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(71) Applicant *(for all designated States except US)*: **ISTITUTO SPERIMENTALE ITALIANO "LAZZARO SPALLANZANI"** [IT/IT]; Via San Barnaba, 32, I-20122 Milano (mi) (IT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **GALLI, Andrea** [IT/IT]; Via S. Pertini, 24, I-24035 Curno (bg) (IT). **BONACINA, Cesare** [IT/IT]; Via Xxv Aprile, 27, I-24058 Romano Di Lombardia (bg) (IT). **BATTAGLIA, Raffaele** [IT/IT]; Via Romualdo Ii Guarna, 8, I-80125 Salerno (sa) (IT). **FERRINI, Gianluca** [IT/IT]; Iii Trav. C. Pavese, 2, I-80018 Mugnano Di Napoli (na) (IT). **MAURO, Marco** [IT/IT]; Via Filippo Cavolino, 7 (scala A), I-80137 Napoli (na) (IT).

(74) Agents: **ROBBA, Pierapolo** et al.; Interpatent S.r.l., Via Caboto, 35, I-10129 Torino (IT).

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(54) Title: A METHOD AND AN APPARATUS FOR CHARACTERIZING AND SEPARATING SPERMATOZOA WITH SUSPENDED LEVER MICROMETRIC SENSORS

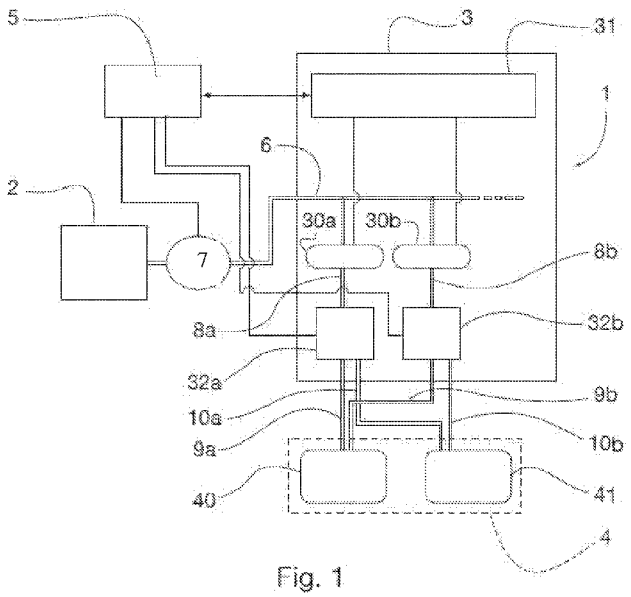

Fig. 1

(57) Abstract: Method and corresponding apparatus for characterizing animal spermatozoa, intended to distinguish and separate spermatozoa carrying the Y chromosome from those carrying the X chromosome, said method being based on the known phenotype differences between the two types of spermatozoa and said apparatus being based on the use of microcantilevers, comprising one or more suspended lever micrometric structures (30a, 30b), single or arranged in parallel, with an integrated microfluidic circulation system (301) in which a fluid is circulated containing a mixed sperm cell population; the differentiation of the spermatozoa can occur: 1) in a static manner, via detection of the bending of the microcantilever at the passage of the spermatozoa in the integrated circulation system; 2) in a dynamic manner, via detection of the variation of the phase difference between an actuation signal for oscillating the microcantilever and the response signal of the transducer, or of the variation of the resonance frequency of the microcantilever.

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"A method and an apparatus for characterizing and separating spermatozoa with suspended lever micrometric sensors"

DESCRIPTION

Field of the Art

The present invention has as object a method and an apparatus for the sexing of animal spermatozoa.

By sexing of the sperm cell, it is intended the operation of distinguishing spermatozoa carrying the X chromosome from those carrying the Y chromosome, which has as objective the determination of the sex of the unborn offspring by using the artificial fertilization (AF) technique. In the zootechnical field, AF has allowed optimizing the selection of the animals by useful characteristics; the techniques of preselection of the sexes represent a further improvement for breeders interested in milk production as well as in meat production. In this manner, it is possible to program the inseminations of the cows, from which calves are obtained intended for farm replacement or to be introduced into genetic centers. The latter permits beef bulls to inseminate the other cows, which allow obtaining feedlot calves of higher value.

Prior Art

Over time, there have been various technological approaches to sexing, but the industrial development of the methods for preselecting spermatozoa was made possible by flow cytofluorometry, which today represents the only commercially viable technique. The high sensitivity of the cytofluorometer allows measuring the only experimentally ascertained difference between spermatozoa carrying the X chromosome and those carrying the Y chromosome: the different DNA quantity, associated with the different size of the sex chromosomes (difference of about 3 - 5%, variable according to the species, the X chromosome being larger).

The flow cytofluorometer is a system which comprises one or more laser sources, a hydrodynamic system that inserts the cells of the sample to be analyzed into an isotonic liquid flow that encounters the laser beam, light detectors (photodiodes and/or photomultipliers) and a processor for analyzing the data in real time.

The analysis is based on the evaluation of the quantity of light emitted by the cells which encounter the laser beam. The cells are previously colored with fluorochromes, which emit fluorescence when hit by the light of a given wavelength (in this case represented by the laser beam). The fluorescence, after having been amplified by the photomultipliers and converted into a digital signal, is analyzed by software.

After the encounter with the laser beam, the driving fluid generates drops that incorporate single sperm cells; each drop is electrically charged in a positive or negative manner as a function of the fluorescence measurement of the sperm cell contained therein. Then, the charged drops pass through two high-voltage plates, where they will be deflected into two collectors (together with the sperm cell contained therein) on the basis of the charge sign.

By coloring the spermatozoa with a fluorochrome that is stoichiometrically linked with the DNA (such as Bisbenzimide Hoechst 33342, which is bonded to the adenine- and thymine-rich regions of the DNA) and defining the selection (sorting) as a function of the double modality of the frequency distribution of the DNA content of the spermatozoa (Johnson et al., 1989, Biol. Reprod., 41:199-203), it is possible to divide the spermatozoa into two populations: those carrying the X chromosome and those carrying the Y chromosome (Garner et al., 1983, Biol. Reprod., 28:312-321; Johnson, 1991, Reprod. Dom. Anim., 26:309-314; Johnson et al., 1989, Biol.

Reprod., 41:199-203; Morell et al., 1988, Vet. Rec., 122:322-324).

In the French patent No. FR 2699678 B1, a sexing method is described in which the selection (sorting) of the cells occurs without electric charging the same, as a function of the particular characteristics of the employed cytofluorometer (PAS III, Partec).

In the English patent No. GB 2145102 B, a method for sexing via flow cytofluorometry is described which makes use of the electric charging of the spermatozoa carried out in a suitable driving liquid.

A semen sexing method by means of flow cytofluorometry, based on the same principle of separation based on the charge, is described in the International Patent application No. WO 90/13303, which uses an Epics V (RTM, Coulter) flow cytofluorometer. The sexing method proposed is based on a series of improving modifications of the instrument (modification of the needle of the flow cell) such to create a flow with a greater percentage of cells oriented with the greater surface perpendicular to the laser beam. In addition, the photodiode is substituted, which is normally assigned to evaluating the light diffracted at 0° - 18° (size evaluation of the particles), with a photomultiplier also capable of detecting low levels of fluorescence (Johnson and Pinkel, 1986 (Cytometry, 7:268-273). A second series of instrumental modifications was then prepared, comprising a new flow cell (Johnson and Welch, 1999, Theriog., 52:1323-1341) with a consequent increase of the percentage of correctly-oriented cells from 25% to 70%. The application of such cell to a high-speed selection cytofluorometer, obtained by applying pressures up to 60 PSI to cells in the flow (MoFlo® Cytomation Inc. - high speed cell sorter), allowed obtaining an instrument capable of theoretically selecting and separating up to 9 million sperm cells per hour with a purity

of 75-80% (U.S.A. patents No. 5150313, 5602039, 5602349, 5643796 and International Patent application No. WO 96/12171). The collected seminal material must then be washed by means of centrifugation, suitably diluted, packaged in 0.25 ml “paillettes” and frozen according to very detailed protocols, as defined in the International Patent application No. PCT/US2003/030814. By using sexed frozen bovine semen dozes in AF (Seidel et al., 1999, Theriog., 52:1407- 1420), acceptable fertility values were obtained, even it was necessary to operate in a shrewd manner regarding the instrumentation, the deposit site of the semen in the female genitals and the animal type (heifers) (International Patent application published with No. WO 99/33956).

Nevertheless, the cytofluorometric method does not lack drawbacks, the main disadvantage regarding the number of spermatozoa separable per unit of time, which remains substantially low even if considerable increases have been achieved. Indeed, if one wishes to obtain a purity level of the enrichment equal to 90% with spermatozoa that have good vitality, suitable for freezing, it is not possible to obtain more than 5,000 spermatozoa per second. This value leads to a limited production of sexed frozen semen doses, even using a reduced total concentration per dose.

Moreover, the use of fluorescent substances does not rule out possible mutagenic actions with respect to the DNA content of the spermatozoa.

Finally, the detection of the fluorescence, and thus the quality of the results, can be affected by the orientation of the spermatozoa along the line of distribution.

Over time, various methods have been tested for sexing spermatozoa alternative to cytofluorometry, which are based on the phenotype differences between spermatozoa carrying the X chromosome and those carrying the Y chromosome.

The methods for sexing which use centrifugation and sedimentation techniques are based on the assumption that the X and Y spermatozoa have different density. In 1982, Meistrich (as reported by Windsor at al., 1993, Reprod. Fertil. Dev., 5, 155-171), considering the known DNA difference between bovine sperm cells X and Y, estimated a density difference equal to 7 x 10^{-4} g/cm^3, i.e. 0.06% of the density of a sperm cell X.

Over the years, much research was conducted with the purpose of separating the X spermatozoa from the Y spermatozoa via centrifugation in high-density gradient solutions.

Kaneko et al., 1983, Fertil. Steril., 40(5):661-5, compared the efficiency of a Ficoll solution with a Percoll solution (with density gradient from 1.06 to 1.11 g/ml) for separating human X and Y spermatozoa. After a centrifugation at 250 x g for 20 minutes, in the Percoll solution the authors reached a percentage of Y spermatozoa of about 73% in the lighter fraction of 1.06 g/ml and a fraction of about 27% in the sediment.

Schwiderski et al., 1991, and Blottner et al., 1993, have used centrifugation in a high-density gradient Percoll solution for separating bovine X and Y spermatozoa. They obtained an enrichment of 75% of the fraction of X spermatozoa in the lower part and 75% of Y in the upper part.

Generally, the experiments known in the literature do not ensure method repeatability and above all the possibility of a commercial system for sexing the spermatozoa is not discussed.

In the International Patent application published with No. WO 2004/072220 A2, a method is described for sexing mammal spermatozoa based on the technique of centrifugation in high-density-gradient solutions, as well as producing and commercializing semen doses enriched with X and Y spermatozoa. The authors sustain the possibility to produce

from 8 to 15 million spermatozoa from a subpopulation (X or Y) in 20-30 minutes of centrifugation. The patented method substantially depends on collaboration with companies specialized in producing and freezing semen.

Currently, experimental studies have been carried out suitable for verifying the actual existence of morphological differences between the heads (where the DNA is contained) of spermatozoa carrying the X chromosome and the heads of spermatozoa carrying the Y chromosome. Research has particularly focused on the study of the volumetric size of the heads, through interference microscopy and image analysis techniques (van Munster et al., 1999, Cytometry, 35:125-128; van Munster, 2002, Cytometry, 47:192-199). The results of such research showed a volume difference between the heads of the X spermatozoa and those of the Y spermatozoa which coincides with the difference in DNA content (about 4%). Even so, at present the techniques employed for sorting based on volumetric measurements have never reached sexed sample purity levels and sorting speeds comparable with those of the ordinary cytofluorometric technique.

Up to now, however, experimental results have not been verified on the mass difference between the two spermatozoa types, with the use of direct methods.

Nevertheless, the present inventors have carried out the first experimental measurement on the mass of a single sperm cell, through the use of quartz crystal microbalances (QCM - Quartz Crystal Microbalances). The average mass value of a single bovine sperm cell resulted equal to $1.95 \pm 0.04 \times 10^{-11}$ g. Considering the measured mass value, the mass difference between spermatozoa carrying the X chromosome and spermatozoa carrying the Y chromosome can be evaluated around a value comprised between 0.5 pg and 1 pg (1 pg = 10^{-12} g).

From the scientific literature, density and volume phenotype differences are known between spermatozoa carrying the X

chromosome and those carrying the Y chromosome, but techniques and methods are not known which allow being able to implement a sexing device alternative to ordinary cytofluorometry.

Godin et al., 2007, Appl. Phys. Lett., vol. 91, No. 12, describe the device, known as "Suspended Microchannel Resonator" (SMR), capable of measuring the mass, the density and the size of cells and nanoparticles. The device comprises a microfluidic channel incorporated in a microcantilever, placed in oscillation at the resonance frequency. The passage of a particle, inside the fluid circulating in the microchannel, causes a variation of the SMR oscillation characteristics, which can be associated with the physical characteristics of the moving particle. The research demonstrated the possibility to measure the density with a resolution of 10^{-4} g/cm^3.

In the article of Burg et al., 2007, Nature, 446:1066-1069 and in the International Patent application published with No. WO 2008/045988 A1, the measurement of mass with an SMR device is described. A sample particle moving inside the fluid circulating in the microchannel causes a variation of the resonance frequency proportional to the mass of the particle. The authors demonstrated the possibility to measure masses to femtogram resolution (1 fg = 10^{-15} g).

The resolution of the SMR allows obtaining a method and a device capable of carrying out the sorting of the spermatozoa, according to a scheme very similar to that of ordinary flow cytofluorometry.

In the document IT TO20080101 A1, the current inventors described a method and corresponding apparatus for sexing animal spermatozoa, based on the use of microcantilevers as mass sensors.

In the present patent application, a method is proposed for sexing spermatozoa which does not require device calibration,

aimed at determining the relation between variation of the resonance frequency and moving mass; the method also does not require complex processing of the signal coming from the sensor, which comprises a microchannel integrated in a cantilever. As a function of the known phenotype differences, the present invention allows characterizing and distinguishing the spermatozoa, based on a threshold value pre-established on a sample of known type, and it implements the proposed method in a sexing apparatus.

Description of the Invention

In light of that discussed and the scientific results, the invention, in a first aspect, provides a method for characterizing and distinguishing the spermatozoa carrying the X chromosome from those carrying the Y chromosome, and, in a second aspect, implements such method in an apparatus for sexing the spermatozoa. The method determines a threshold value, established by measuring the variation of the physical characteristics of a suspended lever micrometric structure with integrated fluidic microchannel, such variation caused by the passage of a single sperm cell.

Cantilevers (or microcantilevers) are micromechanical sensors substantially constituted by a suspended lever of micrometric size, composed of monocrystalline silicon, SiN, polymer materials or other materials. By means of suitable detection systems, the cantilevers transduce a force acting on their end - e.g. due to the presence of an additional mass - into a bending (static functioning). If instead they are suitably stimulated to vibrate, the presence of the additional mass on the cantilever induces a variation both of the phase difference between the actuation signal and the response signal, and of the resonance frequency (dynamic functioning). As is known from scientific literature, cantilevers have been proposed as mass sensors with sensitivities that allow detecting variations even below one attogram (1 ag = 10^{-18});

see for example Li et al., 2007, Nature Nanotechnology, 2:114-120. Sensitivity generally varies as a function of the mechanical and/or geometrical properties, e.g. the size, shape and materials constituting the cantilever.

A review of the construction techniques, of the functioning principles and of the use modes of microcantilevers, especially in the biological field, can be found in Goeders et al., 2008, Chem. Rev., 108:522-542 e Lavrik et al., 2004, Rev. of Sci. Instr., 75:2229-2253.

According to a first aspect of the invention, a fluid with known density is circulated in a fluidic microcirculation channel integrated with the microcantilever, and having size such to allow only one sperm cell pass at a time. The fluid contains a sample of spermatozoa of known type (X or Y spermatozoa), and a threshold value is determined by measuring one of the variations of the sensor's physical characteristics, induced by the passage of a single sperm cell. Subsequently, the same fluid is circulated in the microchannel containing a mixed sperm cell population. The differentiation and characterization occurs by comparing the measurement of the aforesaid physical characteristic of the sensor with the pre-established threshold value.

Such differentiation can occur in static functioning, by detecting microcantilever bending variations, or it can occur in dynamic functioning, by detecting the variations of the phase difference between actuation signal and response signal, or the variations of the resonance frequency of the microcantilever.

According to another aspect of the invention, an apparatus is provided for attaining the sexing method, which implements the previously described method and comprises: a mixed sperm cell population source; at least one suspended lever micrometric structure with integrated microfluidic circulation channel, adapted to receive a single sperm cell;

signal detection and analysis means and means for separating the spermatozoa into a population containing the X chromosome and a population containing the Y chromosome.

In addition, according to another aspect of the invention, for the purpose of obtaining a high yield, a plurality of said structures can be used, each structure composed of a single cantilever or of an integrated formation of parallel-arranged cantilevers which simultaneously receive the fluid containing the spermatozoa to be sexed.

The structure or the plurality of structures can be made to function in active or passive mode, and the method for differentiating the spermatozoa results identical to that employed for single microcantilevers.

Summary Description of the Figures

The invention will now be described in more detail with reference to the enclosed drawings, provided as a non-limiting example, in which:

- Fig. 1 is the general representation of a block diagram of the sexing apparatus according to the invention, in which a plurality of sensitive elements are employed;
- Fig. 2 shows a cantilever as used in the present invention;
- Fig.3 shows a formation of cantilevers as used in the present invention;
- Figs. 4A and 4B show the modes for distinguishing between spermatozoa carrying the X chromosome or the Y chromosome, in static mode;
- Fig. 5 shows the mode for distinguishing between spermatozoa carrying the X chromosome or the Y chromosome, in dynamic mode and as a function of the variation of the phase difference between input signal and output signal; and
- Fig. 6 shows the block diagram of the sexing device which implements the method described in the present invention, and shows as example the schematic diagram of the technique for

measuring the variation of the phase difference between the actuation signal and the response signal as attained in the present invention.

Detailed Description of the Invention

Fig. 1 shows a block diagram of an apparatus for identifying and sexing spermatozoa.

The apparatus, indicated with 1 overall, mainly comprises:

- a source 2 of a mixed sperm cell population;
- a sexing module 3 in turn comprising:
  - one or more elements (two in the figure) 30a, 30b sensitive to the mass of the single spermatozoa;
  - a reading system 31 for detecting the response of the sensitive elements 30a, 30b to the passage of the spermatozoa; and
  - one or more elements 32a, 32b for separating, based on the presence of the X chromosome or the Y chromosome, the spermatozoa which have passed through the sensitive elements 30;
- a system 4 for storing the sexed spermatozoa;
- a control unit 5.

In the figure, the double lines indicate the path of the spermatozoa, while the single lines indicate the path of the reading and command signals. The source 2 of the mixed sperm cell population is constituted, as is normally the case in sexing techniques, by an isotonic fluid containing the spermatozoa, and is connected to the sexing module 3 by means of a distribution system, schematized by the distribution line 6, on which a pump 7 is inserted that is controlled by the control unit 5 in a manner so as to suitably regulate the fluid flow rate.

In the preferred embodiment of the present invention, the sensitive elements 30 are based on so-called cantilevers or microcantilevers, with integrated microfluidic system inside of which the spermatozoa coming from the source 2 circulate.

The sensitive element or each sensitive element can be constituted by a single cantilever, such as that represented in Fig. 2, or by an integrated formation of cantilevers, like that represented in Fig. 3. The cantilevers or the formations of cantilevers can be made to operate in active or passive mode.

The reading system 31 detects the mechanical stresses induced by the alteration of the local equilibrium properties of the cantilever at the passage of a sperm cell inside the fluid circulating in the integrated microfluidic channel. In particular, the reading system 31 detects, in passive functioning mode, the bending of the cantilevers, while in active functioning mode it detects the variation of the phase difference between input signal and output signals or the variation of the resonance frequency.

As regards the active functioning mode, in Fig. 6 the block diagram is shown of the sexing apparatus implementing the method established through the measurement of the phase difference variation, or the resonance frequency variation, employed in the preferred embodiment of the invention. In such embodiment, the microcantilever has a fluidic microcirculation channel, which allows the passage of a single sperm cell at a time, which is connected in input to a mixed sperm cell source and at the output to a switch mechanism. Such switch mechanism normally places the channel in communication with a Y sperm cell storage element, and in accordance with an explicit command places the channel in communication with an X sperm cell storage element.

The microcantilever is actuated, e.g. using a piezoelectric element, at a frequency close to the resonance frequency. The oscillation of the microcantilever is detected through a transduction system, e.g. through optical reader. The input signals (actuation) and output signals (vibration of the cantilever) are sent to a phase comparator and subsequently

to a controller which compares the measurement of the phase difference with the threshold value, established according to the process described in the invention.

The passage of the single sperm cell in the fluidics of the microchannel causes the temporary variation of the microcantilever equilibrium conditions, observable as a transitory variation of the phase difference between input and output signal, whose maximum intensity is determined by the physical and geometrical characteristics of the particle circulating in the channel.

At the passage of a sperm cell of unknown type, the controller compares the measured value with the threshold value, and sends an activation signal to the switch when the surpassing of the aforesaid threshold value has been verified, in a manner so as to place the output channel in communication with the storage system for the spermatozoa carrying the X chromosome.

In the present invention, according to a first aspect, a process is established for characterizing and distinguishing those spermatozoa carrying the Y chromosome from those carrying the X chromosome from among a mixed sperm cell population. The method comprises the step of establishing a threshold value, by measuring the average value of the maximum variation of the phase difference or of the resonance frequency at the passage of a sample population of Y spermatozoa. The passage of an X sperm cell will determine a signal that exceeds the threshold established during calibration.

The method established according to the present invention does not require a calibration aimed at determining the relation between the variation of the resonance frequency and the moving mass. In the method that is the object of the present invention, the calibration phase is instead aimed only at determining a threshold value, with respect to which

is possible to obtain, from the analysis of a mixed sample, two separate distributions corresponding to X and Y sperm cell types.

In the preferred embodiment of the present invention, the microcantilever is placed under vibration at a frequency close to the resonance frequency and the transitory variation is measured of the phase delay between the actuation signal and the response signal, induced by the passage of a sperm cell in the fluidic microchannel. The measurement of the phase delay does not require complex processing of the signal and allows increasing the sampling speed and thus the sorting speed of the spermatozoa.

The separation elements 32a, 32b are each associated with a cantilever 30a, 30b. They receive from the associated cantilever, by means of a line 8a, 8b, the fluid containing the succession of spermatozoa subjected to measurement. Depending on whether the measurement established that a sperm cell present on the line 8 is a carrier of the X chromosome or Y chromosome, such sperm cell is sent to the storage system 4.

The separation elements 32 are, for example, valve elements with an input connected to the line 8 and two outputs connected, by means of respective lines 9a, 9b and 10a, 10b, to two storage units 40, 41, respectively for the chromosomes X and Y. The units 40, 41 form the storage system 4 in their entirety. For example, the separation elements 32 are configured in a manner so as to normally connect the input 8 to the output 10 which leads to the storage unit 41 of the spermatozoa carrying the Y chromosome, and to connect the input 8 to the output 9 which leads to the storage unit 40 of the spermatozoa carrying the X chromosome upon receiving an explicit command of the control unit 5.

The control unit 5 carries out the processing of the signals provided by the reading system 31, necessary for determining

the passage of a sperm cell carrying the X chromosome or Y chromosome. In addition, it will manage and synchronize the actuation times of the separation elements 32 based on the flow rate of the system, on the processing times of the sensor 30 responses and on the transit speed of the spermatozoa through the lines 8.

Fig. 2 illustrates a single cantilever sensor with optical reading. The sensor 30 can be schematized by a support or fixed part 300 to which one end of the suspended lever 301 is fixed, whose free end 302 is made reflective with regard to laser radiation 310 coming from the reading system 31.

Along the periphery of a lever 301, a microfluidic circulation channel 303 is made, along which the spermatozoa move. The channel 303 is isolated from the outside environment and is sized in a manner so as to ensure the passage of a single sperm cell at a time. The channel 303 comprises an input branch 303A, connected to the distribution line 6, and an output branch 303B, connected to the line 8 (Fig. 1).

Fig. 3 shows a formation 330 of sensors integrated on a common support, still indicated with 300. Each sensor of the formation is identical to the single sensor of Fig. 2, and equivalent elements in the two figures are indicated with the same reference numbers, with the addition of single or multiple primes for distinguishing elements belonging to different sensors in the formation.

In order to better comprehend the ideas behind the invention, the mechanism is now described for sexing a mixed sperm cell population in the case of dynamic functioning, in which one must measure the variation of the phase difference between input and output signal, as described in Fig. 5. The control unit 5 (Fig. 1) is adjusted to a specific sensor response threshold level, calibrated on the average peak value of the signal induced by the detection of a sperm cell carrying the

Y chromosome. All the spermatozoa carrying the Y chromosome, which circulate through the single cantilevers 30, will be poured into the respective distribution line 9 of the spermatozoa Y (which is normally held open, i.e. directly connected to the output line 8 of the cantilever through the respective separation element 32) and through such line they will reach the storage unit 41. The transit of a sperm cell carrying the X chromosome, due to its different physical and geometrical properties, will induce an increase in the response of the cantilever and a consequent exceeding of signal threshold level. In this case, the control unit 5 will actuate the separation element 32 in a manner so as to divert the flow towards the line 9 for collecting the X spermatozoa, for sending to the storage unit 40.

It is clear that that described is only given as a non-limiting example and that variants and modifications are possible without departing from the protective scope of the invention. In addition, even if an apparatus is described in detail for distinguishing spermatozoa carrying the X chromosome from those carrying the Y chromosome, based on the measurement of the variation of the phase difference between input and output signals, it is clear that the reading system 31 and the control unit 5 can operate on different physical quantities in accordance with the type of sensor employed and the modes of use. Furthermore, reading methods can be employed that are different from the optical literature.

These alternatives are well known to those skilled in the art. For example, the three documents US 2007/089515, EP 1533611 and WO 2007/081902 mentioned above describe systems in which the variations of the resonance frequency are measured for cantilevers functioning in active mode, and US 2007/089515 and EP 1533611 describe systems in which a response of electrical type is detected.

The possible alternative methods to optical reading are described hereinbelow:

- piezoresistive method: a microcantilever is employed with piezoresistive material spread on the surface, and the value of the electrical resistance of the piezoresistive element is measured using a Wheatstone bridge. When the microcantilever oscillates or is bent, the variation of the resistance of the piezoresistive element causes the flow of a current in the two legs of the Wheatstone bridge, allowing the quantification of the microcantilever bending or oscillation frequency;

- piezoelectric method: the microcantilevers are actuated by applying an alternating voltage to the piezoelectric material layer which covers them. The deformations of the cantilever are detected because they induce a current variation in the piezoelectric material, due to the direct piezoelectric effect.

- capacitive method: a capacitor is used formed by two electrodes, one of which mounted on a fixed support and the other mounted on the movable surface of the microcantilever. When the cantilever is bent, the capacitance between the two electrodes changes, allowing the quantification of sensor deflection.

The methods just described are advantageous in sexing methodology, both because the microcantilever sensor and the reading electronics can be integrated in a single chip and because it is possible to easily arrange the sensors in arrays.

The invention effectively solves the problems of the prior art indicated above. The proposed method in fact allows carrying out tests on the spermatozoa without a preliminary preparation of the samples, as occurs in the case of cytofluorometry. The spermatozoa are not subjected to any chemical treatment, whose mutagenic consequences are not yet

clearly determined. The proposed technology is not affected by problems of orientation of the spermatozoa along the distribution line.

In addition, unlike the cytofluorometric technique, the sexing speed of the spermatozoa, i.e. the number of spermatozoa that can be separated per unit of time, will not be determined only by the speed of the fluid inside the sexing system (for which there is a limit). Due to the typical size of the cantilevers, the invention in principle allows an unlimited increase of the selection speed, obtained simply with the use of a greater number of cantilevers or formations of cantilevers and a consequent increase of the distribution lines.

CLAIMS

1. Method for characterizing and separating spermatozoa carrying the Y chromosome from those carrying the X chromosome, by means of a suspended lever micrometric structure (30; 30a, 30b), with integrated fluidic circulation microchannel (303; 303', 303'', 303''') in which a fluid is circulated containing a mixed sperm cell sample, comprising the following steps:

a) measuring the variation of at least one property of the suspended lever micrometric structure (30; 30a, 30b), induced by the passage of a single sperm cell of known type;

b) determining a threshold value for measuring the variation of at least one property of the suspended lever micrometric structure (30; 30a, 30b), induced by the passage of a single sperm cell of known type;

c) measuring the variation of at least one property of the suspended lever micrometric structure, induced by the passage of a single sperm cell of unknown type;

d) characterizing and separating spermatozoa carrying the X chromosome from those carrying the Y chromosome by comparing the measurement detected in step c) with the threshold value determined step b).

2. Method according to claim 1, wherein the property of the suspended lever micrometric structure is the bending.

3. Method according to claim 1, wherein the suspended lever (301) micrometric structure (30; 30a, 30b) is placed under vibration, by means of a suitable actuator, and the property measured is the resulting mechanical vibration frequency.

4. Method according to claim 1, wherein the suspended lever (301) micrometric structure (30; 30a, 30b) is placed under vibration, by means of a suitable actuator, and the property measured is the phase difference between the actuation signal and the oscillation signal of the lever induced by the actuator.

5. Method according to any preceding claim 1 to 4, wherein more than one suspended lever micrometric structure (30; 30a, 30b) is employed.

6. Apparatus for characterizing and separating spermatozoa, which implements at least one method according to claims 1 to 5.

7. Apparatus for characterizing and separating spermatozoa, which implements the method according to claim 4, comprising:

a) at least one suspended lever micrometric structure;

b) at least one actuator;

c) means for detecting and analyzing the system.

8. Apparatus according to claim 7, wherein the detection and analysis means comprise at least one laser source and at least one photodiode.

9. Apparatus according to claim 7, wherein the detection and analysis means comprise at least one piezoresistive element integrated with the suspended lever micrometric structure (30; 30a, 30b).

10. Apparatus according to claim 7, wherein the detection and analysis means comprise at least one piezoelectric element integrated with the suspended lever micrometric structure (30; 30a, 30b).

11. Apparatus according to claim 7, wherein the detection and analysis means comprise at least one capacitor, wherein one of the plates coincides with the suspended lever micrometric structure (30; 30a, 30b).

12. Apparatus according to claim 7, wherein the actuation means comprise at least one piezoelectric element.

13. Apparatus according to claim 7, wherein the actuation means comprise at least two materials with different thermal expansion coefficient integrated in the suspended lever micrometric structure (30; 30a, 30b).

14. Apparatus according to claim 7, wherein the actuation means comprise at least one electrostatic element.

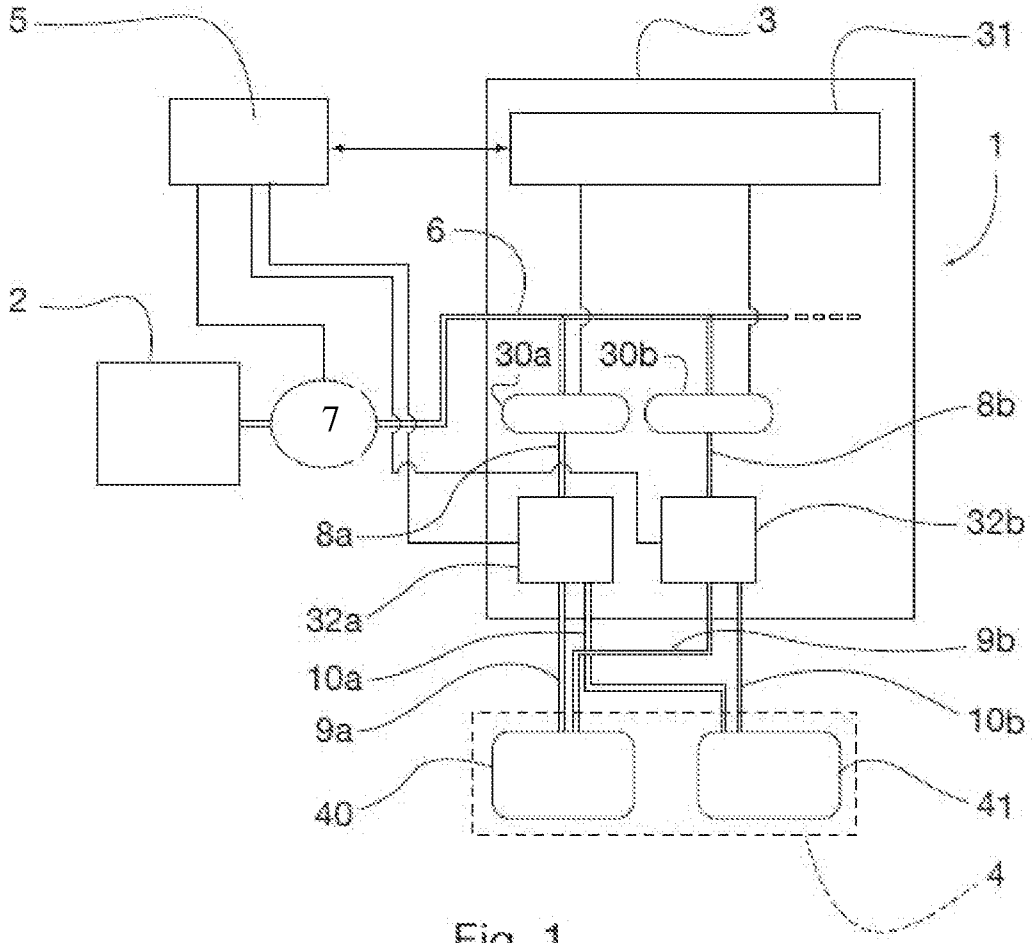
5
3
31
1
6
2
30a
30b
7
8b
8a
32b
32a
9b
10a
10b
9a
40
41
4

Fig. 1

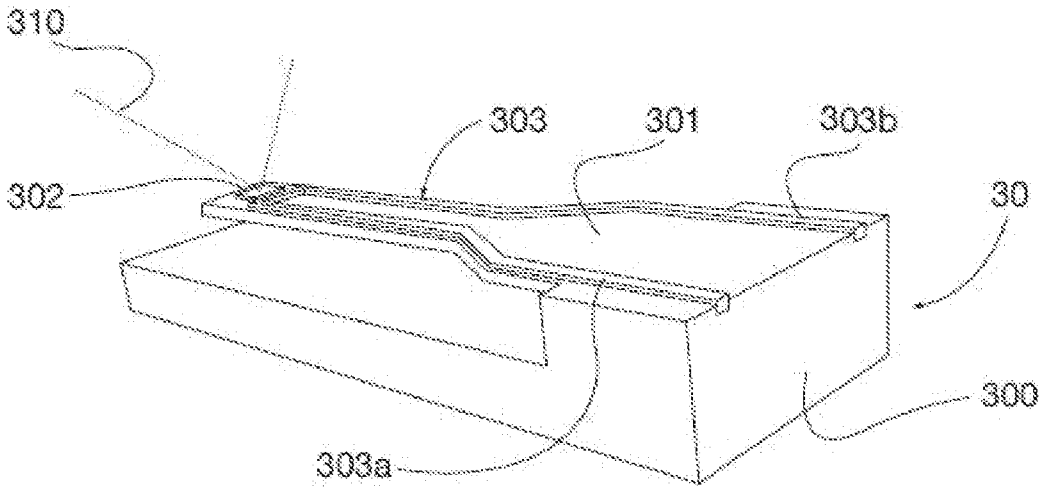
310
303
301
303b
302
30
300
303a

Fig. 2

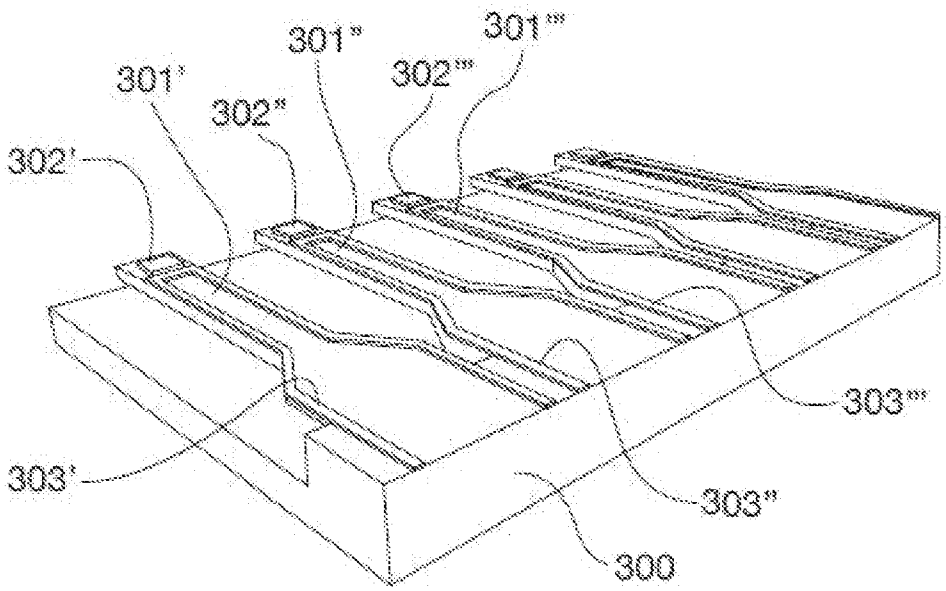

Fig. 3

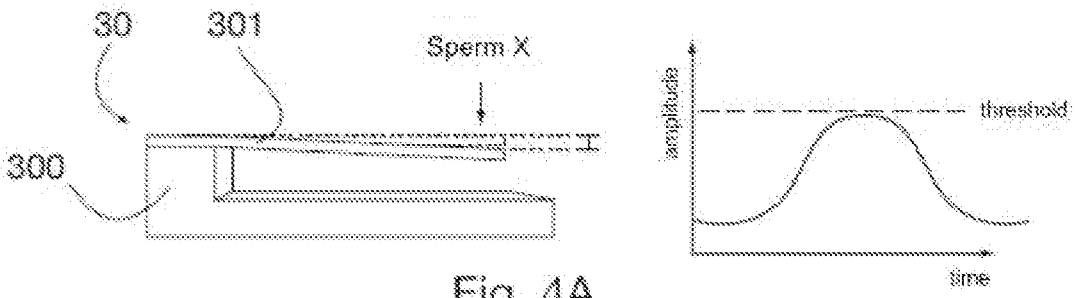

Fig. 4A

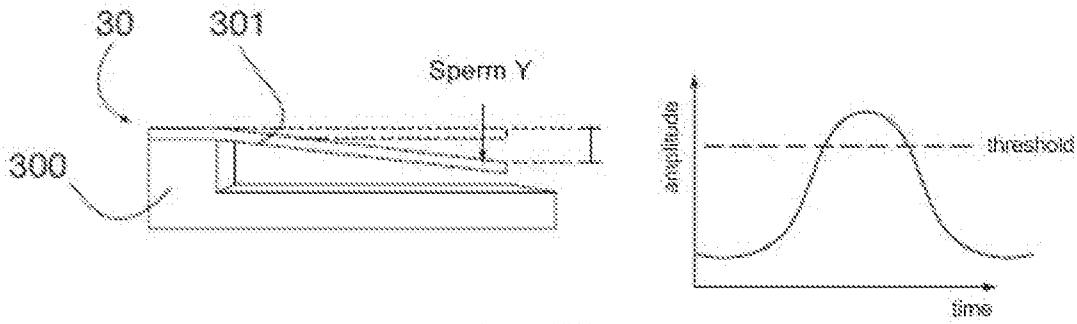

Fig. 4B

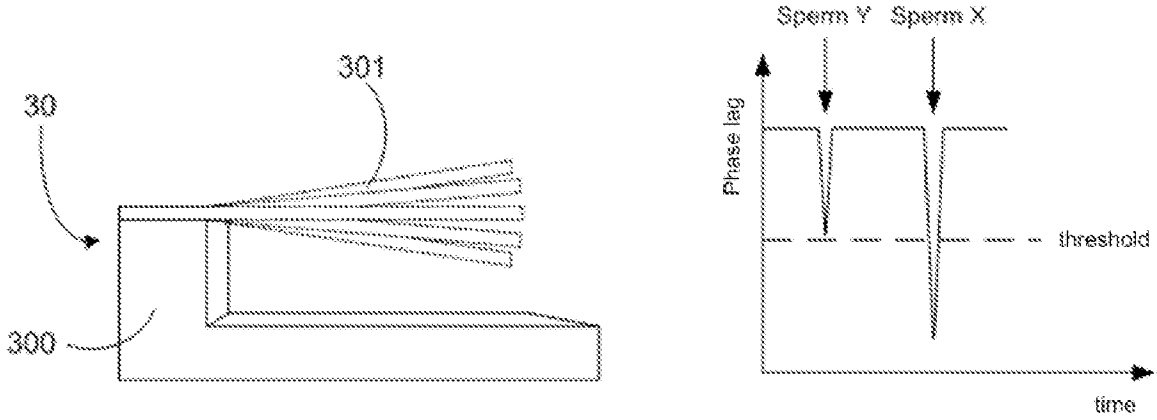
30
301
300
Sperm Y
Sperm X
Phase lag
threshold
time

Fig. 5

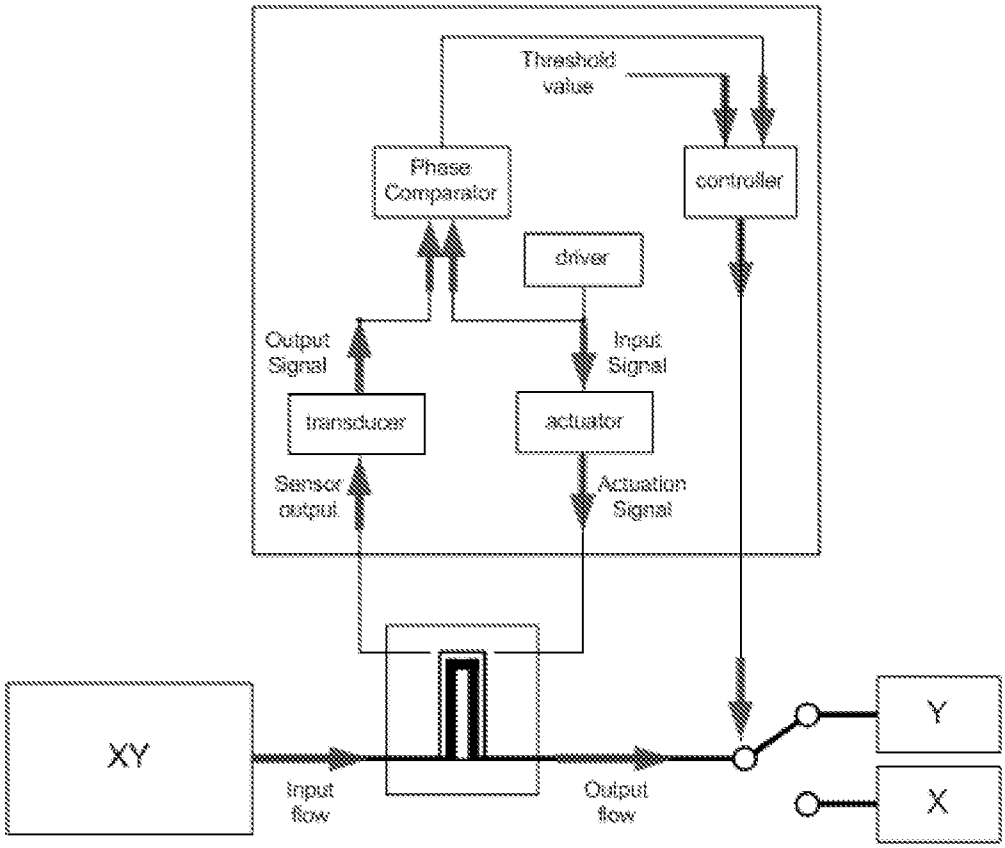
Threshold value
Phase Comparator
controller
driver
Output Signal
Input Signal
transducer
actuator
Sensor output
Actuation Signal
XY
Input flow
Output flow
Y
X

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2011/053302

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N5/02 G01N9/00 G01N15/10 C12N5/00
ADD. G01N15/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N C12N B01L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IT TO20 080 101 A1 (ISTITUTO SPERIMENTALE ITALIANO LAZARO SPALLANZA) 9 August 2009 (2009-08-09) cited in the application the whole document -----	1-14
Y	WO 2004/072220 A2 (FUNDACAO DE AMPARO A PESQUISA [BR]; LIMA VERA FERNANDA MARTINS HOS [BR) 26 August 2004 (2004-08-26) abstract page 2, line 29 page 5, line 23 - page 6, line 16 page 7, lines 18-21 page 9, lines 14-34 ----- -/--	1-14

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 October 2011	27/10/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	van Lith, Joris

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2011/053302

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GODIN MICHEL ET AL: "Measuring the mass, density, and size of particles and cells using a suspended microchannel resonator", APPLIED PHYSICS LETTERS, AIP, AMERICAN INSTITUTE OF PHYSICS, MELVILLE, NY, US, vol. 91, no. 12, 21 September 2007 (2007-09-21), pages 123121-123121, XP012099327, ISSN: 0003-6951, DOI: DOI:10.1063/1.2789694 the whole document	1-14
A	WO 2009/055658 A1 (MASSACHUSETTS INST TECHNOLOGY [US]; MANALIS SCOTT R [US]; BRYAN ANDREA) 30 April 2009 (2009-04-30) page 4, lines 14-23; figures 1-2	1-14
A	US 2009/044608 A1 (BABCOCK KENNETH [US] ET AL) 19 February 2009 (2009-02-19) abstract; figure 1	1-14
A	US 2010/154535 A1 (MANALIS SCOTT R [US] ET AL) 24 June 2010 (2010-06-24) abstract; figure 1	1-14

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Information on patent family members

International application No
PCT/IB2011/053302

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		WO 2008045988 A1	17-04-2008
US 2010154535 A1	24-06-2010	NONE	