



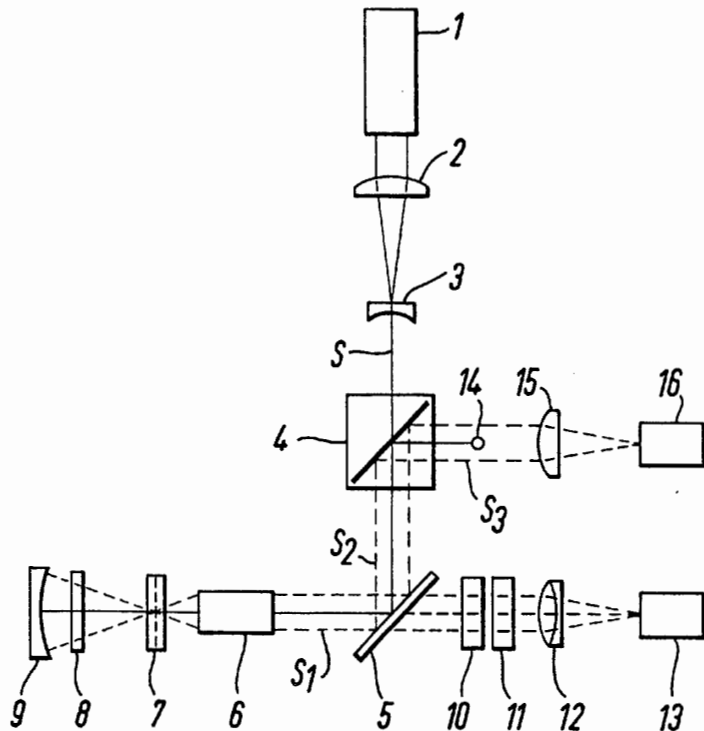
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁵ : G01N 15/14, 21/47, 21/64</p>	<p>A1</p>	<p>(11) International Publication Number: WO 94/29695 (43) International Publication Date: 22 December 1994 (22.12.94)</p>
<p>(21) International Application Number: PCT/NO94/00106 (22) International Filing Date: 8 June 1994 (08.06.94) (30) Priority Data: 932088 8 June 1993 (08.06.93) NO (71)(72) Applicants and Inventors: GJELSNES, Oddbjørn [NO/NO]; Svaneveien 29A, N-1187 Oslo (NO). TANGEN, Reidar [NO/NO]; Herregårdsveien 57B, N-1168 Oslo (NO). (74) Agent: ONSAGERS PATENTKONTOR A.S.; P.O. Box 265 Sentrum, N-0165 Oslo (NO).</p>	<p>(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>In English translation (filed in Norwegian).</i></p>	

(54) Title: LIQUID FLOW CYTOMETER

(57) Abstract

A liquid flow cytometer for counting and classification of particles such as biological cells and other microscopic particles in liquids, where the cytometer comprises an optical system for irradiation and detection in a metering zone (7), thus enabling optical dispersion and fluorescence caused by the particles to be simultaneously detected when the particles pass the metering zone. The optical system comprises in a first beam path (S₁) a mirror reflector (9) with its focal point in the metering zone (7), a quarter-wave plate (8) between the metering zone (7) and the mirror reflector (9), a lens (6) with its focal point in the metering zone (7) and located on the opposite side of it together with a dichroic mirror (5) behind the lens and provided in such a manner that it gives reflection to a second beam path (S₂). In the second beam path (S₂) a beam splitter (4) is provided between the dichroic mirror (9) and a known per se light source (1), and produces a third beam path (S₃). The cytometer comprises a first detector (13) provided in the first beam path (S₁) behind the dichroic mirror (5) and arranged for the detection of fluorescence emitted by the particles in the metering zone (7), together with a second detector (16) provided in the third beam path (S₃) and arranged for the detection of optical dispersion caused by the particles in the metering zone (7).



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Liquid flow cytometer

The invention concerns a liquid flow cytometer for counting and classification of particles such as biological cells or other microscopic particles in liquids, where the cytometer comprises an optical system for irradiation and detection in a metering zone, thus enabling optical dispersion and fluorescence caused by the particles to be simultaneously detected when the particles pass the metering zone.

The use of cytometers based on optical dispersion is known in the prior art for counting and classification of microscopic particles in a liquid. This prior art is illustrated schematically in figs. 1-3, where fig. 1 shows the flow system with a flow cell, fig. 2 shows a hydrodynamic focusing device assigned to the flow cell, and fig. 3 shows the detector device for optical dispersion caused by particles in the flow cell. In fig. 1 the sample is fed into a flow cell together with a transport liquid in the form of filtered water which is pumped into the flow cell from a container. The transport liquid and the sample, i.e. those particles which have to be counted and classified pass through the flow cell, a pump system and a filter which removes particles, after which the transport liquid, i.e. filtered water, is passed back to the container. In the flow cell there is provided a hydrodynamic focusing device in flow connection with the actual flow cell which is composed of a capillary tube. The hydrodynamic focusing device is in the form of a conical nozzle which tapers towards and leads into the capillary tube. The sample is passed into the nozzle through a thin tube in the centre of the nozzle and is carried along with the transport water in such a manner that the sample is accelerated towards the mouth of the nozzle and the capillary tube. By directing a focused beam of preferably monochromatic, coherent light onto the flow cell, the particles in the liquid will cause a scattering or dispersion of the light. The scattered light is focused by a lens device on to a photodetector and the intensity of the detected, scattered light will be a measure of the degree of dispersion which in turn is related to the number of particles in the metering zone and their size. In order to prevent the light from the laser beam from entering the detector there is positioned between the flow cell and the focusing device a beam stop which forms a shield against the direct radiation from the laser, but which substantially does not impair the scattered light.

A cytometer which is substantially based on the above-mentioned principles and is suitable for detection, counting and classification of biological cells and other microscopic particles in liquids is known from Norwegian patent application No. 91 424, which hereby is incorporated as reference.

5 There are also known cytometers and similar instruments for the detection of particles based on the use of fluorescence which is emitted by the particles when they are irradiated with light from a light source. One example of a detector of this kind is known from EP-A1-0539 743, in which there is disclosed a collimator in the form of a parabolic reflector 40 which permits a more efficient use
10 of bandpass filters in order to block scattered radiation during detection.

The need for stable, reliable and sensitive analytical instruments for the determination of microbial and cellular content in liquids such as water, beverages, oils and bodily fluids is constantly increasing.

15 By means of liquid flow cytometry biological particles which are in suspension can be counted and classified in a rapid and reliable manner. As mentioned above, according to this method the particle sample is fed along with a transport liquid and care is normally taken to ensure that the particles pass substantially one at a time through the flow cell where they are subjected to the impact of an intense beam of light, the result of which is that the particles can be counted
20 individually and measured with regard to light scatter and fluorescence. At present, liquid flow cytometry is routinely employed principally for the analysis of white blood cells (leukocytes), and in the field of bacteriology liquid flow cytometry has only been used for research purposes.

25 Cells such as leukocytes are very large in proportion to microbes such as, e.g., enterobacteria. Consequently the requirement for stability and sensitivity for a liquid flow cytometer which is to be routinely employed in the field of microbiology is therefore much greater than for a cytometer for use in haematology.

30 Thus a first object of the present invention is to provide a liquid flow cytometer which is sufficiently stable and sensitive to enable it to be generally employed in the field of microbiology, but which can naturally also be used in the known manner for counting and classification of blood cells in mammals.

A second object of the present invention is to provide a liquid flow cytometer which provides an improved irradiation of the particles and a better collection both of scattered light and fluorescent light caused by the particles, thus enabling the sensitivity to be increased.

- 5 A further object of the present invention is to provide a liquid flow cytometer which can be used simultaneously for the detection of both optical dispersion and fluorescence, without the need for a complicated optical system for this purpose.

10 Yet another object of the present invention is therefore to simplify the optical system and to integrate detectors and optical system, thus providing a liquid flow cytometer with fewer optical components and reduced physical dimensions, which in turn will help to improve the instrument's stability and also lead to lower production costs.

15 The above-mentioned and other objects are achieved according to the present invention with a liquid flow cytometer which is characterized in that the optical system in a first beam path comprises a mirror reflector with its focal point in the metering zone, a quarter-wave plate between the metering zone and the mirror reflector, a lens with its focal point in the metering zone and located on the opposite side of it, together with a dichroic mirror behind the lens and
20 arranged in such a manner that it gives a partial reflection substantially orthogonally to the axis of the lens, the reflection direction being in a second beam path of the optical system, and in the second beam path a beam splitter located between the dichroic mirror and an as per se known light source, the beam splitter producing a third beam path substantially orthogonally to the second
25 beam path, that the cytometer further comprises a first detector provided in the first beam path behind the dichroic mirror and arranged for the detection of fluorescence emitted by the particles in the metering zone, together with a second detector provided in the third beam path and arranged for the detection of optical dispersion caused by the particles in the metering zone.

- 30 Further features and advantages are presented in the attached independent claims.

The fluid flow cytometer according to the present invention will now be described in more detail in connection with an embodiment and with reference

to the drawing, where figs. 1-3 as mentioned above illustrate the prior art, while fig. 4 schematically illustrates an optical system for a fluid flow cytometer according to the present invention, and fig. 5 shows in rather more detail those components which form part of the fluid flow cytometer in fig. 4.

5 The optical system of a fluid flow cytometer according to the present invention is illustrated in fig. 4. Light from a laser 1 is focused and collimated to a beam S which passes through a beam splitter 4 in a beam path S_2 and is reflected by a dichroic filter or mirror 5 through the metering zone or flow cell 7 which here is in the form of a capillary tube and into an excitation amplifier. The capillary
10 tube 7 and excitation amplifier 9 are located in an additional beam path S_2 . The laser beam S passes through the flow cell 7 and returns from the excitation amplifier 9, since it is focused on the flow cell 7 by the excitation amplifier 9, and a lens 6 provided between the dichroic mirror and the flow cell respectively. Thus the laser beam passes twice through the flow cell and any particles in this
15 will now cause a light scatter and possibly also fluoresce. The fluorescent light passes through the dichroic mirror and continues in the beam path S_1 to a fluorescence detector, while the scattered light is reflected back to the beam path S_2 and on to the beam splitter where it is exposed to a further reflection of yet another beam path S_3 and ends in a detector for light scatter.

20 With this embodiment the object is achieved that two different detection principles can be employed in one and the same optical system, while at the same time an intensification is achieved of the light which is used for the two detection mechanisms.

The liquid flow cytometer according to the present invention is illustrated in
25 some more detail in fig. 5 where the three beam paths are indicated in the same way as in fig. 2. A light source 1, preferably a diode laser, emits a monochromatic, coherent beam S of polarized light and this beam S is focused and collimated by a lens device 2,3, which comprises a planoconvex lens 2 and planoconcave lens 3. The beam S passes through a polarizing beam splitter 4
30 which can be designed as a cubiform body and strikes a dichroic mirror or filter 5 which produces a beam path S_1 , substantially orthogonally to the beam path S_2 . The laser beam S reflected by the dichroic filter 5 is focused on the metering zone or flow cell 7 by a lens 6 which can be an ordinary microscope objective and thereafter passes a quarter-wave plate 8 located in the beam path S_1 , which
35 plate causes a polarizing rotation of the laser light by $\pi/4$. The light then strikes

an excitation amplifier in the form of the mirror reflector 9 which like the lens 6 has its focal point in the metering zone 7, the mirror reflector 9 which can preferably be a spherical concave mirror, once again reflecting the light through the quarter-wave plate 8, where it once more receives a polarizing rotation of $\pi/4$ and passes through the metering zone 7. The light from the laser 1 has thereby passed twice through the metering zone 7 and each time is scattered by the particles in the metering zone or causes these to emit fluorescent light. Both the scattered light and the fluorescent light pass through the lens 6 and strike the dichroic mirror 5. The scattered light which is rotated 90° in relation to the laser beam's polarization angle, is reflected by the dichroic mirror 5 and passed back along the beam path S_2 to the beam splitter 4 where due to the altered polarization plane it is reflected to a further beam path S_3 . In the beam path S_3 there is provided a beam stop 14 which causes the narrow, collimated laser beam to be blocked, while the scattered light passes substantially unimpaired to a lens device 15, e.g. in the form of a planoconvex lens with a long focal length, which focuses the scattered light on a detector 16 for detection of the optical dispersion. A photodiode, for example a PIN diode, is preferably used as the detector 16.

If those particles which have to be detected are fluorescent, they will as mentioned also emit fluorescent light. If particles are not intrinsically fluorescent, they can be tagged with one or more fluorochromes which have an absorption corresponding to the wavelength of the light source and in both cases the particles will fluoresce when they pass through the metering zone 7 and are irradiated by the laser beam. The fluorescent light is intercepted directly by the lens 6 and indirectly via the mirror reflector 9. The fluorescent light will have a longer wavelength than the excitation light, i.e. the laser beam, and passes unreflected through the dichroic mirror 5 and continues on the beam path S_1 to a fluorescence detector 13 which preferably employs a photodiode and particularly a silicon avalanche diode. In order to eliminate any remains of scattered light which have passed through the dichroic mirror 5 and continued on the beam path S_1 , there are provided optical filters 10,11 in the beam path S_1 , between the dichroic mirror 5 and a lens device 12 which focuses the fluorescent light on the detector 13. The optical filters 10,11 have a passband which lets through the fluorescent light which has a longer wavelength than the laser light. The lens device 12 for focusing can preferably be an achromatic lens with a long focal length.

Since both the scattered light and the fluorescence from the particles which have to be detected are directly intercepted via the lens 6 and indirectly via the mirror reflector 9, thereby passing the metering zone 7 twice, the effective light collection is increased as well as the intensity of both the light dispersion and the fluorescence. It should be noted that the lens 6 will intercept both scattered and fluorescent light in two different polarization planes, the light which has passed through the quarter-wave plate 8 and is reflected back by the mirror reflector 9 being rotated by $\pi/2$ in relation to the laser beam's original polarization plane. This allows the detection to be performed in an optional polarization plane. As mentioned a photodiode in the form of an avalanche diode can be used in the detector 13, this being an advantage if the fluorescent light is weak in addition to making the use of a photomultiplier for light intensification superfluous. It should also be noted that in the design of the liquid flow cytometer according to the present invention, due to the double scattering in the metering zone a scatter signal of high intensity is achieved. Background noise is largely eliminated and a liquid flow cytometer is thus provided which is suitable for use in microbiology and which has the necessary stability, reliability and sensitivity.

Designed according to the present invention, the liquid flow cytometer has small dimensions and is simple to use. The analysis time is less than 10 s and the sample volume normally used is approximately 0.5 ml.

The liquid flow cytometer according to the present invention is suitable for analysis and counting of biological microparticles with diameters from 0.2-30 μ in concentrations of between 10^3 and 10^7 pr. ml.

PATENT CLAIMS

1. A liquid flow cytometer for counting and classification of particles such as biological cells or other microscopic particles in liquids, where the cytometer comprises an optical system for irradiation and detection in a metering zone (7), thus enabling optical dispersion and fluorescence caused by the particles to be simultaneously detected when the particles pass the metering zone, characterized in that the optical system in a first beam path (S_1) comprises a mirror reflector (9) with its focal point in the metering zone (7), a quarter-wave plate (8) between the measuring zone (7) and the mirror reflector (9), a lens (6) with its focal point in the metering zone (7) and located on the opposite side of it together with a dichroic mirror (5) behind the lens and provided in such a manner that it gives a partial reflection substantially orthogonally to the axis of the lens, the reflection direction being in a second beam path (S_2) of the optical system, and in the second beam path (S_2) a beam splitter (4) located between the dichroic mirror (5) and a known per se light source (1), the beam splitter (4) producing a third beam path (S_3) substantially orthogonally to the second beam path (S_2), that the cytometer further comprises a first detector (13) provided in the first beam path (S_1) behind the dichroic mirror (5) and arranged for the detection of fluorescence emitted by the particles in the metering zone (7), together with a second detector (16) provided in the third beam path (S_3) and arranged for the detection of optical dispersion caused by the particles in the metering zone (7).
2. A cytometer according to claim 1, characterized in that the mirror reflector (9) is a spherical concave mirror.
3. A cytometer according to claim 1, characterized in that the beam splitter (4) is a polarizing beam splitter.
4. A cytometer according to claim 3, characterized in that a suitable lens device (2,3) is provided in the second beam path (S_2) between the polarizing beam splitter (4) and the light source (1) for focusing of light from the light source (1) on to the beam splitter (4).
5. A cytometer according to claim 4, characterized in that the light source (1) is a diode laser provided in the second beam path (S_2).

6. A cytometer according to claim 5,
characterized in that between the first detector (13) and the dichroic filter (5)
there is provided an optical filter (10,11) together with a suitable lens device
(12) for focusing of fluorescent light which passes the dichroic mirror (5) and on
5 to the first detector (13).

7. A cytometer according to claim 6,
characterized in that the first detector (13) comprises a photo diode, preferably
in the form of an avalanche diode.

8. A cytometer according to claim 1,
10 characterized in that between the second detector (16) and the beam splitter (4)
there is provided a beam stop (14) and a suitable lens device (15) for focusing
of scattered light reflected by the beam splitter (4) and on to the second detector
(16).

9. A cytometer according to claim 7,
15 characterized in that the second detector (16) comprises a photo diode, preferably
in the form of a PIN diode.

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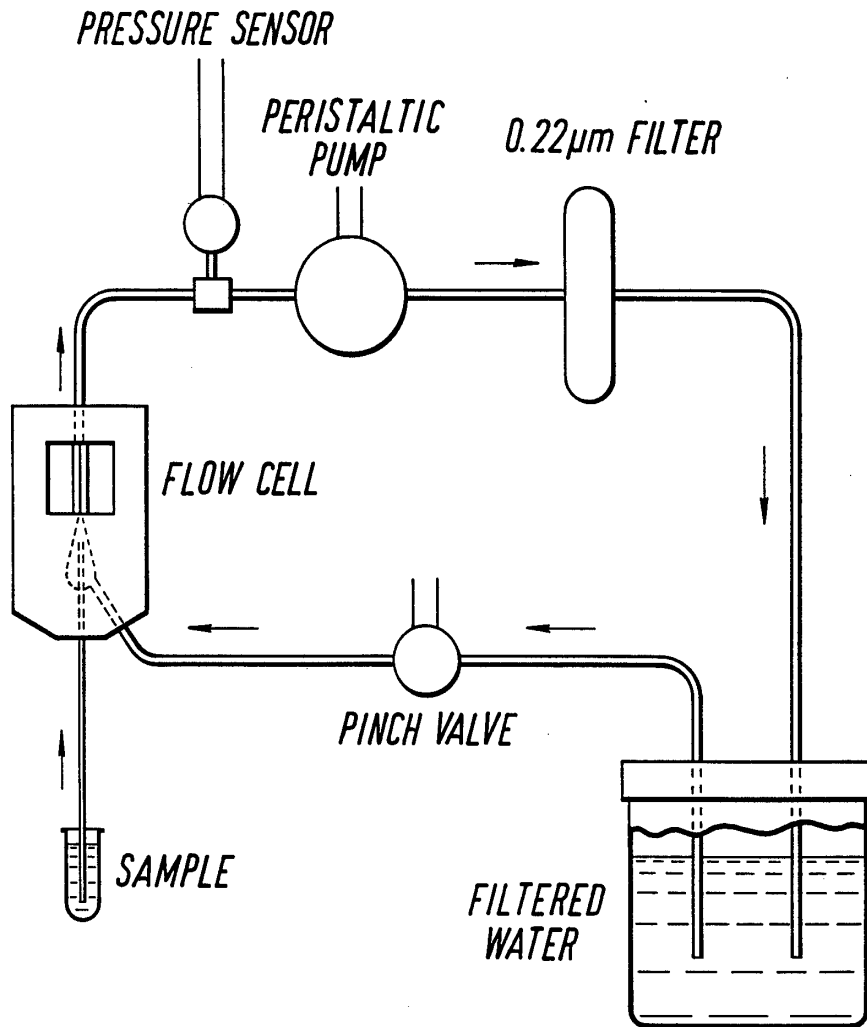


Fig. 1 (PRIOR ART)

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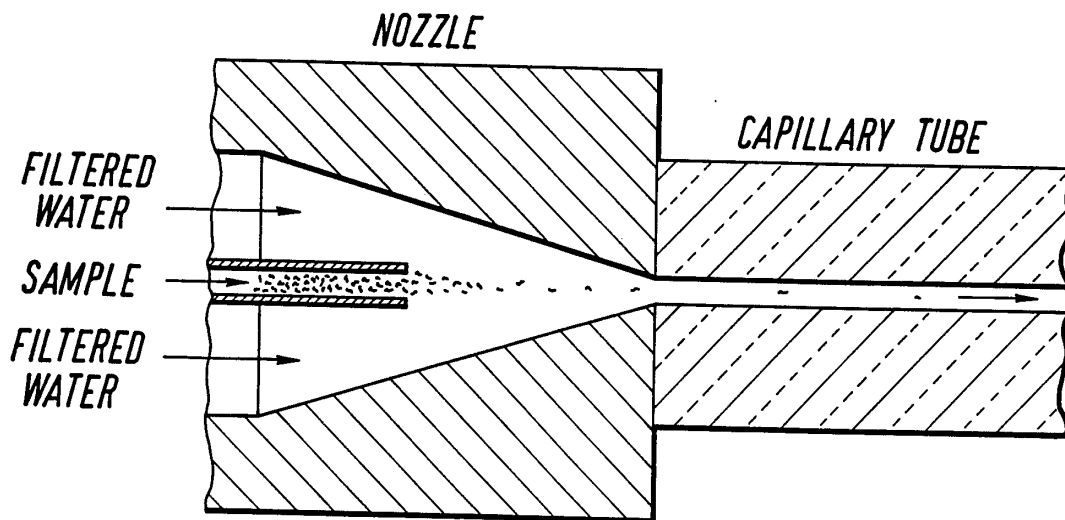


Fig.2 (PRIOR ART)

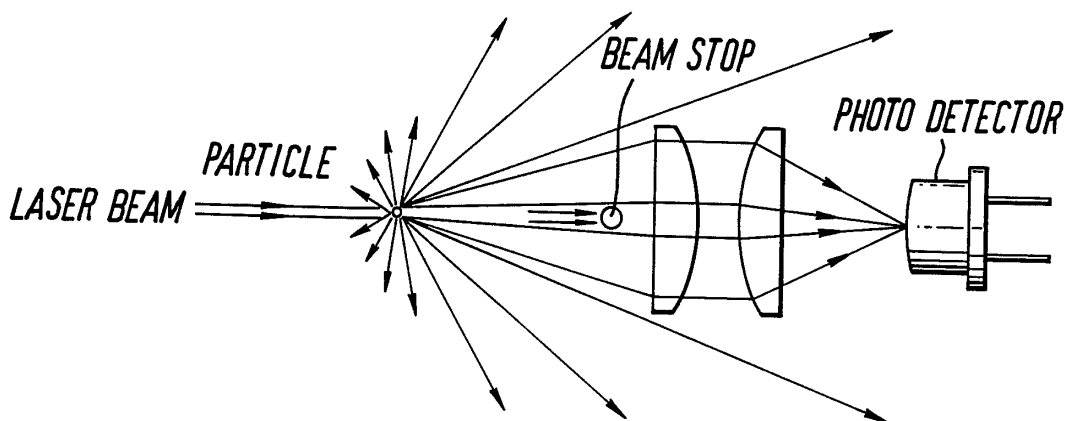


Fig.3 (PRIOR ART)

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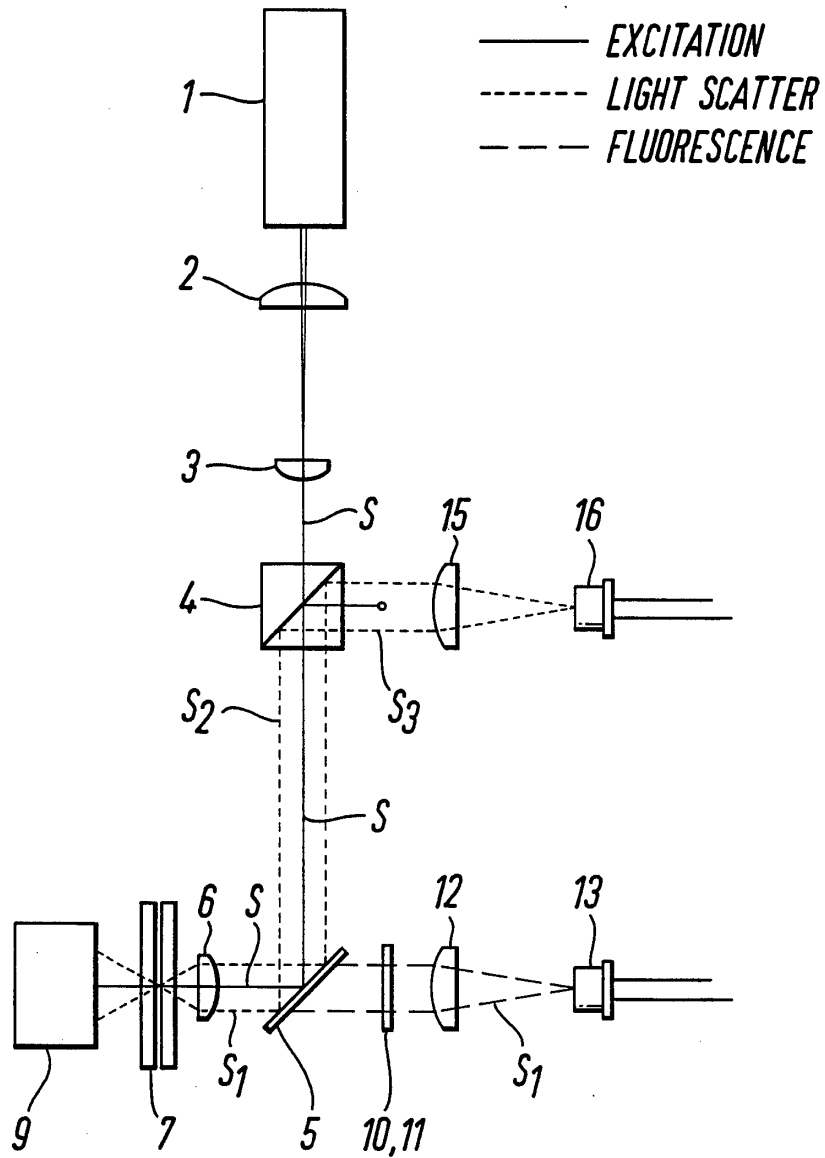


Fig. 4

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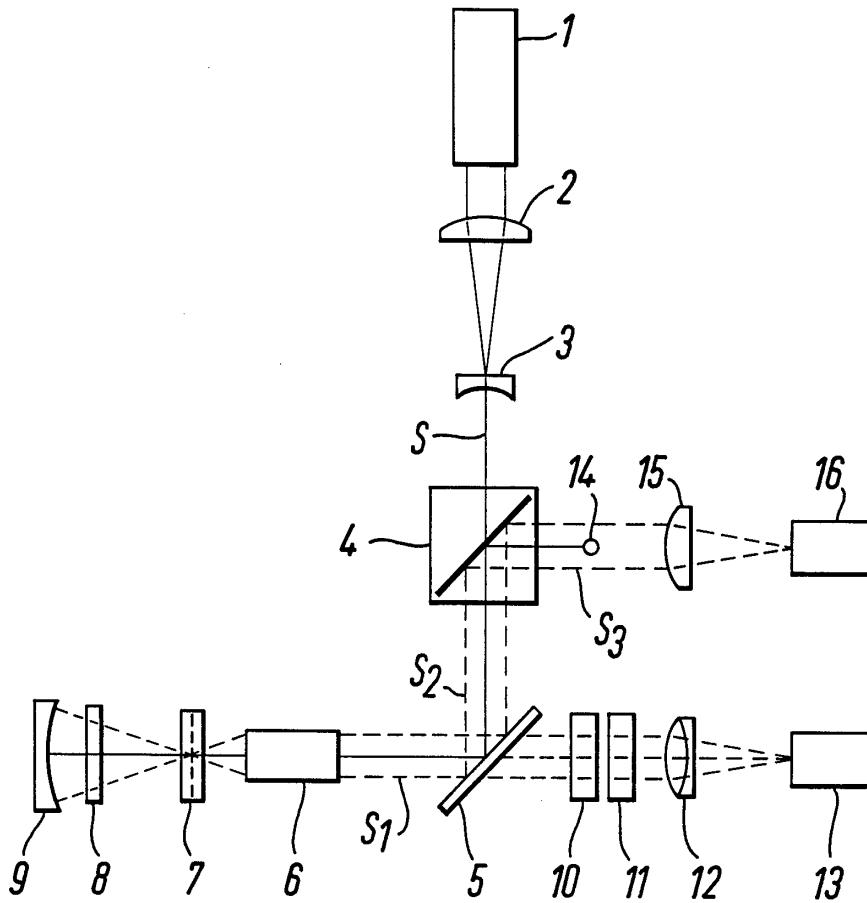


Fig. 5

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 94/00106

A. CLASSIFICATION OF SUBJECT MATTER		
IPC5: G01N 15/14, G01N 21/47, G01N 21/64 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC5: G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
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C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4662742 (VERNON CHUPP), 5 May 1987 (05.05.87) --	1-9
Y	EP, A2, 0538551 (TOA MEDICAL ELECTRONICS CO., LTD.), 28 April 1993 (28.04.93), column 5, line 1 - line 46 --	1-9
A	GB, A, 2238612 (SKATRON A/S), 5 June 1991 (05.06.91) --	1-9
A	EP, A1, 0442025 (TOA MEDICAL ELECTRONICS CO., LTD.), 21 August 1991 (21.08.91) --	1-9
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
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26 Sept 1994		27 -09- 1994
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INTERNATIONAL SEARCH REPORT

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 5179026 (SYOUHEI MATSUDA ET AL), 12 January 1993 (12.01.93), figure 1(a),1(b) --	1-9
A	WO, A1, 9307471 (BECKMAN INSTRUMENTS, INC.), 15 April 1993 (15.04.93) -- -----	1-9

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INTERNATIONAL SEARCH REPORT
Information on patent family members

27/08/94

International application No.

PCT/NO 94/00106

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GB-A- 2238612	05/06/91	BE-A- 1004550 DE-A- 4037376 FR-A,B- 2655147 NL-A- 9002563 US-A- 5185265	08/12/92 13/06/91 31/05/91 17/06/91 09/02/93
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