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LINDSEY A C ET AL: "HYSTEROSCOPIC INSEMINATION OF LOW NUMBERS OF FLOW SORTED FRESH AND FROZEN/THAWED STALLION SPERMATOZOA", EQUINE VETERINARY JOURNAL, R & W PUBLICATIONS, SUFFOLK, GB, vol. 34, no. 2, 1 March 2002 (2002-03-01), pages 121-127, XP009049239, ISSN: 0425-1644
PHILIP J. DZIUK: "Factors that influence the proportion of offspring sired by a male following heterospermic insemination", ANIMAL REPRODUCTION SCIENCE, vol. 43, no. 2-3, 1 June 1996 (1996-06-01) , pages 65-88, XP55033320, ISSN: 0378-4320, DOI: 10.1016/0378-4320(95)01463-2
EVENSON D P ET AL: "Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility", THERIOGENOLOGY, LOS ALTOS, CA, US, vol. 41, no. 3, 2 February 1994 (1994-02-02), pages 637-651, XP023189163, ISSN: 0093-691X, DOI: 10.1016/0093-691X(94)90174-H [retrieved on 1994-02-02]
FLINT ET AL: "Fertility assessment through heterospermic insemination of flow-sorted sperm in cattle.", JOURNAL OF ANIMAL SCIENCE, vol. 81, no. 7, 1 July 2003 (2003-07-01), pages 1814-1822, XP55033261, ISSN: 0021-8812
SUH T K ET AL: "Pressure during flow sorting of bull sperm affects post-thaw motility characteristics.", THERIOGENOLOGY, vol. 59, no. 1, 1 January 2003 (2003-01-01), page 516, XP002388724, & ANNUAL CONFERENCE OF THE INTERNATIONAL EMBRYO TRANSFER SOCIETY; AUCKLAND, NEW ZEALAND; JANUARY 11-15, 2003 ISSN: 0093-691X

Fortsættes ...

DESCRIPTION

I. TECHNICAL FIELD

[0001] Sperm cell insemination samples having selectably controlled sperm cell fertility characteristics produced through entrainment in a fluid stream having correspondingly selectably adjustable flow characteristics and methods of assessing comparative fertility of sperm cell insemination sample.

II. BACKGROUND

[0002] Pre-selection of sex has been accomplished, in many species of livestock following the development of safe and reliable methods of separating sperm cells into enriched X chromosome bearing and Y chromosome bearing populations. See for example methods and apparatus disclosed by WO 00/06193; WO 02/043574; WO 01/85913; WO 99/33956; WO 01/40765; WO 98/34094; WO 99/42810; WO 02/043486.

[0003] A significant problem with sex selected sperm cells may be that separation of sperm cells at rates sufficient to produce sex selected insemination samples or sex selected inseminates which are viable or sufficiently fertile for commercial application by conventional technology has necessitated increasing fluid stream pressure of flow cytometers or flow sort instruments to about 50 pounds per square inch. With respect to sperm cells of many species of mammals entrained in fluid streams having flow characteristics resulting from this application of pressure the viability, motility, or other fertility characteristics altered.

[0004] Another significant problem with sex selected sperm cell inseminates or sex selected sperm cell insemination samples can be the vast difference in sperm cell fertility characteristics which can vary greatly between samples. As such, success of artificial insemination performed under substantially identical conditions can result in correspondingly different pregnancy rates.

[0005] Another significant problem with existing sperm cell sex selection technology can be the lack of au assay from which fertility of sex selected sperm cells can be compared directly in-vitro (for example, in conjunction with artificial insemination procedures) and in-vivo (for example, in conjunction with IVF procedures).

[0006] The instant invention addresses the variety of problems associated with reduced sperm cell fertility spermatozoa that have been separated into enriched X-chromosome bearing and Y-chromosome bearing populations and the lack of heterospermatic assays to compare function and fertility of separated or sorted sperm cells, and in particular flow sorted sperm cells.

III. DISCLOSURE OF THE INVENTION

[0007] The invention in its broadest sense is as defined in the independent claims.

[0008] Accordingly, the broad object of the disclosure provides devices and methods of using such devices to control sperm cell fertility characteristics of sperm cells isolated from semen obtained from a male of species of mammal, such as motility, viability, fertilization rate, cleavage rate, blastocyst rate, or the like.

[0009] Providing controlled sperm cell fertility in accordance with the invention can be achieved with the sperm cells obtained from numerous and varied species of non-human including without limitation, mammals selected from the group consisting of a bovine species of mammal, an equine species of mammal, an ovine species of mammal, a canine species of mammal, a feline species of mammal, a swine species of mammal, a marine species of mammal, a deer species of mammal, a primate species of mammal, a goat species of mammal, or a species of mammal listed by Wilson, D. E. and Reeder, D.M., Mammal Species of the World, Smithsonian Institution Press, (1993).

[0010] With respect to certain aspects of the disclosure, controlled sperm cell fertility characteristics comprises affirmative selection of fertility characteristics in advance of isolating sperm cells from the semen of the male of the species of mammal and application of the disclosure to alter sperm cell fertility characteristics to provide the fertility characteristics desired. With respect to other aspects of the disclosure, sperm cell treatment conditions are selected within a broader range of sperm cell treatment

conditions that can be used treat sperm cells of a particular species of mammal to obtain sperm cells having controlled fertility characteristics. Controlled fertility characteristics can comprise a desired proportion of motile sperm cells, intact acrosomes, viable sperm cells within a population of treated sperm cells; or can comprise a desires cleavage rate of oocytes or rate of blastocyst formation when treated sperm cells are utilized to fertilize oocytes in vitro; or can comprise a desired Pregnancy rate or sex ratio of offspring when treated sperm cells are utilized for artificial insemination. With respect to certain aspects of the disclosure, a greater proportion of motile sperm cells, a greater proportion of viable sperm cells, a greater proportion of intact acrosomes, or a greater number of fertile sperm cells within a treated sperm cell population can be achieved compared to conventional treatment of the same sperm cell population. Certain aspects of the disclosure allow provision of sperm cells having controlled fertility characteristics which are not substantially different than, or are substantially comparable to, the fertility characteristics of sperm cells in fresh ejaculated semen. In other instances, application of certain aspects of the disclosure can if desired result in sperm cells having controlled fertility characteristics which are substantially different than those of sperm cells of fresh ejaculated semen. In particular, certain aspects of the disclosure can be used to provide bovine sperm cells having controlled fertility characteristics or can be used to provide equine sperm cells having controlled fertility characteristics, which if desired can be provided with fertility characteristics substantially comparable to the fertility characteristics of bovine sperm cells or equine sperm cells within freshly ejaculated bovine or equine semen.

[0011] Another bread object of the disclosure can be to provide sperm cell insemination samples having controlled sperm cell fertility characteristics, such sperm cell insemination sample, without limitation, can be configured for artificial insemination of a female of a species of mammal, in vitro fertilization of oocytes, or intracytoplasmic injection of sperm cells, or the like.

[0012] Another broad object of the disclosure can be to provide methods of sex selecting sperm cells that can provide affirmative control of sperm cell fertility characteristics such as motility, viability, fertilization rate, cleavage rate, blastocyst rate, or the like. One aspect of this broad object of the disclosure can be to provide flow cytometry or cell sorting devices or methods of flow cytometry or cell sorting which allows affirmative control of the fertility characteristics of sex selected sperm cells.

[0013] Another object of the disclosure can be to provide sex selected bovine sperm cell insemination samples having controlled sperm cell fertility characteristics configured for artificial insermination of a female of a bovine species of mammal containing between about 100,000 and about 3,000,000 sex selected bovine sperm cells having controlled sperm cell fertility characteristics.

[0014] Another object of the disclosure can be to provide sex selected equine sperm cell insemination samples having controlled sperm cell fertility characteristics configured for artificial insemination of a female of an equine species of mammal costaining between about 5,000,000 and about 50,000,000 sex selected equine sperm cells having controlled sperm cell fertility characteristics.

[0015] Another significant object of the disclosure can be to provide devices or methods of maintaining controlled sperm cell fertility characteristics of sperm cells with respect to processing of sperm cells, storage of sperm cells, or use of sperm cells, including, but not limited to, insemination of female mammal(s) or fertilization of ocyte(s).

[0016] Another significant object of the disclosure can be to provide methods of artificially inseminating females of a species of mammal with sperm cell insemination samples having controlled sperm cell fertility characteristics. With respect to certain aaspects of the disclosure, methods of insemination with a low or reduced number of sperm cells having controlled fertility characteristics compared to the usual number or typical number of sperm cells used in such artificial insemination procedures whether or not such sperm cells are separated into enriched X chromosome bearing or Y chromosome bearing sperm cell populations.

[0017] Another broad object of the invention is to provide a method as recited in the claims of assessing comparative fertility of sperm cell populations. Certain embodiments of the invention provide a method as recited in the claims of assessing comparative fertility of sperm cells from different males of a species of mammal when sperm cells from each male are exposed to substantially the same flow cytometric treatment. Other aspects of the disclosure provide a method of assessing comparative fertility of sperm cells from the same male of a species of mammal which are exposed to different flow cytometric treatments. Certain aspects of the disclosure provide methods of showing comparative fertility of sperm cells having controlled fertility characteristics.

[0018] Naturally, further significant objects of the invention are made clear in the proceeding description of the invention.

IV. BRIEF DESCRIPTION OF DRAWINGS

[0019]

Figure 1 is a schematic diagram of a sorter system according to a flow cytometer separation technique for the present disclosure.

Figure 2 is a diagram of the entrained cells in the free fall area of a typical flow cytometer.

V. MODE(S) FOR CARRYING OUT THE INVENTION

[0020] A semen or sperm cell process system to maintain, enhance, assay, test, or determine the biological, chemical, physical, physiological, or functional attributes of sperm cells within the context of various collecting, handling, storage, transportation, separation, or insemination procedures.

[0021] An aspect of the disclosure can comprise obtaining sperm cells from a species of mammal as broadly defined above. The sperm cells can then be entrained in a fluid stream having flow characteristics. The fluid stream within a conduit has flow characteristics influenced by the rhealogical properties of the fluid stream, the configuration or geometries of the conduit in which the fluid stream flows, as well as external forces applied to the fluid stream such as hydrostatic pressure, oscillatory vibrations, piezoelectric vibrations, oscillations in heat, or the like.

[0022] Importantly, these flow characteristics of the fluid stream contribute to the amount of pressure required to move fluid within the conduit. As a non-limiting example, in flow cytometry fluid moves within a relatively large cross sectional area and then within a relatively small cross sectional area past an analysis interface to a final collection point.

[0023] This type of configuration or geometry along with rheologic properties of the fluid stream can create localized forces such as compressive forces, sheer forces, or the like, which can have influenceon the physical integrity of particles such as sperm cells entrained in the fluid stream.

[0024] With respect to those embodiment of the invention which include steps involving flow cytometry or cell sorting as a means to analyze, separate based upon a sperm cell characteristic, sex select, or other wise process sperm cells, a conceptual nonlimiting flow cytometer or cell sort instrument is shown by Figure 1.

[0025] A flow cytometer or cell sort instrument includes all or a portion of the components shown by Figure 1 including with out limitation, a sperm cell source (1) which acts to establish or supply sperm cells to analyze, separate, control fertility characteristics, or be otherwise treated.

[0026] Sperm cells are deposited within a nozzle (2) in a manner such that sperm cells are surrounded by a sheath fluid (3). Any sheath fluid compatible with the flow cytometer or flow sort instrument and which provides an acceptable environment for sperm cells during flow analysis or processing can be utilized with the invention, including without limitation, sheath fluids which contain, individually or in various combinations, a phosphate buffered saline, a citrate solution (such as a 2.9% sodium citrate solution), or a HEPES buffered solution.

[0027] The sheath fluid (3) is usually supplied by some sheath fluid source (4) so that as the sperm cell source (1) supplies sperm cells, the sheath fluid (3) is concurrently fed through the nozzle (2). In this manner, the sheath fluid (3) forms a sheath fluid environment for the cells. Since the various fluids are provided to the flow cytometer at some pressure, they flow out of nozzle (2) and at the nozzle orifice (5).

[0028] By providing some type of oscillator (6) which may be very precisely controlled through an oscillator control (7), pressure waves may be established within the nozzle (2) and transmitted to the sheath fluid exiting the nozzle (2) at nozzle orifice (5). Since the oscillator (6) thus acts upon the sheath fluid (3), the stream (8) exiting the nozzle orifice (5) eventually and regularly forms drops (9). Because the cells are surrounded by a sheath fluid environment, the drops (9) may contain within them individually isolated sperm cells (10).

[0029] Since the droplets (9) generally contain isolated sperm cells (10), the flow cytometer or cell sorter instrument can distinguish between and separate droplets based upon a distinguishing sperm cell characteristic(s) of the sperm cell contained within a droplet (9). This is accomplished through a sperm cell sensing system (11). The sperm cell sensing system evolves at least some type of detector (12) which responds to sperm cells contained within each droplet (9).

[0030] One type of sperm cell sensing system (11) is as discussed at length in US 5,135,759 to Johnson. As the Johnson patent explains for sperm cells, the cell sensing system (11) may cause an action depending upon the relative presence or relative absence of a particular dye which may be excited by some stimulant such as the beam of a laser (13). While each type of sperm cell can be stained with a dye, the differing length of the X-chromosome and the Y-chromosome causes different levels of staining. Thus, by sensing the degree of dye present in each sperm cells it is possible to discriminate between X-chromosome bearing sperm and Y-chromosome bearing sperm by their differing emission levels. Alternate optics, detection and sperm cell analysis systems are known which can also be used in accordance with the invention and it is intended that the description provided by the Johnson patent is for illustrative purposes so that the numerous and varied uses of the invention as defined in the claims can be understood. See also, WO 01/85913.

[0031] In order to achieve the ultimate separation and isolation of the appropriate cells in a flow cytometer or cell sort instrument separation technique, the signals received by sensor (12) are fed to some type of sorter discrimination system (14) which very rapidly makes the decision and can differentially charge each droplet (9) based upon whether the desired cell does or does not exist within that droplet (9). In this manner the sorter discrimination system (14) acts to permit the electrostatic deflection plates (15) to deflect droplets (9) based on whether or not they contain a sperm cell having certain sperm cell characteristics. As a result, the flow cytometer or cell sorter instrument acts to separate the cells by causing them to land in one or more collection containers (16). Thus by sensing some property of the sperm cells the flow cytometer or cell sorter instrument can discriminate between cells based on a particular characteristics and place them in the appropriate collection container (16). In certain flow cytometers or cell sorter instruments, the X-bearing sperm droplets are charged positively and thus deflect in one direction, the Y-bearing sperm droplets are charged negatively and thus deflect the other way, and the wasted stream (that is unsortable cells) is uncharged and thus is collected in an undeflected stream into a suction tube or the like.

[0032] Now referring primarily to Figure 2, the process can be even further understood. As shown in that figure, the nozzle (2) emits a stream (8) which because of the oscillator (7) (not shown in Figure 2) forms drops (9). Since the cell source (1) (not shown in Figure 2) may supply sperm cells (10) which as described by Johnson can be stained (or in certain embodiments of the invention unstained as when using DIC technology), the light emission generated by the beam generated by laser (or illumination source when using DIC technology) (13) incident upon the dye (the sperm head when DIC technology is utilized) is differentially determined by sensor (12) so that the existence or nonexistence of a charge on each drop (9) as it separates from stream (8) can be controlled by the flow cytometer. This control results in positively charged, negatively charged, and uncharged drops (9) based upon their content. As shown in Figure 2, certain drops are shown as deflected drops (17). These deflected drops (17) are those containing sperm cells (10) which can be one or the other sex. They are then deposited in the appropriate collector (16) thereby generating a population of sex selected sperm cells.

[0033] Wether the fluid stream occurs within the context of a flow cytometer, cell sorter, or other device which entrains sperm cells within a fluid stream, the flow characteristics of the stream can be characterized and adjustment means for altering flow characteristics of the fluid stream can be introduced to increase or decrease forces such as compressive forces, sheer forces, or the like, such that particles entrained in the fluid stream can be physically, physiologically, functionally, or mechanically altered.

[0034] As such a selectably adjustable range of fluid stream characteristics for a flow path can be generated using the adjustment means and can be expressed as an incremental measure. For example, alteration of fluid stream characteristics within a flow cytometer or cell sort instrument context can be incrementally adjusted and measured in pounds per square inch and typically allow the incremental increase or decrease in fluid stream pressure between about 20 pounds per square inch and 100 pounds per square inch with a commensurate increase or decrease in fluid stream or sheath fluid velocity.

[0035] In accordance with certain embodiments of the invention, sperm cells of a particular species of non-human mammal are entrained in a fluid stream having adjustable fluid stream flow characteristics. Fluid stream flow characteristics are then selectably adjusted over an incrementally measured range in which the entrained sperm cells remain viable. Sperm cell fertility characteristics are then assessed for each of a plurality of sperm cell samples taken in correspondence to each of a plurality of flow characteristics generated within the measured range.

[0036] Subsequently, sperm cell fertility characteristics with respect to sperm cells from a species of mammal or individual members of a species of mammal can be controlled and sperm cell insemination samples can be generated having the desired sperm cell fertility characteristics.

[0037] For example, sperm cells from each of six bulls were stained with 125 µm Hoechst 33342 for 45 min at 34°C, and bulk-sorted (passed through a flow cytometer or cell sorter instrument without sorting into subpopulations) or sorted with a flow cytometer having a nozzle with an internal diameter of 70 µm into X-chromosome bearing or Y-chromosome bearing (or both)

populations at about 95% accuracy with the fluid stream having a pressure of at 30 pound per square inch, 40 psi, or 50 psi. Lowering the fluid stream pressure from 50 psi to 30 psi reduced sorting rate by only 2 to 3%.

[0038] The sperm cells were subsequently cooled to 5°C and concentrated by centrifugation, loaded into 0.25 ml straws with about 2 x 10^6 total sperm cells per 100 µl column, and frozen using a vapor freezing method along with unsorted controls. The sperm cells in the straws were subsequently thawed.

[0039] Sperm cells were then evaluated with respect to various sperm cell fertility characteristics blindly by two observers at 30 and 120 min post-thaw for progressive motility, as well as by flow cytometry 105 min post-thaw, for percent live sperm cells by PI stain, and by CASA analysis 120 min post-thaw using the Hamilton Thorne system. The entire procedure was twice replicated.

[0040] Factorial ANOVA indicated that both bull and pressure effects were significant (P<005, Table 1).

Table 1. Responses of sperm post-thaw to different system pressures during sorting.[a]

Pressure (psi)				
Response	50	40	30	Unsorted Control
30 min motility (%)	44.7	48.6	49.6	52.1
120 min motility (%)	34.5	40.8	42.7	40.8
Live sperm (%)	51.7	55.7	57.8	58.5
CASA total motility (%)	25.1	37.2	40.9	34.8
CASA ALH*	6.0	7.6	7.8	8.8

[a] All statistically significant, P<005.
* Amplitude of lateral head displacement.

Higher numbers mean less stiff and more normal motility. There were typical differences among bulls in all responses. However, the bull by treatment interactions were small with one exception, meaning findings apply similarly to most bulls in the population. The flow characteristics of the fluid stream adjusted incrementally to increase pressure affected substantially all sperm cell fertility characteristics measured, however, only highly statistically significant ones are in Table 1.

[0041] As can be understood, there was significant change in sperm cell fertility characteristics between sperm cell samples taken at about 50 psi and at about 40 psi, and then a much smaller change between about 40 psi and about 30 psi, indicating that the effect on sperm cell fertility characteristics can not be assumed linear. For bovine sperm cells exposed to the flow characteristics described at 30 psi, sperm cell fertility characteristics were substantially the same as nonsorted controls or comparable to nonsorted controls, and for a few responses better, if sperm cells are to be used for insemination or artificial insemination of females of the bovine species. Similar procedures were conducted with sperm cells obtained from stallions with similar results and conclusions.

[0042] Sperm cell fertility characteristics can be controlled and with respect to sperm cells obtained from mammals. For most species of mammals altering fluid stream characteristics to incrementally reduce fluid stream pressure, whether in the context of flow cytometry or otherwise, can result in a graded series of corresponding sperm cell samples having altered sperm cell fertility characteristics which may be used for a variety of procedures including artificial insemination, in vitro fertilization, or intracytoplasmic injection as described below.

[0043] The invention provides alternate tests as recited in the claims to assess binomial responses such as pregnant/not pregnant, which typically require large numbers of animals per treatment to obtain statistical significance unless treatment differences are fairly large. One embodiment of the invention which can amplify differences in sperm cell fertility characteristics of sex selected sperm due to treatment differences comprises competitive, or heterospermic, fertilization, mixing sperm of different males before insemination, and determining the proportion of embryos, fetuses or offspring derived from each male, as recited in the claims.

[0044] For example, fertility after sex selection of sperm cells by flow cytometry or by cell sorting for DNA content at two different fluid stream pressures can be assessed using heterospermic insemination using sex as the genetic marker. Sperm cells from each of two bulls was sorted into X-chromosome bearing or Y-chromosome populations, or both, at about 95% accuracy with the fluid stream pressure adjusted to either 30 psi or 50 psi. After concentrating sperm cells post-sort by centrifugation, 1 x 10^6 X-chromosome bearing sperm cells sorted at 30 psi were placed in 0.25-mL straws with 1 x 10^6 Y-chromosome bearing sperm cells sorted at 50 psi for each bull, as well as the converse in other straws: 1 x 10^6 Y-sperm at 30 psi plus 1 x 10^6 X-sperm at 50 psi.

These sperm cells, along with unsorted controls, were then frozen, thawed some months later, and inseminated into the body of the uterus of 85 Holstein heifers either 12 or 24 h after observed estrus with subgroups balanced across two inseminators.

[0045] Two months post-insemination, 81% of the 43 heifers becoming pregnant had fetuses of the sex (determined by ultrasound) corresponding to the sex of sperm processed at 30 psi. This differed from the 50:50 sex ratio expected ($P<0.01$), if there was no difference in sperm cell fertility characteristics of sperm cells sorted at the two pressures. The pregnancy rate with sex selected sperm at 2 x 10^6 sperm per dose was 51% (43/85); this was similar to the controls of 20 x 10^6 unsexed sperm per dose from the same ejaculates, 39% (9/23).

[0046] Another aspect of the disclosure provides a method altering the cleavage rate and rate of blastocyst formation using sperm cells having controlled sperm cell fertility characteristics. Two bovine sperm cell samples each having controlled fertility characteristics were generated by flow sorting bovine sperm cells at 40 psi and 50 psi respectively. Dose response of sperm cell concentration in the fertilization medium was conducted with X-chromosome bearing sperm cells and Y-chromosome bearing sperm cells from each sperm cell sample. Thus, a multifactorial procedure comprising 2 fluid stream pressures, 3 sperm cell concentrations (1, 0.33 and 0.11 x 10^6 sperm/ml), 6 bulls and 2 sexes can be conducted.

[0047] About 2,000 oocytes were aspirated from about 2mm to about 8mm follicles from slaughterhouse ovaries. Chemically defined media (CDM) were used throughout as described by Journal of Animal Sciences, 78:152-157 (2000). Maturation took place in M-CDM supplemented with 0.5% FAF-BSA, 15 ng/ml NIDDK-oFSH~20, 1 µg/ml USDA~LH~B~5, 0.1 µg/ml E_2, 50 ng/µl EGF and 0.1 mM cysteamine for 23 h at 38.8°C and 5% CO_2 in air. Sorted sperm cells frozen with 2 x 10^6 cells per straw were thawed and centrifuged at 400 g through 2 ml 45% and 2 ml 90% Percoll gradients for 20 min. Then the supernatant was discarded and 2 ml of FCDM supplemented with 0.5% FAF~BSA, 2 mM caffeine and 0.02% heparin was added to the sperm pellet and centrifuged at 500 g for 5 min. The supernatant was discarded leaving approximately 50 µl of sperm suspension. Matured oocytes were washed once in FCDM and transferred in groups of 15 in 5 µl into 25~µl drops of FCDM under mineral oil. Fertilization took place by adding 10 µl of sperm suspension per drop for 18 h at 38.8°C, 5% CO_2 in air. Presumptive zygotes were cultured in CDM1 for 2 d and CDM2 for 4.5 d at 38.5°C, 5% O_2, 5% CO_2 and 90% N2. On day 7.5, blastocyst development was evaluated: Quality 1 to 4 (1 being excellent and 4 being poor) and stage of development, 6 to 8 (6 full, 6.5 expanding, 7 expanded, 7.5 hatching and 8 hatched blastocysts). Data (Table 1) were analyzed by ANOVA and first deviation after are sin transformation.

[0048] Cleavage (53.6 and 43.6%) and blastocyst (18.2 and 14.7%) rates were higher for procedures utilizing sperm cells having controlled sperm cell characteristics obtained at about 40 psi than at about 50 psi ($P<0.01$). There was no interaction between dose and pressure; therefore, there was a similar advantage to lower pressure at each sperm concentration. A clear dose response of sperm cell concentration for cleavage and blastocyst production was found (Table 2). Also, there were large differences among bulls ($P<0.01$) for both responses, and there was a bull x dose interaction ($P<0.01$) for % cleaved. The data indicate that the sperm dose should be >1.0 x 10^6/ml for some bulls. Embryo quality was higher ($P<0.01$) for Y-sperm than X-sperm (1.12 vs 1.57). Others have noted this for IVF embryos when embryos were sexed, and this effect now is confirmed with sexed sperm.

Table 2. Cleavage (%, C) and blastocysts (%, B) per oocyte data presented by bull.

	Bull													
	H023		H024		H025		H026		H027		H028		Avg	
Sperm conc. (10^6)	C	B	C	B	C	B	C	B	C	B	C	B	C	B
0.11	18	1	6	1	4	11	36	15	20	7	54	15	30[a]	8[a]
0.33	44	4	7	2	72	31	62	21	29	11	68	18	47[b]	14[b]
1.0	56	18	35	14	85	43	83	34	72	27	85	29	69[c]	28[c]

a,b,c Values without common superscripts within groups differ, P<0.01.

**Fertility of sorted sperm has been low compared to unsorted control sperm, due partly to mechanical damage during sperm sorting by flow cytometry. Lowering system pressure improved both sperm quality and fertility in IVF. The present study evaluated the effect of system pressure during sperm sorting and extended maturation of oocytes on development of embryos after ICSI. Sperm from each of 3 bulls were stained with 125 µM Hoechst 33342 for 45 min at 34°C, sorted into X- and Y-chromosome bearing populations at 95% accuracy with the pressure of SX MoFlo® sorters at 40 or 50 psi, and then cryopreserved. Fifty bovine oocytes obtained from slaughterhouse ovaries were placed per well with 1 ml of CDM1 supplemented with 0.5% FAF-BSA, 2mM glucose, 50 ng/ml EGF, 15 ng/ml NIDDK-oFSH-20, 1 µg/ml USDA-LH-B-5, 1 µg/ml E2 and 0.1 mM cysteamine, and then matured for 24h or 30h at 38.5°C, 5% CO_2 in air. Cumulus cells of matured oocytes were removed by vortexing, and oocytes with a polar body were selected. Motile sperm from sorted frozen-thawed semen were recovered by centrifugation through 2 ml each of 45 and 90% Percoll, and the concentration adjusted to 4 x 10^6/ml. Matured oocytes were divided into two injection groups, ICSI and sham injection using a Piezo injection system. The outer diameter of the sperm injection pipette was 8-10 µm. All manipulations were performed at room temperature (24-25°C). After injection, oocytes were activated with 5 µM ionomycin for 4 min, cultured in 50 µl of CDM1 at 38.5°C under 5% CO_2, 5% O_2 and 90% N_2, and assessed for cleavage at 72 h post-injection. Uncleaved oocytes from ICSI and sham injected groups were stained with orcein and evaluated for fertilization status. Cleaved embryos were further cultured and blastocyst development was evaluated on day 7.5 after injection. Data were subjected to ANOVA; the are sin transformation was used for percentage data.

[0049] With 24h matured oocytes, there were no differences (P>0.1) between sperm sorted at 40 versus 50 psi for cleavage or blastocyst rates, nor was there pressure x bull interaction. There where significant effects of bulls for all responses studied (P<0.05). When injected with sperm sorted at 40 psi, oocytes matured for 30h resulted in a higher cleavage rate than 24h matured oocytes (22.9 versus 12.2%, P<0.05), with no difference (P>0.1) in blastocyst rate. Overall blastocyst development was higher in ICSI than in sham injection (7.5 versus 1.3%, P<0.05). When uncleaved oocytes from 24h maturation were evaluated for fertilization status, ICSI showed higher percentage with 2 polar bodies and/or decondensed sperm compared to sham injection (15.7 versus 1.7%, P<0.05). With 30h matured oocytes there was no difference in fertilization status between those two groups. We conclude that there was no difference in cleavage or development to blastocysts after ICSI using motile sperm that had been sorted at 40 vs 50 psi.

[0050] In another embodiment of the invention, heterospermic insemination using sex as the genetic marker can be used to rank fertility of males and optionally to rank fertility of sperm treatments not involving sperm sexing. Current in vitro tests of sperm function are not highly correlated with male fertility, and homospermic inseminations require hundreds of inseminations per treatment to obtain accurate fertility data. Heterospermic insemination, mixing the sperm of two or more males, provides an accurate estimation of relative fertility in most species examined (DZIOK P.J. (1996), animal reproduction science, Vol.43, pp. 65-88).

[0051] Frozen, flow-sorted sperm from 4 groups of 4 bulls were thawed and inseminated into heifers 12b or 24h following onset of estrus in all combinations of 3 bulls within groups (ABC, ABD, ACD, BCD). Equal numbers of progressively motile sperm were inseminated from each bull, totaling 600,000 motile sperm post-thaw. Half of each inseminate was deposited into each uterine horn. Embryos were collected nonsurgically 14.5 to 20 days following estrus. Collections yielded 165 elongating embryos from 332 heifers (48%). Polymorphic DNA markers were used to genotype embryos to determine the sire of each embryo biopsy. After genyotyping, 118 of the 165 embryos could be assigned a specific sire. Heterospermic indices for ranking each bull group were calculated using the maximum likelihood analysis theorem. Each bull within groups was ranked based on these indices (Table 1). In group 1, the fertility of the poorest bull was significantly lower (P<0.05) than two other bulls. In group 2, the dominant bull had the highest index (P<0.05). Similar distinctions could be made in groups 3 and 4. However, in three of the groups the fertility of some bulls was not clearly high or low (P>0.05).

Table 1. Heterospermic indices ± SE for individual bulls within groups.

Group 1	Group 2	Group 3	Group 4
1.47 ± .41a	2.43 ± .43a	1.68 ± .44a	0.92 ± .36a,b
0.44 ± .27a,b	0.22 ± .15b	1.09 ± .39a,b	0.46 ± .20a
1.84 ± .46a	0.90 ± .35b	0.83 ± .31a,b	2.02 ± .40b
0.25 ± .17b	0.45 ± .23b	0.40 ± .22b	0.59 ± .24a
a,b Indices without common superscripts differ, P<0.05.			

[0052] with these procedures, an average of 30 genotyped embryos per group of 4 bulls enabled detection of bulls with clearly differing fertility. Sperm treatments also could be evaluated with this technique. This in vivo test requiring few females rapidly provides information concerning which bulls have relatively high or low fertility.

[0053] The population of calves obtained by artificial insemination of females with sex selected sperm cells in accordance with the invention are virtually identical to controls using unsex selected sperm cells. Furthermore, artificial insemination of females with sex selected sperm resulted in approximately 90% of calves of the planned sex. As described above, sperm cells can be sex selected on the basis of DNA content by flow cytometry or by cell sorting after staining with H33342. The sex selected sperm cells can then be cryopreserved as described in Therlogenology, 52:7375.

[0054] Estrus can be synchronized in heifers and cows of various beef and dairy breeds, either by feeding 0.5 mg melengestrol acetate (MGA) daily for 14 d followed by 25 mg prostaglandin F_2 (PGF_2) im 17 to 19 d later or injection of 25 mg PGF_2 im at 12-d intervals. Insemination with either frozen-thawed sex selected insemination samples or frozen-thawed sperm from the same ejaculate have been accomplished at either 12 hours or 24 hours after initial observation of estrus. For each breeding group, about 2/3 of the inseminations were with sexed sperm while control sperm were used in the remainder. Pregnancy and fetal sex were diagnosed by ultrasound 2 months later.

[0055] Cattle were managed at 13 farms through calving and weaning under differing levels of management (N = 49 to 228 per farm). Data collected included gestation length, birth weight, calving ease (1 = normal to 4 = Caesarian), weaning weight, neonatal dearths, and deaths from birth to weaning. Not all farms recorded birth and weaning weights. Data were subjected to factorial analysis of variance with factors: management groups, sorted versus control sperm, and sex of calves. The are sin transformation was used for percentage data. Least-square means are in Table 1.

Table 1. Calving results from sexed and control calves

Treatment		Gestation	Neonatal	Calving	Birth	weight Live	at Weaning
	N	length (d)	death (%)	ease	(kg)	weaning (%)	weight (kg)
Sexed[a]	574	279	3.9	1.31	34.3	92.0	239
Control	385	279	5.9	1.30	34.1	88.9	239
No significant differences (P>0.1) for any response.							

[0056] There were no differences (P>0.1) between calves from sexed versus control groups for any response studied, nor were there significant interactions. There were significant effects of management groups for all responses studied (P<0.001 for all except % alive at weaning, P<0.02). Also, there were significant differences (all P<0.001) between female and male calves for birth weight: 32.2 and 35.5 kg; weaning weight 232 and 246 kg; calving difficulty: 1.20 and 1.42; and gestation length: 278 and 280 d.

[0057] The sex ratio of the control calves was 51.0% males (N=382). X sort sperm resulted in 87.7% females, while the Y sort sperm produced 93.6% males (N=94). A few calves that were dead at birth did not have sex recorded and are not included.

[0058] The recent development of flow cytometric reparation of stallion spermatozoa has resulted in the production of normal foals with preselected sex. (Lindsey A.C., Morris L.H., Allen W.R., Schenk J.L. Squires E.L., Bruemmer J.E. Equine vet J. 2002, 34: 128 - 132). For this technology to be accessible, semen will be transported from the flow cytometer to the mare. This study examined the longevity and acrosome status of fresh stallion spermatozoa after sex preselection. Three ejaculate from each of 7 stallions were collected by artificial vagina and shipped to the laboratory at 20°C for 2~6h in a skim milk-glucose extender (1:1 v/v). The semen was centrifuged at 400g for 10min and the seminal plasma removed. The sperm pellet was resuspended to 100 $x10^6$ /ml in Kenneys modified Tyrodes medium (KMT), stained with Hoechst 33342 (5mg/ml, Sigma-Aldrich, St. Louis MO), incubated for 30min and subjected to flow cytometry. The sorted spermatozoa were centrifuged and resuspended to 40 $x10^6$ml in KMT m 250µl aliquots for 48h storage at either 4°C or 20°C. The total progressive motility (TPM) and the acrosome status of the spermatozoa were evaluated prior to sorting and at 0, 2, 12, 24, 36 and 48h after sorting. The TPM was evaluated microscopically and acrosomes stained with FITC-PNA (Sigma-Aldrich) and classified as intact, patchy or lost. The effect of stallions, time and storage temperatures were analyzed using the Proc GLM procedure and least means comparisons made (SAS Institute).

[0059] There was an effect of stallion (p = 0.03) on sperm motility and on the proportion of intact acrosomes over time. Staining

and incubating the spermatozoa with Hoechst 33342 resulted in a decrease in the proportion of intact acrosomes (Table 1). However, the proportion of intact acrosomes observed after sorting was higher than in the sperm population prior to sorting. The proportion of intact acrosomes declined ($p < 0.0001$) as the lost acrosomes increased ($p < 0.0001$) during 48h after sorting, but there was no effect of time on the proportion of patchy acrosomes. There was a significant effect of sperm storage temperature after sorting such that that storage for 12h at 20°C resulted in higher mobility than storage at 4°C. Sex-sorting spermatozoa by flow cytometry results in the selection of a population of spermatozoa which can maintain acrosome integrity for 24h, equivalent to fresh spermatozoa. The maintenance of sperm longevity for 12h after FACS separation should enable sex-sorted spermatozoa to be shipped to mares located some distance from the site of the flow cytometer.

Table 1 The mobility and acrosome status of flow sorted spermatozoa over time

Stage of Processing		TPM	Acrosome intact
Prestain		50.7 ± 10.2	57.1 ± 28.2
Post stain		42.0 ± 17.1	42.3 ± 26.7
Post incubation		48.0 ± 15.7	34,9 ± 27,1[a]
0h	Post sort	49.9 ± 18.3	60.2 ± 22.3[b]
2h	4°C	34.3 ± 19.3	47.1 ± 28.3
	20°C	40.8 ± 21.8	53.9 ± 24.5
12h	4°C	8.5 ± 12.8[a]	59.8 ± 19.7
	20°C	27.0 ± 21.0[b]	66.6 ± 12.0
24h	4°C	4.8 ± 12.9	56.2 ± 16.8
	20°C	17.2 ± 17.9	64.3 ± 16.4
48h	4°C	0.0 ± 0.0	32.7 ± 25.0
	20°C	5.1 ± 8.6	41.6 ± 24.0

[a,b]Values within a column with different superscripts are significantly different ($p<0.05$).

Foals of predetermined sex have been accurately and reliably produced in a research setting (Lindsey et al., Equine Vet. J. 2002, 34: 128-132). Sex-sorted sperm would be more efficiently utilized by the industry, however, if frozen/thawed sex-sorted sperm were available. The objective of this study was to compare the motion characteristics of sperm that had been stored for 18h at 15°C, flow-sorted, and then frozen, to sperm that had been cryopreserved immediately following shipment at 18h at 15°C. Two ejaculates were used from each of five stallions. Following collection, sperm for both treatments were extended to $25x10^6$/mL in a Kenney + Modified Tyrodes (KMT) medium and stored in a water bath at 15°4 for 18 h. After storage, sperm were allowed so reach ambient temperature (~22°C) prior to centrifugation at 600g for 10 min. Seminal plasma was removed and the sperm pellet resuspended to 500 x 10^6/ml in KMT. An aliquot of sperm was removed (Control) from this samples, extended to 87 x 10^6/ml in a skim-mild, egg yolk freezing extended (4% glycerol; FR5), and allowed to slow cool to 5°C for 90 min, before freezing in liquid nitrogen vapor. A. second aliquot (Flow-sorted) of sperm was extended to 100 x 10^6/ml in KMT, strained with Hoechst 33342 (5mg/ml, Sigma-Aldrich, St. Louis MO), incubated for 30 minutes, and subsequently sorted by flow-cytometry. Sorted sperm was centrifuged at 850g for 20 min, resuspended to 87 x 10^6/ml in FR5, and allowed to cool slowly to 5°C for 90 min prior to

cryopreservation. Sperm for both treatments were packaged in 0.25-ml straws, and each straw contained 20 million sperm. Sperm were evaluated (blindly) for visual pregressive motility (2 observers) at 30 and 90 min post-thaw. An aliquot of sperm from each straw was diluted in both KMT and in KMT containing 2mM caffeine. Samples were allowed to equilibrate for 5-10 min at 37°C prior to evaluation. A second straw of each treatment was evaluated (with and without caffeine) using the Hamilton-Thorn Motility Analyzer (CASA). Results are in Table 1. Differences in motion parameters were determined by ANOVA. According to most measured responses, flow-sorting was detrimental to sperm motility. Additionally, 2 mM caffeine improved many sperm responses, There was an interaction whereby caffeine improved some responses more for sorted sperm than for control sperm. Therefore, the damage caused by sorting can be partially compensated for by caffein. It is possible that similar compensation may occur in the mare reproductive tract. Studies are currently in progress to compare the fertility of stored, cryopreserved stallion sperm to that of sperm that has been stored and sorted prior to cryopreservation.

	Vis	Vis	CASA	CASA							
Treatment	30	90	Tot	Prog	VAP	VSL	VCL	ALH	BCF	STR	LIN
C-Control	50n	47s	64b	60a	94a	80c	164a	6.19b	33a	83b	50b
Control	45a	40b	50h	44b	32a	69c	144b	5.73a	33a	82b	50d
C-Sorted	32b	31c	32c	21c	48b	38b	98b	4.75b	41b	73b	39b
Sorted	18c	16d	24d	12d	39b	30b	80b	4.51b	37c	69b	38b
a,b,c,dValues in the same column without common superscripts differ (P,0.05). C-treatments stimulated with 2mM caffeine.											

[0060] Cow elk 3-6-yr of age in Colorado and Minnesota were synchronized for estrus in September by insertion of a progesterone CIDR into the vagina for 12-14 d. Upon removal of the CIDR, 200 IU of eCG was administered intramuscular and elk were timed-inseminated 60 h later. Fresh semen was collected via electro-ejaculation from a 5-yr old bull elk and slowly cooled over 4 h to about 20°C for transportation as a neat ejaculate to the sperm-sorting laboratory. The ejaculate was concentrated to 1 x 10^9 sperm/ml for straining by centrifuging 1.5 ml aliquots for 10 sec at 15,000 x g. Semen was incubated in 112 µM Hoechst 33342 at 200 x 10^6 sperm/ml in a TALP medium for 45 min at 34°C, and then diluted to 100 x 10^6/ml for sorting. Sperm were sorted on the basis of differing DNA content of X and Y chromosome-bearing sperm. X chromosome-bearing elk sperm contained 3.8% more DNA than Y chromosome-bearing sperm. Sperm were flow-sorted over a 4 h period using MoFlo®SX operating at 50 psi with a TRIS-based sheath fluid. The 351 and 364 bands of an argon laser, emitting 150 mW, excited Hoechst 33342 dye bound to DNA. Both X and Y chromosome bearing sperm were collected (~92% purity as versified by reanalyzing sonicated sperm aliquots for DNA) were collected at ~4,700 sperm/sec into tubes containing 2 ml of 20% egg yolk-TRIS extender. Sorted volumes of 15 ml were sequentially collected. Approximately 110 x 10^6 sperm of each sex were sorted and cooled to 5 °C over 90 min. An equal volume of glycerol (12%) containing extender was added to the sorted volume at 5 °C. Sorted sperm aliquots containing 30-ml were concentrated by centrifugation at 4 °C for 20 min at 850 x g. Sperm pellets were pooled, adjusted to 21.7 x 10^6 sperm/ml and loaded into 0.25-ml straws. Each straw, containing 5 x 10^6 total sperm, was frozen in liquid nitrogen vapor. As a control 5 x 10^6 total sperm from the same ejaculate were frozen in 0.25 ml straws at the same time as the sexed sperm. After thawing for 30 sec at 37 °C, 65% and 60% of sperm (control and sexed, respectively) were progressively motile as determined by visual estimates. Cows at 3 different locations and management schemes were inseminated using routine trans-cervical semen deposition in the uterine body. Pregnancy was determined 40-d post insemination by assaying blood for Pregnancy-Specific Protein B (Bio Tracking, Moscow, Idaho). Ten cows at one location were in poor condition at the time of insemination and no pregnancies were achieved with sexed of control sperm. The pregnancy rate at the other locations with sexed sperm (61%; 11/18) was similar to that for control inseminates (50%; 3/6). These pregnancy rates (sexed and controls) resulted from fewer sperm than are used in normal elk artificial insemination. Nine of eleven (82%) of sexed calves were of the predicted sex.

[0061] As can be easily understood from the foregoing, the basic concepts of the present invention as recited in the claims may be embodied in a variety of ways. The disclosure involves both a sperm cell process system including both techniques as well as devices to accomplish sperm cell processing. In this application, various sperm cell processing techniques are disclosed as part of the results shown to be achieved by the various devices described and as steps which are inherent to utilisation. They are simply the natural result of utilizing the devices as intended and described. In addition, while some devices are disclosed, it should be understood that these not only accomplish certain methods but also can be varied in a number of ways. Importantly, as to all of the foregoing, all of these facets should be understood to be encompassed by this disclosure.

[0062] The discussion included in this specification is intended to serve as a basic description. The reader should be aware that

the specific discussion may not explicitly described all embodiments possible; many alternatives are implicit. Where the invention is described in device-oriented terminology, each element of the device implicitly performs a function.

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- WO0006193A [0002]
- WO02043574A [0002]
- WO0185913A [0002] [0030]
- WO9933956A [0002]
- WO0140765A [0002]
- WO9834094A [0002]
- WO9942810A [0002]
- WO02043486A [0002]
- US5135759A [0030]

Non-patent literature cited in the description

- **WILSON, D. E.REEDER, D.M.**Mammal Species of the WorldSmithsonian Institution Press19930000 [0009]
- Journal of Animal Sciences20000000vol. 78, 152-157 [0047]
- **DZIOK P.J.**animal reproduction science, 1996, vol. 43, 65-88 [0050]
- **LINDSEY A.C.MORRIS L.H.ALLEN W.R.SCHENK J.L.SQUIRES E.L.BRUEMMER J.E.**Equine vet J., 2002, vol. 34, 128-132 [0058]
- **LINDSEY et al.**Equine Vet. J., 2002, vol. 34, 128-132 [0059]

Patentkrav

1. Fremgangsmåde til vurdering af fertiliteten af sædceller fra en han af en ikke-human pattedyrsart, der omfatter:

a. opnåelse af sædceller fra mindst to hanner af en ikke-human pattedyrsart;

b. eksponering af sædcellerne fra hver af de mindst to hanner af den ikke-humane art for den samme flowcytometriske behandling, der omfatter kønsselektering af sædceller fra hver af de mindst to hanner;

c. inseminering af mindst én hun af den ikke-humane pattedyrsart med en blanding af det samme antal af de kønsselekterede sædceller fra hver af de mindst to hanner af den ikke-humane pattedyrsart, der har været eksponeret for den samme flowcytometriske behandling, hvor blandingen omfatter en X-kromosombærende population af kønsselekterede sædceller fra én af de mindst to hanner og en Y-kromosombærende population af kønsselekterede sædceller fra en anden af de mindst to hanner;

d. indsamling af embryoer fra den mindst ene hun af den ikke-humane pattedyrsart;

e. bestemmelse af, hvilken af de mindst to hanner af den ikke-humane pattedyrsart, der er far til hvert embryo, ved anvendelse af køn som en genetisk markør; og

f. rangering af komparativ fertilitet af de mindst to hanner af den ikke-humane pattedyrsart baseret på komparative antal af embryoer, som hver af de mindst to hanner er far til.

2. Fremgangsmåde til vurdering af fertiliteten af sædceller fra en han af en ikke-human pattedyrsart, der omfatter:

a. opnåelse af sædceller fra mindst to hanner af en ikke-human pattedyrsart;

b. eksponering af sædcellerne fra hver af de mindst to hanner af den ikke-humane art for den samme flowcytometriske behandling, der omfatter kønsselektering af sædceller fra hver af de mindst to hanner;

c. befrugtning af oocytter *in vitro* med en blanding af det samme antal af de kønsselekterede sædceller fra hver af de

mindst to hanner af den ikke-humane pattedyrsart, der har været eksponeret for den samme flowcytometriske behandling, hvor blandingen omfatter en X-kromosombærende population af kønsselekterede sædceller fra én af de mindst to hanner og en Y-kromosombærende population af kønsselekterede sædceller fra en anden af de mindst to hanner;

d. indsamling af embryoer frembragt ved *in vitro*-befrugtningen af oocytterne;

e. bestemmelse af, hvilken af de mindst to hanner af den ikke-humane pattedyrsart, der er far til hvert embryo, ved anvendelse af køn som en genetisk markør; og

f. rangering af komparativ fertilitet af de mindst to hanner af den ikke-humane pattedyrsart baseret på komparative antal af embryoer, som hver af de mindst to hanner er far til.

DRAWINGS

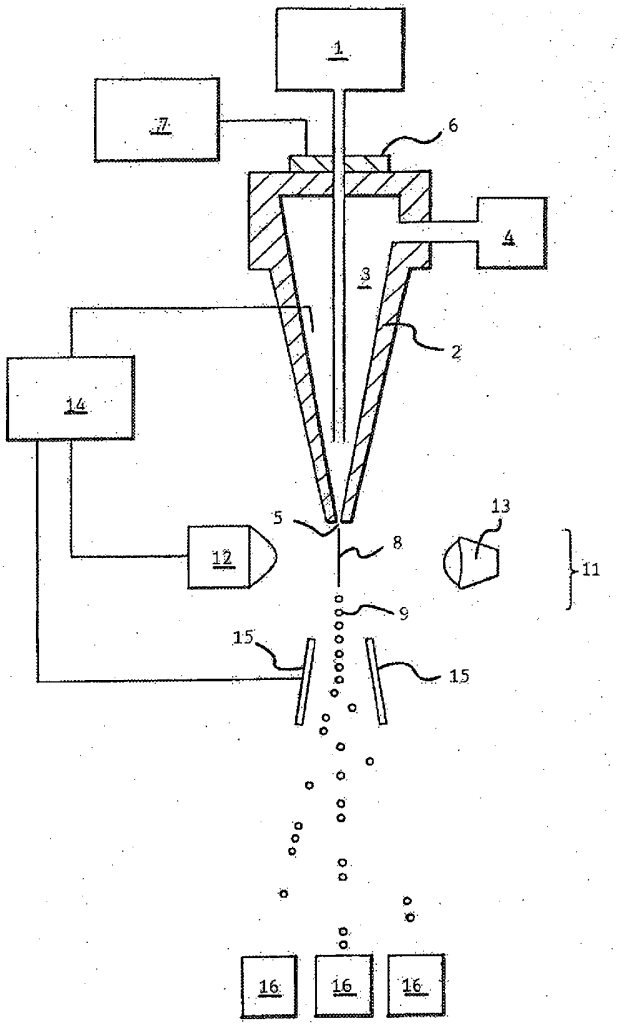


FIG.1

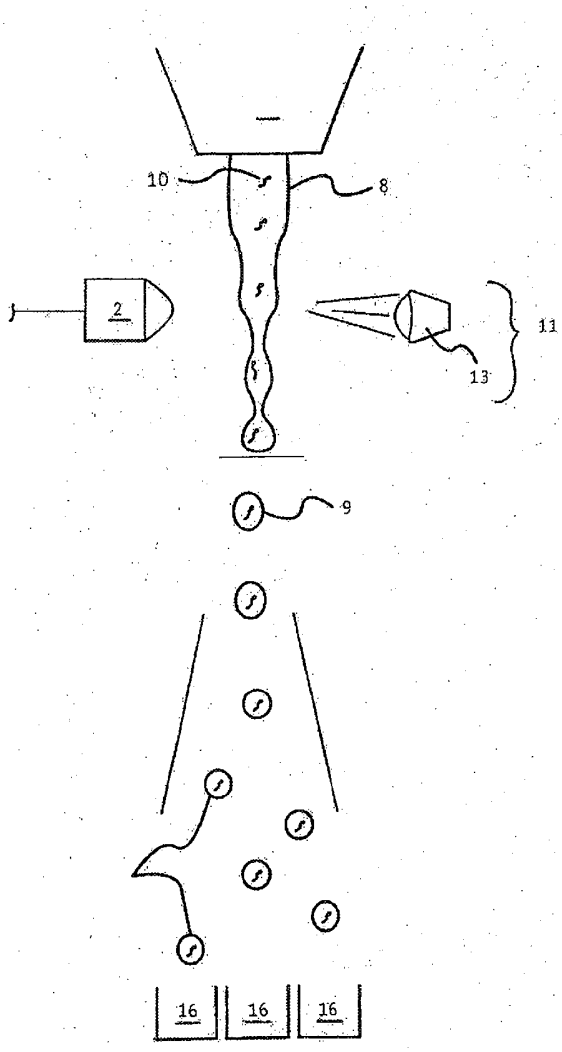


FIG.2