(51) International Patent Classification 5 : G01N 15/14, 21/85, 21/64	**A1**	(11) International Publication Number: **WO 92/08120** (43) International Publication Date: 14 May 1992 (14.05.92)

(71) Applicant *(for all designated States except US)*: MACQUA-RIE UNIVERSITY [AU/AU]; Balaclava Road, North Ryde, NSW 2113 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : PIPER, James, Austin [NZ/AU]; 32 Greenhaven Drive, Pennant Hills, NSW 2120 (AU). NARAI, Joseph, Alan [AU/AU]; 1 Mondial Place, West Ryde, NSW 2114 (AU). RAMSAY, Donald, James [AU/AU]; 129 Murray Farm Road, Beecroft, NSW 2119 (AU).

(54) Title: PULSED LASER FLOW CYTOMETRY
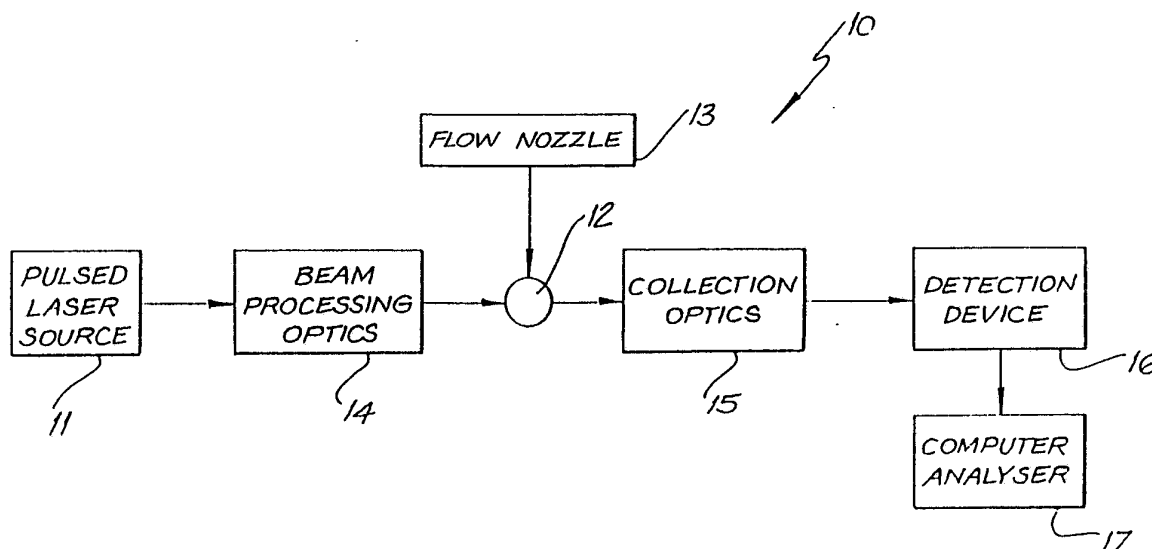
(57) Abstract

Flow cytometry comprising the steps of: producing a flow of cells of velocity (v), in single file, through an interaction region (12) of length (d), where (d) is defined by the height of a pulsed laser source (11) of height (d), which beam shines onto the flow of cells which beam defines the interaction region; pulsing the laser source at a repetition rate of (f) onto the flow of cells where the time between pulses (1/f) is approximately equal to the transit time (t) of a cell in the interaction region; collecting (15) and detecting (16) the light scattered by or fluoresced from a cell in the interaction region; analysing (17) the detected light to determine the presence or other characteristics of said cell.

+ DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic	SE	Sweden
CH	Switzerland		of Korea	SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU+	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

PULSED LASER FLOW CYTOMETRY

TECHNICAL FIELD

The invention pertains to flow cytometry and more particularly to a pulsed laser light source in a flow cytometer.

5 BACKGROUND ART

Flow cytometry is a technique for rapid measurement of biological and physical properties of cells and particles. It involves analysis of directly scattered or Stokes-shifted light (fluorescence) from cells in a fast-flowing fluid stream, illuminated by a strong light source (usually a

10 laser). Information concerning physical properties of the cells including, shape and size may be derived from the directly scattered light. Cells may be labelled with fluorescent probes to determine biological properties such as, DNA, RNA and protein content. A variety of properties may be studied simultaneously using multiple wavelength excitation.

15 The technology associated with flow cytometry has arisen over the last twenty years and recently many new applications have evolved including AIDS, Hepatitis B and Cancer detection. A flow cytometer is now a standard feature of many hospitals, research and clinical laboratories. A flow cytometer consists of several components, each of which is described below.

20 1. A flow system which causes particles in a fluid to be hydrodynamically focused and transported single file through an analysis region, where they are irradiated with an intense light beam. The resulting scattered and/or fluorescent light gives details about particle characteristics.

25 2. A light source and focusing system providing an intense light beam (usually a laser) focused to the analysis region within the fluid stream, so that each particle is irradiated as it passes through the beam.

3. A detection system to capture, the light either scattered or fluoresced from each particle, then to generate a corresponding electrical

30 signal.

4. An analysis system to process the electrical signals received and determine the desired information about the particles characteristics.

All current laser flow cytometers, including systems available commercially from a number of international suppliers, employ

35 continuous-wave lasers as the excitation source. Most commonly these are continuous wave argon or krypton ion lasers operating in the green, red or sometimes ultraviolet, or low power helium-cadmium lasers operating in the violet or ultraviolet. The main problems associated with these laser

sources are the high initial purchase and installation costs and high operating costs arising from the large power and cooling water services required. Another problem is the relatively short laser tube lifetime especially for ultraviolet operation of the argon and krypton ion lasers.

5 Even small scale air-cooled argon ion lasers (used in small bench-top flow cytometers) are expensive and have large power supply requirements and a short operating life. Multiple wavelength excitation requires two or more ion lasers and is extremely problematic in terms of system reliability, cost and alignment and there is little practical prospect of extending the

10 wavelength capabilities of these devices.

A second disadvantage of present technology is the difficulty in alignment. The laser tube must be aligned with the focusing optics and any slight movement in any of the components can cause large variations in the data obtained. For multiple lasers, this difficulty in alignment is

15 magnified by the number of lasers used. For information regarding cytometry, the reader is referred to Practical Flow Cytometry by H.M. Shapiro (1985), Flow Cytometry: Instrumentation and Data Analysis by Marvin A. Van Dilla et.al. (eds.) (1985), and Cytometry The Journal of the International Society for Analytical Cytology, which works are incorporated

20 by reference herein.

DISCLOSURE OF THE INVENTION

It is an object of the invention to substantially ameliorate some of the disadvantages of the prior art.

Accordingly, a flow cytometer having a flow system is provided

25 characterized by a pulsed laser light source, beam processing optics, collection optics and a detection device, the beam processing optics adapted to deliver a beam whose size and profile are tailored to a transit time of a cell sample in the flow system and the pulse rate of the laser.

In addition a method of cytometry is disclosed, comprising the steps

30 of producing a flow of cells, in single file, of velocity (v) through a region of length (d); shining a pulsed laser beam of pulse repetition frequency (f) and diameter (d) onto the region; collecting the scattered or fluoresced light emitted by the cells in the region; and analysing the collected light with a pulse height analyser.

35 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of pulsed laser flow cytometry according to the present invention.

Figure 2 is a schematic diagram illustrating one embodiment of the present invention including fibre optic delivery of the laser beam.

Figure 3 is a schematic diagram illustrating lens beam processing according to the present invention.

5   Figure 4 illustrates prism/diode array detection which is utilizable with the teachings of the present invention.

Figure 5 is a schematic diagram illustrating grating/diode array detection.

Figure 6 is a schematic diagram illustrating temporally multiplexed

10   laser flow cytometry according to the present invention.

Figure 7 is a schematic diagram illustrating another embodiment of temporally multiplexed laser flow cytometry according to the present invention.

BEST MODE AND OTHER EMBODIMENTS OF THE INVENTION

15   Progress in the development of practical high repetition rate pulsed lasers have allowed the inventors to consider the applications of these sources to laser flow cytometry. Immediately available are high pulse rate (5-20kHz) copper vapour lasers generating in the green, yellow and, by various wavelength conversion techniques, the ultraviolet and red. Even

20   more importantly, all-solid-state lasers using semiconductor diode lasers to pump crystalline solid state (eg neodymium: YAG) lasers whose output may be frequency-doubled to generate high repetition rate pulsed output in the visible are now becoming available in small integrated packages. These latter devices are expected to increasingly dominate in laser applications

25   requiring long-term reliability in the low-power regime; their compact size and projected low cost are especially appropriate to applications requiring multiple sources as in flow cytometry. On a more fundamental level, high repetition rate pulsed lasers have particular advantages in application as flow cytometry sources including high instantaneous illumination

30   intensities (giving high signal levels), relaxed beam focussing requirements, good beam uniformity and especially wavelength versatility which can be effectively utilised by temporal multiplexing techniques.

Application of pulsed lasers to flow cytometry requires a significant change in the approach to the basic physical problem, however. Since the

35   optical pulse duration of appropriate pulsed lasers (eg. copper laser or diode-pumped solid-state lasers) is typically only a few tens of nanoseconds and the duty cycle even for high pulse rate lasers is only 1 in 10^4, a major difficulty apparently arises in ensuring each cell is

- 4 -

illuminated as it passes through the interaction region. This difficulty
has previously been taken to rule out the use of pulsed lasers for flow
cytometry since sophisticated cell position-detecting electronics appeared
to be necessary. However, the inventors have demonstrated that pulsed
5 lasers operating at high pulse rates (~ 10 kHz) can be quite simply
matched to the droplet flow rates (also ~ 10^4 per second) usually
employed, given appropriate adjustments in focal spot size of the
illuminating beam at the interaction region. In essence, only approximate
synchronisation of the laser pulse rate and droplet flow rate is necessary
10 if an extended focal spot size is used so that the transit-time across the
interaction region is of the order of the interpulse period. The
uncertainty in the position of the cell when illuminated does not lead to
significant problems with scattered light detection. In general for a cell
or particle of velocity v moving through an interaction region of length d
15 (equal to the beam diameter, also d) the transit time t equals d/v. The
time between pulses 1/f (where f is the laser repetition rate) is
preferably equal to the transit time t. Therefore the beam diameter d is
preferably equal to vt, the cell velocity v multiplied by the time between
pulses.

20   As shown in Figure 1, a pulsed laser flow cytometer 10 utilises a
pulsed laser source 11 to illuminate cells or particles in an interaction
region 12. The cells or particles are delivered to the interaction region
12 by a flow nozzle 13. Any conventional flow nozzle may be utilized, for
example a standard flow nozzle in which the sample stream is surrounded by
25 a buffer sheath before ejection from the nozzle, which flow nozzle utilises
a simple hypodermic pump system. Beam processing optics 14 are interposed
between the pulsed laser source 11 and the interaction region 12. The beam
processing optics may be based on lenses but is preferably a fibre optic
system. Both lens based and fibre optic beam processing optics will be
30 explained in further detail. The light emerging from the interaction
region 12 is collected by collection optics 15. The collection optics may
be in the form of a 50 mm diameter, 50 mm focal length lens with a
horizontal beam stop 5 mm high, across the collection lens to stop the
incident beam from being collected. The light collected by this lens is
35 therefore generated by the particles or cells flowing in the stream carried
by the flow nozzle 13. The collection optics 15 images the collected light
onto a detection device 16 such as a photomultiplier tube where an

- 5 -

electrical signal is generated for each incident pulse. The electrical signals generated by the detection device 16 are processed to generate useful information, such as a standard pulse height analyser package in an IBM™ compatible computer 17. It will be understood that the combination

5   of linear array detectors and digital electronics can be used to provide data from which various characteristics of the cell flow can be

  ascertained. Cell presence, cell position, cell size, cell shape and nuclear volume for example, can all be ascertained from the scattered or fluoresced light. Information regarding the application of array detectors

10 is available from manufacturers such as Spiricon, Big Sky and Exitech.

      The results obtained may be plotted on a graph showing the pulse height (or intensity of the light pulse) along the horizontal axis and the number of counts (or number or pulses at the corresponding intensity). The system depicted in Figure 1 may also be utilized to collect light at angles

15 other than directly on axis with the incident beam. Typically, a standard flow cytometer will have collection at 0 and 90° and multiple fluorescence collection at 90°. Other collection angles may be used along or in combination.

      Figure 2 depicts a pulsed laser flow cytometry system in more

20 detail. In this instance, a copper vapour laser 20 generates 30 nanosecond pulses at a repetition rate of 10kHz. A fibre coupler 21 is used to couple the beam into a 100 micrometer optical fibre 22. The light emerging from the fibre 22 is focused onto a 1 mm pinhole 23 using a graded index lens 24. The pinhole removes stray or unwanted light emerging from the fibre.

25 The light emerging from the pinhole 23 is collected by a 25 mm diameter, 60 mm focal length achromatic lens 25 and imaged onto the flow stream 26 in the interaction region 27. As previously mentioned, the light emerging from the interaction region 27 is collected via a 50 mm diameter, 50 mm focal length lens 28 with a horizontal beam stop across the collection lens

30 to stop the incident beam from being collected. The collection lens 28 images the light onto a photomultiplier 29 where an electrical signal is generated for each incident pulse, which signals are processed by a standard pulse height analyser package in a computer 30. It should be understood that diode pumped lasers such as a NdYAG laser would be

35 considered a suitable alternative to the copper vapour laser 20. It is also worth noting that the photomultiplier tube 29 may be replaced with photo diodes or linear photodiode arrays, which alternatives will be

further described. The optical fibre 22 disclosed with reference to Figure 2, transfers the light from the laser to the interaction region 27 and gives a uniform beam profile. For multimode fibre, the emerging profile is "top hat" and is ideal for pulsed and continuous wave flow cytometry. For

5 single mode fibre, the emerging profile is gaussian which is suitable for continuous wave cytometry only. In the alternative to the fibre optic processing means 22 disclosed in Figure 2, a lens beam system is also a viable alternative. Such a system is disclosed in Figure 3. In a lens beam system, a condensing lens 31 focuses the light from the laser source

10 20 onto a mask or pinhole 32 of a suitable diameter. The mask prevents unwanted or stray light from reaching the interaction region and gives a uniform "top hat" profile. An imaging lens 33 focuses the emerging light onto the sheath in the interaction region 27.

At least two distinct detection systems may be used to gain spectral

15 information from pulsed laser flow cytometry. A prism/diode array detection system is depicted in Figure 4. A grating/diode array detection system is depicted in Figure 5. With reference to the prism/diode array detection system depicted in Figure 4, light from the interaction region is focused by a collection lens 40 onto a linear array detector 41 via a prism

20 42. The prism 42 will disperse the light. This means that different wavelengths will be focused onto different elements of the linear array 41. For each laser pulse, the array will be read out yielding information regarding the spectral (wavelength) content of the collected light. With reference to the grating/diode array detection system depicted in Figure 5,

25 light from the interaction region 27 is focused by the collection lens 50 onto one or more array detectors 51 by a diffraction grating 52. The defraction grating 52 will reflect light in different directions depending upon the wavelength and the incident angle. The light reflected from the zeroth order (the central) reflection will be directed to the same position

30 independent of the wavelength and a single element detector can be used to collect the light. The light reflected from the first order (first reflection either side of the center) reflection will be directed to differing positions depending on the wavelength. Thus, linear arrays can be positioned here and operated as for the prism example.

35 The adaptation of pulsed lasers and fibre optic light delivery to flow cytometry also makes possible temporal multiplexing as schematically illustrated in Figures 6 and 7.

As shown in Figure 6, a pulsed laser flow cytometer may incorporate separate pulsed lasers 60, one for each wave length desired. They are synchronised by an external trigger 61, delaying each source by an appropriate time so that each wavelength will arrive at the interaction

5 region 27 sequentially. Separate fibres 62 for each pulsed laser source 60 take each lasers output and couple the various outputs into a single optical delivery system 63. The delivery optics 68 take the emerging light and focus it into the interaction region 27. The collection optics 64 deliver the emitted light to a gated detection device 65. The gated

10 detection device 65 also receives a signal from the trigger box 61, which synchronisation signal 66 is used to initialise the gated detection device and syncronise its readout.

As shown in Figure 7, a single pulsed multi wavelength laser source 70 such as a copper vapour laser is utilised. The copper vapour laser can

15 emit at 511 and 578 nm. The emitted light passes through two or more dichroic beams splitters 71 each of which reflect only a particular wavelength. The light is sent through couplers 72 into separate optical fibre delay lines 73. Each of the optical fibre delay lines is preferably of a different length so that the optical signal reaches the interaction

20 region 27 at different times. The differing length delays each of the pulses by 3.3 nanoseconds from every metre of fibre length. The collection optics 64 and gated detection device arrangement 65 are substantially similar to the arrangement disclosed with reference to Figure 6.

The advantage of temporally multiplexing the lasers rather than

25 physically separating them is that only a single detection and illumination system is required. With existing multiple wavelength systems, each of the laser sources, focusing optics and detection optics must be aligned individually. This can take several hours even for an experienced operator. This individual alignment is not required in a temporally

30 multiplexed system. Only one optical system is required for illumination and one for detection.

As a further aspect of the invention, there may be optionally provided, an array detector to detect beam intensity variations (temporal and spacial) and an array detector to detect light emerging from the

35 analysis region or interaction region 27. The signal from the array detector compensates the beam profile detector signal, producing a signal independent of the illuminating beam profile. In this way, beam intensity

- 8 -

variations, both spatially (across the analysis region) and temporally (jitter from the laser light source), can be simply compensated for in real time. This reduces the need for exact alignment and high quality beam processing optics.

5   For each of the abovementioned systems, the preferred source beam is of low divergence and "top hat" profile. Both of these factors contribute to the resolution of the system. Linear array detectors can be used to correct for irregularities in the beam profile. It will be understood that an ideal "top hat" profile is never exactly achieved. The actual beam

10 profile however can be measured using the linear array detector. This measurement can be compared with, subtracted from or otherwise used to offset the scattered or fluoresced readings so as to yield a compensated or corrected measurement. The linear array can also be used to yield a one dimensional image of the cell as it passes through the interaction region

15 or, to give an indication of cell position for cell sorting later in the cell flow stream. A two dimensional linear array can be used to yield a two dimensional image of the cell.

While the invention has been described with reference to particular components, instruments and details of construction these should be

20 understood as having been provided by way of example and not as limitations to the scope or spirit of the invention.

INDUSTRIAL APPLICABILITY

The device of the present invention is ideally suited to cell counting, cell sorting and cell identification in hospitals, research

25 facilities and clinical laboratories.

CLAIMS:

1.   A method of flow cytometry comprising the steps of:

producing a flow of cells of velocity (v), in single file, through an interaction region of length (d), where (d) is defined by the height of a pulsed laser source of height (d), which beam shines onto the flow of cells and which beam defines the interaction region;

pulsing the laser source at a repetition rate of (f) onto the flow of cells where the time between pulses (1/f) is approxiamtely equal to the transit time (t) of a cell in the interaction region;

collecting and detecting the light scattered by or fluoresced from a cell in the interaction region; and

analysing the detected light to determine the presence or other characteristics of said cell.

2.   The method of claim 1, further comprising the step of:

adjusting the source height (d) to be approximately equal to the cell velocity (v) multiplied by the time between pulses (1/f).

3.   The method of either of claims 1 or 2 wherein:

the source has a top hat profile.

4.   The method of any of claims 1 to 3 further comprising the step of:

compensating for a source profile by using one or more array detectors which detect the unscattered beam profile and scattered or fluoresced light signal after which a detection signal may be corrected according to the beam profile.

5.   The method of claim 4, further comprising the step of:

using one or more linear array detectors to generate a signal indicative of cell position in the interaction region.

6.   The method of claim 4, further comprising the step of:

using the one or more array detectors are used to provide information as to the cross-sectional intensity of a cell in the interaction region.

7.   The method of any of claims 1 to 6 wherein

prior to entering the interaction region, the laser source comprises two or more combined, pulsed laser beams synchronised to pass through the region at different times; and further comprising the steps of:

using a gated detection device to detect the scattered or fluoresced light; and

synchronising the two or more pulsed beams, using a trigger with the gated detection device.

- 10 -

8.   The method of claim 7, wherein:

the two or more laser beams are of different wavelength.

9.   The method of either of claims 7 or 8, wherein:

the two or more laser beams are triggered simultaneously and fed into separate fibre optic delay and delivery optics.

10.   The method of any one of claims 1 to 6 wherein:

the laser source comprises a multi-wavelength laser whose beam is split using a beam splitter into separate beams of individual wavelengths, then introduced into fibres or delay lines, then recombined before shining onto the interaction region;

whereby the scattered or fluoresced light is detected with a one or more gated devices which are synchronised with the multi wavelength lasers.

11.   The method of any of claims 1 to 10 wherein:

collecting is accomplished by collecting optics which further comprise a diffraction grating or prism to separate collected wavelengths and dispense same onto a one or more gated array detectors.

12.   An apparatus for flow cytometry comprising:

one or more pulsed laser light sources, the beam or combined beams of which are fed through beam processing optics, which optics lead the pulses into a cell flow system comprising an interaction region;

collection optics for gathering scattered or fluoresced light;

one or more detection devices;   and

a signal processor for analysing the signals produced by the detection device or devices.

13.   The device of claim 12, where:

the length of the interaction region is approximately equal to the velocity of cells in the flow system times the time between pulses and where the interaction region is defined by the height of the laser pulses which impinge on the cell flow.

14.   The apparatus of either of claims 12 or 13 wherein:

a single multi-wavelength laser source is used and further comprising one or more beam splitters and an equal number of coupled fibre optic delay lines which are recombined prior to delivery into the interaction region;

whereby two or more detection devices are gated to be synchronised with the delayed beams emerging from the delay lines.

15.   The apparatus of claim 12, wherein:

two or more pulsed laser sources, each with its own fibre delay line are recombined prior to reaching the interaction region;   and

- 11 -

the detection devices are gated and synchronised by a trigger device which also operates the two or more pulsed laser sources.

16. The apparatus of claim 15, wherein:

the two or more sources are of different wavelength.

17. The apparatus of any one of claims 12 to 16 where the collection optics further comprises a prism or diffraction grating for frequency separating the gathered light and dispensing same to two or more gated linear arrays.

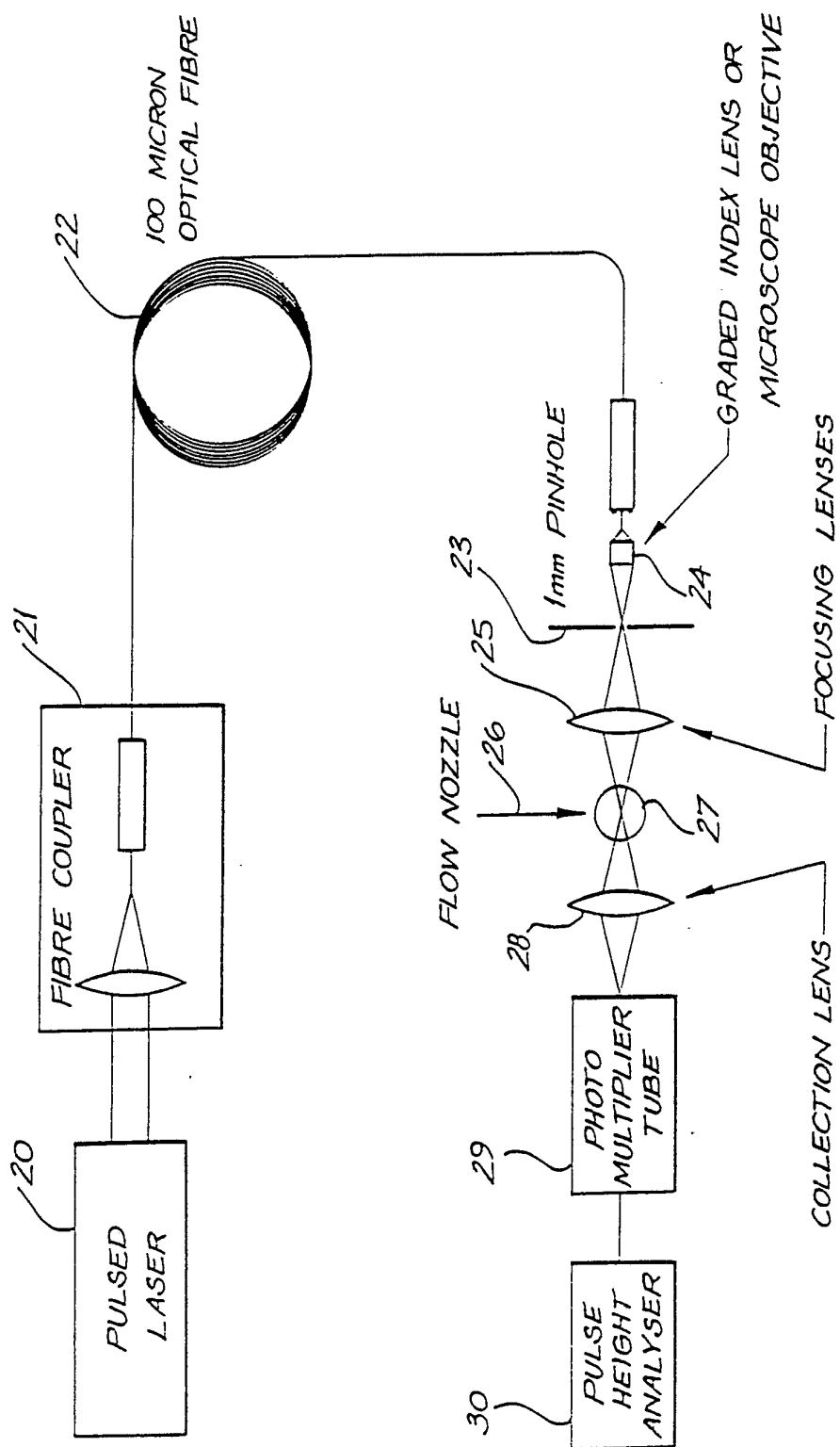18. A flow cytometer substantially as hereinbefore described with reference to the drawing figures.
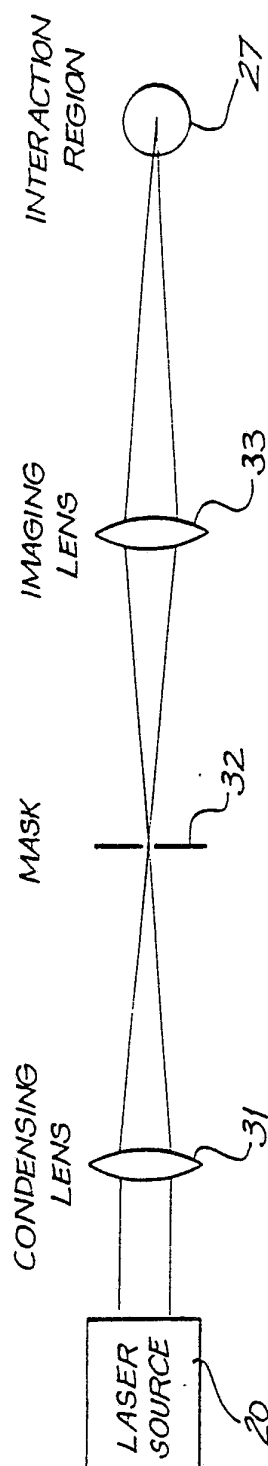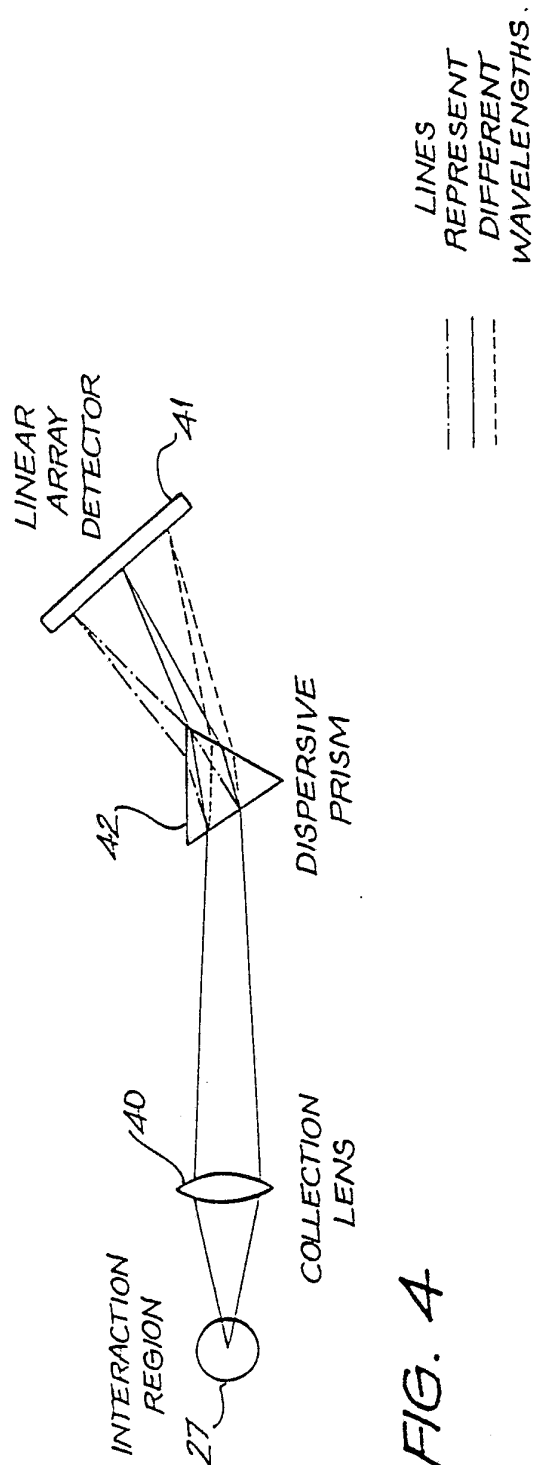
FIG. 1

FIG. 2

FIG. 3

LINES
REPRESENT
DIFFERENT
WAVELENGTHS.

LINEAR
ARRAY
DETECTOR

41

42

DISPERSIVE
PRISM

40

27

INTERACTION
REGION

COLLECTION
LENS

FIG. 4

GATED
LINEAR
ARRAY
DETECTOR

51

52

DIFFRACTION
GRATING

PHOTODIODE
DETECTOR

51

GATED
LINEAR
ARRAY
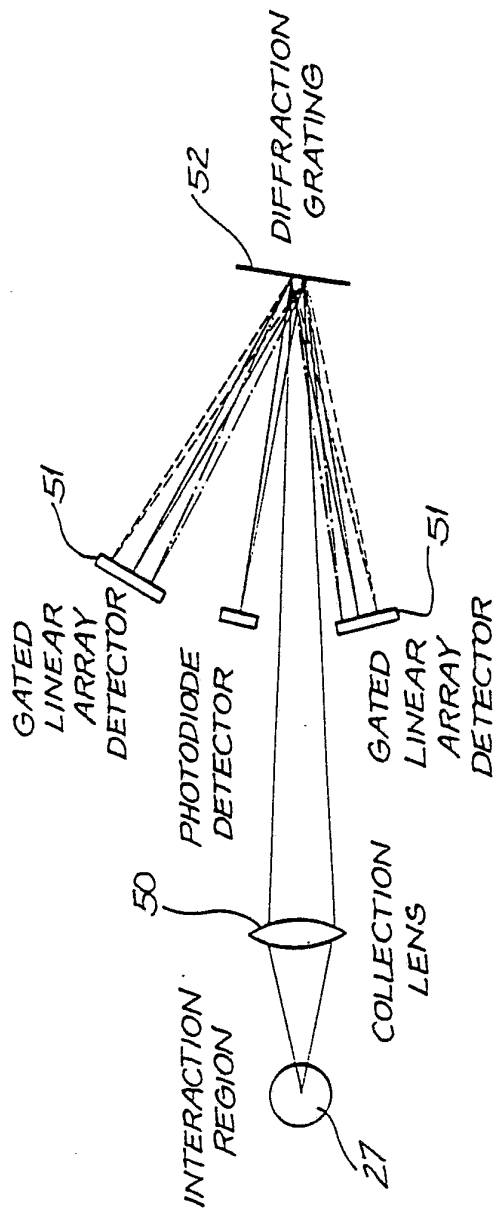DETECTOR

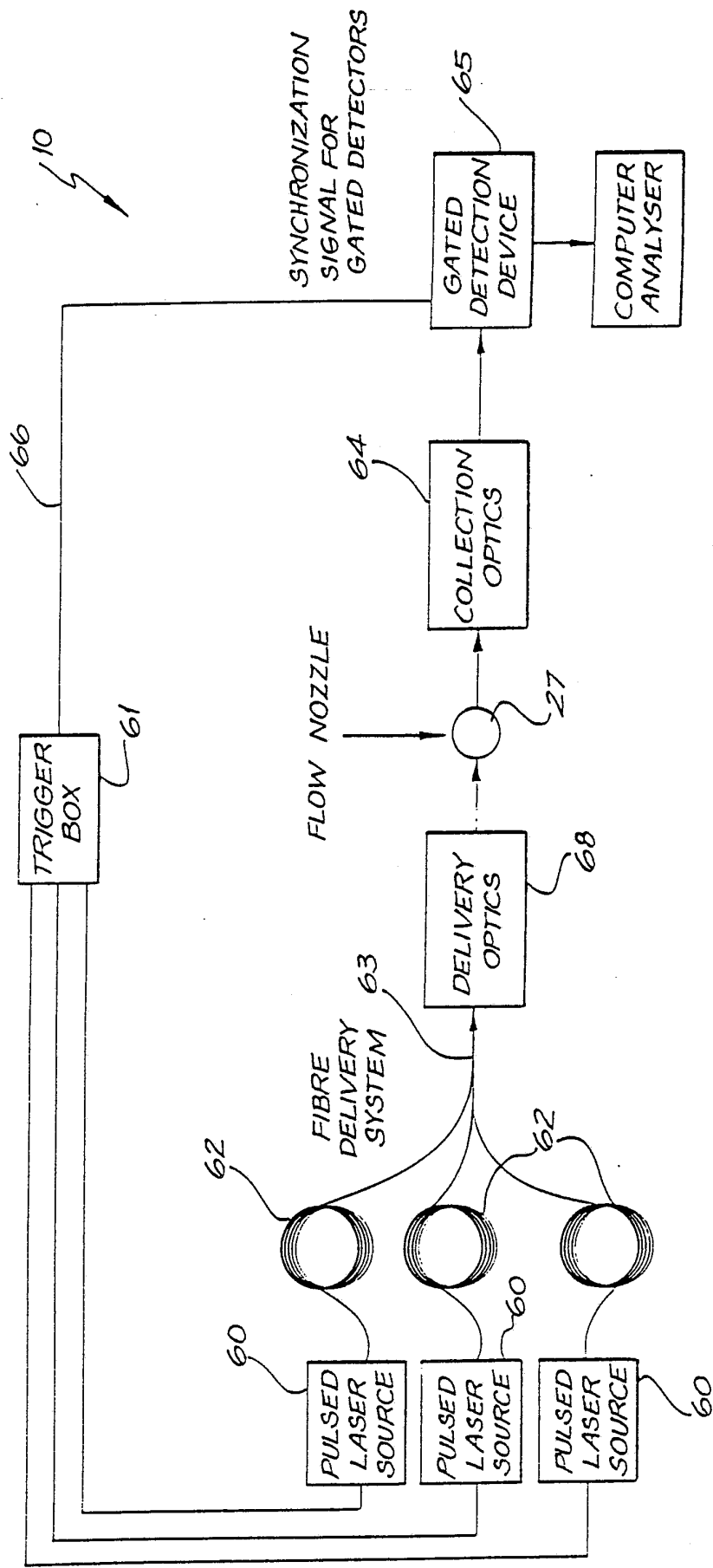50

27

INTERACTION
REGION

COLLECTION
LENS
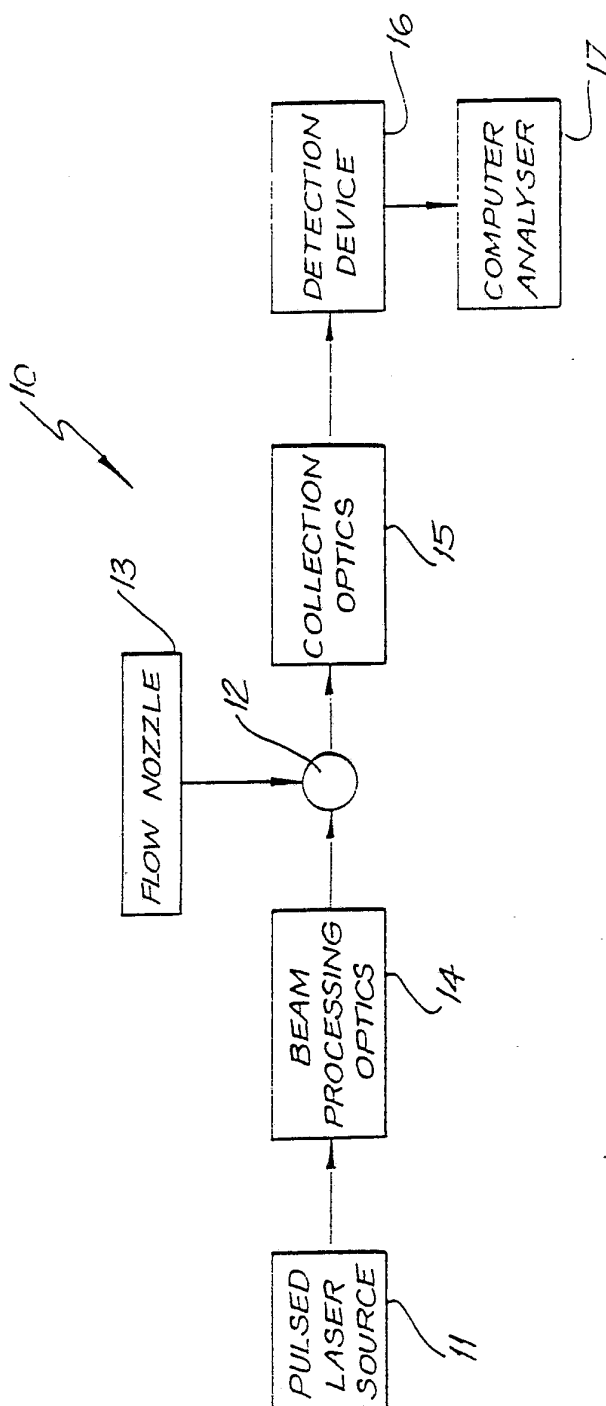
FIG. 5

FIG. 6
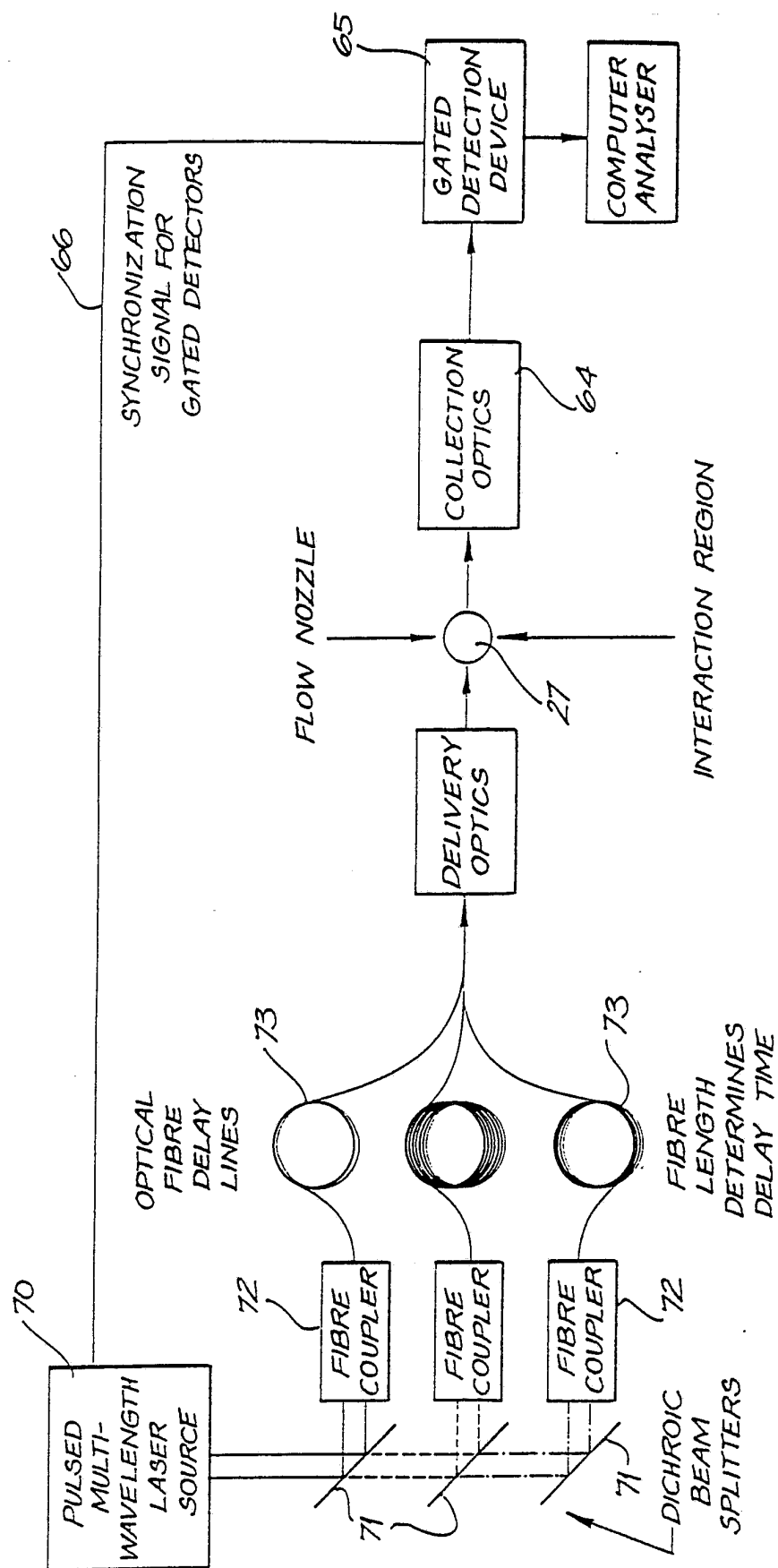
FIG. 7

INTERNATIONAL SEARCH REPORT

I.   CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁶

According to International Patent classification (IPC) or to both National Classification and IPC
Int. Cl.⁵ G01N 15/14, 21/85, 21/64.

II.   FIELDS SEARCHED

Minimum Documentation Searched ⁷	
Classification System	Classification Symbols
IPC	G01N 15/02, 15/07, 15/14, 21/53, 21/64, 21/85.

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸
AU:IPC as above

III.   DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category*	Citation of Document, ¹¹ with indication, where appropriate of the relevant passages ¹²	Relevant to Claim No ¹³
X	US,A, 4900933 (NESTOR et al) 13 February 1990 (13.02.90) (see abstract)	12
A	Derwent Abstract Accession No 86-111827/17, Class S03, SU,A, 1182342 (ZHULANOV YUV) 30 September 1985 (30.09.85) (see abstract)	
A	US,A, 4786165 (YAMAMOTO et al) 22 November 1988 (22.11.88) (see column 2 lines 9-20)	
A	EP,A, 0369654 (KOWA COMPANY LTD) 23 May 1990 (23.05.90) (see abstract)	
P,A	Derwent English Language Abstract S03, 91-233388/32 JP,A, 03-150445 (CANON K.K.) 26 June 1991 (26.06.91) (see abstract).	

---

*	Special categories of cited documents : ¹⁰	"T"	Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	Document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior t the international filing date but later than the priority date claimed		
		"&"	document member of the same patent family

IV.   CERTIFICATION

Date of the Actual Completion of the International Search 23 January 1992 (23.01.92)	Date of Mailing of this International Search Report 30 January 92
International Searching Authority AUSTRALIAN PATENT OFFICE	Signature of Authorized Officer P.F. GOTHAM

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member					
US 4900933	EP	259951	JP	63070151	EP	283289
	JP	1063843				
US 4786165	DE	3705876	JP	63094156		
EP 369654	JP	2134540				