High-frequency electric field trapping of individual human spermatozoa

Günter Fuhr1,4, Torsten Müller1, Vera Baukloh2 and Kurt Lucas3

1Humboldt-Universität zu Berlin, Institut für Biologie, Invalidenstr. 43, 10115 Berlin, 2Praxisgemeinschaft Prof. Leidenberger und Partner and 3Eppendorf-Netheler-Hinz-GmbH, Hamburg, Germany
4To whom correspondence should be addressed

We present a new touch-free technique for trapping, positioning and selecting human spermatozoa. This can be done in free solution (culture medium) by high-frequency electric fields. Ultramicroelectrodes fabricated by photo- and electron-beam lithography on quartz and glass substrates were used to create field cages or long field channels. If the conductivity of the external salt solution is higher than the average value of sperm cell conductivity, negative polarization and negative dielectrophoresis occur. As a result, the induced cell polarization leads to forces repelling spermatozoa from the electrodes towards the field minimum. Using four planar electrodes a field funnel can be formed in which an individual spermatozoon is retarded while swimming. The same can be done more effectively in three-dimensional cages created by an octopole electrode system. In these systems, rapidly swimming spermatozoa could be trapped for several seconds but some spermatozoa stop moving if exposed to field strengths of more than 500 V/cm at frequencies in the MHz range. However, in stripwise and interdigitated electrodes, rapidly swimming sperm cells could be very well positioned in front of a break-electrode by a combination of electric field trapping and field induced laminar fluid streaming. This technique can be applied to bring individual spermatozoa to a defined position for characterization followed by sampling with capillaries.

Key words: AC-electric field/characterization/human spermatozoa/microstructures/physiological conditions

Introduction

For about 20 years AC-electric fields have been used to manipulate cells in aqueous solutions (Pohl, 1978). However, the field forces are too weak to influence swimming cells like paramecia (Fuhr and Hagedorn, 1987) or spermatozoa. From a physical point of view AC-fields induce charges at the interfaces of the cell, i.e. at the plasma membrane and inner organelle membranes. The interaction of these charge accumulations with the electrode charges leads to forces acting on dielectric objects. In homogeneous fields, cells are elongated or compressed but not moved. In field gradients, directed motion occurs (Pohl, 1978). However, DC-excitation and low frequency fields (below several hundred kHz) cannot be applied. The first leads to electrolysis, the second to unphysiologically high induced membrane potentials (Zimmermann and Neil, 1996).

A further limitation is the applicable conductivity range. In electrode structures with typical dimensions of several millimetres, field strengths of more than 10 V/cm can only be applied to solutions with conductivities of less than 0.1 S/m (Bernhardt, 1992). Otherwise, boiling occurs within seconds or less. This situation is completely changed by the introduction of micro-electrodes processed by photolithography on glass or silicon substrates (Washizu, 1989; Fuhr et al., 1991). Temperature rises are proportional to the conductivity and (with most chamber geometries) to the square of the electrode distance. With electrode systems on the 10 micrometre scale, conductivities as high as 5 S/m can be used even at field strengths of 100 kV/m (Fuhr et al., 1994). Most physiological solutions are less conductive than this (e.g. Roswell Park Memorial Institute (RPMI) 1640, Dulbecco’s modified Eagle’s medium (DMEM), phosphate-buffered saline (PBS) solution 1–2 S/m) and can, therefore, be used. Additionally, higher field strength can be applied to miniaturized electrode systems as the breakdown voltage of the solution is also increased. This is of great practical importance, since the forces acting on cells are proportional to the square of the field strength. Therefore, in microstructures much stronger forces can be induced without causing damage.

It is possible to generate attracting or repelling polarization forces. Attraction means movement of cells toward higher field strength, which ends at the surface of the electrodes (this is positive dielectrophoresis — pDP (Pohl, 1978). However, this type of force is not suited for sperm manipulation since electrode contact should be avoided. Repelling forces move cells toward field minima. These can be in free solution far from the electrodes (Pohl, 1978; Jones, 1986; Schnelle et al., 1993). Repelling forces (so called negative dielectrophoresis — nDP) occur if the conductivity of the solution is higher than the average conductivity of the cell. This is always true in physiological media with high ionic content. The forces can be further increased if strong field gradients and electronic resonances are used to produce well-defined field minima (Gimsa et al., 1996).

Micro-devices may solve the problem of moving, retarding and trapping rapidly swimming cells like spermatozoa. The aim of this paper is to demonstrate how micro-devices can be used for single spermatozoon positioning.
Sperm trapping

Figure 1. (A) Two octopole electrode arrangements processed by photolithography. Each of the four electrodes per quadruple could be individually addressed by electric signals. The aim of this structure is to create a field funnel or a cage to trap a single spermatozoon. (B) Interdigitated electrodes fabricated by electron-beam lithography. Since the electrode widths and gaps are very small (each 3 µm), extremely high field strengths can be used. The aim of this structure is to direct the motion of swimming spermatozoa parallel to the electrode strips (1) and to hold them in a defined position in front of the break-electrodes (2).

Figure 2. Hybrid system as used in the experiments. A channel of 15–25 µm height was formed by spacers and by covering the electrode structure by a 200 µm thick glass plate. The electrodes were energized from left and right. Sperm suspension was streamed slowly (by the electric field itself or from outside) through the channel and exposed to the electric field. In the case of three-dimensional microsystems, the cover slip was replaced by an additional electrode chip mounted overhead. The whole hybrid chip was mounted into a leadless ceramic carrier.

Figure 3. Equipotential lines between four electrodes arranged as quadruple. Due to the higher conductivity around the spermatozoa at frequencies in the MHz range, negative dielectrophoresis tends to focus the spermatozoa toward the field minimum in the central part between the electrodes. However, only slowly moving spermatozoa could be forced along the path denoted with (I). Rapidly swimming spermatozoa could only be deflected from their starting trajectory (II).

Materials and methods

Fabrication of microstructures
Gold electrodes were processed by photo- and electron-beam lithography on glass and silicon substrates as described elsewhere (Fuhr et al., 1995). The fabricated structures are shown in Figure 1. The basic structures were mounted to form three-dimensional and closed hybrid microsystems as shown in Figure 2. The cell suspension could be inserted through micro-channels. Cells were observed with a conventional microscope (for cell sampling, an inverted microscope was used).

Field generation
We used frequencies higher than 5 MHz to minimize induced membrane potentials (Fuhr et al., 1987). At such frequencies the induced transmembrane potential is less than 10 mV due to the slow charge relaxation time of the membrane. Depending on the microstructure used, the amplitude of square wave signals (peak to peak — pp) was between 1 Vpp and 5 Vpp. A signal generator (HP-
Figure 4. Two field cages (octopole electrode systems), each with a trapped spermatozoon (frequency: 8 MHz, amplitude: 5 Vpp).

8116A, Hewlett Packard, Böblingen, Germany) was connected to the chips with 50 Ω resistors at each electrode. The hybrid system was bonded into a leadless ceramic carrier and inserted into a microscope.

Sperm preparation
Ejaculates from donors with conventional sperm parameters within the normal range as defined by WHO (1993) were used. Preparation of a subpopulation of highly motile spermatozoa was performed by Percoll density gradient centrifugation (Leventhal et al., 1987; Pardo et al., 1988). In short, 1.5 ml of the ejaculate liquefied at room temperature for 30 min was layered onto a two-step Percoll gradient [1 ml 80% Percoll in IVF medium (lower layer), 1 ml 40% Percoll (upper layer); MediCult, Copenhagen, Denmark] and centrifuged at 300 g for 10 min. The supernatant was removed, the remaining pellet resuspended in 2 ml IVF medium and centrifuged again at 300 g for 10 min. The final pellet was resuspended in 1 ml IVF medium and further diluted with culture solution to the sperm densities used in the experiments.

Results
Trapping of spermatozoa in a field funnel or a three-dimensional field cage
Individual sperm cells could be trapped for minutes in quadruple structures with electrode gaps of less than 40 µm. A typical field distribution of a quadruple and octopole system is shown in Figure 3.

The situation was similar in three-dimensional field cages consisting of eight electrodes arranged as a cube. Interestingly, a large number of spermatozoa which moved on path (I) stopped any motion on the octopole electrode surface and, therefore, could be trapped for some minutes (Figure 4). More than 90% of the trapped cells restarted motion seconds to minutes after field applications of <20 s. The temperature increase induced by the field (controlled by an integrating thermo-element) was smaller than 5°C. Non-motile spermatozoa were also trapped in the cages. By slow outside and non-electrically driven streaming of a sperm suspension through a field cage arrangement as shown in Figure 4, slow or non-moving spermatozoa particularly accumulated in the field minima.

Figure 5. (A) Electrode signals and trajectories (I) of sperm motion over planar interdigitated electrodes processed on silicon. (B) Field distribution (contour-plot of the square of E rms (root mean square)) and position of trapped spermatozoa. Darker areas correspond to higher field strength.

Trapping of spermatozoa in stripwise arranged electrodes
Fast swimming spermatozoa could be accurately trapped in stripwise arranged electrodes as shown in Figure 5. The electrode system was planar (electrode height: 500 nm). The small electrode gap (3 µm) and the applied voltages of between 1 Vpp and 5 Vpp produced extremely high field strengths (several hundred kV/m up to MV/m). The field also induces a fluid streaming of up to 50–100 µm/s directed parallel to the electrode strips (Figure 5A). This effect is caused by thermal gradients in the solution near the electrode surfaces (for more information see Fuhr et al., 1991).
The combination of the field-induced polarization forces and field-mediated hydrodynamic streaming led to a separation with a single spermatozoon between each electrode strip (1). The spermatozoa swam with velocities of ~40–70 µm/s to within several µm of the break-electrodes and held a stable position for several minutes.

The behaviour of spermatozoa can be summarized as follows (Figure 5B). (i) They swam parallel to the strips of electrode (1) against the induced liquid streaming. (ii) Depending on its motility, an individual spermatozoon found a stable position in front of the break-electrodes (2). This is the equilibrium point between the sperm forces, the repelling electric field force and the hydrodynamic force. Therefore, rapidly swimming spermatozoa stopped near the break-electrode, whereas slower swimming cells came to rest further away. (iii) Most spermatozoa swam for minutes at the equilibrium position but could be freed if a second spermatozoon migrated along the electrode strips (1) behind the trapped spermatozoon. (iv) In contrast to the trapping in field cages, spermatozoa do not stop their motion in interdigitated electrodes. The temperature increase was also lower (~2°C) since the small strips were processed on silicon/silicon oxide which has a high thermal conductivity.

A sequence demonstrating the sperm behaviour is shown in Figure 6. After switching off the field the trapped spermatozoa were freed immediately.

Other electric field effects on model cells (mouse fibroblasts)

A fundamental question is: if electric field traps induced physiological changes in cells, would their use be limited? Since we had no possibility of carrying out fertilization experiments within an animal model, we used mouse fibroblasts as model objects. Their proliferation rate and motility are sensible parameters to test field influences similar to spermatozoa (Fuhr et al., 1994).

The interdigitated electrode structure was carefully cleaned and sterilized. The whole system was closed and thermostated at 37°C. The same fields were applied over 2–3 days, that is much longer than used in the sperm experiments. In Figure 7 a chip is shown after cell cultivation. Cells grew in the area of the overlapping electrodes and were viable as observed by fluorescence microscopy. Due to the strong repelling forces in the central part of the structure fibroblasts could not adhere there. It is noteworthy that cells grew very close to the trapping point of the spermatozoa (area of dashed line). The only effects we found of MHz-range fields were unspecific temperature effects (heating of the solution and the cells) if the starting temperature was near 40°C. As the tolerance limit of fibroblasts is near 42°C the electric field induced a temperature increase of about 2°C. Further experiments on mouse fibroblasts carried out in a four-electrode chamber with a similar set up and field conditions of 50 kV/m and 5 MHz showed that cell motility, division rate and other physiological parameters like growth rate were not significantly affected by the electric field (Fuhr et al., 1994).

Discussion

We have shown that strong field gradients produced in micro-electrode structures can be used to control sperm trajectories and to trap individual cells for minutes in physiological solutions. Electric field forces alone seem to be insufficient for well-defined manipulation. However, in combination with the liquid streaming which is induced (created by the electric field or from outside), it allows reliable manipulation of spermatozoa and exact compensation of their motion.

Microstructures processed on glass or silicon allow the necessary high fields to be created in physiological solutions of conductivities up to 5 S/m. Only under these circumstances are the repelling forces large enough to compensate for the motion of rapidly swimming cells.

The observed temporary immobilization of spermatozoa trapped in field cages is, as yet, unexplained. One possible reason is the heating of the cell body by the field. However, all experiments were done at room temperature (25°C) and the temperature rise was <5°C (typically 2°C). Therefore, direct electric field influences seem a more likely explanation. The condensed mitochondrial system with high and low conductive double membrane systems could possibly be disordered by the field-induced charges at the interfaces. To our knowledge this kind of field effect is not described in the literature and should be investigated in more detail.
The cellular paralysis does not occur in the interdigitated electrode structure. The situation is indeed different to that in the field cages: as long as the spermatozoon is swimming between the strips of electrode 1 it is not exposed to strong electric fields since both electrodes (left and right) are at the same potential. Near the break-electrodes the strong fields act mainly on the head of the spermatozoon, not on the whole cell body as in field cages. Since the heating of the solution is comparable in both cases, the hypothesis of a direct field influence on the motile system of the cell is plausible.

Conclusions
The method described here opens up some practical possibilities. Many IVF groups use a single spermatozoon to be injected by means of tiny glass capillaries inserted directly into the cytoplasm of mature oocytes (Lanzendorf, 1995; Ubaldi et al., 1995; Palermo et al., 1996).

Although these procedures are well established it would be helpful to characterize sperm motility and morphology before release and injection. However, that is difficult or has to be done in a very short time since spermatozoa swim with velocities of several 10 µm/s. The microsystems described here open up new ways to bring individual spermatozoa to a defined position, to hold them stationary and to observe or characterize them. In particular, the beat frequency of the flagellum and other parameters can be observed for minutes while the spermatozoon is swimming without change of position. Our systems are far from being optimized. It is technically relatively easy to insert micro-channels between the strip electrodes (1) of the interdigitated system and to build up three-dimensional hybrid systems with openings to micro-capillaries for sperm capture and release. Often electric fields are assumed to be destructive or damaging to cells. We have shown that at high frequencies (MHz-range) field strengths up to 100 kV/m are well tolerated. However, before further use of this technique more survival experiments and tests on preservation of fertilizing abilities under prolonged field application should be performed.

Note that laser-generated optical traps were proven to be an alternative technical approach to manipulate single spermatozoa (Tadir et al., 1994; Enginsu et al., 1995; König et al., 1996). Laser trapping and electric field caging can easily be combined, since both forces are uncoupled and are of the same order of magnitude. In contrast to laser traps, where the cell is focused toward the region of highest light intensity, in a field cage the particle moves toward the field minimum.

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References


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Appendix

Calculation of the induced membrane potential

An electric field (DC or AC) induces charge accumulations at dielectric interfaces. In spermatozoa, the membrane is the dielectric interface with the strongest differences in the permittivity and conductivity in comparison to the surrounding solution and the cytoplasm. Therefore, the highest potential difference is induced across this thin layer. To know this value is important for any manipulation of cells in electric fields.

To get a rough approximation the sperm head can be modelled as a sphere (cytoplasm) enveloped by the membrane. The flagellum is nearly unaffected and therefore can be neglected. As shown in Figure A1 the induced membrane potential depends on the applied field frequency and the conductivity of the external medium. At low frequencies (up to 100 kHz) the membrane loading is maximal. At higher frequencies, the field changes faster than the accumulated charges can relax and the transmembrane potential decreases. The rising medium conductivity shifts this drop towards higher frequencies of the applied electrical field. Frequencies higher than 10 MHz lead to membrane potentials of <20 mV. If we assume the endogenous membrane potential to be around –80 mV, this loading seems to be acceptable. The best frequency range for sperm manipulation lies above 50 MHz.