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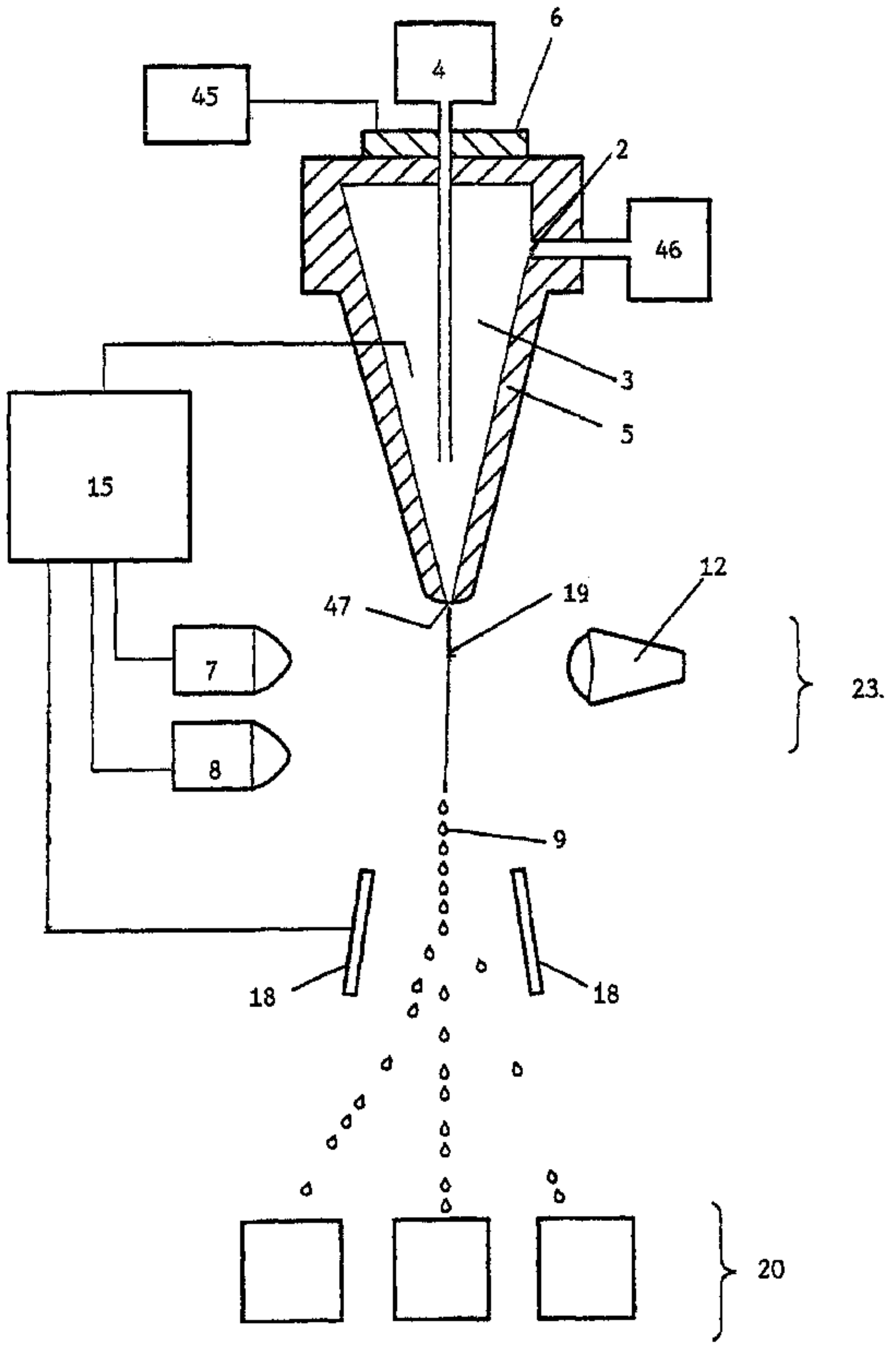
(54) **Titre : TRI EFFICACE DE CELLULES HAPLOIDES POUR SYSTEMES DE CYTOMETRIE EN FLUX**
(54) **Title: EFFICIENT HAPLOID CELL SORTING FOR FLOW CYTOMETER SYSTEMS**



(57) **Abrégé/Abstract:**

A flow cytometry system (1) for sorting haploid cells, specifically irradiatable sperm cells, with an intermittingly punctuated radiation emitter (56). Embodiments include a beam manipulator (21) and even split radiation beams directed to multiple nozzles (5). Differentiation of sperm characteristics with increased resolution may efficiently allow differentiated sperm cells to be separated higher speeds and even into subpopulations having higher purity.

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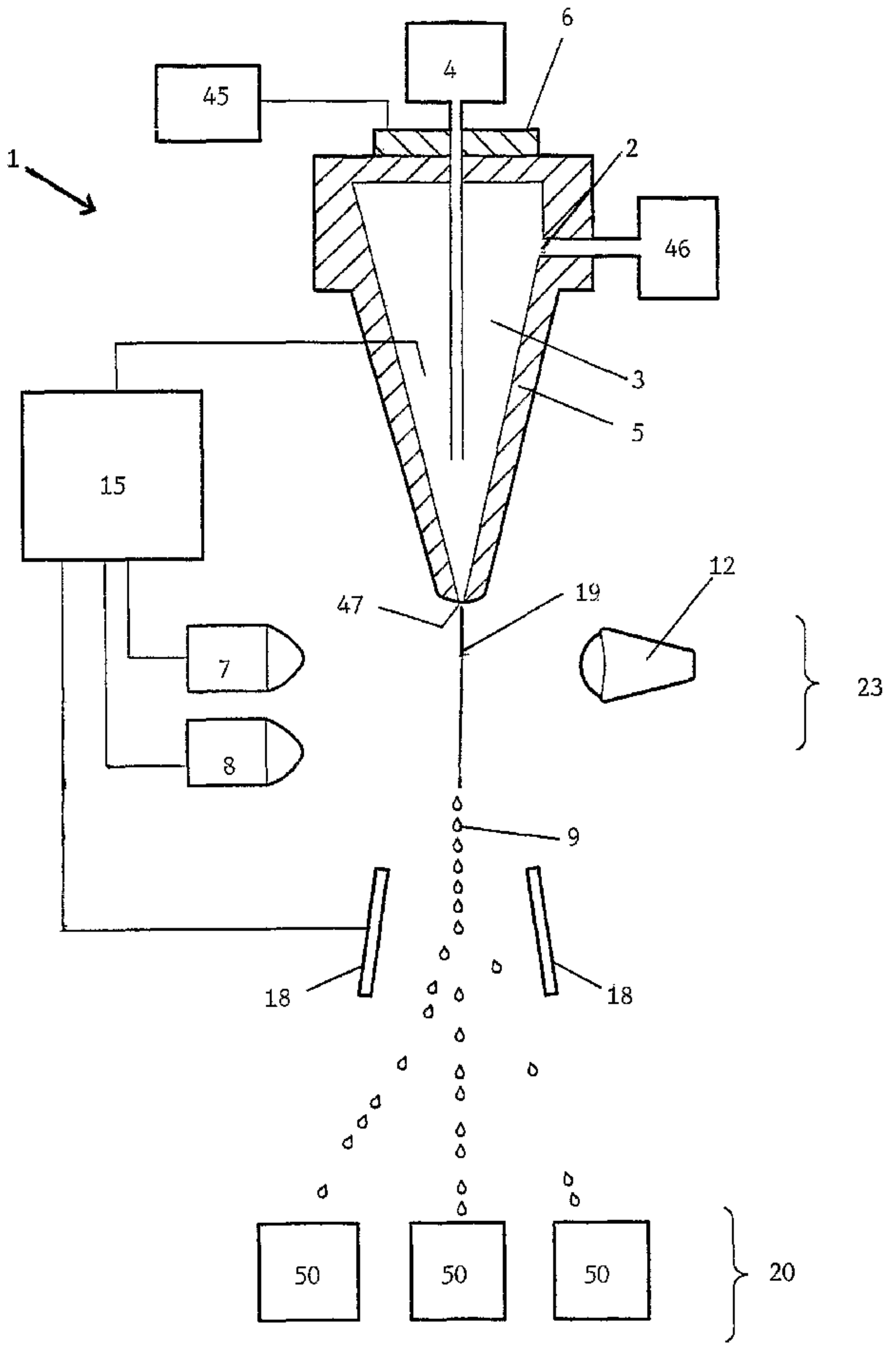
(54) Title: EFFICIENT HAPLOID CELL SORTING FOR FLOW CYTOMETER SYSTEMS



(57) Abstract: A flow cytometry system (1) for sorting haploid cells, specifically irradiatable sperm cells, with an intermittingly punctuated radiation emitter (56). Embodiments include a beam manipulator (21) and even split radiation beams directed to multiple nozzles (5). Differentiation of sperm characteristics with increased resolution may efficiently allow differentiated sperm cells to be separated higher speeds and even into subpopulations having higher purity.

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EFFICIENT HAPLOID CELL SORTING FOR FLOW CYTOMETER SYSTEMS

I. FIELD OF THE INVENTION

The present invention relates to a flow system for particle analysis. More specifically, the invention relates to the use of a pulsed laser on a flow system for particle analysis which results in more accurate quantification of measurable properties of individual particles. It may be of particular interest in analyzing populations of very similar particles, at high speeds, allowing more efficient separation of particles into two or more different populations. The invention is particularly useful in the application of separating live X-chromosome bearing and Y-chromosome bearing sperm of all mammals at higher speeds, better purities and with equal or better sperm health outcomes, meaning less damage to sperm. The invention may contribute significant improvements to the economics of sperm sorting.

II. BACKGROUND OF THE INVENTION

Lasers can be used to deliver light to biological or non-biological particles and emission spectra can be used for the analysis of particle characteristics. In some instances, this can be applied such as where a particle is self florescent or self color absorbing, is associated by affinity, avidity, covalent bonds, or otherwise to another molecule which may be colored or fluorescent, may be associated to another molecule which is colored or fluorescent through a specific biological or modeled macromolecular interaction, such as an antibody binding event or a nucleic acid oligomer or poynucleic acid hybridization event, may obtain color or fluorescence such as through an enzymatic synthesis event, an enzymatic attachment or cleavage reaction, enzymatic conversion of a substrate, association of a florescent molecule with a nearby quencher, the reaction of a product in certain local proton (pH) or NADH or NADPH or ATP or free hydride (H-) or bound hydride R-(H-) concentrations, or may gain color or fluorescense by way of a variety of methods to associate emitted or absorbed light (electromagnetic radiation EMR).

Conventional lasers can generate a strong, perhaps intense, source of light. Through coherence properties of the beam such light may travel very long distances, perhaps across reflective mirrors which may change the angle of the light illumination beam, perhaps through prisms or refractive objects or lenses which may split it into two or more beams of equal or differing intensity, or may defocus, perhaps expand, or focus, perhaps concentrate, the beam. Such light may also be affected by filters which may reduce the net energy of the beam. Most lasers also allow the modulation of light intensity, perhaps watts, in the beam by adjustment of an input current from a power supply to the light generating element.

In some applications, conventional lasers used in the analysis and quantification of biological objects can be combined with sensitive light detectors that may be as simple, such as a photographic film or paper, or may be more complex, such as a photomultiplier tube. Often, a light detector may collect only information about a cumulative amount of light, perhaps electromagnetic radiation, EMR, or it may collect and report on the dynamic changes in intensity of light or EMR hitting all of, or portions of, localized regions of, or positions on the detector surface. The light detector may also involve use of a photoelectric coupling device, which may allow the energy of photons absorbed on the EMR by the light detector to be converted to current proportional to the incident light or EMR on the light detector surface. The photoelectric coupling device can even be integrated into an electronic circuit with an amplifier which may increase the signal or create gain such that the fluctuations or perhaps summation of amplified current may be available to an analog or digital logic circuit. Designs may also transmit a signal or data set to a user of a particle analysis instrument and this signal may be proportional to the *static, cumulative,* or perhaps even dynamic intensity of the light or EMR incident upon the detector.

In certain uses of laser light to analyze biological particles, a detector may measure the change in intensity of the source light after incidence upon a particle(s) being analyzed using a reference beam which takes a path without incidence upon the particle(s). In other uses of laser light, modified or unmodified particles take up a fraction of the illumination light or EMR and may emit light of a different frequency. In many cases, the presence of emission light or EMR of a certain wavelength can be used to identify or to quantify characteristics associated with specific particles, or quantitatively measure the amount or number of the specific biological particles present in the sample or in a specific region of or position in the sample.

In some cases, it can be useful to accurately determine very small differences in the illumination light or emission light from two very similar biological particles (for example an X-chromosome bearing sperm cell versus a Y-chromosome bearing sperm cell). These small differences can be analyzed by way of serial presentation of perhaps 50,000 separate emission events per second in a liquid stream. These can also be thousands of separate emissions from molecules (nucleic acids or proteins as examples) on an array field allowing analysis of genetic, genomic, proteomic, or glycomic libraries.

The traditional type of laser used for the analysis of particles in flow cytometry is a continuous wave (CW) laser. Often this provides a beam of constant intensity. However, in some instances, CW lasers can have particular disadvantages for applications as discussed here. The beam can result in modification or destruction of the sample being observed. For example, with respect to sperm cells, irradiation can result in lower fertility of the sperm cells. Second, in some instances when the laser beam continuously operates, it may be desirable to have a method of interrupting the beam if it is moved from a first location of incidence to a second location of incidence without illumination of intermediate areas.

In U.S. Pat. No. 5,596,401 to Kusuzawa, a pulsed laser may be used for imaging an *object, such as a cell, in a flow cytometer.* This disclosure may be related to improvements in the capture of images from particles such as coherence lowering modulations. Kusuzawa *may teach a use of a continuous wave laser for particle detection* and imaging.

In U.S. Pat. No. 5,895,922 and U.S. Patent Application No. 2003/0098421 to Ho, pulsed laser light may be used to illuminate and detect hazardous biological particles dispersed in an airflow stream. The invention may include an ultraviolet laser light and looking for the emission of fluorescence from potentially hazardous biological particles. This disclosure may teach the disadvantages of a laser diode apparatus.

U.S. Pat. No. 6,177,277 to Soini, describes employing a two-photon excitation and/or confocal optimal set-up. The invention may relate to the use of confocal optics to reduce an analysis volume to about 10% of standard analysis volume in a flow cytometer. A pulsed laser may provide short pulses of intense light and may allow the simultaneous absorption of two photons so that a wavelength of illuminating light beam may be longer than an emitted single photon bursts. Background signal may be reduced by use of a filter. The invention may include dual signal processing. The invention as described in Soini, may be beneficial in the analysis of small particles such as erythrocytes and bacterial cells.

In U.S. Pat. No. 6,671,044 to Ortyn, a special analysis optics and equipment may be used in an imaging flow cytometer. The Ortyn disclosure may include analyzing a sex of fetal cells in maternal blood as a method for determining the sex of a child during early pregnancy. Ortyn may indicate that analysis rates from an imaging flow cytometer may be restricted to theoretically maximizing at 500 cells per second.

With respect to particle analysis using laser light, the present invention discloses technology which addresses each of the above-mentioned problems.

For the purposes of this invention, a rapidly pulsed, high intensity pulsed laser may be used. This laser may deliver short pulses of high intensity perhaps lasting about 5-20 picoseconds, followed by intervals between pulses which are 100-1000 times as long as the pulses or about 0.5-20 nanoseconds. The light may have very high peak intensities over the period of about 5-20 picoseconds, and low net energies over the period of about 2-10 microseconds.

Flow cytometry, using a high-speed cell analysis, or high-speed cell analysis and sorting instrument, often relies on a laser light source to illuminate a stream of fluid in which particles are entrained. Particles may be caused to flow by a point of illumination at a rapid rate, often in the range of 500 to 100,000 particles per second. Often the light from the illuminating laser source is of constant intensity. The particles in the analysis stream may be of the same size, and may spend the same amount of time within the area of illumination. The amount of light illuminating each particle in a large population of particles analyzed in series may be identical. A detector may be capable of measuring scattered light, or other types of light emitted by the particle as a result of auto-fluorescence or fluorescence associated with a chemical dye, dye complex, or conjugated dye which may be targeted to one or more types of molecular species contained on or within particles in the population and can determine the identity of a particle and, in some cases, make a measurement of the quantity of a specific molecular target associated with the particle. A specific molecular structure on or even within a particle may be characterized and a quantitative measurement of the amount of associated molecular structure on or even within a particle, may yield information which may be used as a basis for sorting out or separating one type of particle from another.

In a flow cytometer, there may be a very short time duration between the exact moment that a particle is illuminated and the exact moment that a physical manipulation or an electrical condition, may be triggered to elicit separation of a specific particle from a stream containing various particles. An example of a physical manipulation may be charging of a droplet. A specific duration may be called a drop delay period, and the duration may be perhaps as brief as about 100 microseconds or perhaps as long as about 10 milliseconds, and may even be about 1 millisecond. In the case of particle sorting, information may be detected from each particle, computational analysis of the information may be determined, and comparison of the computation to a gating value or perhaps even a selection criteria may be accurately performed within a time period shorter than a duration of the drop delay.

Flow cytometer systems may be useful for measuring an average amount of a specific molecule present on or even within a population of particles. Past systems may not have measured the exact amount of a specific molecule on or even within a population of particles. Factors which can contribute to inaccurate measurements of single particles may include the saturation of a stain or even a conjugate to a particle, variation in the quanta of illumination light, effects from the shape of a particle, and perhaps even electronic noise in the detection apparatus.

An example of a particularly challenging problem is the sorting of X-chromosome bearing and Y-chromosome bearing sperm of mammals at high processing rates and high sorting purities. The population of sperm in most mammals is about 50% X-chromosome bearing and about 50% Y-chromosome bearing. A stain, such as Hoechst 33342, may form complexes with double stranded DNA. A measurement of total Hoechst 33342-DNA complex in each sperm may correlate to the total amount of DNA in each sperm. In general, mammals have larger X chromosomes than Y chromosomes and may have a differential between total DNA contents of X-chromosome bearing over Y-chromosome bearing sperm for various mammals. Such differentials may include: human having about

2.8%; rabbit having about 3.0%; pig having about 3.6%; horse having about 3.7%; cow having about 3.8%; dog having about 3.9%; dolphin having about 4.0%; and sheep having about 4.2%. The differentials may correlate to a relative difference of intensities emitted from a stained sperm being sorted for the purpose of separation of X-chromosome bearing and Y-chromosome bearing sperm.

Significant achievements have been made in developing staining conditions to stain DNA in live sperm with Hoechst 33342, such as, the use of dual orthogonal detection systems to determine sperm orientation, the use of hydrodynamic fluidics to increase the numbers of correctly oriented sperm, the setting of gain on detectors, and even the use of high-speed electronics. In the most efficient use of said achievements, it may be possible in most mammals to simultaneously sort sperm into two populations, X-chromosome bearing, and Y-chromosome bearing, at rates of 2500 per second or higher. It may also be possible to sort sperm to purities of 90% or even higher. There may be, however, a distinct problem in that at rates faster than 2500 per second, the purity of the sample may decrease.

This problem may be understood due to the observation that the co-efficient of variation (CV) in possibly even the best sperm sorting procedures may be between about 0.7%-1.5%, and with poor conditions can even be between about 2%-5%. Since the difference in DNA between X-chromosome bearing sperm and Y-chromosome bearing sperm in mammals may be as low as 2.8% as seen in humans and as high as 7.5% as can be seen in chinchillas, the CV may be lower than the DNA differential in order to achieve a large enough separation of the two populations. Humans have one of the lowest known DNA differentials and may have some of the lowest known maximum purities in sorting. It may be desirable to improve procedures which can reduce the CV.

A method which has been shown to improve the CV may be to use higher intensities of laser light illumination. For example, it is known to use continuous wave lasers to sort various sperm species with between about 100-200 milliwatts of laser illumination, and possibly with about 150 milliwatts. It has been observed that doubling or tripling the intensity and increasing the power to about 300-500 mW can improve the CV. An improved CV can be most apparent by analysis of the "split" between two peaks on a histogram. Yet, there may be problems associated with an increase of intensity or perhaps even an increase of power with a continuous wave laser. In the case of analyzing a Hoechst 33342 DNA complex with a continuous wave laser, the light source may be near a UV spectrum and may have some ionizing effect upon the DNA complex. Ionizing may then cause changes to the DNA. Accordingly, sperm sorted with high intensities continuous wave lasers such as 300-500mW may not be as fertile. Another problem may include the energy that it may take to power a continuous laser to deliver about 150mW of energy at near UV spectrum. Continuous wave lasers may require 10,000 mW or perhaps even more of power. Since there may already be a large amount of electrical power required to run a continuous wave laser at 150mW, a much larger amount of power may be required to run a continuous wave laser at higher powers. Furthermore, a tube life of ion lasers may be reduced when operating at higher powers. An additional problem with the use of continuous wave lasers may be that the CV may drop significantly when using lower powers such as between about 20-80 mW.

In embodiments, the present invention provides flow cytometer designs which may incorporate the use of 2 or more flow nozzles, and even as many as dozens of flow nozzles, possibly operated by a single sorting instrument. Fields such as microfluidics, optics, electronics, and even parallel processing may be explored. In other embodiments the present invention includes the use of beam splitters to create multiple light beams. Yet, a major problem facing the development of reliable flow analysis and flow sorting in parallel may be the high intensity of laser light needed for analysis at each nozzle. This problem is particularly relevant for applications which require a very low CV in measurement of identical particles.

There is a need to provide flow systems for the analysis and sorting of particles that require a low CV value, yet may require higher laser light intensities, yet higher intensities may have negative effects on sperm and require higher power. In the search for solutions to the problems in flow systems for the analysis and sorting of particles, the field of pulsed lasers represents a possible solution.

Surprisingly, even though sperm sorted on a high speed flow cytometer may be damaged by UV light between about 300-500 mW, it is now shown in this invention that powers between about 100-500 mW may not be damaging to sperm if they are delivered in pulses. In embodiments, this may include a pulse having a peak intensity possibly as much as 1000 times higher than the intensity of a continuous wave laser. Pulsed lasers may be designed as quasi-continuous wave lasers and may have fast repetition rates such as between about 50 – 200 Megahertz and even up to 80 Megahertz. In embodiments, pulses may be between about 5-20 picoseconds. Pulsed lasers may be ideal for providing pulsed light to a stream of particles being analyzed in a flow cell or a flow cytometer. Particles analyzed in flow cytometers with event rates possibly between 10,000 – 100,000 Hertz, and even between 20,000 – 60,000 Hertz, may be illuminated from a few hundred pulses from a laser having repetition rates near 80,000 Hertz. Each pulse may provide an intense amount of energy.

There may be certain industrial uses of flow cytometers, as preparative instruments, which may be economically limited by the traditional methods of processing. It may be desirable to provide systems which facilitate parallel processing for industries such as those that rapidly process mammalian ejaculates for the production of large numbers of live sperm for insemination, those that process blood samples for the recovery of specified cells such as fetal cells, white blood cells, stem cells, hematopoetic cells from bone marrow, and the like. In an embodiment of the present invention, special forms of pulsed laser light can allow a single laser to illuminate a plurality of nozzles, perhaps even while not reducing the CV of the samples analyzed.

As a result, by the use of special forms of pulsed laser light, further improvements in the speed and sample purity can be seen. These types of lasers may be essential in the design and development of new flow cytometers perhaps having multiple sorting streams as well.

III. SUMMARY OF INVENTION

Accordingly, a broad object of the invention may provide a particle analysis system having a pulsed laser which can be operated at a low power.

Another broad object of the invention can be to provide a particle analysis system having a pulsed laser which may allow detection of smaller differences in illumination or emission to differentiate a particle characteristic.

Yet another broad object of the invention can be to provide a particle analysis system having a pulsed laser which allows differentiated particles to be separated into subpopulations having a higher incidence of the desired characteristic.

Another broad object of the invention can be to provide a particle analysis system having a pulsed laser which allows multiple particle differentiation systems to be run simultaneously using a single laser.

Another broad object of the invention can be to provide a particle analysis system having a pulsed laser which affords greater resolving power than conventional particle analysis systems using a CW laser.

Another broad object of the invention can be to provide a particle analysis system having a pulsed laser which generates from fluorochromes upon irradiation greater light intensity than conventional particle analysis systems using a CW laser.

Another broad object of the invention can be to provide a particle analysis system having a pulsed laser which allows differentiated particles to be separated into subpopulations at a greater rate than conventional particle analysis systems using a CW laser.

Another broad object of the invention can be to provide a particle analysis system having a pulsed laser which allows sperm cells of any species of mammal to be differentiated with increased resolution into X-chromosome or Y-chromosome bearing subpopulations. The benefits of this object of the invention may allow differentiation of sperm cells having: less DNA bound fluorochrome, less residence time in staining protocols, greater elapsed storage time prior to sorting, or perhaps even less affinity to stain due to having been frozen prior to staining protocols.

Another broad object of the invention can be to provide a particle analysis system having a pulsed laser which allows sperm cells to be separated into X-chromosome or Y-chromosome bearing subpopulations having higher purity or separated into X-chromosome or Y-chromosome bearing subpopulations at a greater number per second.

Yet another broad object of the invention can be to provide a miniaturized and parallel flow cytometer which allows a multiple of nozzles sorting in tandem to be positioned on the same apparatus, that may allow increases in the production rate of sorting, by increasing the number of nozzles which are sorting on a single apparatus.

In accordance with one aspect of the present invention, there is provided a method of particle separation using flow cytometry comprising the steps of: establishing a sheath fluid; flowing said sheath fluid into a nozzle; injecting irradiatable particles into said sheath fluid; forming a stream of irradiatable particles surrounded by the sheath fluid at an exit of the nozzle; subjecting said irradiatable particles in the stream to pulsed coherent radiation; detecting and evaluating an amount of fluorescence emitted from each of said irradiatable particles in response to the pulsed coherent radiation; forming a plurality of droplets entraining the irradiatable particles; charging the droplets based upon the amount of fluorescence detected from each of the entrained particles; isolating said charged droplets from said stream; deflecting said charged droplets; sorting said droplets; and collecting the sorted particles.

In accordance with a further aspect of the present invention, there is provided a flow cytometry system for sperm comprising: a sheath fluid port to introduce a sheath fluid; a sample injection element having an injection point through which irradiatable sperm cells may be introduced into said sheath fluid; a nozzle located in part below said injection point; an oscillator to which said a sheath fluid is responsive; a pulsed coherent laser; a sperm cell fluorescence detector; a processing unit connected to said sperm cell fluorescence detector; a drop charge circuit to apply an electrical condition to a stream of said irradiatable sperm cells and sheath fluid; a first and second deflection plate each disposed on opposite sides of a free fall area in which a drop forms, wherein said first and second deflection plates are oppositely charged; and a sperm cell collector.

In accordance with a further aspect of the present invention, there is provided a A flow cytometry system comprising: at least one sheath fluid port to introduce a sheath fluid; at least one sample injection element having an injection point through which an irradiatable sample may be introduced into said sheath fluid; at least two nozzles located in part below said at least one injection point; an oscillator to which said sheath fluid is responsive; a pulsed coherent radiation emitter; a particle sample cell fluorescence detector; processing unit connected to said particle sample fluorescence detector; a drop charge circuit to apply an electrical condition to a stream of said irradiatable sample and sheath fluid; a first and second deflection plate each disposed on opposite sides of a free fall area in which a drop forms, wherein said first and second deflection places are oppositely charged; and a particle sample collector.

In accordance with one aspect of the present invention, there is provided a method of particle separation using flow cytometry comprising the steps of: establishing a sheath fluid; flowing said sheath fluid into at least one nozzle; injecting irradiatable sperm cells into said sheath fluid, wherein said sperm cells include X-chromosome bearing sperm and Y-chromosome bearing sperm; forming a stream of irradiatable sperm cells surrounded by the sheath fluid at an exit of the at least one nozzle; subjecting said irradiatable sperm cells in the stream to multiple pulses of pulsed coherent radiation; determining a summated total energy of the fluorescence emitted from each of said irradiatable sperm cells in response to the pulsed coherent radiation; distinguishing between a X-chromosome bearing sperm and a Y-chromosome bearing sperm based up said summated total energy of the fluorescence emitted; detecting and evaluating an amount of fluorescence emitted from each of said irradiatable particles in response to the pulsed coherent radiation; forming a plurality of droplets entraining the irradiatable sperm cells; charging the droplets based upon the amount of fluorescence detected from each of the entrained sperm cells; isolating said charged droplets from said stream; deflecting said charged droplets; sorting said droplets; and collecting the sorted sperm cells.

In accordance with one aspect of the present invention, there is provided a flow cytometry system for sperm comprising: a sheath fluid port to introduce a sheath fluid; a sample injection element having an injection point through which irradiatable sperm cells may be introduced into said sheath fluid; a nozzle located in part below said injection point; an oscillator to which said a sheath fluid is responsive; a pulsed coherent laser; a sperm cell fluorescence detector; a processing unit connected to said sperm cell fluorescence detector for determining a summated total energy of the fluorescence emitted from each of said irradiatable sperm cells subjected to multiple pulses from the pulsed coherent laser and distinguishing between a X-chromosome bearing sperm and a Y-chromosome bearing sperm based up said summated total energy of the fluorescence emitted; a drop charge circuit to apply an electrical condition to a stream of said irradiatable sperm cells and sheath fluid; a first and second deflection plate each disposed on opposite sides of a free fall area in which a drop forms, wherein said first and second deflection plates are oppositely charged; and a sperm cell collector.

In accordance with one aspect of the present invention, there is provided A flow cytometry system comprising: at least two nozzles for producing at least two streams of irradiatable sample having X chromosome bearing sperm and irradiatable Y chromosome bearing sperm; an oscillator associated with each nozzle to which each stream is responsive; a pulsed coherent radiation emitter;a beam splitter for directing a portion of radiation emitted from the pulsed coherent radiation emitter to each stream produced from the at least two nozzles; a quantitative fluorescence detector; processing unit connected to said quantitative fluorescence detector for determining a summated total energy of the fluorescence emitted from each of said irradiatable sperm cells subjected to multiple pulses from the pulsed coherent laser and distinguishing between a X-chromosome bearing sperm and a Y-chromosome bearing sperm based up said summated total energy of the fluorescence emitted; a drop charge circuit to apply an electrical condition to a stream of said irradiatable sample; a first and second deflection plate each disposed on opposite sides of a free fall; an area in which a drop forms, wherein said first and second deflection places are oppositely charged; and a particle sample collector.

In accordance with an aspect of the present invention, there is provided a method of sperm cells separation using flow cytometry comprising the steps of:

establishing a sheath fluid;

flowing said sheath fluid into at least one nozzle;

injecting irradiatable sperm cells into said sheath fluid, wherein said sperm cells include X-chromosome bearing sperm and Y-chromosome bearing sperm;

forming a stream of irradiatable sperm cells surrounded by the sheath fluid at an exit of the at least one nozzle;

subjecting said irradiatable sperm cells in the stream to multiple pulses of pulsed coherent radiation;

detecting the fluorescence emitted from each of said irradiatable sperm in response to the pulsed coherent radiation;

determining a summated total energy of the fluorescence emitted from each of said irradiatable sperm cells in response to the pulsed coherent radiation;

distinguishing between a X-chromosome bearing sperm and a Y-chromosome bearing sperm based up said summated total energy of the fluorescence emitted;

forming a plurality of droplets entraining the irradiatable sperm cells;

charging the droplets based upon the amount of fluorescence detected from each of the entrained sperm cells;

isolating said charged droplets from said stream;

deflecting said charged droplets;

sorting said droplets; and

collecting the sorted sperm cells.

In accordance with another aspect of the present invention, there is provided a flow cytometry system for sperm comprising:

a sheath fluid port to introduce a sheath fluid;

a sample injection element having an injection point for introducing irradiatable sperm cells into said sheath fluid;

a nozzle located in part below said injection point;

an oscillator to which said a sheath fluid is responsive;

a pulsed coherent laser;

a sperm cell fluorescence detector;

a processing unit connected to said sperm cell fluorescence detector for determining a summated total energy of the fluorescence emitted from each of said irradiatable sperm cells subjected to multiple pulses from the pulsed coherent laser and distinguishing between a X-chromosome bearing sperm and a Y-chromosome bearing sperm based up said summated total energy of the fluorescence emitted;

a drop charge circuit to apply an electrical condition to a stream of said irradiatable sperm cells and sheath fluid;

a first and second deflection plate each disposed on opposite sides of a free fall area in which a drop forms, wherein said first and second deflection plates are oppositely charged; and

a sperm cell collector.

In accordance with another aspect of the present invention, there is provided a flow cytometry system comprising:

at least two nozzles for producing at least two streams of irradiatable sperm cells having X chromosome bearing sperm and irradiatable Y chromosome bearing sperm;

an oscillator associated with each nozzle to which each stream is responsive;

a pulsed coherent radiation emitter;

a beam splitter for directing a portion of radiation emitted from the pulsed coherent radiation emitter to each stream produced from the at least two nozzles;

a quantitative fluorescence detector;

processing unit connected to said quantitative fluorescence detector for determining a summated total energy of the fluorescence emitted from each of said irradiatable sperm cells subjected to multiple pulses from the pulsed coherent laser and distinguishing between a X-chromosome bearing sperm and a Y-chromosome bearing sperm based up said summated total energy of the fluorescence emitted;

a drop charge circuit to apply an electrical condition to a stream of said irradiatable sample;

a first and second deflection plate each disposed on opposite sides of a free fall;

an area in which a drop forms, wherein said first and second deflection places are oppositely charged; and

a sperm cells collector.

In accordance with another aspect of the invention, there is provided a method of sperm cells separation using flow cytometry comprising the steps of:

establishing a sheath fluid;

flowing said sheath fluid into at least one nozzle;

staining irradiatable sperm cells with a fluorochrome and injecting said stained irradiatable sperm cells into said sheath fluid, wherein said sperm cells include X-chromosome bearing sperm and Y-chromosome bearing sperm;

forming a stream of irradiatable sperm cells surrounded by the sheath fluid at an exit of the at least one nozzle;

subjecting said irradiatable sperm cells in the stream to multiple pulses of pulsed coherent radiation;

detecting the fluorescence emitted from each of said irradiatable sperm in response to the pulsed coherent radiation;

determining a summated total energy of the fluorescence emitted from each of said irradiatable sperm cells in response to the pulsed coherent radiation;

distinguishing between a X-chromosome bearing sperm and a Y-chromosome bearing sperm based on said summated total energy of the fluorescence emitted;

forming a plurality of droplets entraining the irradiatable sperm cells;

charging the droplets based upon the amount of fluorescence detected from each of the entrained sperm cells;

isolating said charged droplets from said stream;

deflecting said charged droplets;

sorting said droplets; and

collecting the sorted sperm cells.

In accordance with another aspect of the invention, there is provided a flow cytometry system for sperm comprising:

a sheath fluid port to introduce a sheath fluid;

a stain for staining irradaitable sperm cells;

a sample injection element having an injection point for introducing said stained irradiatable sperm cells into said sheath fluid;

a nozzle located in part below said injection point;

an oscillator to which said a sheath fluid is responsive;

a pulsed coherent laser;

a sperm cell fluorescence detector;

a processing unit connected to said sperm cell fluorescence detector for determining a summated total energy of the fluorescence emitted from each of said irradiatable sperm cells subjected to multiple pulses from the pulsed coherent laser and distinguishing between a X-chromosome bearing sperm and a Y-chromosome bearing sperm based on said summated total energy of the fluorescence emitted;

a drop charge circuit to apply an electrical condition to a stream of said irradiatable sperm cells and sheath fluid;

a first and second deflection plate each disposed on opposite sides of a free fall area in which a drop forms, wherein said first and second deflection plates are oppositely charged; and

a sperm cell collector.

In accordance with another aspect of the invention, there is provided a flow cytometry system comprising:

at least two nozzles for producing at least two streams of irradiatable stained sperm cells having X chromosome bearing sperm and Y chromosome bearing sperm;

an oscillator associated with each nozzle to which each stream is responsive;

a pulsed coherent radiation emitter;

a beam splitter for directing a portion of radiation emitted from the pulsed coherent radiation emitter to each stream produced from the at least two nozzles;

a quantitative fluorescence detector;

processing unit connected to said quantitative fluorescence detector for determining a summated total energy of the fluorescence emitted from each of said irradiatable sperm cells subjected to multiple pulses from the pulsed coherent laser and distinguishing between a X-chromosome bearing sperm and a Y-chromosome bearing sperm based on said summated total energy of the fluorescence emitted;

a drop charge circuit to apply an electrical condition to a stream of said irradiatable stained sperm cells;

a first and second deflection plate each disposed on opposite sides of a free fall;

an area in which a drop forms, wherein said first and second deflection places are oppositely charged; and

a sperm cells collector.

In accordance with another aspect of the invention, there is provided a method of sperm cells separation using flow cytometry comprising the steps of:

establishing a sheath fluid;

flowing said sheath fluid into at least one nozzle;

forming a stream including irradiatable sperm cells surrounded by the sheath fluid at an exit of the at least one nozzle, the irradiatable sperm cells being stained with Hoechst 33342;

subjecting said irradiatable sperm cells in the stream to multiple pulses of pulsed coherent radiation, wherein the pulsed coherent radiation is delivered at a power between 100mW and 500mW;

detecting the fluorescence emitted from each of said irradiatable sperm in response to the pulsed coherent radiation;

determining a summated total energy of the fluorescence emitted from each of said irradiatable sperm cells in response to the pulsed coherent radiation; and

distinguishing between a X-chromosome bearing sperm and a Y-chromosome bearing sperm based on said summated total energy of the fluorescence emitted.

In accordance with another aspect of the invention, there is provided a flow cytometry system for sperm comprising:

a sheath fluid port to introduce a sheath fluid;

a stain for staining irradiatable sperm cells;

a sample injection element having an injection point for introducing said stained irradiatable sperm cells into said sheath fluid;

a nozzle located in part below said injection point;

an oscillator to which said a sheath fluid is responsive;

a pulsed coherent laser, having an average power between 100mW and 500mW;

a sperm cell fluorescence detector; and

a processing unit connected to said sperm cell fluorescence detector for determining a summated total energy of the fluorescence emitted from each of said irradiatable sperm cells subjected to multiple pulses from the pulsed coherent laser and distinguishing between a X-chromosome bearing sperm and a

Y-chromosome bearing sperm based on said summated total energy of the fluorescence emitted.

In accordance with another aspect of the invention, there is provided a flow cytometry system comprising:

at least two nozzles for producing at least two streams of irradiatable stained sperm cells having X chromosome bearing sperm and Y chromosome bearing sperm;

an oscillator associated with each nozzle to which each stream is responsive;

a pulsed coherent radiation emitter having an average power between 100mW and 500mW;

a beam splitter for directing a portion of radiation emitted from the pulsed coherent radiation emitter to each stream produced from the at least two nozzles;

a quantitative fluorescence detector; and

a processing unit connected to said quantitative fluorescence detector for determining a summated total energy of the fluorescence emitted from each of said irradiatable sperm cells subjected to multiple pulses from the pulsed coherent laser and distinguishing between a X-chromosome bearing sperm and a Y-chromosome bearing sperm based on said summated total energy of the fluorescence emitted.

Naturally, further independent objects of the invention are disclosed throughout other areas of the specification.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides a block diagram of an embodiment of a particle analysis system invention which includes beam manipulators such as optical elements, splitters, filters, directional or the like.

Figure 2 provides other embodiments of beam manipulators.

Figure 3 represents a fluidically connected system according to certain embodiments of the invention.

Figure 4 is a simplified representation of a representative pulse of radiation that may used in some embodiments.

Figure 5 provides illustrations of characteristics of a pulsed illumination beam.

Figure 6 provides illustrations which may differentiate characteristics of a pulsed radiation beam from a conventional continuous wave radiation beam.

Figure 7 shows an embodiment of the invention representing a sorting process having multiple nozzles.

Figure 8 is a drawing of a flow sort embodiment of the invention.

Figure 9 provides an expanded diagram showing various embodiments of a multiple nozzle assembly.

Figure 10 depicts embodiments of certain time intervals and light energy quantities which may be derived from particular properties of pulsed light.

Figure 11 is a comparison of a pulsed laser radiation beam and a continuous wave laser radiation beam.

Figure 12 is a representation of an embodiment for a sensing routine.

Figure 13 is a representation of a comparison of aggregate data from various trial data.

Figure 14 provides histograms and bivariant plots of X-chromosome bearing and Y-chromosome bearing subpopulations of sperm cells using a flow sort embodiment of the invention which provides a 20 mW pulsed laser beam incident to the sperm cells analyzed.

Figure 15 provides histograms and bivariant plots of X-chromosome bearing and Y-chromosome bearing subpopulations of sperm cells using a flow sort embodiment of the invention which provides a 60 mW pulsed laser beam incident to the sperm cells analyzed.

Figure 16 provides histograms and bivariant plots of X-chromosome bearing and Y-chromosome bearing subpopulations of sperm cells using a flow sort embodiment of the invention which provides a 90 mW pulsed laser beam incident to the sperm cells analyzed.

Figure 17 provides histograms and bivariant plots of X-chromosome bearing and Y-chromosome bearing subpopulations of sperm cells using a flow sort embodiment of the invention which provides a 130 mW pulsed laser beam incident to the sperm cells analyzed.

Figure 18 provides histograms and bivariant plots of X-chromosome bearing and Y-chromosome bearing subpopulations of sperm cells using a flow sort embodiment of the invention which provides a 160 mW pulsed laser beam incident to the sperm cells analyzed

Figure 19 *provides histograms of sperm cells analyzed with a flow sort* embodiment of the invention compared to a histogram of sperm cells from the same sample analyzed with conventional CW flow sort technology.

V. DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As mentioned earlier, the present invention includes a variety of aspects, which may be combined in different ways. The following descriptions are provided to list elements and describe some of the embodiments of the present invention. These elements are listed with initial embodiments, however it should be understood that they may be combined in any manner and in any number to create additional embodiments. The variously described examples and preferred embodiments should not be construed to limit the present invention to only the explicitly described systems, techniques, and applications. Further, this description should further be understood to support and encompass descriptions and claims of all the various embodiments, systems, techniques, methods, devices, and applications with any number of the disclosed elements, with each element alone, and also with any and all various permutations and combinations of all elements in this or any subsequent application.

Referring primarily to Figure 1, the present invention provides, in embodiments, a radiation emitter used with particle analysis systems. In some embodiments of the invention, a radiation emitter (12) or even an intermittingly punctuated radiation element may convert electrical current into photons of radiation of a specific wavelength and may generate radiation or even laser light for analysis of non-biological or biological particles. Radiation (11) may enter a chamber or region (22) in which it may be modulated or even *modified, such as but not limited to establishing a coherent wave form. Radiation may be* modified by a beam manipulator (21) such as optical elements before it may illuminate a *particle or even a particle sample(s).*

In some embodiments, beam manipulators (21) such as optical elements may be used and may be located along a light path. Beam manipulators may include mirrors, optically reflective or even refractive mirrors, partially mirrored surfaces, deflectors, beam splitters, refractive objects, lenses, filters, prisms, lenses, or the like. Beam manipulators may modify or even modulate a pulsed laser light by focusing, condensing, de-focusing, expanding, splitting, or the like. Radiation may be split into multiple beams of equal or perhaps even unequal intensity. A radiation beam manipulator may influence a radiation beam such as by adapting a beam or changing a beam as a particular situation may be needed.

With respect to some embodiments of the invention, positional control elements (24) can provide positional control over a mirror, lens, prism, filter, or other optically relevant components. A positional control element may influence the angle of reflection or even refraction of a beam, and may even ultimately direct a final position of a pulsed laser light beam on a particle or particle sample(s). A positional control element may be a mechanical device which may move a mirror along a path, or it may even be a ratcheting or even a stepped device which can provide large numbers of predefined angles for an optical element. A positional control element(s) (24) may be used at any point in a pulsed laser and even a pulsed light assembly or apparatus to modify a beam of pulsed laser light. An oscillator may provide a constant vibration in an optical element and may define a frequency and amplitude.

In order to provide splitting of radiation into at least two light beams, the present invention may include a beam splitter. This may subject sperm cells to a reduced power of radiation than a power of radiation with was originally emitted from a laser source such as radiation emitter. Examples of reduced radiation may include splitting a radiation beam in half, in a fourth of said originally emitted power or even in an eighth of the originally emitted power, as may be represented by Figure 2. Of course, there are many options in which to reduce power and all are intended to be included within the scope of this disclosure. With a pulsed laser, for example producing a 1000mW output split into 8 equal beams of approximately 120mW, it may be possible to have 8 flow cytometry nozzles sorting together from one light source.

As to other embodiments of the invention, independent beams of pulsed laser light may be equal or may even be unequal in intensity. Split beams can be derived from a source beam of pulsed laser light by the action of optical splitters. As shown by Figure 2, a beam splitter (31) such as a prism can divide a beam of radiation into at least two beams of the same frequency and pulse rate, but may have equal or even unequal intensities. Split beams of light may have intensities which can be each less intense than, or even additively close to an equivalent to the original beam which entered a splitter or prism. A complex beam splitter or prism, which with multifaceted, three dimensional geometries can split a beam of pulsed laser light into more than one, certainly two, possibly dozens or even hundreds of independent beams of pulsed laser light.

Complex beam splitters (34) may include a three dimensional packed array of simple splitters or even prisms which can create combined three dimensional geometries of refraction and reflection and can even split a beam of radiation into more than one, certainly two, possibly dozens or even hundreds of independent beams of pulsed laser light. This may differ from a complex beam splitter in that an array of simple splitters and even prisms may allow individual geometries of each component, refractive index of each component, which may allow a much larger number of options. A rotational beam splitter (35) can be rotated on an axis, potentially at very high speeds, such that an extremely large number of pulsed laser light beams may be created to the point where one can no longer speak of actual beams, but rather of each pulse moving in an independent direction, slightly different that the prior pulse. Beam splitters or optical splitters may be used at any point along beam of pulsed laser light.

Another example of a beam manipulator (21) may include filters can be placed in a beam path to modulate or modify a property of a pulsed laser light, and may even reduce the net energy of a particular beam. A large number of filters may be used in series or even in parallel across many different beams of pulsed laser light.

A radiation emitter (12) or even a intermittingly punctuated radiation emitter may be embedded in an integrated fashion into a particle analysis or particle separation system. Alternately, with respect to some embodiments of the invention, a pulsed laser source may be independent and splitters, as described above, may be used to split an original beam of light to provide illumination light to numerous independently operating particle analysis components.

It is understood that a fundamental unit of illumination may be one single pulse of a laser light, and that each pulse or laser light may be split numerous times. Through splitting, filtration or even both, a net energy of any given pulse illuminating a biological object or sample may be perhaps as small as a single photon of light or perhaps as large as an original pulse energy emitted from a laser unit.

It is also understood that the use of splitters, which can divide laser light beams into two or more beams, may increase the number of light beams that can illuminate particles, such as biological objects. In embodiments, a number of pulsed light beams per second that can be directed toward particles can be multiplied with a splitter. It is also understood that the pulses per second may be altered to a desired number of pulses per second, timing of the pulse, and even position the pulse. A pulsed light may be distributed by an apparatus to possibly millions of particles or particle sample(s) which may be located in different positions. Through the use of harmonically synchronized oscillations, rotations, and even geometries, many pulses per second may be delivered to the same particle, sample, or even biological objects. It is particularly understood that, in embodiments, systems may be established with recurring illumination events.

An embodiment of the present invention may include a system fluidicly connected system, such as a flow cytometer system, as seen in figure 3. This may be representative of a multiple number of flow cytometry systems that are linked as one system.

Radiation emitters and even intermittingly punctuated radiation emitters, as described in more detail below, may provide one, two, three or perhaps even more radiation beams having specific frequencies, wavelengths, intensities, and even watts to illuminate the type of particles to be analyzed. An intermittingly punctuated radiation emitter may multiply subject radiation to, for example, sperm cells for a first amount of time and may multiply terminate radiation of sperm cells for a second amount of time (41). This may be represented by Figure 4.

In embodiments, a first amount of time (40) may include an amount of time radiation occurs and this time may be between about 5 to about 20 picoseconds. A second amount of time (41) may be a radiation off time and may be between about 0.5 to about 20 nanoseconds. Of course, other amounts of time for each of a first amount and a second amount of time may be used and all are understood to be included within the scope of this invention. A cycle of a first and second time may be understood as a repetition (42). Each repetition may include a time of about 2 to about 10 microseconds, yet the repetition may vary. In embodiments, a repetition rate may include between about 50 to about 200 MHz and may even include a rate up to about 80 MHz. Other repetition rates are possible and all are meant to be included within the scope of this disclosure. In embodiments, a radiation emitter may be a Nd:YAG, Nd:YVO_4, or the like radiation emitters.

Figure 4 shows parameters taken into consideration when discussing the differences between continuous wave (CW) lasers or even gas and pulsed lasers or even solid state lasers. As shown by Figure 4, an intermittingly punctuated radiation emitter (56) may emit radiation that may not be continuous, yet be in short regular pulses which may have a duration time or a first amount of time (40). Following a pulse, there may be a dark period or a second amount of time (41) in which no light may be generated. The total elapsed time between two pulses, a repetition rate, may therefore be the duration time in addition to the dark period. A pulse line width and dark period may be similar in length, and it could be postulated that a laser may actually be illuminating a sample for somewhat less than "half of the time". Alternatively, a pulse line width may be much smaller than a dark period, and thus a sample may be illuminated for only a very small fraction of the time.

Peak energy (36) and a full amount of energy or joules delivered from one pulse of laser light is represented in Figure 5. Fractional amounts (37) of that energy can be split as described above or put through a neutral density filter. Importantly, one may diminish the amount of light in one pulse used to illuminate a particle by dividing or filtering the beam. For example, a 350mW beam can be split into 10 equal beams of approximately 35mW to run 10 independent analysis machines from a single source laser. In practice, the quality of analysis at 35mW must afford information regarding the characteristics of the particle illuminated, and for commercial applications perhaps afford at least the same amount of information as when particles illuminated with a CW UV laser running at the standard of 150mW. Figure 5 may further help in an understanding of the block diagram represented in figure 4. Each radiation pulse may be reduced in energy as previously discussed.

An energy density or even watts may be needed to achieve maximum light emission (38) from a particle upon illumination as shown by Figure 6. It has been contemplated that light input of a continuous wave laser, however, may be so low that a particle may never be fully saturated with illumination (43). An emission light (44) from particles illuminated by a CW laser at a given energy intensity may be constant, as the source light may be constantly refreshing the particle to a certain partial saturation value. By comparison, emission light (39) from the same particle which has been illuminated with pulses may be greater than from a CW laser. Pulses may be short and may have illumination light intensity many orders of magnitude higher than the illumination level of a CW laser. It has also been speculated that a fate of light emission from a particle or particle sample(s) during the dark period may be dependent upon the half-life of emission for the illuminated particle and may even be dependent upon the length of time of the dark period. In the case where a half life may be as long or longer than the dark period, the emission could remain close to maximum during the entire time across all pulses delivered to the sample.

It should also be pointed out that if instead of reducing the input energy by splitting or filtering, one instead uses movement of mirrors and reflectors, one may reduce the number of pulses delivered to a biological sample to as low as one pulse, while retaining the very strong intensity of the pulse. Thus, it is a unique aspect of the invention to provide movement of the full strength pulsed laser beam across a plurality of particles which may for example be entrained in a fluid stream which passes through a flow cytometry nozzle or located on an array or matrix (such as a DNA or protein microarray), or a combination of both, as would be the case of a single laser being oscillated so that it illuminates a small number of flow cytometry nozzles in close proximity.

Now referring primarily to Figure 8, irradiatable sperm cells may be introduced through a sperm sample injection element (4) which may act to supply irradiatable sperm cells for flow cytometry analysis. Irradiatable sperm cells may be deposited within a nozzle (5) in a manner such that the particles or cells are introduced into a sheath fluid (3). A nozzle may be located in part below an injection point of sperm cells. A sheath fluid (3) may be supplied by a sheath fluid source (46) through a sheath fluid port (2) so that irradiatable sperm cells and sheath fluid may be concurrently fed through a nozzle (5). Accordingly, the present invention may provide establishing a sheath fluid and flowing a sheath fluid into a nozzle, and injecting irradiatable sperm cells into a sheath fluid as shown in Figure 8.

Further, in embodiments, the present invention may include subjecting irradiatable sperm cells radiation. Radiation may be produced from a radiation emitter (12) as discussed previously. In embodiments the radiation emitter may be a intermittingly punctuated radiation emitter or may even be a continuous wave laser.

In embodiments, the present invention may include multiply subjecting sperm cells to radiation having a wavelength appropriate to activate fluorescence in a sperm cell. The invention may include a fluorescence activation wavelength. Such wavelength may include about 355 nm. Of course, this may include any wavelength that may be needed to activate fluorescence. Such other wavelengths may include 350 nm, 360 nm and other wavelengths and all are meant to be included within the scope of this disclosure.

In embodiments, the present invention may include sufficiently hitting a sperm with radiation to cause an irradiatable sperm to emit fluorescence. This may include providing radiation at certain wavelength, power, energy and the like that is enough to cause an irradiatable sperm to emit fluorescence.

In embodiments, the present invention may provide for exciting irradiatable sperm cells that have been subjected to radiation. When in an excited state, the cells may emit fluorescence. In embodiments, irradiatable sperm cells may be multiply excited with radiation. This may include radiation that is emitted from a intermittingly punctuated radiation emitter.

A pulse of laser light may illuminate particles or even a particle sample(s) at a specific location with an EMR frequency or Hertz, timing such as a clock, intensity or even watts, and even net energy or joules. The particles may absorb the pulsed light, may get excited and may even emit light of the same frequency as that of the pulsed laser light, such as a scatter or may even emit a light of a difference frequency or even fluorescence. The exact nature of the amount of energy absorbed by a particle may be related to the chemical properties of the particle, the chemical properties of any objects attached to or closely associated with the particles, the physical chemical properties of the particle environment , such as biological segregations including membranes, organelles, solutes, pH, temperature, osmolality, colloidal character, or the like, and may even be related to the frequency and intensity of the laser light illuminating the particle. An EMR light emission from a particle, characterized by a wavelength and quantity, can provide highly accurate information about the status of a particle when a pulse of illuminating light is incident. Depending on the nature of the particle and the particle environment, the particle may then emit a florescent light signal, and may do so over a certain period of time defined by a half-life of emission. Typically, a number of pulses of laser light can illuminate a particle or particle sample in a specified period of time, and there can be a corresponding dynamically changing emission of light over the same period, or a time period after illumination.

Emitted fluorescence from each of the sperm cells may be detected with a detection system (23). A detection system may include a fluorescence detector (7) which may be connected to a processing unit (15). While processing the emitted fluorescence, the present invention may include evaluating the emitted signals. Evaluation of emitted fluorescence may include how much fluorescence may be emitted possibly by comparison between the cells or may even possibly be compared to a predetermined number. The present invention may include, in embodiments, selecting an electrical condition to be associated with each of the sperm cells in a sheath fluid flow. An electrical condition may be a charge, voltage or any electrical condition. A drop charge circuit (8) may charge a stream of cells and sheath fluid based upon deduced properties of each of the excited cells. For example, this may be to charge all of the X-chromosome bearing sperm cells with a positive charge, and charging all of the Y-chromosome bearing sperm cells with a negative charge. Of course, while the disclosure focuses primarily upon sperm cells, other particles may be analyzed as discussed in the various embodiments disclosed.

A charged drop may be formed and isolated in a free fall area. A drop may be based upon whether a desired cell does or does not exist within that drop. In this manner the detection system may act to permit a first and second deflection plates (18) to deflect drops based on whether or not they contain the appropriate cell or other item. The deflection plates may be disposed on opposite sides of a free fall area in which a drop may form and the deflection plates may be oppositely charged. As a result, a flow cytometer may act to sort cells by causing them to land in one or more collectors. Accordingly, by sensing some property of the cells or other items, a flow cytometer can discriminate between cells based on a particular characteristic and place them in the appropriate collector. In some embodiments, X-bearing sperm droplets are charged positively and deflected in one direction, and Y-bearing sperm droplets are charged negatively and deflected the other way. A wasted stream which may be unsortable cells may be uncharged and may be collected in collector, an undeflected stream into a suction tube or the like.

In this manner, a sheath fluid may form a sheath fluid environment for the sperm cells to be analyzed. Since the various fluids are provided to the flow cytometer at some pressure, they may flow out of nozzle (5) and exit through a nozzle orifice (47). By providing some type of oscillator (6) which may be very precisely controlled through an oscillator control (45), pressure waves may be established within the nozzle and transmitted to the fluids exiting the nozzle at nozzle orifice. Since an oscillator may act upon the sheath fluid, a stream (19) exiting the nozzle orifice (47) can eventually and regularly forms drops (9). Because the particles or cells are surrounded by the fluid stream or sheath fluid environment, the drops (9) may entrain within them individually isolated particles or cells, such as sperm cells with respect to some embodiments of the invention.

In other embodiments, since the drops can entrain particles or cells, the flow cytometer can be used to separate particles, cells, sperm cells or the like based upon particle or cell characteristics. This is accomplished through a particle or cell detection system (23). The particle or cell detection system involves at least some type of detector (7) which responds to the particles or cells contained within fluid stream. The particle or cell sensing system may cause an action depending upon the relative presence or relative absence of a characteristic, such as fluorochrome bound to the particle or cell or the DNA within the cell that may be excited by an irradiation source such as a radiation emitter (12) generating an irradiation beam to which the particle can be responsive. While each type of particle, cell, or the nuclear DNA of sperm cells may be stained with at least one type of fluorochrome different amounts of fluorochrome bind to each individual particle or cell based on the number of binding sites available to the particular type of fluorochrome used. With respect to spermatozoa, the availability of binding sites for Hoechst 33342 stain is dependant upon the amount of DNA contained within each spermatozoa. Because X-chromosome bearing spermatozoa contain more DNA than Y-chromosome bearing spermatozoa, the X-chromosome bearing spermatozoa can bind a greater amount of fluorochrome than Y-chromosome bearing spermatozoa. Thus, by measuring the fluorescence emitted by the bound fluorochrome upon excitation, it is possible to differentiate between X-bearing spermatozoa and Y-bearing spermatozoa.

As a result, the flow cytometer acts to separate the particle or cells by causing them to be directed to one or more collection containers. For example, when the analyzer differentiates sperm cells based upon a sperm cell characteristic, the droplets entraining X-chromosome bearing spermatozoa can be charged positively and thus deflect in one direction, while the droplets entraining Y-chromosome bearing spermatozoa can be charged negatively and thus deflect the other way, and the wasted stream (that is droplets that do not entrain a particle or cell or entrain undesired or unsortable cells) can be left uncharged and thus is collected in an undeflected stream into a suction tube or the like as discussed in United States Patent No. 6,149,867 to Seidel. Naturally, numerous deflection trajectories can be established and collected simultaneously.

Irradiatable sample cells may include a sample cell that is capable of emitting rays of light upon illumination. This may or may not include having stain molecules attached to a sample particle. Some particles may have features that allow them to emit fluorescence naturally without having to add a stain to them.

Laser light incident upon the particle(s) being analyzed may generate at least one, or perhaps even two, three or more beams of scattered light or emitted light having specific frequencies, wavelengths, intensities and perhaps even watts. All or a portion of the scattered light or even emitted light may be captured by a detector. A detector may include a photomultiplier tube or the like detectors.

A detection system may be used to detecting an amount of emitted fluorescence from each of the sperm cells in a flow cytometry system. A detection system or even a sperm cell fluorescence detector may include a photomultipler tube. In other embodiments a detection system may include an optical lens assembly, a photomultipler tube and even some sort of analysis system such as a computer. An optical lens assembly may collect emitted fluorescence and transport a collected signal to a photomultipler tube. The signal detected by a photomultipler tube may then be analyzed by a computer or the like devices.

A single or perhaps even a multiple digital or analog detector(s) may receive all or even a portion of the scattered light or emission light. Analog or digital processor(s) may convert the signals detected by the detector(s) into analog current or even digital information. The information may accurately represent the identity, frequency, quantity, and even joules of light or EMR received by the detector.

In embodiments, a detector may generate a current or digital signal corresponding to the amount of light quanta hitting the detector. Certain embodiments of the invention, can provide a one dimensional detector which summates all light of the specified wavelength incident upon the detector surface during a specified period of time. The duration or even a total time of detection may be as simple as a fully additive collection or even integration over an entire analysis time of the sample, or it may be a dynamic data set which may record an emission of light from a biological object(s) or sample(s) over a time period. That time period may be as short as the time between two pulses of the pulsed laser light, or it may be as long as many millions of pulses. It is understood that such a detector may become two dimensional when the second dimension of time is considered.

In other embodiments, a two or three dimensional detector may comprise a flat panel, a three dimensional matrix of unit cells or even pixels which can detect an emission light once or more times and can record or report the interaction specific to that individual unit cell. The information relevant to the operator of an apparatus may be a summation or display of the results of many unit cells. This type of detector may include, but is not limited to, photographic film, photographic paper, or even a microchip capable of sending data for imaging on a television screen or computer screen. It is understood that a two dimensional detector may become three dimensional, and three dimensional detector may become dimensional when the additional dimension of time may be considered.

A signal generated by a detector can be processed to provide simple outcomes such as photos. A signal generated by a detector may even be able to allow analysis of many thousands or even millions of biological objects in real time with computer graphics which can give representations to allow a user to modulate or modify an analysis process in real time. In other embodiments, it may be desirable to sort with a magnetic detection.

In embodiments, the present invention may include quantitatively detecting an amount of emitted fluorescence from each of the sperm cells. The quantity of the emitted fluorescence may be detected with a sperm cell fluorescence quantitative detector. Of course this may include other samples. In sperm cells, a X chromosome bearing sperm and a Y chromosome bearing sperm may be distinguished because an X chromosome bearing sperm may emit a different amount of fluorescence than a Y chromosome bearing sperm. In other embodiments, if using other samples besides sperm cells, the present invention may provide distinguishing between a first population of particles and a second population of particles due to a difference in an amount of fluorescence emitted from each population of particles. A distinguishing analysis may be calculated with a detector.

Operating system controlled computer(s) or even graphic user interface controlled computers may use data from an analog or even digital processor(s). A computer may facilitate direct feedback control of a laser and even analytical equipment. A computer may even provide data to support a workstation which may give an operator(s) of an analytical or separation equipment images that may relate to a behavior of the system. This may allow control of the behavior of the system for optimal analysis, quantification, and even separation of a biological object(s) or even a sample(s).

Auxiliary computational, command equipment or even control equipment may provide local network control of analysis, quantification and separation apparatus. Control equipment may communicate in local area networks (LAN) or even wide area networks (WAN) to provide local or perhaps even distant operators capability to initiate, monitor, control, troubleshoot, download data or even instructions, upload data or instructions, terminate, and the like. Control equipment may allow operation of one, two, three or even more apparatus.

Computational subcomponents may correspond to a command and even a control which may be integrated into a pulsed laser design and construction. In some embodiments, computational subcomponents may be independent or even integrated parts of an apparatus and may reside outside a housing of a pulsed laser.

In embodiments, the present invention may provide staining a sperm cell with a fluorescent dye. A stained sperm cell (or in other embodiments, a stained sample) may be stained with fluorochrome and in yet other embodiments, may be stained with Hoechst bisbenzimide H33342 fluorochrome.

In some instances, a large amount of dye may be used to stain a sperm cell. Sperm cells contain deoxyribonucleic acid and deoxyribonucleic acid may have many binding sites that stain (dye) molecules may bind with. Due to the nature of sperm cells, a stained sperm cell may have many molecules of a dye attached to each binding site of a sperm.

In embodiments, the present invention may include staining a sperm sample for a reduced staining time. The staining time may vary due to the type of stain used and even due to the type of sample used, here sperm. Typically, staining sperm with Hoechst 33324 may take about 40 minutes. under other constant conditions. Some examples of a reduced staining time may include the following:

- less than about 40 minutes.
- less than about 35 minutes;
- less than about 30 minutes;
- less than about 25 minutes;
- less than about 20 minutes;
- less than about 15 minutes;
- less than about 10 minutes; and
- less than about 5 minutes.

Of course, other stain times are certainly possible and all should be understood as represented within the scope of this invention.

In embodiments, the present invention provides distinguishing between a X chromosome bearing sperm and a Y chromosome bearing sperm in a flow cytometer system. A X chromosome bearing sperm may emit a different fluorescence from said Y chromosome. For example, a X chromosome bearing sperm may contain more DNA than a Y chromosome bearing sperm, thus a X chromosome bearing sperm may bind to more dye. When illuminated, a X chromosome bearing sperm may emit a greater fluorescence than a Y chromosome bearing sperm. The difference may provide the ability to distinguish the two chromosome bearing sperms.

The present invention may include, in embodiments, minimally staining sperm cells with a fluorescent dye. A minimum sperm stain may include allowing less stain to bind to each sperm. For example, it may take a certain amount of stain to complete attach stain molecules to each binding site of a sperm cell. It may be an efficient option if less amount of stain could be used while maintaining the ability to achieve a desired result, such as the ability to distinguish between two different cells after radiation. In embodiments, the present invention may include providing a percentage of stain. While a percentage of stain may be as low as 10%, other examples may include about 90%, about 80%, about 70% and about 60%. All stain percentages are understood as included within the scope of this disclosure.

A benefit with respect to sorting sperm cells using a pulsed laser can be a reduction in the amount of stain taken up by sperm cells during staining. Because stains or dyes, such as Hoechst 33342, bind with DNA within sperm cells, stain has been considered a factor detrimental to the viability or fertility of sperm cells. Using a pulsed laser flow sort invention, the amount of stain taken up by sperm cells during staining can be reduced by 20% over the amounts used with conventional CW laser cell sorting technology with similar or better resolution of X-chromosome bearing and Y-chromosome bearing subpopulations. In certain sperm cell samples the amount of stain taken up by the sperm cells can be reduced to as little as 60% of the amount used with the same cells sorted by conventional CW cell sorting technology.

Any kind of sample or particle may be used in a flow cytometry system. A sample may include usable cells, reproductive cells, haploid cells, sperm cells, delicate sample, non-biological particles, biological particles, or any kind of cell that can be used with a flow cytometer system. A useable cell may be a cell that can be used for further processing or analysis after completion through a cytometry system. Specifically, in embodiments, this may include providing a viable cell. Reproductive cells may include cells that can be used to reproduce an organism or even a mammal and the like. Haploid cells may include those cells that have a single set of chromosomes, such as sperm cells. A delicate sample may include a sample that is fragile or may even be easily damaged such as reduction in viability. A delicate sample may have increased sensitivity to certain environments such as the type of stain, the sorting process and other environments.

Particles can be non-biological particles such as plastic beads, biological particles such as diploid cells, haploid sperm cells, or the like. It is to be understood that particles are not limited to cells or beads but can also include other non-biological particles, biological particles, and the like. Particles may include, without limitation: the individual binding sites or attachment sites of a molecule on the surface of a cell or other molecule; large molecules (possibly whether on the surface of a cell or within a cell) such as proteins, single stranded DNA, double stranded DNA, mRNA, tRNA, DNA-RNA duplexes, combined protein nucleic acid structures such as a ribosomes, telomerases or the like, DNA or RNA polymerases, samino acid synthetase; the active site of an enzyme such as luciferase, peroxidase, dehydrogenase or even cytochrome oxidase which may require cofactors such as ATP, NADH or NADPH; free or bound hydride (H-); or even any structure biological or non-biological that can be entrained in a fluid stream and made incident to an illumination beam to generate scattered light or even emission light.

In another embodiment that may contribute to efficiency in a flow cytometry system, the present invention may include subjecting sperm cells to low power radiation. While the range of power that may be used with a flow cytometry system may vary, some possibilities for low power may include:

- less than 300 milliwatt;
- less than 350 milliwatt;
- less than 200 milliwatt;
- less than 175 milliwatt;
- less than 100 milliwatt;
- less than 88 milliwatt;
- less than 50 milliwatt; and
- less than 25 milliwatt.

Again, other powers of radiation are certainly possible and all should be understood as represented within the scope of this invention.

In some examples, a Vanguard Laser may be used. The Vanguard Laser is manufactured by Spectra-Physics and can emit 80 million pulses per second (80 MHz). LaserForefront, Spectra-Physics, No. 30 (2001). Each pulse may have line width of about 12 picoseconds, and a repetition rate of about 10 nanoseconds. This may mean that to an approximation, during a single repetition of 10 nanoseconds, the pulsed laser may illuminate a target for about 12 of 10,000 picoseconds or about 0.12% of the total time. In other words, a sample being illuminated by a pulsed laser may be spending approximately 99.88% of the time in the dark. This may also mean that since a pulsed laser may be delivering 350 milliwatts (mW) of total power, during the short 12 picosecond pulse, an average of 280 Watts may be delivered to a particle. This may be 800 times more intense than a light from a continuous wave (CW) laser running at 350mW. Since reliable sperm sorting can be performed at 150 mW on a standard CW UV laser, which may represent a factor of 653 fold, it could be hypothesized that it may be possible to run a pulsed laser at as low as 150/650 or 0.23 mW and still have light intense enough to illuminate a sperm.

In embodiments, the present invention may include utilizing at least one shared resource to process sperm cells. This may help in efficiency of sperm sorting in flow cytometry systems. A shared resource may include a computer system, a sheath fluid, an integrated multiple nozzle device, and the like. In embodiments, a shared resource may include utilizing one radiation source. Radiation may be split into at least two beams and each beam may be directed toward an nozzle and the sample being sorted. In embodiments, the present invention may include one radiation emitter and a beam splitter or may even include one intermittingly punctuated radiation emitter and a beam splitter. A beam splitter may be any kind of beam splitter as previously discussed.

As discussed above, the use of refractive, or semi-reflective splitters provides multiple beams of pulsed laser light derived from the original source light. These beams may have diminished intensity from the original beam, but may be able to each be used to analyze separate particles or particle sample(s). Also discussed above, each beam may be dedicated to an independent analytical or analytical/separation device (for example, but not limited to, a sperm sorting flow cytometer or cell sorter). In some embodiments of the invention, each light beam corresponding to an independent instrument can be split into two light beams of equal intensity and one light beam made incident upon the particle to be analyzed, and the other light beam can provide a reference beam. By comparing the two beams, the absorption of source light by the particle may be measured. Another unique and important attribute of using a single pulsed laser to supply light to dozens or hundreds or thousands of independent analysis or separating machines may be that the entire complex of instruments served by the single light will be using the same light, and to the extent that all machines are performing identical or highly similar activities, it is possible to use the data from all machines as internal references and standards to each other, and by using computers or both which can give local (LAN) as well as distant (WAN) access to the data, to allow operators or persons at a distance to monitor the performance of each machine in real time.

While multiple nozzles may be integrated into one device, separate flow cytometers having only one nozzle may be lined up so that radiation may be directed to or even split between each nozzle.

In embodiments, the present invention may include flowing at least one sheath fluid and sperm cells into at least two nozzles. By multiplying the number of nozzles operating on a single flow cytometer, the amount (number of particles) analyzed and sorted per unit time may be increased. In the case where the operation of the flow cytometer may be in a production setting representing a saleable product, multiple nozzle may increase the number of units produced in a single shift by a single operator, and thus a reduction in the costs of each unit produced.

By operating a number of nozzles on the same device, a controlling instrumentation used on the flow cytometer and operators of the flow cytometer may use statistical analysis of the performance (operation data) of a multiple of nozzles and may use this data for feedback control of single nozzles within the population of the nozzles being used. By operating a number of nozzles on the same device, a single light source providing a multiple of beams (one or more for each nozzle) may reside on the same mounting as the nozzles and thereby reduce the complexities of light paths related to nozzles running on individual mountings, which may be independent of the mounting of the primary illumination source. By operating a number of nozzles on the same device illuminated by multiple beams from a single light source providing the capital, operating, parts, service, and maintenance costs from a single laser may be distributed across a multiple of productive sorting nozzles, and, therefore, reduce costs per unit produced which are allocated to the laser operation.

Figure 7 shows multiple nozzles (5) which can provide charged drops (9). The multiple nozzles and collector (20) may be arranged so that a number of selected containers may be less than a number of nozzles. Selected containers may include containers having collected one specific type of cell, such as all the X chromosome bearing sperm cells. In this figure, the sorted X chromosome sperm cells may be represented by the containers (32). Here there are three selected containers of a selected cell that have been sorted from four nozzles.

In other embodiments, the present invention provides utilizing collected sorted sperm for insemination of female mammals and may even provide for a mammal produced through use of a sorted sperm cells produced with a flow cytometer system according to any of the embodiments as presented in this disclosure.

In other embodiments, the present invention may include individually controlling or even compositely controlling at least two nozzles. Each nozzle may individually adjusted according to a desired function with an individual nozzle control, or a plurality of nozzles may be adjusted compositely with a single nozzle control device that may be connected to each of the nozzles.

Another way to increase efficiency in a flow cytometry system, the present invention includes rapidly sorting said sperm cells. This may be achieved with a rapid sperm sorter or even a rapid particle sample sorter. Sperm may be sorted at any rate. Such possibilities for a sort rate may include:

- greater than 500 cells per second.
- greater than 1000 cells per second;
- greater than 1500 cells per second;
- greater than 2000 cells per second; and
- greater than 3000 cells per second.

Other sort rates are certainly possible and all should be understood as represented within the scope of this invention.

In embodiments, the present invention may include a particle sample collector such as a sperm cell collector. A collector may be multiple containers, a combined collector having individual container, or any type of collector. For example and as shown in figure 9, a combined collector (63) may include a collector for one type of particle (32), such as X chromosome bearing sperm populations, a container for a second type of particle (33) such as Y chromosome bearing sperm populations, and may even have a third container (64) to collect those drops which may not have been charged.

It may be important in designing illumination beams to consider that the closer the illumination source (laser) may be to an analysis point, the less effect any form of movement such as vibrations may have on the path of the beam. It may be desirable to provide an system which reduces the distance between and location of all nozzles to within a very small distance of each other (for example all within 15 cm), and greatly simplifies and enables the use of multiple beams from a single laser light source.

Figure 9 shows an exploded diagram of components of a flow cytometry system combined into a parallel construction where a multiple of nozzles may be operated on a single apparatus. Although the diagram depicts six nozzles, it is exemplary, such that it might as easily have only 2 or 3 nozzles, or may have as many as 8 or 10 or 12 or even 24 nozzles side by side.

A multiple of incoming laser beams or radiation (11), which in most embodiments could be equal beams derived by splitting from an original source beam located close to the nozzles, shines onto an analysis point which is defined by the intersection of the beam or radiation (11) with a narrow stream of fluid which emits from the nozzle (5). In some embodiments, the analysis point may be at the focal point (60) of a reflective parabolic dish (61) which may reflect emitted light (62) up to the detection surface (58). Unabsorbed laser light which may not be absorbed may be absorbed by a heat sink, or it may be measured by an additional detector to determine an exact real time intensity of the beam. Each nozzle may be equipped with an oscillator (6) which may provide a force causing the stream emitting from the nozzle tip or orifice (47) to break into droplets at defined frequencies such as in the 10,000 - 100,000 Hz range. Droplets may be charged, and by action of a magnetic field may be separated. In the case of sorting live mammalian sperm for the presence of X or Y chromosomes, there can be 3 streams of droplets: a stream containing primarily X chromosome bearing sperm, which may by example be collected in one container (32) on one side of a collector (20), a stream containing primarily Y chromosome bearing sperm, which may by example be collected in another container (33) on the other side of a collector (20), and a stream containing sperm which may be dead and which may be collected a third container (64) in the middle of a collector (20). In other embodiments, features such as a detection surface (58), parabolic dishes (61), collectors (20) and in some embodiments nozzles (5) and oscillator(s) (6), may be fabricated into single parts or group subassemblies, which may be sandwiched together to manifest the actual sorting nozzle architecture.

In other embodiments of the present invention, detection surfaces may have diameters (57) similar to the diameter of microtiter plate wells, which can be about 5-8 millimeters, and can have distances (59) between two neighboring flow cytometry nozzle tips which are equal to the distance between two wells, which may be about 12-18 mm.

Now, referring primarily to figure 10, certain time intervals and light energy quantities which may be derived from the particular properties of light provided by a pulsed laser are shown. The standard lasers used in most flow cytometry and particularly in sperm sorting have been ion tube continuous wave (CW) lasers which emit a fairly constant light flux, pulsed lasers may deliver the same rate of net light. For example, as watts is defined as joules per second, we may consider the period of 1 second. It may be exemplary that for many applications in flow cytometry, as many as 10,000-100,000 individual events may be analyzed in 1 second, so that each event requires illumination energies of approx 1/10,000-1/100,000 joules.

In contrast, the pulsed laser may emit the same net light in regular pulses. In figure 10, which represents an arbitrary time axis, each pulse of laser light (68) can emit a certain energy, and have a certain illumination pulse duration. When a pulse may illuminate a particle, a fluorescent emission pulse may occur from that particle which can be represented by an emission curve (65). An emission curve can represents a classic exponential decay function where maximum emission is at the start and the rate of emission (decay) is along a co?esponding half life curve. Based on some definition of final decay, for example to the point where emission is 1/1000 of original emission, or about 10 half lives, an emission pulse duration (67) can be established. There may also be a period commencing from the final decay point occu?ing at the end of the time summated by illumination pulse duration and emission pulse duration (67) and the beginning of the next illumination pulse, which can be defined as the resting period. The total sum of these periods may the period between pulses which may be the interpulse period and is typically the inverse of the frequency of the laser.

Using a detection surface, it may be possible to analyze the light output emitted from a particle emission pulse and specifically measuring the summated total of energy from the emission pulse, which may be an integration of the area (66) under the decay curve (65). This measurement (70) may be stored as an analog electrical charge in a charge storage device such as an appropriate capacitor, of it may be converted to a digital value (70) which can be stored in a digital memory device. Given that the emission pulse occurs as a dynamic emission event, which through a photodiode/amplifier system may be translated in realtime to an electrical current (or voltage differential), measurements of current or voltage at multiple points during the particle emission may allow the derivative of the decay curve to be determined (71). These can be useful values in statistical analysis of multiple identical illumination events of the same particle.

In flow cytometry, which is a broad field in which the instant invention may be used, particles which are being illuminated by a laser are commonly flowing in a fluid stream or a flow cell past a fixed point upon which a laser beam is focused. In figure 11, a rate of flow of particles past the point of illumination can be a function of the volume flux of the stream, and the concentration of the particles. An illumination period (72) of time in which the particle is being illuminated may be determined by the flow rate and the size of the particle in the direction of the flow. When the particle may be illuminated by a pulsed laser generating individual emission pulses (73) which can occur in interpulse periods much less than the illumination period (72) of the particle, then a large number of individual emission pulses may be derived from the particle (74). In contrast, when a particle may be illuminated during the same period by a continuous wave (CW) laser, there may be a long emission over the period (75), which can commonly be detected as a peak profile of current over the period. A measurement of the emission from particles illuminated by CW lasers are a single long analog events without natural internal cut points and so either the entire value may be integrated, or the event may be sampled at a discreet multiple of times, or segments of the event are integrated.

In other embodiments, lasers may be used where the illumination pulse duration may be much smaller than an interpulse period which may itself be much shorter than the particle illumination period (72). For example, when the Vanguard Laser is used for the sorting of sperm at approximately 25,000 events per second, the laser which has

80,000,000 illumination pulses per second will deliver approximately 3000 pulses per event, and about 5-10% or 150-300 pulses occur in the particle illumination period (72). Also, the pulse duration is about 10 picoseconds (10-1 1 sec), the interpulse period is about 10 nanoseconds (10-8 seconds), and the pulse emission period is less than 1 nanosecond.

As may be understood from figure 12, an illumination pulse may initiating a sensing routine. The instant invention may use a laser pulses as an internal clock to the entire analysis system. Advantages are that each illumination pulse, which is very brief and very strong, can be used to initiate each clock cycle. Within a single clock cycle, a computational subroutine may run which uses the resting period to calculate specific values for each illumination/emission event, and cache the result prior to the initiation of the next clock cycle. An analysis of individual particles could be manifested over a multiple of clock cycles (for example 150-300), such that statistical analysis of all emission events mapping to a single particle may occur, and averaged values related to the measurement of the quantity particle and the position of the particle may be cached. The period between a multiple of events, which may be dominated by periods without emissions, may be used to map the identity and distances and using the momentary gating criteria to effect the sort. Values of operating parameters and results within each sort may be cached for viewing at the 1 minute level, possibly operator status, and graphic or summations for entire sort runs may be generated. In figure 12, using the example of sperm being sorted using a Vanguard Laser, the clock cycle may be about 10-8 seconds. Each clock cycle encompasses three periods. The illumination pulse of 10-11 seconds, the emission period of 10-9 seconds, and the resting period of 9 X 10-9 seconds. Each clock cycle (77) occurs approximately 300 times in each analysis event of 3 X 10-6 seconds. Time between analysis events averages 2 X 10-5 seconds. The time between each analysis and the sort (79) is approximately 5 X 10-2 seconds. Operators will usually want to observe net historical data over the most recent minute (80) and be able to view the progress/history of data over the entire sample from start of sort (81).

There may be many hierarchical layers of data occuring dynamically. At the same time, with a number of nozzles all sorting the same sample at the same time, there are simultaneous events occurring in each nozzle at each hierarchical layer. As it would be labor intensive and inefficient for the operator to control each nozzle, the statistical analysis and algorithmic mapping should allow the operator to view status, history, and averages of all nozzles in aggregate and note only nozzles which are not functioning near the mean of the group. The operator also needs to use commands to change the sorting, which should effect all nozzles at one time.

The data may also be shared between control functions across multiple nozzles and over time to allow the system to make automatic adjustments such as: adjusting optical mirror positions to assure equal laser light in each beam; tracking the performance of each nozzle to make early identification of nozzle occlusion events; tracking the performance of each nozzle to identify differential flow rates and even comparing one or more semen samples with direct comparisons.

All of these various calculations, in real time, can be calibrated very precisely in time, as they may all use the very high frequency laser clock. Thus, in the parallelized flow cytometer, a pulsed laser may serve as an important integration component for all of the data being generated in a multiple of nozzles.

Referring primarily to figure 13, since it may be desirable to stain samples just prior to sorting, sorting a sample for a period of time before staining a second sample which may have been sorted and repeating this process several times may create samples which have been stained and sorted at different times, but may be pooled as a single batch. Aggregated data (82) for each sample may be compared across a multiple of nozzles and multiple of sorts (83), for example, from the same ejaculate of a certain bull. Comparisons of the same bull over multiple days (84) and comparisons between various bulls (85) can give a history. The data from this history may reside within the operating system and may be used to assist operators in choosing staining concentrations or times, or to help identify ejaculates which are sorting worse than their normal sorting. Also, if high-throughput resort analysis is available, predicted sex ratios versus. actual sex ratios may be determined, and the aggregated sex ratios may be compared to identify settings and methods which different operators may be using, or to identify operators who are consistently getting lower results. Also, trends in the sorting performance may become visible which dictate special maintenance such as cleaning of optics, replacement of nozzle, mirror assemblies, or the like.

Now referring primarily to figures 14 through 18, embodiments of the invention are shown using a pulsed laser in the context of flow sorting of sperm cells. Various results from experiments run at different powers of radiation beams are shown. The different experiments included 20mW, 60mW, 90mW, 130mW and 160mW power and each power was created using neutral density filters. These embodiments can provide high-purity sperm sorting for enrichment of X or Y-chromosome bearing sperm cells which can even be up to 98% in purity.

In yet other embodiments, the present invention may provide collecting at least two populations of sample particles, more specifically, collecting a sorted population of X chromosome bearing sperm and collecting a sorted population of Y chromosome bearing sperm. A collector may be provided to collect each sorted population. Accordingly, the present invention may include a X chromosome bearing sperm collector and a Y chromosome sperm collector. It may be important to sort and collect each population at a high purity. A high purity sorted population of X chromosome bearing sperm and said Y chromosome bearing sperm may include a percentage of purity. Of course, any percentage of purity may exist and some examples may include:

- greater than 85% purity;
- greater than 90% purity;
- greater than 95% purity;
- greater than 96% purity; and
- greater than 98% purity.

Other percentages of purity are certainly possible and all should be understood as represented within the scope of this invention.

Typical pulsed lasers having characteristics similar to that set out by Table 1 or Table 2 can be used with the invention.

TABLE 1.

Vanguard 150mW Output Power

Average Output Power [W]
0.15
UV Beam Size [mm]
1
Energy per Pulse [J]
1.875E-09
Peak Power [W]
234.375
Power per cm^2 [W/cm^2]
2.98E+04

TABLE 2.

Vanguard 350 mW Output Power

Average Output Power [W]
0.35
UV Beam Size [mm]
1
Energy per Pulse [J]
4.375E-09
Power per Pulse [W]
546.875
Power per cm^2 [W/cm^2]
6.96E+04

Figures 14 through 18 show univariate histograms and a bivariate dot plots from sorting of Hoechst 33342 stained bovine sperm cells. Sperm cells sorted were obtained from the same freshly ejaculated bull sperm diluted to 200 x 10^6 sperm cells per mL and incubated in Hoechst 33342 at 34°C for 45 min.

With respect to the particular histograms and bivariant plots shown by Figures 14 through 19, the event rate (illumination of the sperm cells as they pass through the pulsed laser beam) was established at 20,000 events per second. The sort rate (separation of the sperm cells differentiated by analysis) into subpopulations was varied from 850-3500 depending on the power used. The results are also set out by Table 3.

TABLE 3.

Pulsed Laser Power Setting	Purity X%	Purity Y%
20mW pulsed	96.5	91.0
60mW pulsed	93.5	85.5
90mW pulsed	96.0	89.5
130mW pulsed	96.0	91.0
160mW pulsed	97.0	93.0

As can be understood from Table 3, using a pulsed laser sperm cells can be sorted into high purity X-chromosome bearing and Y-chromosome bearing subpopulations. For each laser power setting between 20mW and 160mW sorted subpopulations had a purity of up to 97.0% of the correct sex type.

Now referring primarily to Figure 19, histograms compare the resolution of the same sample of sperm cells using conventional CW flow sorting technology and with an embodiment of the flow sorting invention utilizing a pulsed laser beam. Importantly, pulsed laser illumination of stained sperm cells provides superior resolution of X-chromosome bearing sperm cells and Y-chromosome bearing sperm cells. This is true even when the pulsed laser beam has a power significantly lower than the compared to conventional CW flow sorter technology. Even when the pulsed power is 130 mW, 70 mW or even 20/150 of the power used in the compared to CW flow sorter technology. The histograms of the pulsed laser experiments show a cleaner separation of the two peaks as compared to the continuous wave experiments.

In embodiments, the present invention may include providing a high resolution of a sorted population of sperm cells. A higher resolution may be indicative of the purity of a sorted population. While many different resolution values may exist, some examples of high resolutions may include:

- greater than 7.0;
- greater than 7.5;
- greater than 8.0;
- greater than 8.5;
- greater than 9.0; and
- greater than 9.2.

Of course, other resolution values may exist and all are to be understood as represented within the scope of the invention.

As discussed above, lower laser power analysis of particles, and in this particular embodiment of the invention sperm cells, resolves the long standing problem of having to have a dedicated laser source for each flow sorter. By reducing the laser power required, even without achieving any other benefit, multiple flow cytometers, flow sorters, or cell sorters can be operated using a single laser source. For example, when sorting is accomplished at about 20mW, a single 350mW pulsed laser can be used to provide illumination light for as many as 18 separately functioning flow cytometers or flow sorters used to separate sperm cells on the basis of bearing an X or Y chromosome.

Again referring to Figure 19, another important benefit provided by a pulsed laser invention in the context of sorting sperm cells can be increased resolution of X-chromosome bearing and Y-chromosome bearing subpopulations even when sperm cell samples, *such as those used in this specific example, are stored for long periods of time at* about 5°C, such as 18 hours or longer, or frozen and thawed prior to staining and analysis, *the resolution of sorted sperm cells can improved with the pulsed laser invention* compared to conventional CW laser technology.

In embodiments, the present invention may include sorting sperm cells at a low coincidence rate. Some examples of low coincidence rates may include:

- less than 4400;
- less than 4000;
- less than 3700; and
- less than 3600.

Again, other low coincidence rates are certainly possible and all should be understood as represented within the scope of this invention.

The present invention may include, in embodiments, collecting sorted populations of sperm cells at a high collection rate. A high collection may increase productivity and even efficiency. Some examples of high collection rates may include:

- greater than 2400 sperm per second;
- greater than 2600 sperm per second;
- greater than 2900 sperm per second;
- greater than 3000 sperm per second; and
- greater than 3100 sperm per second.

Other collection rates are possible and all are meant to be included within the scope of this invention.

In yet other embodiments, the present invention may include detecting sperm cells at an event rate of between about 10,000 to about 60,000 sperm cells per second. Of course, an event rate may be greater than 10,000 or even lower than 60,000 cells per second.

A benefit with respect to sorting sperm cells using the pulsed laser invention can be higher sorting speeds. When resolution of a particular sample is increased, the sort rate of subpopulations of sperm cells to a given purity can be increased.

Another benefit with respect to sorting sperm cells using the pulsed laser invention can be a higher purity sort. When resolution of a particular sample is increased, the purity of the subpopulations of sperm cells can be increased.

A pulsed laser invention may be understood to have application with respect to any particular particle characteristic which may be differentiated by change of illumination intensity or by detectable light emission upon illumination with a pulsed light beam. While the applicant has provided specific examples of differentiating the amount of DNA within a cell using the invention, it to be understood that these examples are illustrative of how to make and use the invention with regard to the wide variety of non-biological and biological particles, including, but not limited to, viral particles, polyploid cells, diploid cells, haploid cells (such as sperm cells obtained from any species of mammal such as any type or kind of bovine, ovine, porcine, or equine sperm cells; or sperm cells obtained from any type or kind of elk, deer, oxen, buffalo, goats, camels, rabbits, or lama; or sperm cells obtained from any marine mammal such as whales or dolphins; or sperm cells obtained from any rare or endangered species of mammal; or sperm cells obtained from a zoological species of mammal; or sperm cells obtained from a rare or prize individual of a species of mammal; or sperm cells obtained from an individual of a species of mammal that used to produce dairy or meat products. In embodiments, sperm cells may include any type of sperm cells such as but not limited to, mammals, bovine sperm cells, equine sperm cells, porcine sperm cells, ovine sperm cells, camelid sperm cells, ruminant sperm cells, canine sperm cells and the like.

It is also to be understood that these specific examples provided are not intended to limit the variety of applications to which the invention may be used, but rather are intended to be illustrative how to make and use the numerous embodiments of the invention for application with analytical devices such as flow cytometers, cell sorters, microarray analyzers, imaging microscopes, or microimaging equipment, which may easily be built to contain two or more, and perhaps thousands or even millions parallel channels for analysis, and in such that each of these separate channels is capable to perform the identical or very similar function to a single flow cytometry sorting nozzle, they may be considered "machines", and it is understood mat even a very small device which could be held in a persons hand, may contain many hundreds or many thousands of such "machines" and only be able to function if the use of illumination light is similar or identical to the inventions described herein.

EXAMPLE 1.

Purified fixed bull sperm heads (also described as bull sperm nuclei), stained in standard conditions with DNA binding stain Hoechst 33342, are used as a performance standard to calibrate a sperm sorting flow cytometer prior to the sorting of live sperm. A pulsed laser (Spectrophysics VNGD350-HMD355) delivering 300 mW of energy at 355 nm and 80

MHz illuminates the sample analysis stream in a flow cytometer operating at standard settings and provides the histogram plot shown in Fig. Ex 1. This demonstrates that a standard sperm sorting flow cytometer equipped with the pulsed laser is able to resolve bull sperm nuclei into X-chromosome bearing and Y-chromosome bearing populations using standard conditions.

EXAMPLE 2.

A sample of live bull sperm is stained in standard conditions with DNA binding stain Hoechst 33342. A pulsed laser (Spectrophysics VNGD350-HMD355) delivering 300 mW of energy at 355 nm and 80 MHz illuminates the sample analysis stream in a flow cytometer operating at standard settings sorting said sperm and provides the histogram plot shown in Fig Ex 2. This demonstrates that a standard sperm sorting flow cytometer equipped with the pulsed laser is able to resolve live sperm into X-chromosome bearing and Y-chromosome bearing populations under standard conditions.

The above sample is sorted for collection of X-chromosome bearing sperm, and the sort collection rate is 3800 live X-chromosome bearing sperm second. A resort analysis of the sample prepared in said manner measures the purity of said sorted sample to be 95%. This demonstrates that a standard sperm sorting flow cytometer equipped with the pulsed laser is able to enrich the content of a sperm population from one in which approximately 50% of the sperm are X-chromosome bearing sperm, to one in which 95% of the sperm are X-chromosome bearing sperm.

Said sorted sperm above are further processed by standard methods for packaging into artificial insemination straws, are cryopreserved by the standard freezing method, and are thawed for analysis of motility of the sperm. Percent motility at various points in the procedure is determined to be: After stain 80%, after sorting 70%, after cooling 65%, after freezing and thawing at 0 minutes 45%, 30 minutes after thawing 45%, 120 minutes after thawing 35%. This demonstrates that a standard sperm sorting flow cytometer equipped with the pulsed laser is able to enrich live stained sperm samples which appear normal in respect to the sperm motility.

EXAMPLE 3

A sample of live bull sperm is stained in standard conditions with DNA binding stain Hoechst 33342. A pulsed laser (Spectrophysics VNGD350-HMD355) delivering 300 mW of energy at 355 nm and 80 MHz is equipped with beam splitters and neutral density filters, in five separate conditions, to provide illumination energy beam levels of 160mW (53% of beam power), 130mW (43% of beam power), 90 mW (30% of beam power), 60 mW (20% of beam power), and 20 mW (6.6% of beam power), respectively, to illuminate the sample analysis stream of a sperm sorting flow cytometer operating at standard settings sorting said stained sperm and providing the 5 histogram plots shown in Fig Ex 3. This demonstrates that a standard sperm sorting flow cytometer equipped with the pulsed laser is able to clearly resolve live sperm with energies as low as 60 mW (20% of the beam).

The above samples are sorted at each of the 5 beam energy settings for collection of X-chromosome bearing sperm and Y-chromosome bearing sperm in separate fractions, and the sort collection rate is 850-3500 X-chromosome bearing or Y-chromosome bearing sperm second, depending on the power used, with lower powers associated with lower sort collection rates. A resort analysis of the samples prepared in said manner measures the purity of said sorted samples as shown in Table Ex 3. This demonstrates that a standard sperm sorting flow cytometer equipped with the pulsed laser delivering beam energies in the range of 20 mW to 160 mW is consistently able to enrich the content of a sperm population from one in which approximately 50% of the sperm are X-chromosome bearing sperm, to one in which 95% or higher of the sperm are X-chromosome bearing sperm, and simultaneously to one in which 90% or higher of the sperm are Y-chromosome bearing sperm.

TABLE 4

Beam Energy (Pulsed)	% Purity - X	% Purity - Y
20 mW	96.5	91.0
60 mW	93.5	85.5
90 mW	96.0	89.5
130 mW	96.0	91.0
160 mW	97.0	93.0

EXAMPLE 4

A sample of live bull sperm is stained in standard conditions with DNA binding stain Hoechst 33342. A pulsed laser (Spectrophysics VNGD350-HMD355) delivering 300 mW of energy at 355 nm and 80 MHz is equipped with beam splitters and neutral density filters, in two separate conditions, to provide illumination energy beam levels of 130mW (43% of beam power) and 70 mW (23% of beam power), respectively, to the sample analysis stream of a flow cytometer operating at standard settings sorting said stained sperm. As comparison, the same sample is analyzed on two identical but different flow cytometers operating at standard settings and equipped with a CW (continuous wave) lasers delivering 150 mW in both cases. This demonstrates that even with lower beam energies a standard sperm sorting flow cytometer equipped with the pulsed laser provides superior resolution capability when compared to a same standard sperm sorting flow cytometer equipped with a standard CW laser.

EXAMPLE 5

A sample of live bull sperm is stained in standard conditions with DNA binding stain Hoechst 33342 with the standard concentration of Hoechst 33342 being defined as 100% level of stain (control). Two additional samples are prepared which are identical except that they are stained with 80% or 60% of the amount of Hoechst 33342 stain, respectively. A pulsed laser (Spectrophysics VNGD350-HMD355) delivering 300 mW of energy at 355 nm and 80 MHz is equipped with beam splitters and neutral density filters, in two separate conditions, to provide illumination energy beam levels of 150mW (50% of beam power) and 90 mW (30% of beam power), respectively, to the sample analysis stream of a flow cytometer operating at standard settings sorting said stained sperm with 3 different concentrations of stain used. The resolution between X-chromosome bearing and Y-chromosome bearing sperm for these 6 conditions are provided in the 6 histogram plots shown in Fig Ex 5. This demonstrates that lesser amounts of Hoechst 33342 stain may be used to prepare sperm samples for sorting on a standard sperm sorting flow cytometer, if higher pulsed beam energies are also used.

EXAMPLE 6

Purified fixed bull sperm heads (also described as bull sperm nuclei), stained in standard conditions with DNA binding stain Hoechst 33342, are used as a performance standard to calibrate a sperm sorting flow cytometer prior to the sorting of live sperm. A pulsed laser (Spectrophysics VNGD350-HMD355) delivering 300 mW of energy at 355 nm and 80 MHz and equipped with a beam splitter to provide an illumination energy beam level of 150mW (50% of beam power) illuminates the sample analysis stream of a flow cytometer operating at standard settings. Said stained nuclei are analyzed at 20,000 events/second (a rate comparable to the rate used in live bull sperm analysis), as well as at 59,000 events/second. The resolution between X-chromosome bearing and Y-chromosome bearing bull sperm nuclei for these 2 event rate conditions are provided in the 2 histogram plots shown in Fig Ex 6. This demonstrates, that for ideal particles such as nuclei standards, the event rates of analysis may be increased as much as 3-fold with only modest loss in the resolution between X-chromosome bearing and Y-chromosome bearing bull sperm nuclei.

EXAMPLE 7

Samples of live bull sperm from 4 different bulls were stained in standard conditions with DNA binding stain Hoechst 33342. A pulsed laser (Spectrophysics VNGD350-HMD355) delivering 300 mW of energy at 355 nm and 80 MHz is equipped with beam splitters and neutral density filters, in two separate conditions, to provide illumination energy beam levels of 300 mW (100% of beam power) and 150 mW (50% of beam power), respectively, to the sample analysis stream of a flow cytometer operating at standard settings sorting said stained sperm samples. As comparison, the same samples are sorted on an identical but different flow cytometer operating at standard settings and equipped with a CW (continuous wave) laser delivering 150 mW of energy in the illumination beam. The samples are bulk sorted, which means both X-chromosome bearing and Y-chromosome bearing sperm fractions are pooled. The sorted sperm samples are cryopreserved using standard procedures and the percent of post thaw sperm motilities, as well as the percent of live and dead using PI staining with flow cytometry analysis are scored. The averages for all 4 bulls with the 3 different illumination conditions are shown in Table 5. This demonstrates without statistical significance that all three conditions of illumination yield similar numbers of intact viable sperm after sorting.

TABLE 5

Laser (mW)	% Motility at 0 min post thaw	% Motility at 90 min post thaw	% Live at 0 min post thaw	% Live at 90 min post thaw
CW (150 mW)	50.0	42.5	43.5	40.6
Pulsed (150 mW)	46.3	42.5	40.0	37.9
Pulsed (300 mW)	48.1	36.3	40.3	37.2

EXAMPLE 8

Samples of live bull sperm from 5 different bulls are stained in standard conditions with DNA binding stain Hoechst 33342, with the standard concentration of Hoechst 33342 being defined as 100% level of stain (control). Two additional samples from the same 5 bulls are prepared which are identical except that they are stained with 80% or 60% of the amount of Hoechst 33342 stain, respectively.

A pulsed laser (Spectrophysics VNGD350-HMD355) delivering 300 mW of energy at 355 nm and 80 MHz is equipped with beam splitters and neutral density filters, in two separate conditions, to provide illumination energy beam levels of 150 mW (50% of beam power) and 90 mW (30% of beam power), respectively, to the sample analysis stream of a flow cytometer operating at standard settings sorting said stained sperm samples. As comparison, the same samples are sorted on an identical but different flow cytometer operating at standard settings and equipped with a CW (continuous wave) laser delivering 150 mW of energy in the illumination beam.

For the sorting procedures on all these samples, the average values for resolution (higher values are better), the coincidence rates (lower values are better), the sort collection rates (higher values are better) are compared and shown in Table 6. This demonstrates that sorting efficiencies in all conditions tested with the pulsed laser were equal to or better than the sorting efficiencies achieved using the standard CW laser.

TABLE 6

Stain(%) / Laser (mW)	Resolution	Co-incidence rate	Sort Rate
100 / 150 pulsed	8.0	3570	3160
100 / 90 pulsed	9.3	3560	2610
80 / 150 pulsed	8.2	3600	3160
80 / 90 pulsed	9.6	3500	2480
60 / 150 pulsed	8.6	3600	2940
60 / 90 pulsed	9.8	3520	2450
100 / 150 CW	7.6	4380	2720

The same samples are bulk sorted, which means both X-chromosome bearing and Y-chromosome bearing sperm fractions are pooled. The sorted sperm samples are cryopreserved using standard procedures and the percent of post thaw motilities, as well as the percent of live / dead using PI staining with flow cytometry analysis are scored. The averages for all 5 bulls with the 7 different stain and illumination conditions are shown in Table 7. This demonstrates that sperm viability and live counts in all conditions tested with the pulsed laser were equal to or better than the sperm viability and live counts achieved using the standard CW laser and standard stain.

TABLE 7

Stain(%) / Laser (mW)	% Motility at 0 min post thaw	% Motility at 120 min post thaw	% Live at 30 min post thaw	% Live at 120 min post thaw
100 / 150 pulsed	43.3	34.0	36.8	32.4
100 / 90 pulsed	42.8	33.5	35.3	33.2
80 / 150 pulsed	42.8	33.8	35.1	35.1
80 / 90 pulsed	42.0	35.3	35.2	29.7
60 / 150 pulsed	40.8	31.0	35.8	35.1
60 / 90 pulsed	41.0	32.8	34.4	33.7
100 / 150 CW	39.8	33.3	33.4	28.6

EXAMPLE 9

Samples of live bull sperm from 5 different bulls and 2-6 replicates were stained in standard conditions with DNA binding stain Hoechst 33342 (80%) and bulk sorted under standard conditions in a sperm sorting flow cytometer at event rates of 23,000 sperm / second. A pulsed laser (Spectrophysics VNGD350-HMD355) delivering 300 mW of energy at 355 nm and 80 MHz is equipped with beam splitter to provide illumination energy beam level of 150 mW (50% of beam power) to the sample analysis stream of a flow cytometer operating at standard settings sorting said stained sperm samples.

As a control comparison, same samples of live bull sperm from 5 different bulls and 2-6 replicates were stained in standard conditions with DNA binding stain Hoechst 33342 (100%) and these samples were sorted on an identical but different flow cytometer operating at standard settings and equipped with a CW (continuous wave) laser delivering 150 mW of energy in the illumination beam.

Said various sperm samples were used at concentrations of 200,000 sperm/ml or 1 *million sperm/ml to inseminate matured bovine oocytes in standard procedures of in-vitro* fertilization (IVF). Cleavage and 2 cell rates at 2.75 days post-insemination, blastocyst development rates at 7.75 days post-insemination, total cell numbers in blastocyst and the blastocyst quality at 7.75 days were measured. The average results for 587 oocytes inseminated with sorted sperm prepared from the system equipped with the pulsed laser, and for 558 oocytes inseminated with sorted sperm prepared from the system equipped with the CW laser are shown in Table 8. Note: lower numbers for blastocyst quality are better. This demonstrates that sperm prepared by a standard sperm sorting flow cytometer equipped with the pulsed laser is capable of fertilizing oocytes in standard IVF procedures and exhibits similar cleavage and blastocyst rates, with the mean quality of blastocysts being slightly better when inseminated with sperm sorted using the standard flow cytometer equipped with the pulsed laser.

TABLE 8

Laser (mW)	% Cleaved	% 2 cell	% blastocyst	quality of blastocyst	cell counts in blastocyst
CW (150 mW)	50.7	27.3	6.7	2.7	131.8
Pulsed (150 mW)	49.7	29.5	5.2	2.1	136.2

EXAMPLE 10

Samples of live bull sperm from 3 different bulls, on multiple days, were stained in standard conditions with DNA binding stain Hoechst 33342 (100%) and bulk sorted under standard conditions in a sperm sorting flow cytometer at event rates of 20-23,000 sperm / second. A pulsed laser (Spectrophysics VNGD350-HMD355) delivering 300 mW of energy at 355 nm and 80 MHz is equipped with a beam splitter to provide illumination energy beam level of 150 mW (50% of beam power) to the sample analysis stream of a flow cytometer operating at standard settings sorting said stained sperm samples.

As a control comparison, same samples of live bull sperm from the same 3 different bulls on same days, were stained in standard conditions with DNA binding stain Hoechst 33342 (100%) and these samples were sorted on an identical but different flow cytometer operating at standard settings and equipped with a CW (continuous wave) laser delivering 150 mW of energy in the illumination beam.

Said various sperm samples were used in amounts of 2 million sperm per cyropreserved artificial insemination straw containing 0.25 ml of fluid.

Control straws containing 10 million unsorted sperm, and control straws containing 2 million X enriched sperm sorted using a sperm sorting flow cytometer equipped with a the standard CW laser were used.

In a heterospermic analysis, X-fractions from CW laser sorts were mixed in equal sperm numbers with Y-fractions from pulsed laser sorts to create the #1 comparision. Y-fractions from CW laser sorts were mixed in equal sperm numbers with X-fractions from pulsed laser sorts to create the #2 comparison. Identification of sex in fetuses at 60 days was used as a marker to assign the sex outcome, and accordingly, the likely condition (which laser) can be attributed to successful fertilization. The heterospermic method is particularly useful, as all other factors than sorting procedure are internally controlled in each insemination.

Holstein heifers weighing approximately 750 pounds were synchronized using CIDR/Lutalase. Thereafter observed (AM or PM) for standing heat and were inseminated at 12-24 hours after heat observation. Using 2 inseminators, and a single deep uterine insemination treatment, with 5 test groups spaced approximately one month apart, at a single farm, pregnancy rates and sex of fetus were determined at 60 days post insemination using ultrasonograhy. The results shown in Table 9 demonstrate that the sperm sorted with a standard sperm sorting flow cytometer equipped with a pulsed laser give *essentially identical pregnancy rates as sperm sorted using a standard sperm sorting* flow cytometer equipped with the standard CW laser.

TABLE 9

Sperm dose type	% Conception Rate	Preganancies / Inseminations
Unsexed control containing 10 million total sperm	62.5	55/88
X-sexed control containing 2 million total sperm	56.4	101/179
Heterospermic #1 containing 2 million total sperm	50.0	45/90
Heterospermic #2 containing 2 million total sperm	58.4	52/89
Pregancies attributed to CW laser	49.50%	48/97
Pregancies attributed to pulsed laser	50.50%	49/97

EXAMPLE 11

A sample of live dolphin sperm was collected at poolside, shipped via air freight, and stained with Hoechst 33342 approximately 6 hours after collection. The sorting efficiencies for the single stained sample were then tested on two identical Mo Flo SX sperm sorters, in one case equipped with a pulsed laser (Spectrophysics VNGD350-HMD355) delivering 300 mW of energy at 355 nm and 80 MHz equipped with a beam splitter to provide illumination energy beam level of 150 mW (50% of beam power), and the second case with an Innova 90-6 (CW – continuous wave) laser delivering 150 mW of beam energy. The dolphin ejaculate was stained 3 times, and in each case sorted for approximately 2 hours.

Using the CW laser, with sorter event rates at 30,000/sec an average co-incidence rate of 6430/sec was observed, X-chromosome bearing sperm were collected at an average rate of 3450/sec and a total of 72 million sperm were collected in 7 hours for an average recovery of 10.3 million sperm per hour.

Using the pulsed laser, with sorter event rates at 30,000/sec an average co-incidence rate of 5400/sec was observed, X-chromosome bearing sperm were collected at an average rate of 3930/sec and a total of 79.5 million sperm were collected in 6.33 hours for an average recovery of 12.6 million sperm per hour.

The recovered sperm from both samples has X purities of >95% and post-thaw motility of >50%.

EXAMPLE 12

A sample of live canine sperm was collected from a common dog housed at a kennel and stained about 3 hours later with a non-optimized quantity of Hoechst 33342. The stained sperm were analyzed by a standard sperm sorter equipped with a pulsed laser (Spectrophysics VNGD350-HMD355) delivering 300 mW of energy at 355 nm and 80 MHz equipped with a beam splitter to provide illumination energy beam level of 150 mW (50% of beam power). Approximately 43 % of the sperm were correctly oriented. From the correctly oriented stained canine sperm, approximately 32% were collected as X-chromosome bearing sperm, and approximately 36% were collected as Y-chromosome bearing sperm. Visual inspection by microscope showed high numbers of canine sperm in both samples to be motile.

EXAMPLE 13

The standard CW laser uses a cathode tube which requires an average input of 12 KW of electrical power, and a large volume of cooling water, or a chiller with a load of approximately 15 KW. The pulsed laser (Spectrophysics VNGD350-HMD355 delivering 300 mW of energy at 355 nm and 80 MHz) requires approximately 500 watts (0.5 KW).

The standard CW laser also requires replacement of the cathode tube after approximately 5000 hours of use, at a replacement cost of about $12,000, whereas the VNG pulsed laser is expected to have 30,000+ hours of operation before refurbishment of head element at similar costs.

A commercial operation using the sperm sorting flow cytometers to sort sperm for production of artificial insemination straws, running 24 hours per day, year-round, may be expected to operate lasers for 8,640 hours per year.

Electric utility and water rates in Fort Collins quoted for the year 2004 were used to calculate the operating costs of the standard CW laser, in the first case cooled by utility water and in the second case cooled using electric powered chiller. The pulsed laser requires no cooling. The comparative costs are shown in Table 10. This demonstrates that the pulsed laser has significant benefits in reducing the costs of operation of a sperm sorting flow cytometer.

TABLE 10

Cost component	CW laser with water cooling	CW laser with electric chiller cooling	Pulsed Laser
Electrical Charges	$4,389	$9,828	$183
Water Charges	$6,483	$0	$0
Laser tube or rebuild	$20,736	$20,736	$3,456
TOTAL (1 year)	***$31,608***	***$30,564***	***$3,639***

As can be easily understood from the foregoing, the basic concepts of the present invention may be embodied in a variety of ways. It involves both sorting techniques as well as devices to accomplish the appropriate sorting system. In this application, the sorting techniques are disclosed as part of the results shown to be achieved by the various devices described and as steps which are inherent to utilization. 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.

The discussion included in this application is intended to serve as a basic description. The reader should be aware that the specific discussion may not explicitly describe all embodiments possible; many alternatives are implicit. It also may not fully explain the generic nature of the invention and may not explicitly show how each feature or element can actually be representative of a broader function or of a great variety of alternative or equivalent elements. Again, these are implicitly included in this disclosure. Where the invention is described in device-oriented terminology, each element of the device implicitly performs a function. Apparatus claims may not only be included for the device described, but also method or process claims may be included to address the functions the invention and each element performs. Neither the description nor the terminology is intended to limit the scope of the claims included in this or in any subsequent patent application.

It should also be understood that a variety of changes may be made without departing from the essence of the invention. Such changes are also implicitly included in the description. They still fall within the scope of this invention. A broad disclosure encompassing both the explicit embodiment(s) shown, the great variety of implicit alternative embodiments, and the broad methods or processes and the like are encompassed by this disclosure and may be relied upon when drafting the claims for the full patent application. This patent application seeks examination of as broad a base of claims as deemed within the applicant's right and is designed to yield a patent covering numerous aspects of the invention both independently and as an overall system.

Further, each of the various elements of the invention and claims may also be achieved in a variety of manners. This disclosure should be understood to encompass each such variation, be it a variation of an embodiment of any apparatus embodiment, a method or process embodiment, or even merely a variation of any element of these. Particularly, it should be understood that as the disclosure relates to elements of the invention, the words for each element may be expressed by equivalent apparatus terms or method terms -- even if only the function or result is the same. Such equivalent, broader, or even more generic terms should be considered to be encompassed in the description of each element or action. Such terms can be substituted where desired to make explicit the implicitly broad coverage to which this invention is entitled. As but one example, it should be understood that all actions may be expressed as a means for taking that action or as an element which causes that action. Similarly, each physical element disclosed should be understood to encompass a disclosure of the action which that physical element facilitates. Regarding this last aspect, as but one example, the disclosure of a "sorter" should be understood to encompass disclosure of the act of "sorting" -- whether explicitly discussed or not -- and, conversely, were there effectively disclosure of the act of "sorting", such a disclosure should be understood to encompass disclosure of a "sorter" and even a "means for sorting" Such changes and alternative terms are to be understood to be explicitly included in the description.

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In drafting any claims at any time whether in this application or in any subsequent *application, it should also be understood that the applicant has intended to capture as full* and broad a scope of coverage as legally available. To the extent that insubstantial substitutes are made, to the extent that the applicant did not in fact draft any claim so as to literally encompass any particular embodiment, and to the extent otherwise applicable, the applicant should not be understood to have in any way intended to or actually relinquished such coverage as the applicant simply may not have been able to anticipate all eventualities; one skilled in the art, should not be reasonably expected to have drafted a claim that would have literally encompassed such alternative embodiments.

Further, if or when used, the use of the transitional phrase "comprising" is used to maintain the "open-end" claims herein, according to traditional claim interpretation. Thus, unless the context requires otherwise, it should be understood that the term "comprise" or variations such as "comprises" or "comprising", are intended to imply the inclusion of a stated element or step or group of elements or steps but not the exclusion of any other element or step or group of elements or steps. Such terms should be interpreted in their most expansive form so as to afford the applicant the broadest coverage legally permissible.

The embodiments of the present invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method of sperm cells separation using flow cytometry comprising the steps of:

establishing a sheath fluid;

flowing said sheath fluid into at least one nozzle;

forming a stream including irradiatable sperm cells surrounded by the sheath fluid at an exit of the at least one nozzle, the irradiatable sperm cells being stained with Hoechst 33342;

subjecting said irradiatable sperm cells in the stream to multiple pulses of pulsed coherent radiation, wherein the pulsed coherent radiation is delivered at a power between 100mW and 500mW;

detecting the fluorescence emitted from each of said irradiatable sperm cells in response to the pulsed coherent radiation;

determining a summated total energy of the fluorescence emitted from each of said irradiatable sperm cells in response to the pulsed coherent radiation; and

distinguishing between a X-chromosome bearing sperm and a Y-chromosome bearing sperm based on said summated total energy of the fluorescence emitted.

2. The method according to claim 1, further comprising

forming a plurality of droplets entraining the irradiatable sperm cells;

charging the droplets based upon the amount of fluorescence detected from each of the entrained sperm cells;

isolating said charged droplets from said stream;

deflecting said charged droplets;

sorting said droplets; and

collecting the sorted sperm cells.

3. The method according to claim 1 or 2, further comprising the step of utilizing a beam manipulator.

4. The method according to claim 3, wherein said step of utilizing a beam manipulator comprises the step of utilizing a beam manipulator selected from a group consisting of mirrors, deflectors, beam splitters, prisms, refractive objects, lenses and filters.

5. The method according to any one of claims 1 to 4, wherein said step of detecting and evaluating an amount of fluorescence emitted from each of said sperm cells is performed with a detection system.

6. The method according to claim 5, wherein the detection system comprises a photomultiplier tube.

7. The method according to any one of claims 1 to 6, wherein said step of subjecting said irradiatable sperm cells in the stream to pulsed coherent radiation further comprises the step of using a wavelength appropriate to activate fluorescence in said irradiatable sperm cells.

8. The method according to claim 8, wherein said wavelength is 355nm.

9. The method according to any one of claims 1 to 6, wherein said step of staining said sperm cells occurs for a time period selected from a group consisting of less than about 40 minutes less than about 35 minutes; less than about 30 minutes; less than about 25 minutes; less than about 20 minutes; less than about 15 minutes; less than about 10 minutes; and less than about 5 minutes.

10. The method according to any one of claims 1 to 9, wherein said step of subjecting said irradiatable sperm cells in the stream to pulsed coherent radiation comprises the step of using radiation selected from the group consisting of: less than 300 mW; less than 350 milliwatt; less than 200 milliwatt; and less than 175 milliwatt.

11. The method according to any one of claims 1 to 10, further comprising the step of splitting said pulsed coherent radiation into at least two light beams.

12. The method according to claim 11, wherein said step of splitting said pulsed coherent radiation into at least two light beams further comprises the step of reducing the power of the radiation that was originally emitted and wherein said reduced power is selected from the group consisting of a half, a fourth and an eighth of the originally emitted power.

13. The method according to claim 2, wherein said step of collecting said sorted sperm cells comprises the step of collecting a sorted population of X chromosome bearing sperm and collecting a sorted population of Y chromosome bearing sperm.

14. The method according to claim 13, wherein said step of collecting a sorted population of X chromosome bearing sperm and collecting a sorted population of Y chromosome bearing sperm comprises the step of collecting said populations at a purity selected from the group consisting of greater than 85% purity; greater than 90% purity; greater than 95% purity; greater than 96% purity; and greater than 98% purity.

15. The method according to claim 14, wherein said step of collecting said populations at said purity further comprises the step of a providing a resolution of said sorted sperm said resolution is selected from the group consisting of greater than 7.0; greater than 7.5; greater than 8.0; greater than 8.5; greater than 9.0; and greater than 9.2.

16. The method of any one of claims 1 to 12, further comprising the step of sorting a X-chromosome bearing sperm and/or a Y-chromosome bearing sperm and collecting the sorted sperm.

17. The method according to any one of claim 16, wherein said step of sorting X-chromosome bearing sperm and/or a Y-chromosome bearing sperm and collecting the sorted sperm comprises the step of collecting said populations at a collection rate selected from the group consisting of greater than 2400 sperm per second; greater than 2600 sperm per second; greater than 2900 sperm per second; greater than 3000 sperm per second; and greater than 3100 sperm per second.

18. The method according to any one of claims 1 to 17, wherein said step of detecting the fluorescence emitted from each of said irradiatable sperm cells comprises the step of detecting said sperm cells at an event rate of between about 10,000 to about 60,000 sperm cells per second.

19. The method according to any one of claims 1 to 18, wherein said step of subjecting said irradiatable sperm cells in the stream to pulsed coherent radiation further comprises the step of initiating a sensing routine.

20. The method according to any one of claims 1 to 18, wherein said step of subjecting said irradiatable sperm cells in the stream to pulsed coherent radiation further comprises the step of subjecting said to radiation for a first amount of time between about 5 to about 20 picoseconds.

21. The method according to claim 20, wherein the step of subjecting said irradiatable sperm cells to radiation for a first amount of time between about 5 to about 20 picoseconds further comprises terminating said radiation for a second amount of time wherein the second amount of time between about 0.5 to about 20 nanoseconds.

22. The method according to claim 21, wherein the first amount of time and the second amount of time form a cycle and the cycle can total between about 2 to about 10 microseconds.

23. The method according to claim 22, wherein said cycle has a repetition rate of between 50-200 MHz.

24. The method according to claim 22, wherein said cycle has a repetition rate of up to about 80 MHz.

25. The method according to any one of claim 16, wherein said step of sorting further comprise oscillating said sheath fluid to form droplets and charging said droplets.

26. The method according to any one of claims 1 to 25, wherein said sperm cells are selected from the group consisting of mammals, bovine sperm cells, equine sperm cells, porcine sperm cells, ovine sperm cells, camelid sperm cells, ruminant sperm cells, and canine sperm cells.

27. The method according to any one of claims 1 to 26, wherein said steps of flowing said sheath fluid into the least one nozzle and injecting irradiatable sperm cells into said sheath fluid comprise the step of flowing at least one sheath fluid and said sperm cells into at least two nozzles.

28. The method according to claim 27, further comprising a step of collecting X chromosome bearing sperm populations and Y chromosome bearing sperm populations

in a collector, wherein said collector is selected from the group consisting of multiple containers and a combined collector having individual containers.

29. The method according to claim 28, further comprising the step of providing a number of selected containers less than the number of nozzles.

30. The method according to any one of claims 1 to 29, further comprising the step of utilizing at least one shared resource to separate said sperm cells, the shared resource being selected from the group consisting of a computer system, the sheath fluid, an integrated nozzle device and the pulsed coherent radiation source.

31. The method according to claim 30, wherein when the shared resource is a pulsed coherent radiation source, it is utilized by splitting said radiation into at least two beams; and directing each of said beams to one or more nozzles of identical flow cytometers.

32. The method according to claim 16, further comprising the step of utilizing said collected sorted sperm for insemination of female non-human mammals.

33. The method according to any one of claims 1 to 32, wherein said step of subjecting said irradiatable sperm cells in the stream to pulsed coherent radiation comprises the step of utilizing a pulsed laser.

34. The method according to claim 33, wherein said step of utilizing a pulsed laser comprises the step of a utilizing a Nd:YAG or a Nd:YVO4 laser.

35. The method according to claim 1, wherein said irradiatable sperm cells comprise reproductive or haploid cells.

36. The method according to claim 16, wherein said step of collecting said sorted sperm cells comprises the step of collecting at least two populations of sperm cells.

37. The method according to claim 27, further comprising the step of individually or compositely controlling said at least two nozzles.

38. A flow cytometry system for sperm comprising:

a sheath fluid port to introduce a sheath fluid;

a Hoechst 33342 stain for staining irradiatable sperm cells;

a sample injection element having an injection point for introducing said stained irradiatable sperm cells into said sheath fluid;

a nozzle located in part below said injection point;

an oscillator to which said sheath fluid is responsive;

a pulsed coherent laser, having an average power between 100mW and 500mW;

a sperm cell fluorescence detector; and

a processing unit connected to said sperm cell fluorescence detector for determining a summated total energy of the fluorescence emitted from each of said irradiatable sperm cells subjected to multiple pulses from the pulsed coherent laser and distinguishing between a X-chromosome bearing sperm and a Y-chromosome bearing sperm based on said summated total energy of the fluorescence emitted.

39. The flow cytometry system for sperm according to claim 38, wherein said sperm cell fluorescence detector comprises a sperm cell fluorescence quantitative detector.

40. The flow cytometry system for sperm according to claim 39, wherein said sperm cell fluorescence quantitative detector detects between an X chromosome bearing sperm and a Y chromosome bearing sperm.

41. The flow cytometry system for sperm according to claim 38 further comprising:

a drop charge circuit to apply an electrical condition to a stream of said irradiatable sperm cells and sheath fluid;

a first and second deflection plate each disposed on opposite sides of a free fall area in which a drop forms, wherein said first and second deflection plates are oppositely charged; and

a sperm cell collector.

42. The flow cytometry system for sperm according to any one of claims 38 to 41, and further comprising a radiation beam manipulator.

43. The flow cytometry system for sperm according to claim 42, wherein said radiation beam manipulator is selected from the group consisting of mirrors, deflectors, beam splitters, prisms, refractive objects, lenses and filters.

44. The flow cytometry system for sperm according to claim 38, wherein said sperm cell fluorescence detector comprises a photomultiplier tube.

45. The flow cytometry system for sperm according to any one of claims 38 to 44, wherein said pulsed coherent laser comprises a fluorescence activation wavelength.

46. The flow cytometry system for sperm according to claim 45, wherein said fluorescence activation wavelength comprises 355 nm.

47. The flow cytometry system for sperm according to any one of claims 38 to 46, wherein said pulsed coherent laser comprises radiation at a power selected from the group consisting of less than about 300 mW; less than 350 milliwatt; less than 200 milliwatt; and less than 175 milliwatt.

48. The flow cytometry system for sperm according to any one of claims 38 to 47, further comprising a beam splitter.

49. The flow cytometry system for sperm according to any one of claims 41 to 48, wherein said sperm cell fluorescence detector detects between an X chromosome bearing sperm and a Y chromosome bearing sperm.

50. The flow cytometry system for sperm according to claim 41, wherein said sperm cell collector comprises an X chromosome bearing sperm cell collector and a Y chromosome sperm cell collector.

51. The flow cytometry system for sperm according to claim 49 or 50, further comprising a purity of sorted populations of said X chromosome bearing sperm and said Y chromosome bearing sperm selected from a group consisting of: greater than 85% purity; greater than 90% purity; greater than 95% purity; greater than 96% purity; and greater than 98% purity.

52. The flow cytometry system for sperm according to claim 51, wherein sorting sperm occurs at a resolution rate selected from the group consisting of greater than 7.0; greater than 7.5; greater than 8.0; greater than 8.5; greater than 9.0; and greater than 9.2.

53. The flow cytometry system for sperm according to claim 41, wherein sperm is collected at a coincidence rate selected from the group consisting of less than 4400; less than 4000; less than 3700; and less than 3600.

54. The flow cytometry system for sperm according to claim 41, wherein the sperm collector collects sperm at a collection rate selected from a group consisting of greater than 2400 sperm per second; greater than 2600 sperm per second; greater than 2900 sperm per second; greater than 3000 sperm per second; and greater than 3100 sperm per second.

55. The flow cytometry system for sperm according to any one of claims 38 to 54, further comprising an event rate between about 10,000 to about 60,000 sperm cells per second.

56. The flow cytometry system for sperm according to any one of claims 38 to 55, wherein said pulsed coherent laser comprises a radiation on time between about 5 to about 20 picoseconds.

57. The flow cytometry system for sperm according to claim 56, wherein said pulsed coherent laser comprises a radiation off time between about 0.5 to about 20 nanoseconds.

58. The flow cytometry system for sperm according to claim 57, wherein the radiation on time and the radiation off time form a cycle and the cycle can total between about 2 to about 10 micro-seconds.

59. The flow cytometry system for sperm according to claim 58, wherein said cycle can have a repetition rate of between about 50 to about 200 MHz.

60. The flow cytometry system for sperm according to claim 59, wherein said repetition rate comprises up to about 80 MHz.

61. The flow cytometry system for sperm according to any one of claims 38 to 60, wherein said sperm cells comprises sperm cells selected from a group consisting of mammal sperm cells, bovine sperm cells, equine sperm cells, porcine sperm cells, ovine sperm cells, camelid sperm cells, ruminant sperm cells, and canine sperm cells.

62. The flow cytometry system for sperm according to any one of claims 38 to 61, wherein said nozzle comprises at least two nozzles.

63. The flow cytometry system for sperm according to claim 41, wherein said sperm cell collector is selected from the group consisting of multiple containers and a combined collector having individual containers.

64. The flow cytometry system for sperm according to claim 62 or 63, further comprising a number of selected containers that is-less than the number of nozzles.

65. The flow cytometry system for sperm according to any one of claims 38 to 64, further comprising at least one shared resource to separate said sperm selected from the group consisting of a computer system, the sheath fluid, an integrated nozzle device and the pulsed coherent laser.

66. The flow cytometry system for sperm according to claim 38, wherein said pulsed coherent laser is selected from the group consisting of Nd:YAG and Nd:YVO4 lasers.

67. A flow cytometry system comprising:

at least two nozzles for producing at least two streams of irradiatable stained sperm cells having X chromosome bearing sperm and Y chromosome bearing sperm the irradiatable sperm cells being stained with Hoechst 33342 stain;

an oscillator associated with each nozzle to which each stream is responsive;

a pulsed coherent radiation emitter having an average power between 100mW and 500mW;

a beam splitter for directing a portion of radiation emitted from the pulsed coherent radiation emitter to each stream produced from the at least two nozzles;

a quantitative fluorescence detector; and

a processing unit connected to said quantitative fluorescence detector for determining a summated total energy of the fluorescence emitted from each of said irradiatable sperm cells subjected to multiple pulses from the pulsed coherent laser and distinguishing between a X-chromosome bearing sperm and a Y-chromosome bearing sperm based on said summated total energy of the fluorescence emitted.

68. The flow cytometry system according to claim 67. wherein said pulsed coherent radiation emitter comprises a pulsed laser.

69. The flow cytometry system according to claim 67 or 68, further comprising a sperm cells sorter able to sort sperm cells at a sort rate selected from the group consisting of greater than 1000 cells per second; greater than 1500 cells per second; greater than 2000 cells per second; and greater than 3000 cells per second.

70. The flow cytometry system according to any one of claims 67 to 69, further comprising a radiation beam manipulator.

71. The flow cytometry system according to claim 70, wherein said radiation beam manipulator is selected from a group consisting of mirrors, deflectors, beam splitters, prisms, refractive objects, lenses and filters.

72. The flow cytometry system according to any one of claims 67 to 71, wherein said fluorescence detector comprises a photomultiplier tube.

73. The flow cytometry system according to any one of claims 67 to 72, wherein said pulsed coherent radiation emitter comprises a fluorescence activation wavelength.

74. The flow cytometry system according to claim 73, wherein said fluorescence activation wavelength comprises 355 nm.

75. The flow cytometry system according to claim 67, wherein the power of said pulsed coherent radiation emitter is selected from the group consisting of less than 300 milliwatt less than 350 milliwatt; less than 200 milliwatt; and less than 175 milliwatt.

76. The flow cytometry system according to any one for claims 67 to 75, wherein said sperm cells fluorescence quantitative detector detects between two different sperm cells,

77. The flow cytometry system according to any one of claims 67 to 76, further comprising a sperm cell collector comprising a collector of at least two populations of sperm cells and the cell fluorescence quantitative detector detects between two sperm cells.

78. The flow cytometry system according to claim 77, wherein said collector of at least two populations of sperm cells comprises a X chromosome bearing sperm collector and a Y chromosome bearing sperm collector.

79. The flow cytometry system according to claim 77 or 78, wherein the obtained purity of said sorted populations is selected from a group consisting of greater than 85% purity; greater than 90% purity; greater than 95% purity; greater than 96% purity; and greater than 98% purity.

80. The flow cytometry system according to any one of claims 67 to 79, further comprising sorting a sample at a resolution selected from a group consisting of greater than 7.0; greater than 7.5; greater than 8.0; greater than 8.5; greater than 9.0; and greater than 9.2.

81. The flow cytometry system according to any one of claims 67 to 80, wherein a sample is sorted at a coincidence rate selected from the group consisting of less than 4400; less than 4000; less than 3700; and less than 3600.

82. The flow cytometry system according to claim 67 further comprising:

a drop charge circuit to apply an electrical condition to a stream of said irradiatable stained sperm cells;

a first and second deflection plate each disposed on opposite sides of a free fall;

an area in which a drop forms, wherein said first and second deflection places are oppositely charged; and

a sperm cells collector.

83. The flow cytometry system according to any one of claims 67 to 82, further comprising an event rate between about 10,000 to about 60,000 particles per second.

84. The flow cytometry system according to any one of claims 67 to 83, wherein said pulsed coherent radiation emitter is configured to emit intermittently punctuated radiation having a radiation on time between about 5 to about 20 picoseconds.

85. The flow cytometry system according to claim 84, wherein said pulsed coherent radiation emitter has a radiation off time between about 0.5 to about 20 nanoseconds.

86. The flow cytometry system according to claim 85, wherein the radiation on time and the radiation off time form a cycle and the cycle can total between about 2 to about 10 microseconds.

87. The flow cytometry system according to claim 86, wherein said cycle can have a repetition rate between about 50 to about 200 MHz.

88. The flow cytometry system according to claim 87, wherein said repetition rate comprises up to about 80 MHz.

89. The flow cytometry system according to claim 82, wherein said sperm cells comprises sperm cells selected from a group consisting of mammal sperm cells, bovine sperm cells, equine sperm cells, porcine sperm cells, ovine sperm cells, camelid sperm cells, ruminant sperm cells, and canine sperm cells.

90. The flow cytometry system according to claim 82, wherein said sperm cells collector is selected from the group consisting of multiple containers and a combined collector having individual containers.

91. The flow cytometry system according to claim 90, further comprising a number of selected sperm cells containers that is less than the number of nozzles.

92. The flow cytometry system according to claim 68, wherein said pulsed laser is selected from the group consisting of Nd:YAG and Nd:YVO4.

93. The flow cytometry system according to any one of claims 67 to 92, further comprising an individual or composite nozzle control.

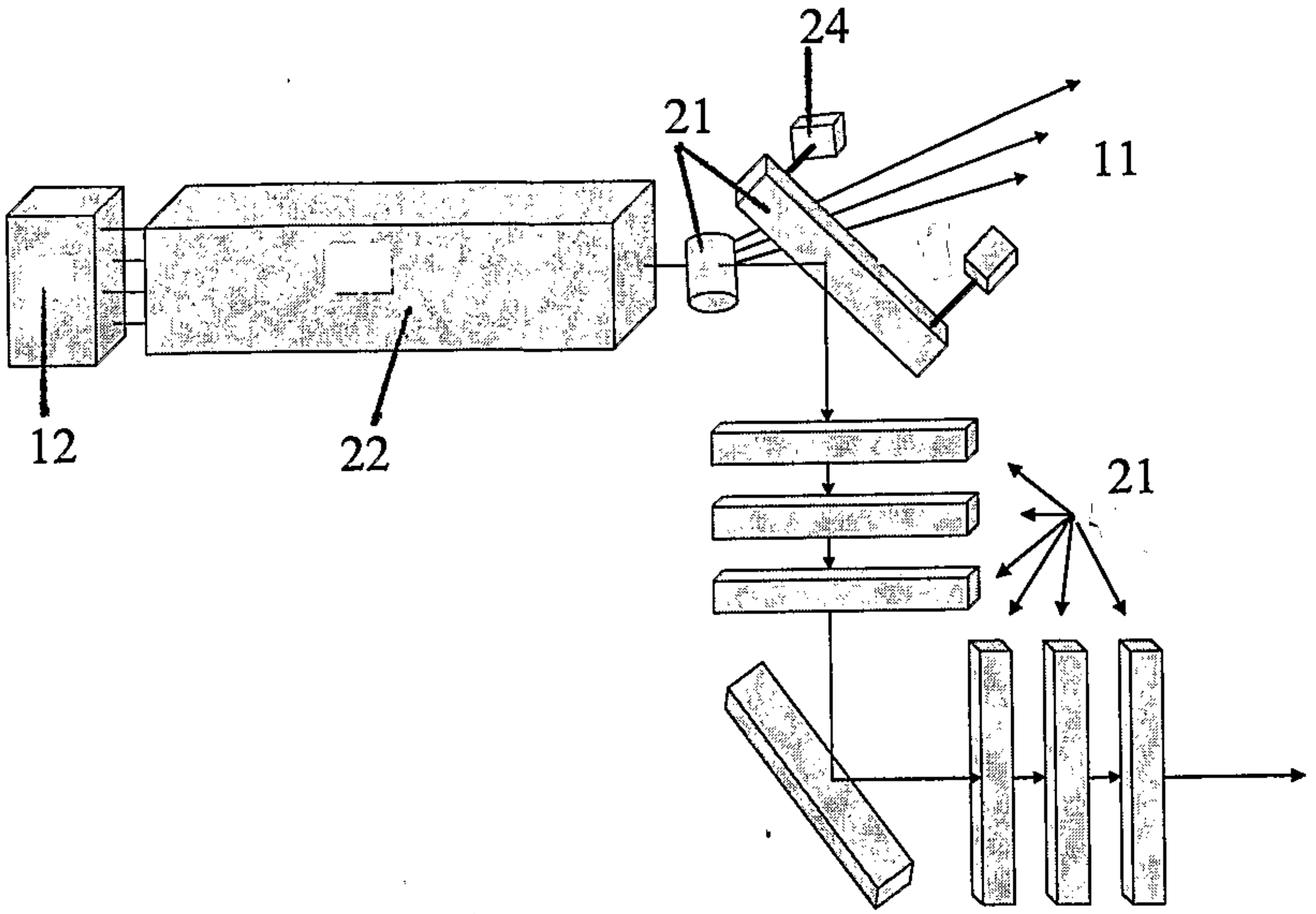


FIG. 1

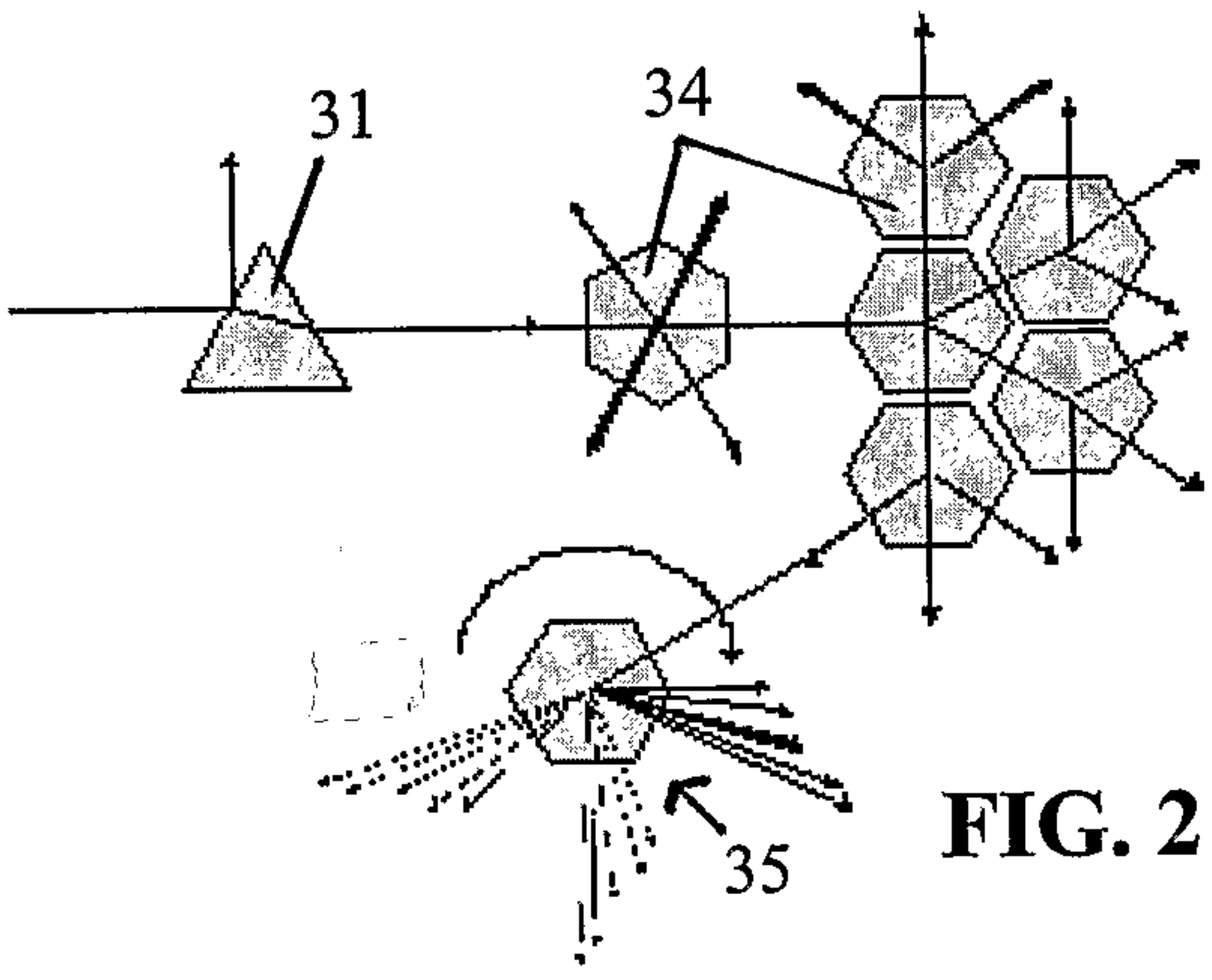


FIG. 2

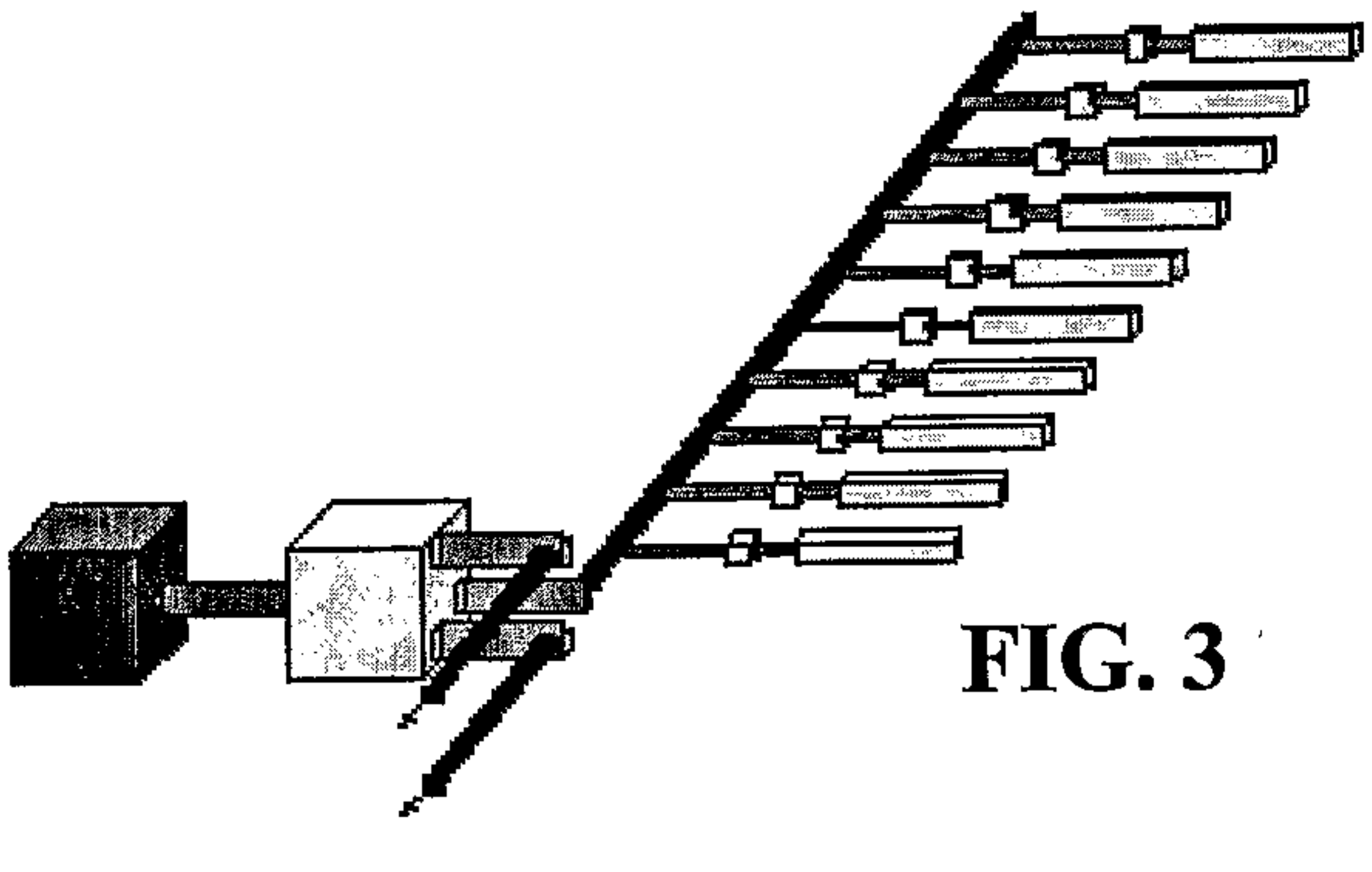


FIG. 3

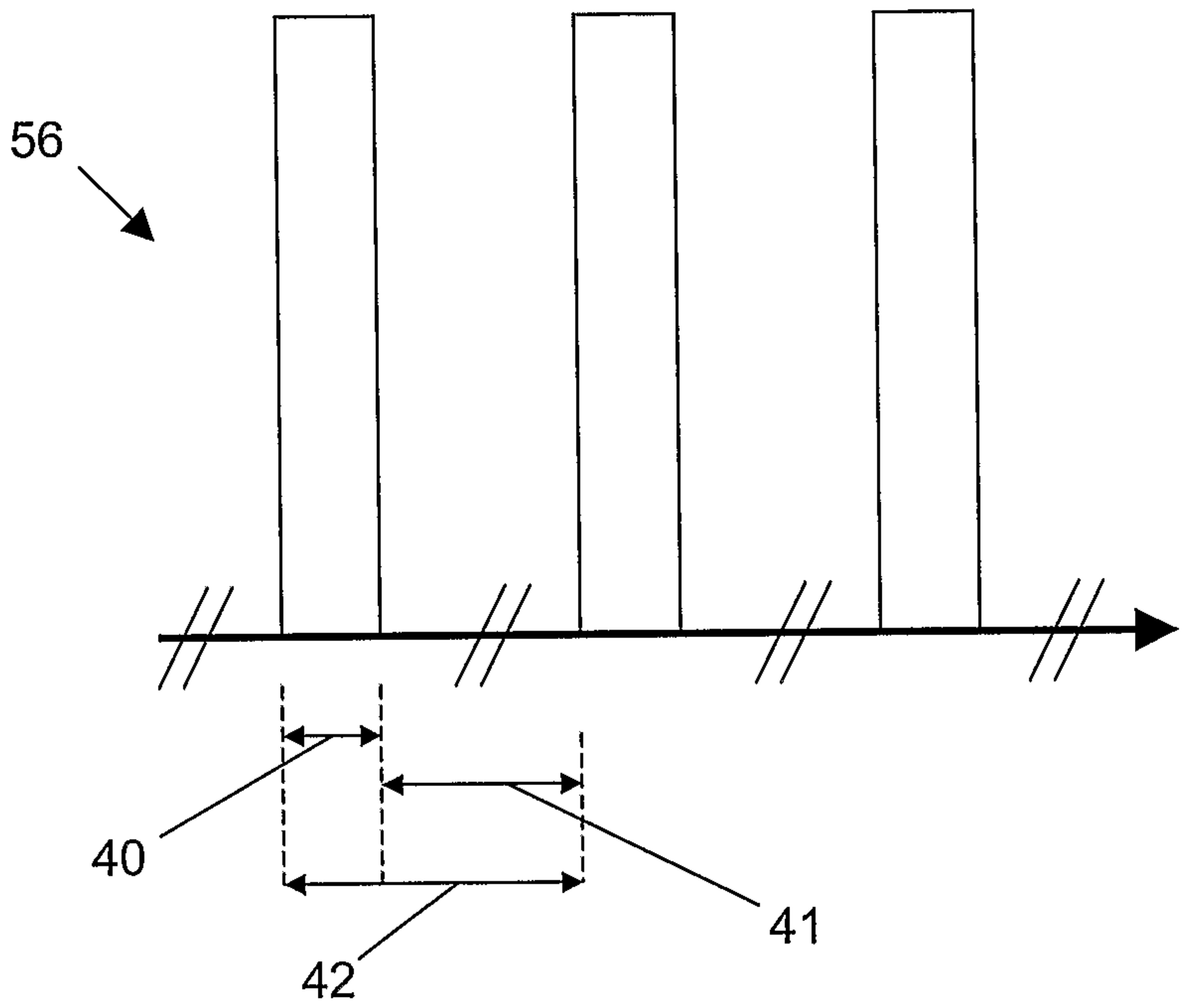


FIG. 4

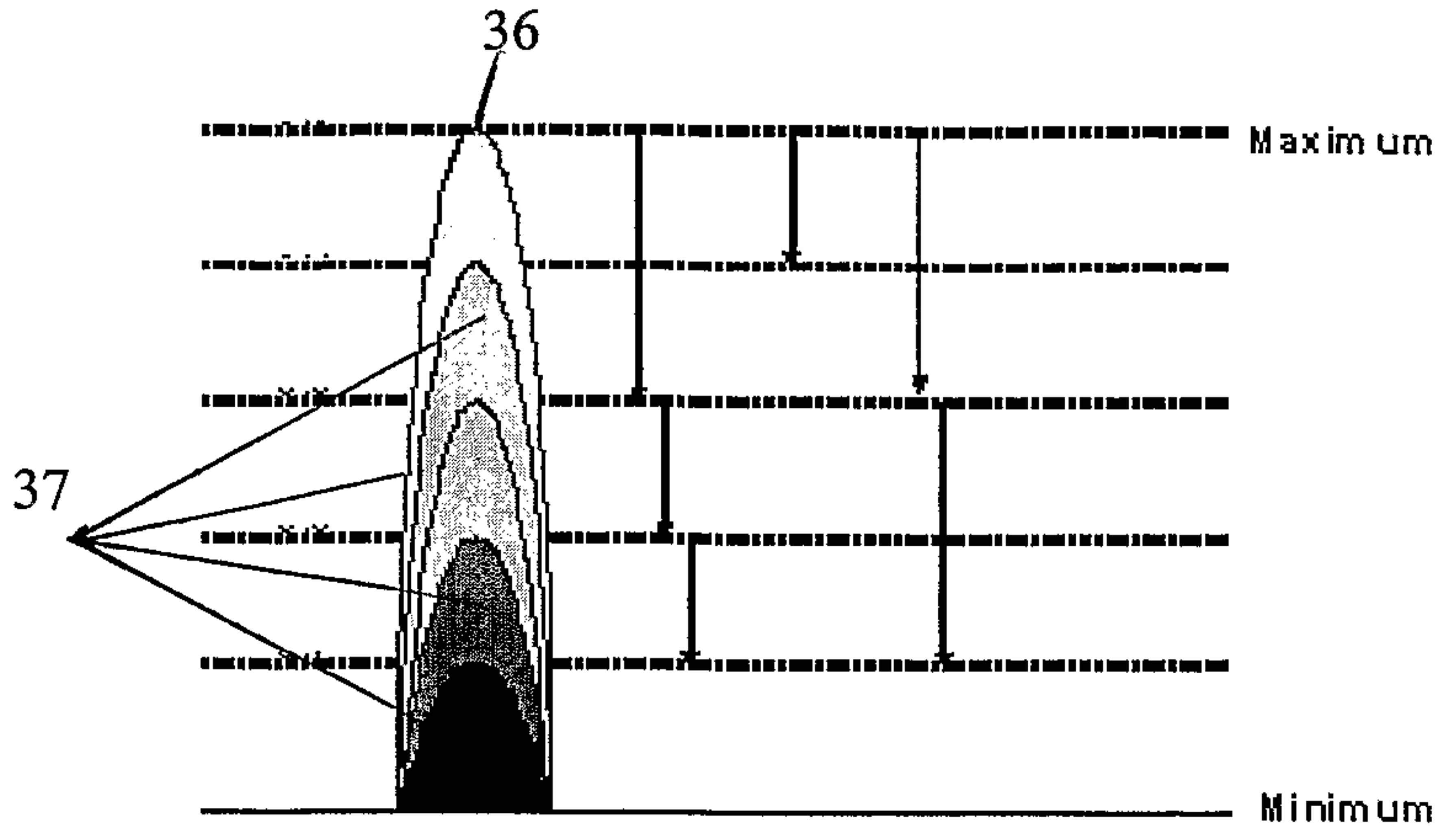


FIG. 5

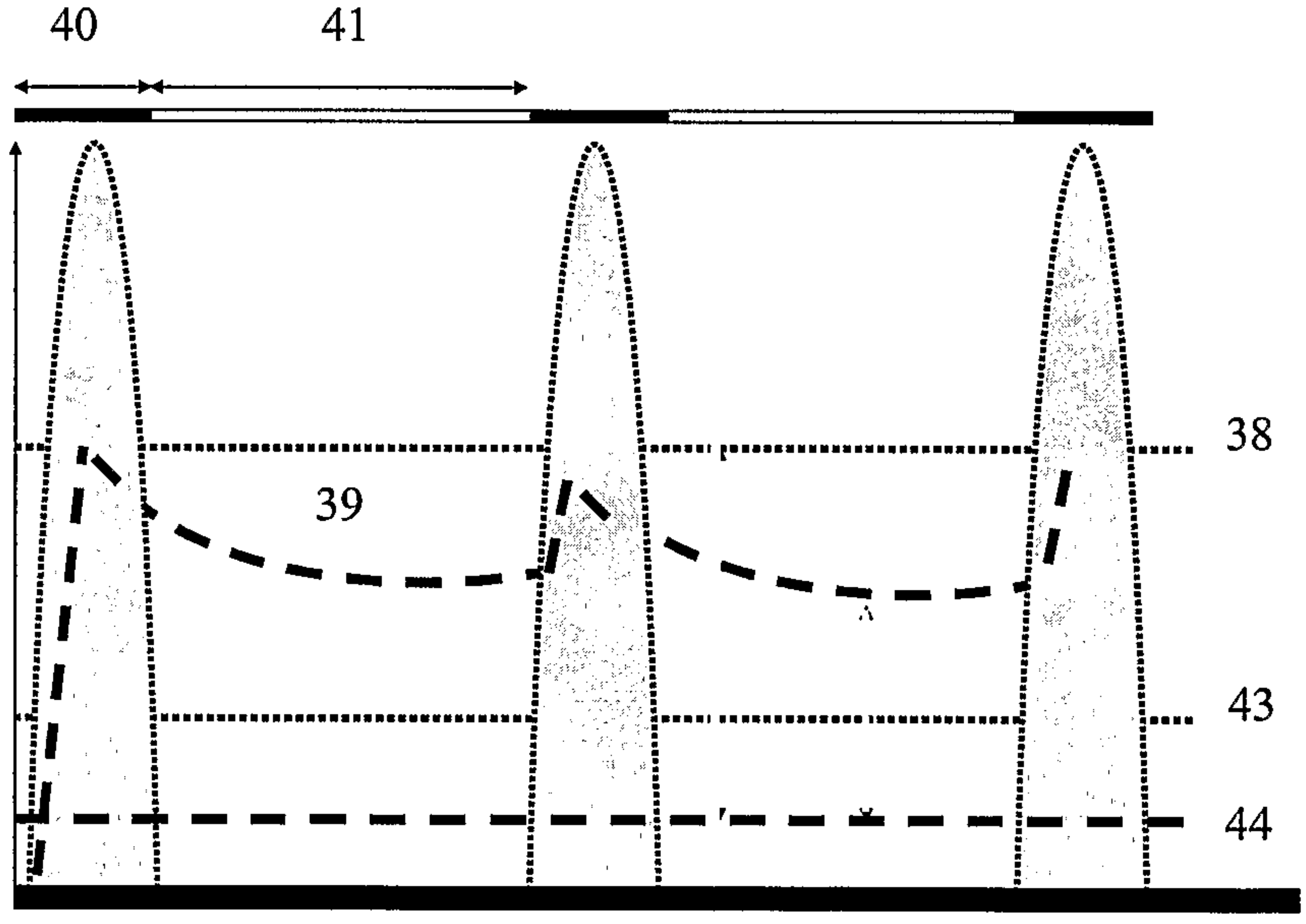


FIG. 6

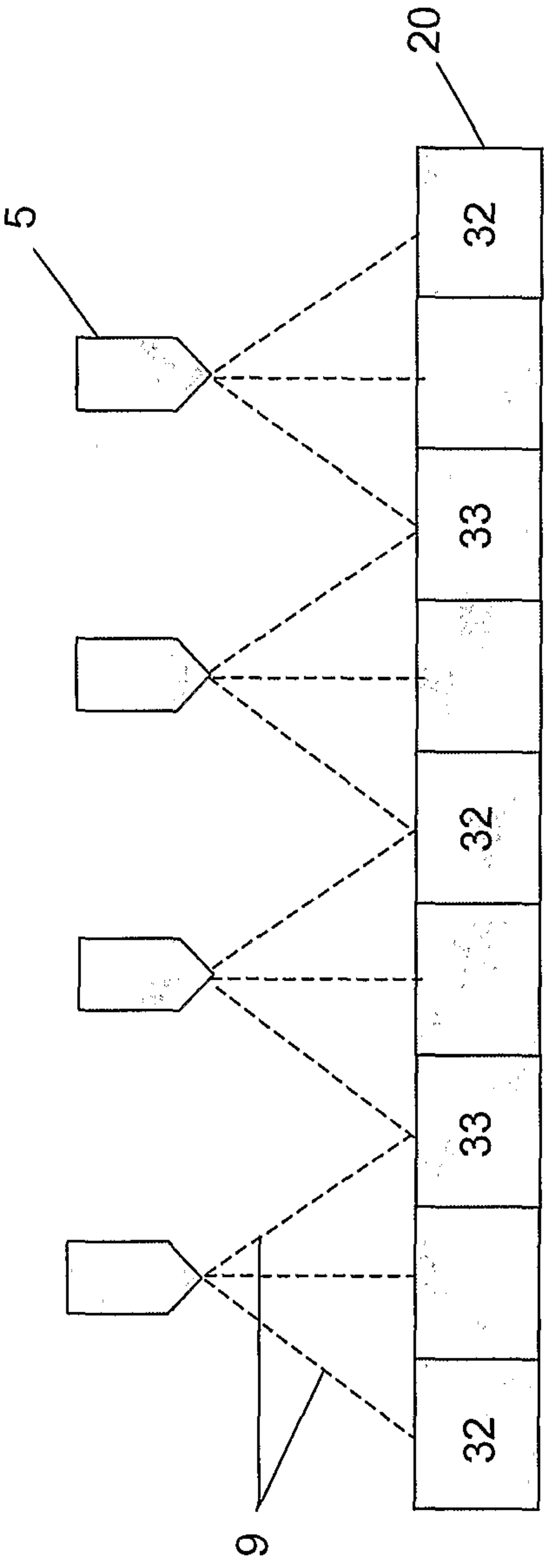


FIG. 7

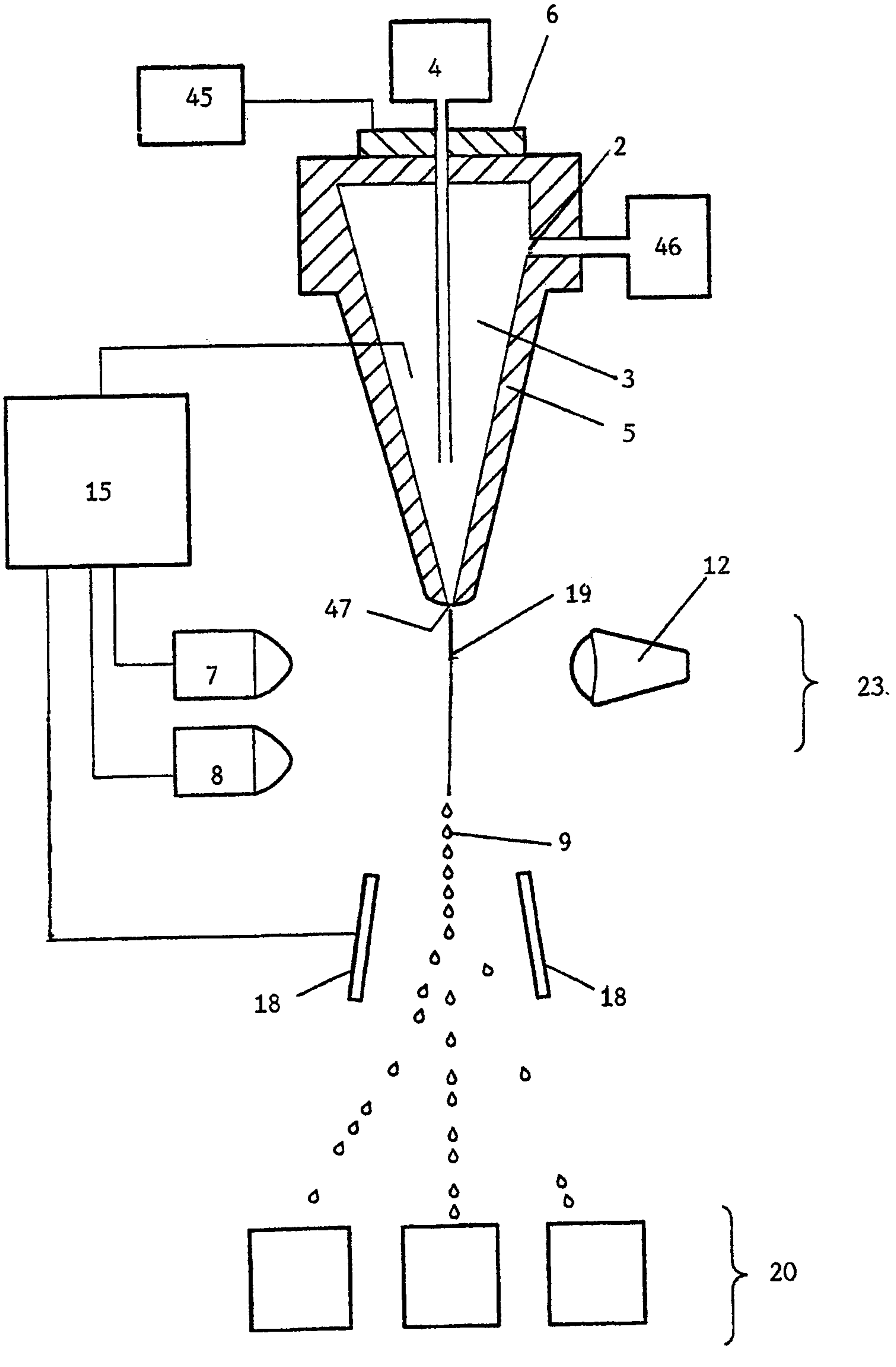


FIG. 8

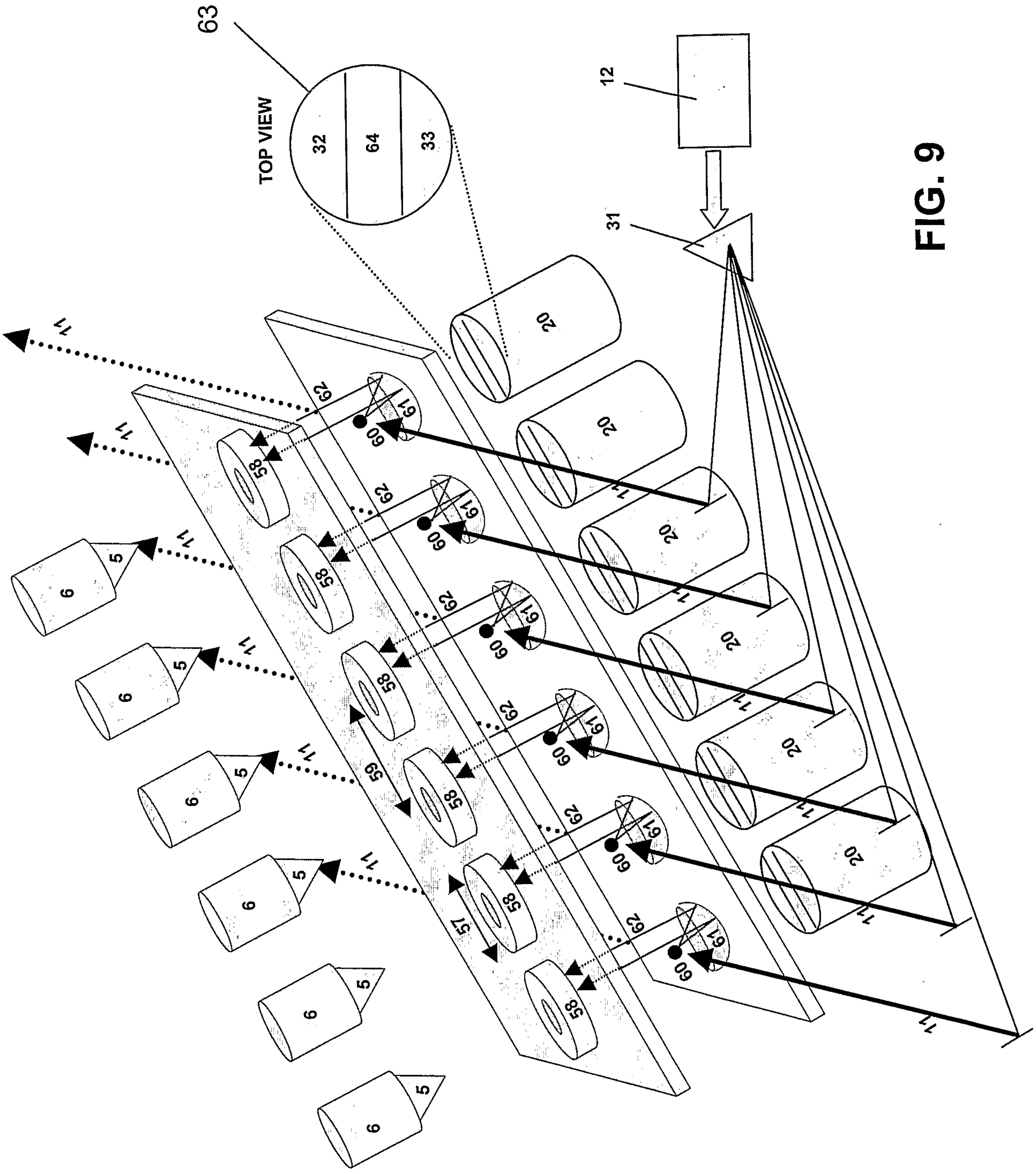


FIG. 9

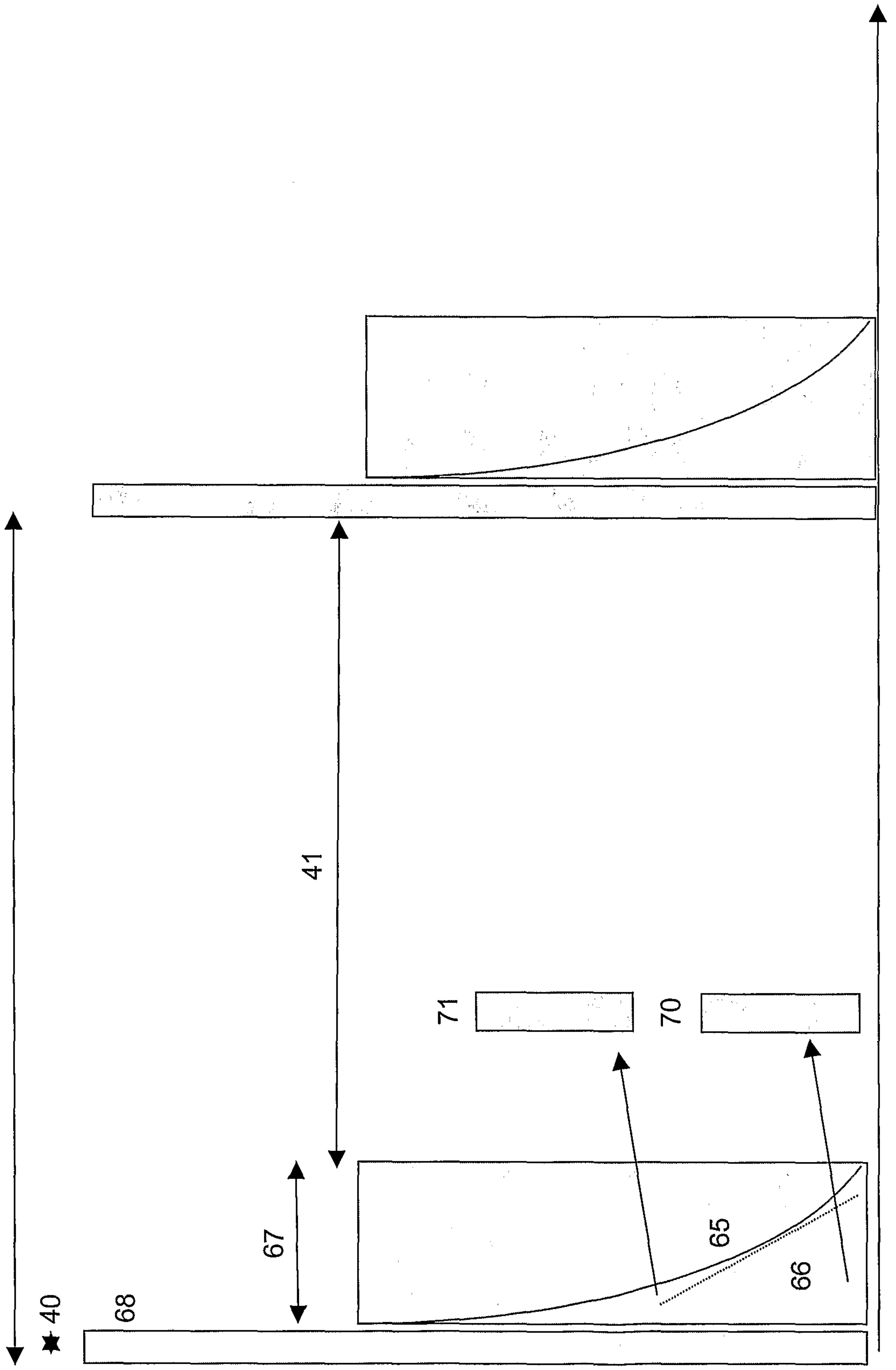


FIG. 10

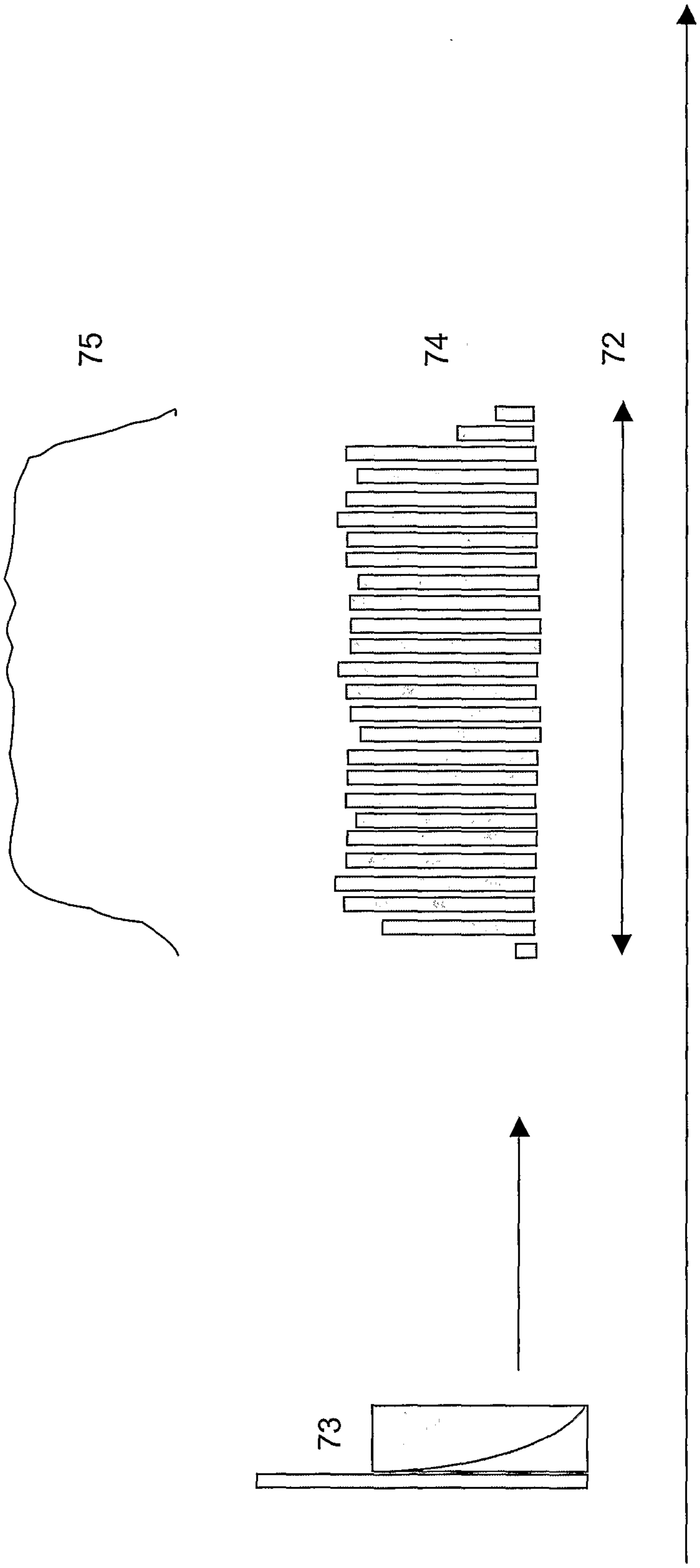


FIG. 11

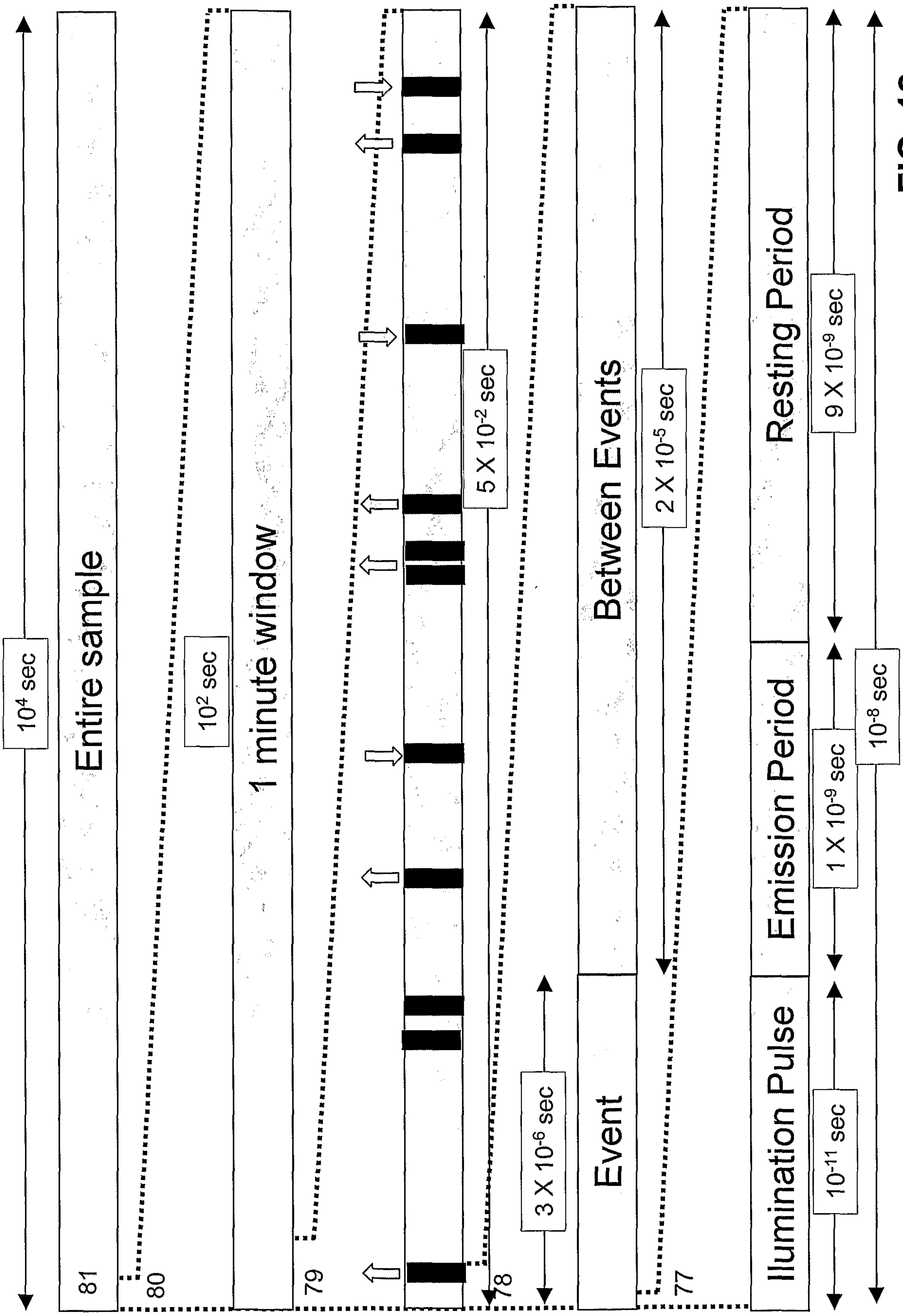

Entire sample
10^4 sec
81
1 minute window
10^2 sec
80
79
5×10^{-2} sec
78
Event
3×10^{-6} sec
Between Events
2×10^{-5} sec
77
Ilumination Pulse
10^{-11} sec
Emission Period
1×10^{-9} sec
Resting Period
9×10^{-9} sec
10^{-8} sec

FIG. 12

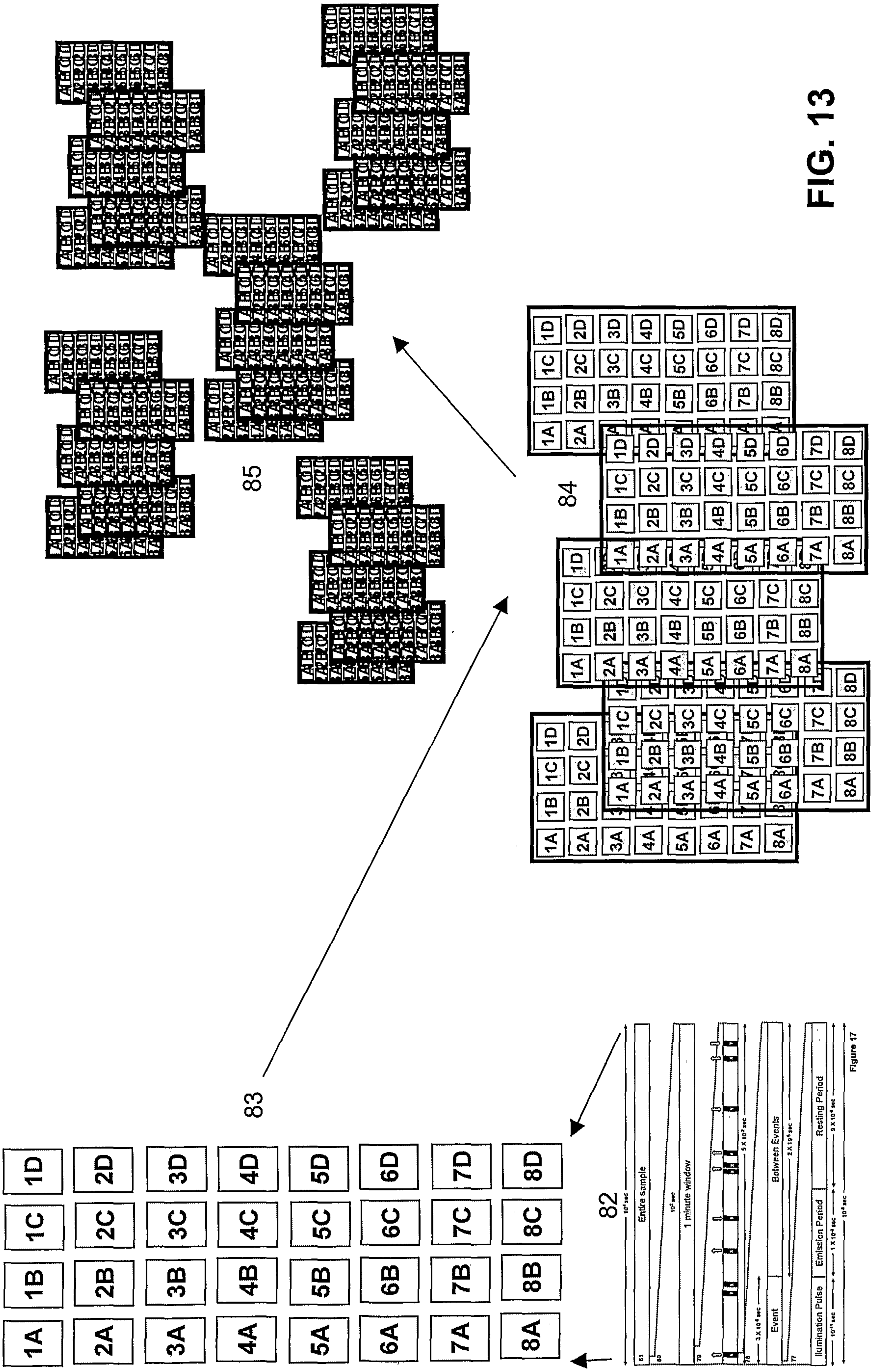

82
83
84
85
Entire sample
1 minute window
Between Events
Event
Illumination Pulse
Emission Period
Resting Period
Figure 17

FIG. 13

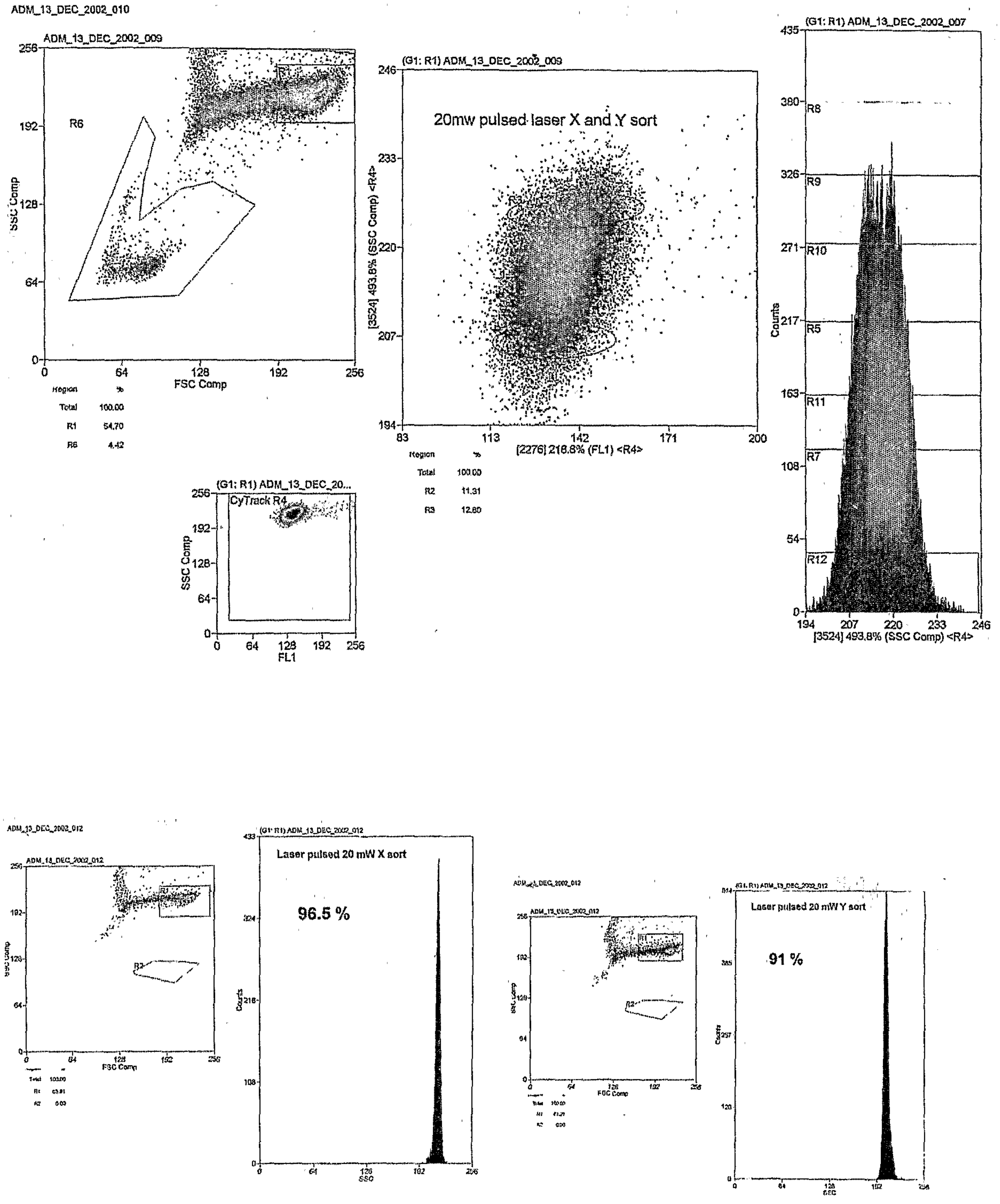

ADM_13_DEC_2002_010
ADM_13_DEC_2002_009
R1
R6
SSC Comp
FSC Comp
0
64
128
192
256
Region %
Total 100.00
R1 54.70
R6 4.42
(G1: R1) ADM_13_DEC_2002_009
20mw pulsed laser X and Y sort
[3524] 493.8% (SSC Comp) <R4>
[2276] 218.8% (FL1) <R4>
194
207
220
233
246
83
113
142
171
200
Region %
Total 100.00
R2 11.31
R3 12.80
(G1: R1) ADM_13_DEC_20...
CyTrack R4
FL1
(G1: R1) ADM_13_DEC_2002_007
435
380
326
271
217
163
108
54
R8
R9
R10
R5
R11
R7
R12
Counts
ADM_13_DEC_2002_012
(G1: R1) ADM_13_DEC_2002_012
Laser pulsed 20 mW X sort
96.5 %
433
324
216
108
SSC
R2
Total 100.00
Laser pulsed 20 mW Y sort
91 %

FIG. 14

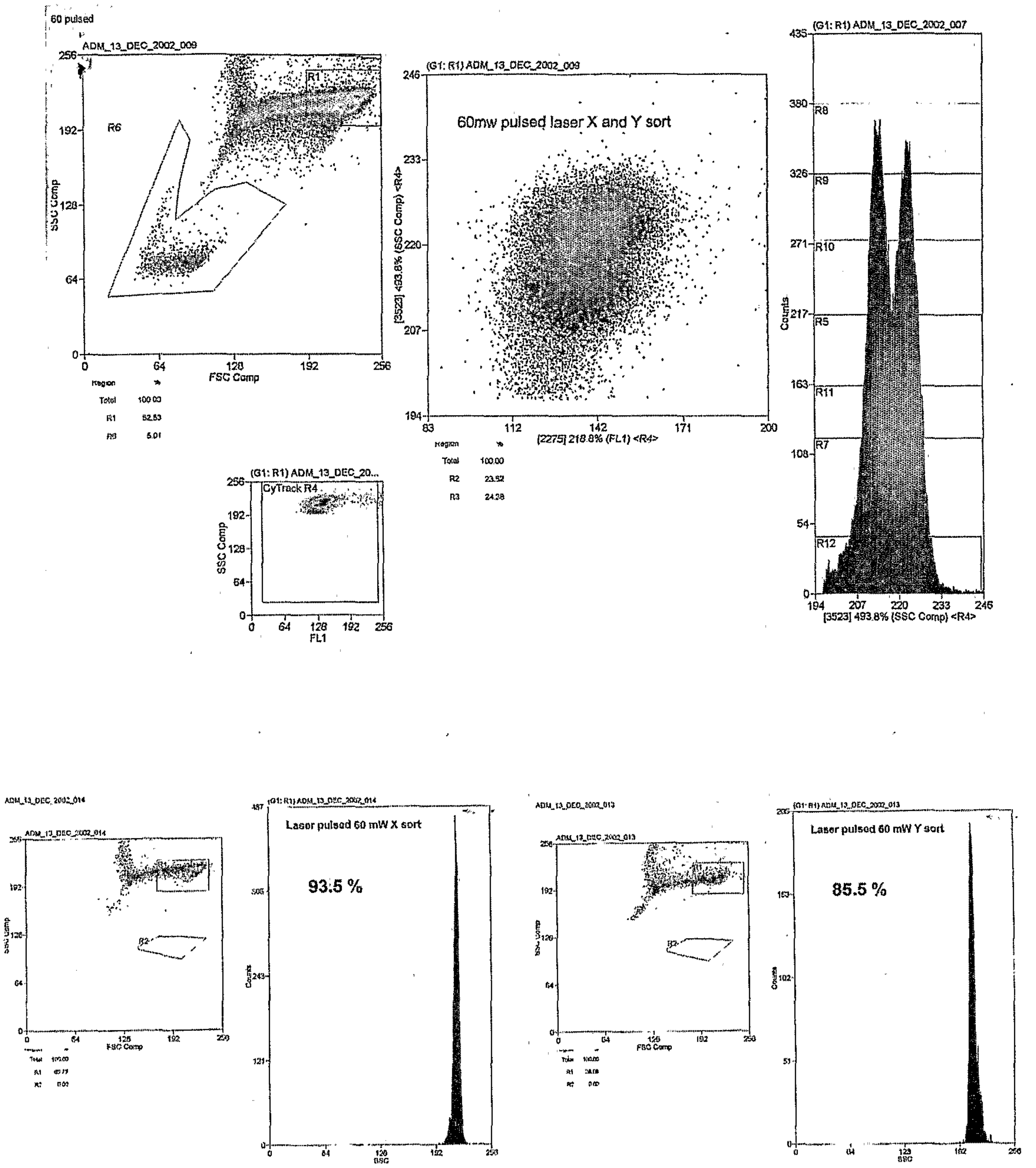

60 pulsed
ADM_13_DEC_2002_009
R1
R6
SSC Comp
FSC Comp
Region
Total 100.00
R1 52.53
R6 5.01
(G1: R1) ADM_13_DEC_2002_009
60mw pulsed laser X and Y sort
[3523] 493.8% (SSC Comp) <R4>
[2275] 218.8% (FL1) <R4>
Region
Total 100.00
R2 23.52
R3 24.28
(G1: R1) ADM_13_DEC_2002_007
R8
R9
R10
R5
R11
R7
R12
Counts
[3523] 493.8% (SSC Comp) <R4>
(G1: R1) ADM_13_DEC_20...
CyTrack R4
SSC Comp
FL1
ADM_13_DEC_2002_014
Laser pulsed 60 mW X sort
93.5 %
Counts
SSC
ADM_13_DEC_2002_013
Laser pulsed 60 mW Y sort
85.5 %
Counts
SSC
FSC Comp

FIG. 15

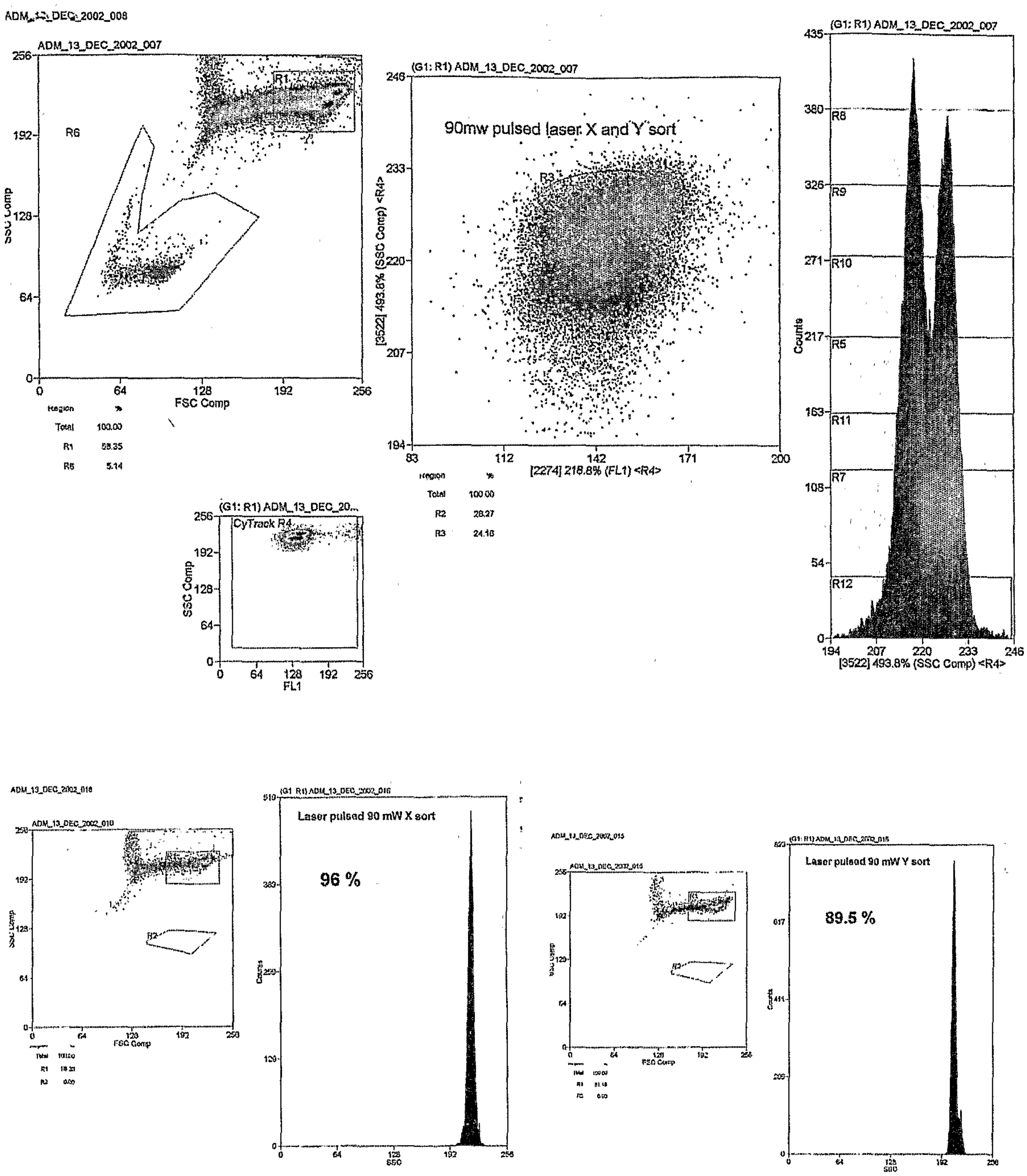

ADM_13_DEC_2002_008
ADM_13_DEC_2002_007
R1
R6
SSC Comp
FSC Comp
0
64
128
192
256
Region %
Total 100.00
R1 58.35
R6 5.14
(G1: R1) ADM_13_DEC_2002_007
90mw pulsed laser X and Y sort
R3
[3522] 493.8% (SSC Comp) <R4>
[2274] 218.8% (FL1) <R4>
83
112
142
171
200
194
207
220
233
246
Region %
Total 100.00
R2 28.27
R3 24.16
(G1: R1) ADM_13_DEC_20...
CyTrack R4
FL1
(G1: R1) ADM_13_DEC_2002_007
Counts
435
380
326
271
217
163
108
54
R8
R9
R10
R5
R11
R7
R12
ADM_13_DEC_2002_010
Laser pulsed 90 mW X sort
96 %
ADM_13_DEC_2002_015
Laser pulsed 90 mW Y sort
89.5 %
R2

FIG. 16

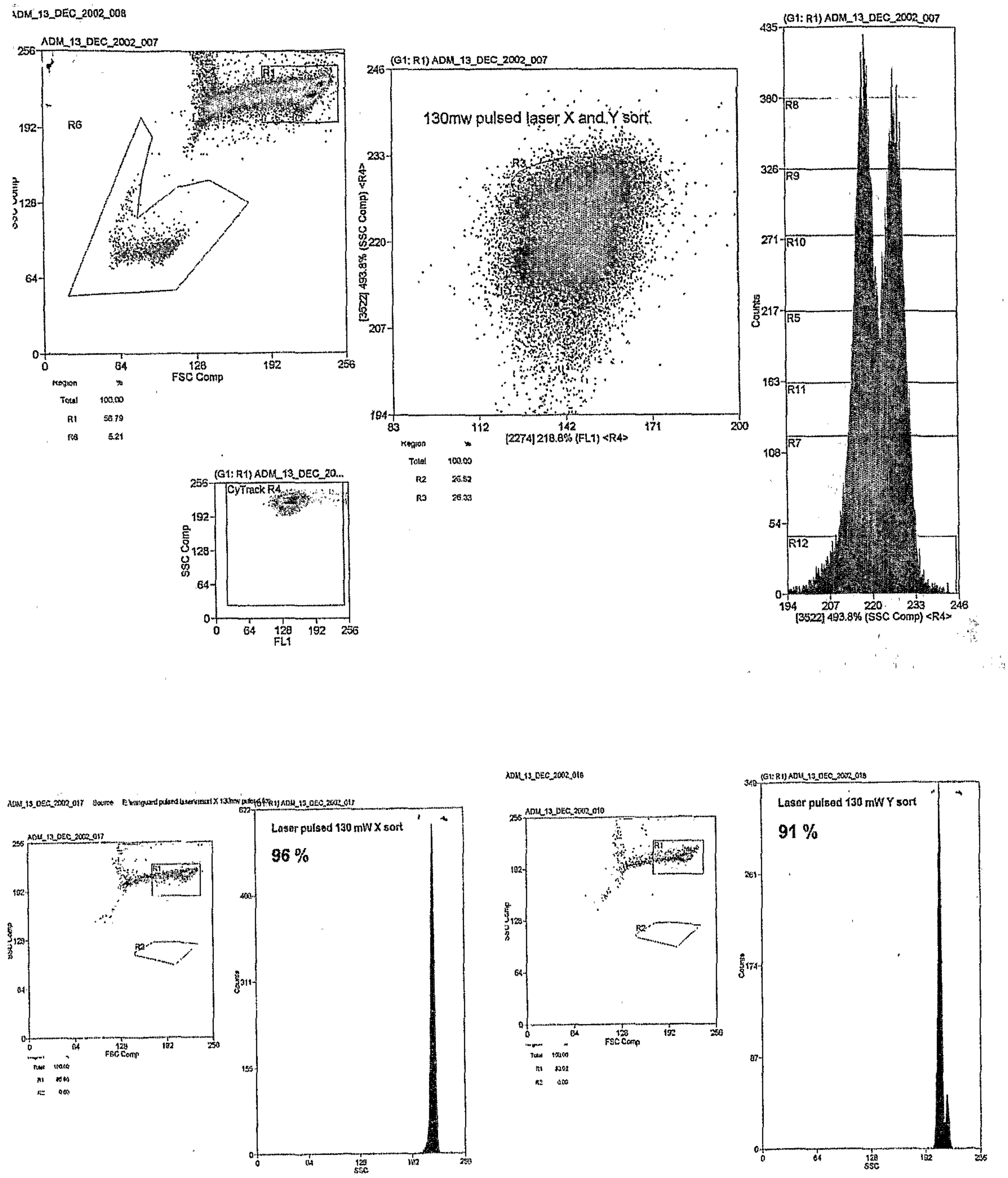

ADM_13_DEC_2002_008
ADM_13_DEC_2002_007
R1
R6
SSC Comp
FSC Comp
Region
Total 100.00
R1 56.79
R6 5.21
(G1: R1) ADM_13_DEC_2002_007
130mw pulsed laser X and Y sort.
R3
[3522] 493.8% (SSC Comp) <R4>
[2274] 218.8% (FL1) <R4>
Region
Total 100.00
R2 26.52
R3 26.33
(G1: R1) ADM_13_DEC_20...
CyTrack R4
FL1
R8
R9
R10
R5
R11
R7
R12
Counts
ADM_13_DEC_2002_017
(G1: R1) ADM_13_DEC_2002_017
Laser pulsed 130 mW X sort
96 %
R2
Counts
SSC
ADM_13_DEC_2002_018
ADM_13_DEC_2002_018
(G1: R1) ADM_13_DEC_2002_018
Laser pulsed 130 mW Y sort
91 %

FIG. 17

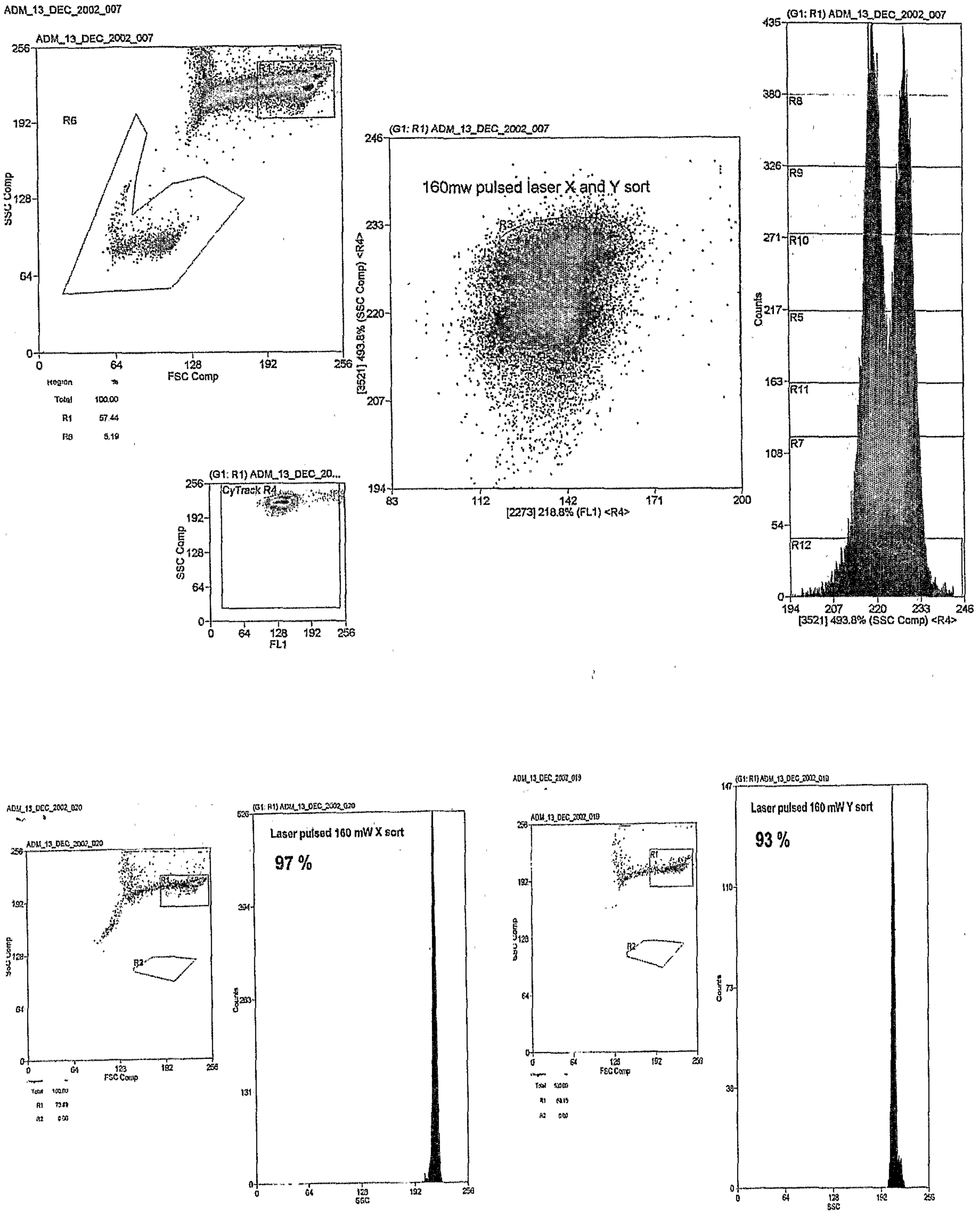
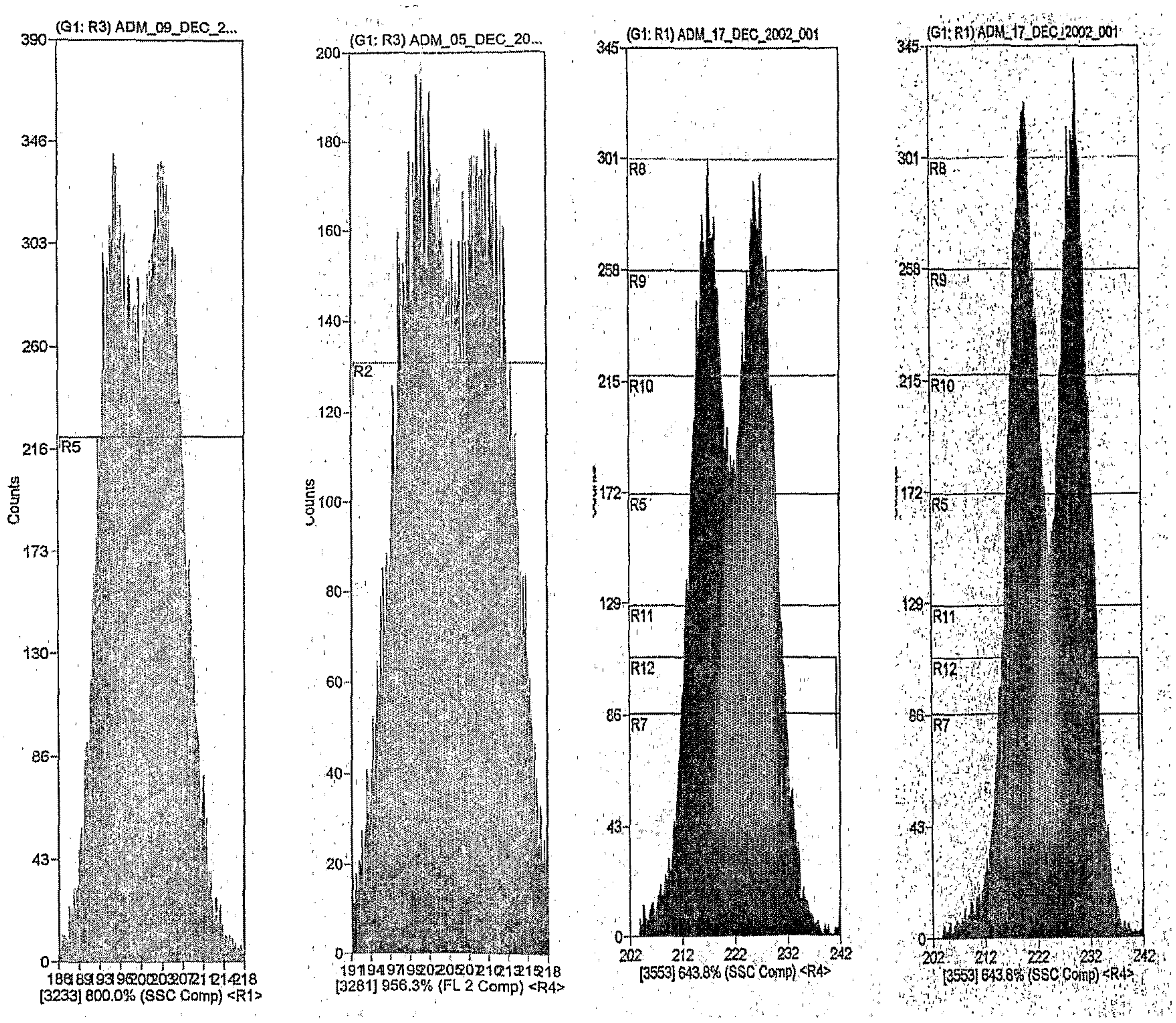

ADM_13_DEC_2002_007
ADM_13_DEC_2002_007
R1
R6
SSC Comp
FSC Comp
0
64
128
192
256
Region
%
Total 100.00
R1 57.44
R6 5.19
(G1: R1) ADM_13_DEC_20...
CyTrack R4
SSC Comp
FL1
(G1: R1) ADM_13_DEC_2002_007
160mw pulsed laser X and Y sort
R3
[3521] 493.8% (SSC Comp) <R4>
[2273] 218.8% (FL1) <R4>
246
233
220
207
194
83
112
142
171
200
(G1: R1) ADM_13_DEC_2002_007
Counts
435
380
326
271
217
163
108
54
R8
R9
R10
R5
R11
R7
R12
[3521] 493.8% (SSC Comp) <R4>
ADM_13_DEC_2002_020
ADM_13_DEC_2002_020
SSC Comp
FSC Comp
R1
R2
Total 100.00
R1 73.49
R2 0.50
(G1: R1) ADM_13_DEC_2002_020
Laser pulsed 160 mW X sort
97 %
Counts
526
394
263
131
SSC
ADM_13_DEC_2002_019
ADM_13_DEC_2002_019
SSC Comp
FSC Comp
R1
R2
Total 100.00
R1 69.15
R2 0.00
(G1: R1) ADM_13_DEC_2002_019
Laser pulsed 160 mW Y sort
93 %
Counts
147
110
73
36
SSC

FIG. 18



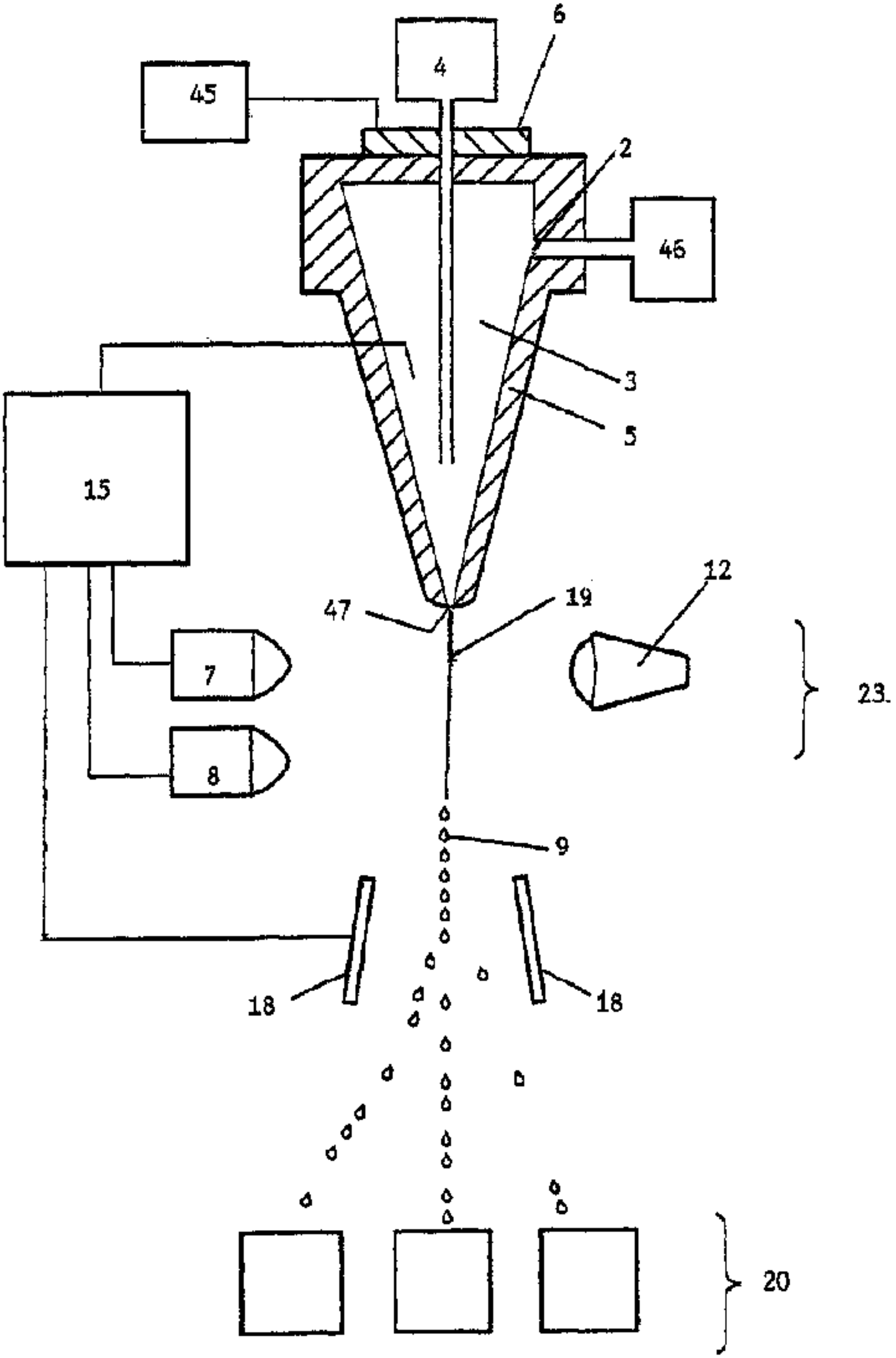
150mW CW
Bonnie

150mW CW
Elliott

70mW
Pulsed
Vanguard

130mW
Pulsed
Vanguard

FIG. 19


6
4
45
2
46
3
5
15
12
47
19
7
23.
8
9
18
18
20