodes head-first based on (A) calculation of the area under the curve (AUC). The first (positive) peak, zero-crossing point and second (negative) peak are denoted as A, C and B, respectively. Peak heights (Y_B) and width (X_B) were used for normalization of the AUC, yielding AUC_n . (B) Based on the threshold value of AUC_n , the amount of positively identified droplet-containing sperm cells (true positive, or TP) and positively identified cells without a droplet (true negative, or TN) were used to calculate the sensitivity and specificity of electric cytoplasmic droplet detection. The receiver operating characteristic curve (ROC) showed an AUC value of 0.85.

Table 1 AUC analysis of sperm cells head-first without (control) and with cytoplasmic droplets (both populations, $n = 18$)

	10^4 AUC	10^4 SD	10^3 AUCn	10^2 SDn
Control	2.96	1.50	5.05	7.25
Droplet	2.6	1.58	6.98	23.6
		$p = 0.53$		$p = 0.003^*$

in Matlab. When comparing the AUC means of the negative peaks of the two populations using a (paired sample) *t*-test, no statistical difference was found ($p = 0.53$). A plausible explanation is the effect of the cell orientation, location and velocity on the AUC. The orientation (*e.g.* cell tilting) and location influence the peak height. Furthermore, the cell velocity has an effect on the peak width. Therefore, the AUC was normalized (AUC_n, Fig. 3A) using the peak height (Y_B) and peak width (X_B). X_B , the time interval between the zero crossing point C and the peak minimum B, was chosen to correct for the cell velocity. Due to the channel constriction close to the first electrode pair, the sperm cells were accelerating when they passed the first electrode pair. At the second electrode pair, their velocity was more constant. Therefore, time interval X_B was chosen for peak normalization. After correction for the peak height and peak width (AUC divided by Y_B and X_B), a significant difference was found between the AUCs of both populations ($p = 0.003$).

A threshold value between the populations of sperm cells with and without cytoplasmic droplets (droplet and control, respectively) was set to calculate the amount of cells, which were positively and negatively identified based on the AUC_n (Fig. 3B, inset table). In this example, this threshold value was set to provide the best trade-off between the sensitivity and specificity. An AUC_n value higher or lower than the threshold value is denoted as AUC_n+ or AUC_n- respectively. In total, 16 out of 18 sperm cells with a cytoplasmic droplet were positively identified (TP), yielding a sensitivity of 0.89. For the control sperm cells, 13 of 18 cells were positively identified as cells without droplet content (TN), yielding a specificity of 0.72. Furthermore, the positive and negative predictive values were 0.76 and 0.87, respectively. The relation between sensitivity and specificity depends on the chosen threshold and this relation is illustrated by a receiver operating characteristic (ROC) curve. This curve shows the trade-off between sensitivity and specificity. The area under the ROC curve (AUC_{ROC}) was found to be 0.85, indicating a predictive value (AUC_{ROC} > 0.5).

With a perfect analysis method, the AUC_{ROC} would approach a value of 1. A computer assisted semen analysis (CASA) system, which uses optical methods for sperm detection and analysis, would achieve a value close to 1. Although CASA systems are versatile tools for sperm analysis, they do not allow sperm manipulation or sorting in any way. A different approach is the use of fluorescence activated cell sorting (FACS) to analyse and sort sperm cells based on cytoplasmic droplet content.

Although these systems are able to perform analysis and sorting at high throughput (28×10^6 sperm cells per hour),³⁰ analysis depends on fluorescent staining. It is hard to stain the cytoplasmic droplet exclusively, since it consists of residual cytoplasm from the sperm head and, currently, no reports have shown FACS of sperm based on cytoplasmic droplet content. Furthermore, the throughput of FACS is too low to fit the demands from the swine livestock industry, which requires approximately 1.5 sperm cells per insemination when

using routine artificial insemination.³¹ Compared to optical systems like FACS, the throughput of electrical methods can be increased more easily by massive parallelization.³²

Several improvements can be performed in future experiments to improve electrical detection of cytoplasmic droplets. Curve fitting using wavelet transform could be a better candidate for analysis of the cytoplasmic droplet content on the single cell level³³ compared to AUC analysis. Furthermore, reducing the measurement cell volume by altering the electrode and channel geometry might improve detection. Moreover, better control over the cell position within the microchannel could reduce the effects of cell orientation, location and velocity on the recorded impedance and could improve droplet detection. Exemplarily, dielectrophoretic focusing is a potential approach.³⁴

3.3. Impedance analysis and sorting of sperm cells

Active sorting of sperm cells is an essential feature of a microfluidic sperm sorter. Although impedance measurement is a widely used technique for label-free and non-invasive analysis of single cells in microfluidic systems, only a few reports show its integration with active sorting capability.²⁰ To our knowledge, no reports have shown application of this approach to a biological sample. Here, we show the development of a label-free cell sorting system based on impedance measurement. For these experiments, sperm cells were analysed using the chip shown in Fig. 5A using the measurement setup illustrated in Fig. S3B.†

3.3.1. Effect of DEP focusing on cell location and velocity. Control over cell location and velocity is necessary to perform accurate measurements of the sperm morphology and to control cell sorting after analysis. As shown before, DEP focusing is used to control these parameters. A frequency of 10 MHz was applied to the focusing electrodes (Fig. 5A). At this frequency and in a high conductive environment (1.4 S m^{-1}), a negative DEP force is exerted on sperm cells.¹² As a result of this DEP force, the sperm cells will move away from high field gradients at the electrode edges to the middle of the microfluidic channel. Furthermore, high frequency excitation is more suitable for non-invasive DEP manipulation of cells compared to DC or low frequency excitation.³⁵

To show the effect of DEP focusing on the cell location and velocity, sperm cells were flown through the microfluidic channel with a flow rate of $0.025 \mu\text{L min}^{-1}$ with and without DEP excitation. Sperm cell trajectories and location were investigated to show the effect of DEP. Without excitation, the sperm cell trajectories were unaffected (Fig. S8A and supplementary video V1†). With excitation, the sperm cells were clearly deflected to the middle of the channel (Fig. S8B and supplementary video V2†). The cell location and velocity were measured optically right after passing the $20 \mu\text{m}$ electrode pair (Fig. 5A). The data are presented as a scatterplot of the cell location *versus* cell velocity with corresponding histograms and boxplots (Fig. 4). Without focusing, image analysis showed a broad distribution in both cell location and

Fig. 4 The effect of DEP focusing on sperm location and velocity. Sperm location and velocity were more uniform after focusing the sperm cells in the middle of the channel. The channel walls are situated at $y = 9$ and $y = 107 \mu\text{m}$ (dashed lines).

velocity. With focusing, the width of these distributions was reduced extensively. However, multiple outliers were observed in the boxplots. These data points were caused by sperm cells which got stuck in the channel walls or sperm cells which pushed themselves away from the middle of the channel by beating their tails.

3.3.2. Impedance-controlled sorting of sperm cells. After detection of a cell with morphological abnormality (*i.e.* cytoplasmic droplet), it should be removed from the sample. Previous results showed the ability of the cell sorter to detect changes in impedance when sperm cells crossed the microelectrodes, to distinguish sperm cells with and without cytoplasmic droplets and to focus the sperm cells using dielectrophoresis to control their location and velocity. A remaining but crucial feature of the microfluidic cell sorter is a sorting algorithm, which accurately controls DEP excitation based on impedance data.

In a proof-of-concept experiment, we aim to sort beads and sperm cells based on impedance. For this study, LabVIEW was chosen and used to design the sorting algorithm. The process flow of this algorithm is shown in Fig. S4†. Whenever a change in impedance is recorded, from which the peak shape matches the peak template, the width and the height of the peak are determined. The peak width is used to calculate the particle's velocity in order to predict the ETA at the sorting electrodes. The optically calculated cell velocity, which is used as a reference, is plotted *versus* the elec-

trically calculated cell velocity (Fig. S10†). To correct for the inaccuracy in measured cell velocity (electrically) when determining the window of DEP excitation, a 30% error margin is built in. This margin indicates that the cell velocity can be underestimated (bottom red line) or overestimated (top red line) by 30% compared to the optically measured velocity. As a result, all sperm cells that arrive at the sorting electrodes within the DEP window (*i.e.* data points that fall within the red hatched region) are effectively sorted.

In this example, $3 \mu\text{m}$ polystyrene beads were sorted from the sperm cells (Fig. 5 and supplementary video V2†). A mixture of sperm cells and beads (2×10^6 cells and beads mL^{-1}) was flown through the microfluidic channel at a flow rate of approximately $0.025 \mu\text{L min}^{-1}$. The impedance change of a population of sperm cells showed a clear difference compared to a population of beads when crossing the $20 \mu\text{m}$ wide electrodes (Fig. S9†), allowing discrimination between both species. Whenever an impedance change of a particle was detected, which fits within the WOI, the DEP electrodes are activated to sort the particle in the top channel. The impedance WOI was set to $4\text{--}8 \Omega$, which matched the impedance change when a bead passed the electrodes. When beads passed the electrodes, the recorded impedance changes ($5.4 \pm 0.7 \Omega$, $n = 15$) fitted within the WOI (Fig. 5B), consequently sorting the beads actively in the top channel at the channel split (green trajectories, Fig. 5C). Whenever sperm

Fig. 5 Discrimination and active sorting of beads and sperm cells in (A) a chip with a 100 μm channel with integrated electrodes for cell focusing, detection and sorting. (B) When a bead passed the detection electrodes and the corresponding impedance change fitted within the window of interest (green window, 4–8 Ω), the DEP electrode pair was activated (blue window) to sort the particle. (C) Analysis of the bead trajectories showed effective deflection of the beads to the top channel by active sorting, whereas sperm cells and debris were drawn into the bottom channel.

cells ($19.6 \pm 5.7 \Omega$, $n = 12$) or debris ($<4 \Omega$) passed the detection electrodes (red triangles and blue squares, respectively), the recorded impedance was above or below the WOI, respectively. As a result, sperm cells and debris were drawn in the bottom channel without being deflected by the sorting electrodes (red and blue trajectories, respectively).

For optical verification, the sorting speed in the described experiment was set to <1 sperm cell s^{-1} using a low bead and sperm concentration and small flow rate. In the reported experiment, the sorting speed is limited to <5 cells s^{-1} when using the peak fitting algorithm at a sampling frequency of 899 Hz when simultaneously recording video data. By increasing this sampling frequency towards the 100–1000 kHz range, the sorting speed of this system could in theory be increased to >1000 cells s^{-1} . With this throughput (1000 cells s^{-1} in 100 parallel channels), a single insemination dose (1.5 billion cells) would be sorted in just over 4 hours, which is close to the required sorting speed. Right now, the sorting system is based on a custom-built LabVIEW program (with a

minimal computational time), which relies on data communication between a Microsoft Windows computer (no real-time operational system) and the impedance spectroscopy via a USB connection (USB latency 1–10 ms). All these factors induce a time delay between the processing of impedance data and excitation of the DEP electrodes, which limit the system's throughput. Therefore, we expect values <50 cells s^{-1} based on hardware limitations.

To meet the demands from the livestock industry, the sorting speed must be increased extensively. In order to achieve this, a more hardware-based solution is required to decrease computation time in order to increase the sorting throughput. A control system based on custom-built analogue hardware could increase the sorting speed to meet the demands in throughput. Furthermore, integration of microfluidics with CMOS technology has shown large potential in analysis and manipulation of cells and particles^{36,37} and may provide the solution towards high-throughput microfluidic analysis and sorting of sperm.

4. Conclusions

In this paper, we presented a novel application of impedance measurement to study sperm cells in a microfluidic system. Differential impedance analysis was employed to successfully study the cell orientation and cytoplasmic droplet content, which is a frequently occurring morphological defect in sperm cells. Furthermore, we developed a label-free cell sorting system in which cells can be sorted using DEP based on impedance data. In a proof-of-concept experiment, we were able to sort plastic beads from sperm cells based on corresponding impedance peaks. Combination of both techniques is a promising approach towards sperm refinement applications in the livestock industry. Besides analysis of morphology (droplet content), impedance analysis might be used to investigate other sperm cell parameters, such as cell viability and the internal environment of the cell.^{15,16,18}

To meet the demands from the cattle industry, analysis and sorting of sperm cells should be performed at high-throughput. Although impedance analysis can be performed at high-throughput, the sorting speed is currently limited. Therefore, a more hardware-based solution is necessary to increase the sorting throughput. Integration of microfluidics with CMOS technology has shown large potential in analysis and manipulation of cells and particles^{36,37} and may provide the solution towards high-throughput microfluidic analysis and sorting of sperm.

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