(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 June 2004 (03.06.2004)

PCT

(10) International Publication Number
WO 2004/046695 A2

(51) International Patent Classification[7]: G01N 21/00

(21) International Application Number:
PCT/US2003/036533

(22) International Filing Date:
14 November 2003 (14.11.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10/295,482 15 November 2002 (15.11.2002) US

(71) Applicant: EKSIGENT TECHNOLOGIES LLC [US/US]; 2021 Las Positas Court, Livermore, CA 94550 (US).

(72) Inventors: RAKESTRAW, David, J.; 3620 Thornhill Drive, Livermore, CA 94550 (US). ARNOLD, Don, W.; 1746 Old Tower Road, Livermore, CA 94550 (US). PAUL, Phillip, H.; 258 Daisyfield Drive, Livermore, CA 94550 (US).

(74) Agents: SHELDON, Jeffrey, G. et al.; Sheldon & Mak, 225 South Lake Avenue, Suite 900, Pasadena, CA 91101 (US).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW.

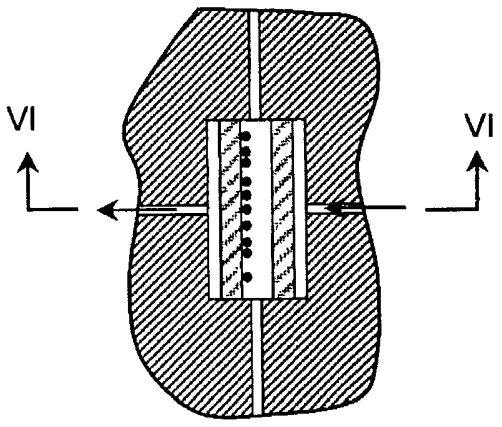
(84) Designated States (regional): ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for all designations*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for all designations*

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROCESSING OF PARTICLES



(57) Abstract: Particles dispersed in a liquid are assembled in a configuration in which all the particles lie in the same plane, and the assembled particles are processed while in that configuration. The assembled particles can for example be simultaneously exposed to electromagnetic radiation which elicits from the particles a response which provides information about the particles. The particles can for example be cells, cell fragments, or analyte-bearing beads of the type conventionally analyzed in a cytometer.

WO 2004/046695 A2

PROCESSING OF PARTICLES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. patent application Serial No. 10/295,482, filed November 14, 2002.

BACKGROUND

The invention relates to the processing of particles, including but not limited to the examination of particles which are conventionally examined by cytometry.

Flow cytometry is widely used in life sciences research and clinical diagnostics for analyzing particles, including cells and beads having analytes attached thereto. A disadvantage of cytometry is that each particle must be examined individually. Other techniques for examining particles are disclosed in, for example, Nature Biotechnology (2000), 18, 630-634, Brenner, S. et al, and Anal. Chem. (2000), 72, 1144-1147, Kitamori, T. et al.

SUMMARY OF THE INVENTION

We have discovered, in accordance with the present invention, that particles dispersed in a liquid can be efficiently processed by assembling the particles in a configuration in

which all the particles lie in the same plane, and processing the assembled particles while they are in that configuration. In a preferred embodiment of the invention, substantially all the particles are simultaneously exposed to electromagnetic radiation which elicits from the particles a response which provides information about the particles. In this preferred embodiment, the invention can be used to obtain analytical information about particles which is similar to the information provided by cytometry, but which can be obtained using apparatus which is simpler to make and use.

In a first preferred aspect, this invention provides a method of processing a sample comprising particles dispersed in a liquid, the method comprising

(A) placing the sample in a processing chamber containing a particle receptor;

(B) after step (A), causing at least some of the particles to be received by the particle receptor so that the received particles are all in the same plane;

(C) after step (B), processing at least some of the particles received by the particle receptor; and

(D) after step (C), removing the particles from the processing chamber.

As discussed below, the particles in the sample may undergo physical and/or chemical change in step (C). The term "removing the particles" is used herein to include removing the particles in the original or in a changed state.

In a second preferred aspect, this invention provides a novel processing chamber suitable for use in the method of the first aspect of the invention, the processing chamber comprising

(i) an elongate passage having a first end and a second end; and

(ii) between the first and second ends, an elongate particle receptor comprising an elongate barrier member which permits passage of liquid but not the passage of particles having a selected size in the range of 0.1 to 50 micron, preferably a selected size in the range of 1 to 20 micron.

In a third preferred aspect, this invention provides apparatus for carrying out the method of the first aspect of the invention, the apparatus comprising a processing chamber according to the second aspect of the invention and means for applying, simultaneously to all the particles received by the receptor, electromagnetic radiation which elicits from the particles a response which provides information about the particles.

DETAILED DESCRIPTION OF THE INVENTION

In the Summary of the Invention above, in the Detailed Description of the Invention and the claims below, and in the accompanying drawings, reference is made to particular features of the invention. It is to be understood that the disclosure of the invention in this specification includes all appropriate combinations of such particular features. For example, where a particular feature is disclosed in the context of a particular aspect or embodiment of the invention, or a particular Figure, or a particular claim, that feature can also be used, to the extent appropriate, in combination with and/or in the context of other particular aspects and embodiments of the invention, and in the invention generally.

The term "comprises", and grammatical equivalents thereof, are used herein to mean that other components, ingredients, steps etc. are optionally present in addition to the component(s), ingredient(s), step(s) specifically listed after the term "comprises". The term "at least" followed by a number is used herein to denote the start of a range beginning with that number (which may be a range having an upper limit or no upper limit, depending on the variable being defined). For example "at least 1" means 1 or more than 1, and "at least 80%" means 80% or more than 80%. The term "at most" followed by a number is used herein to denote the end of a range ending with that number (which may be a range having 1 or 0 as its lower limit, or a range having no lower limit, depending upon the variable being defined). For example, "at most 4" means 4 or less than 4, and "at most 40%" means 40% or less than 40 %. When, in this specification, a range is given as "(a first number) to (a second number)" or "(a first number) - (a second number)", this means a range whose lower limit is the first number and whose upper limit is the second number.

Particles

A wide variety of particles can be used in the present invention, and they can be dispersed in any suitable liquid. Typically, but not necessarily, the particles are from about 0.1 to about 50, for example from about 1 to about 20, micron in diameter. The particles are usually, but need not be, of the same size. The sample can contain for example from about 5 to about 500 particles per microliter. Thus, depending on the size of the sample, the number of particles in the sample can for example be up to 5000, for example 50 to 5000, e.g. 100 to 1000.

The particles can for example be particles of the kind used in cytometry. Such particles include naturally occurring materials, e.g. cells and cell fragments, and particles

which contain one or more sites which have interacted with one or more analytes. The particles may also contain one or more characteristic features which, when the particle is examined under appropriate conditions, identify the particle as belonging to a particular class. Such characteristic features include, for example, the presence of known amounts of one or more dyes which will fluoresce in a characteristic waveband when illuminated by a suitable laser. Other characteristic features include size, shape, reflectivity, density, radioactivity, color, electrical charge, magnetic properties, the presence of optodes, and the presence of fluophores which cease to fluoresce under the examination conditions. Analytes which can be examined in this way include electrolytes, metabolite molecules, enzymes, antibodies, antigens, and polynucleotide fragments.

For disclosure of particles used in cytometry and their examination, reference may be made for example to U.S. Patent Nos. 4,499,052, 4,665,020, 5,736,330, 5,747,349, 5,981,180, 6,057,107, 6,139,800, 6,165,790 and 6,271,042, European patent No. 126,450 and UK patent No. 1,561,042.

Processing Chambers, Particle Receptors and Barrier Members

The processing chamber preferably comprises an elongate passage which includes (i) a sample inlet, and (ii) at least one elongate particle receptor comprising an elongate barrier member which permits passage of the liquid but not of the particles. The use of such a barrier member makes it possible to direct the particle-bearing liquid towards the barrier member, so that the particles are received by the receptor and the liquid passes beyond the barrier member. In some embodiments, two such barrier members form opposite walls of the elongate passage. This makes it possible to reverse the direction in which a positioning liquid flows (either during the examination of a single sample, or for the examination of different samples) or because one of the barrier members has become unusable, for example because it has become contaminated. The use of two opposed barrier members is also useful when an electrical or magnetic field causes different particles in the sample to move in opposite directions.

In one embodiment, the barrier member is a weir, the clearance gap above the weir being less than the size of particles. The particles, when directed against the weir, are received in a line at the top of the weir. In another embodiment, the barrier member has a plurality of conduits which traverse the barrier member and provide the only way in which liquid can pass through the barrier member, the size of the conduits being such that the

particles cannot pass through them. For example, 2x2 micron square conduits will prevent the passage of any particle larger than 2 micron in diameter. The conduits are preferably placed in a line at the top of the barrier member, so that the particles are received in a line at the top of the conduit-containing barrier member.

The elongate passage can be shaped so that particles are guided towards the desired configuration. For example, the barrier member can have a concave arcuate cross-section ending at or near the top of the barrier member, and/or there can be an elongate member set at an angle, e.g. substantially at a right angle, to the face of the barrier member, so as to form an elongate corner which helps to trap the particles in the desired configuration.

The barrier member, or other particle receptor, can optionally comprise a plurality of pockets, each of the pockets being capable of receiving one, but only one, of the particles. Such pockets can be placed on, or be an integral part of, a barrier member. They are preferably placed so that they do not substantially impede particle flow during steps (A) and (D). The pockets may be at least 5%, preferably at least 10%, larger than the maximum dimension of the particles in the sample, but less than twice the smallest dimension of the particles, to prevent two particles from being received by the same pocket. Generally, the pockets have a maximum depth of about 2 to about 20 micron and a width of about 3 to about 15 micron. The number of pockets may be for example from about 100 to about 1500. The number of pockets is for example from 1.5 times to 3 times the number of particles in the sample.

In some embodiments, the elongate passage has a sample inlet at one end and a sample outlet at the other end. In other embodiments, the processing chamber has a single port which is used both as an inlet and as an outlet. The passage can be straight or curved. The cross-section of the passage, which is preferably constant, can be of any shape, e.g. part-round or rectangular (including square). The passage may have, for example, transverse dimensions in the range of about 10 to about 100 micron, e.g. a width of about 40 to about 100 micron and a depth of 10 to 50 micron, such that the particle-bearing liquid can freely flow therethrough, while minimizing the volume of the sample.

The particle receptor is preferably such that substantially all the particles in the sample are received close to one another by the receptor, with substantially no overlap between the particles so that all the particles can be subjected to simultaneous processing, e.g.

interrogation. The particle receptor should be such that the particles can not only be received by the receptor, but also removed therefrom after the particles have been processed.

In a preferred embodiment, a positioning liquid is used to direct the particles towards the receptors. In this embodiment, the processing chamber preferably further comprises an inlet and an outlet for the positioning liquid, the inlet and outlet being placed so that positioning liquid passing between them flows in a direction generally transverse to, preferably substantially at right angles to, the axis of the elongate passage, thus directing the particles towards the particle receptor. The inlet and outlet for the positioning liquid are generally separated from the adjacent side wall by respective auxiliary chambers which act as flow diffusers, so that the positioning liquid acts uniformly over the length of the elongate passage.

The processing chamber can conveniently comprise a bottom member and a top member sealed thereto. One or both of the members is shaped in any convenient way (e.g. by machining, including laser machining, water jet machining or bead jet abrasive machining; injection molding; embossing; or wet or dry chemical etching) to provide the desired components before the members are sealed together. The processing chamber can be made of any convenient material, remembering, however, that when the processing of the particles involves exposing the particles to electromagnetic radiation, and/or results in a signal in the form of electromagnetic radiation, the relevant parts of the processing chamber must be sufficiently transparent to that radiation, e.g. will transmit at least 40%, preferably at least 60%, of that radiation.

Causing Particles to be Received by Receptors

As noted above, in a preferred embodiment of the invention, the particles of the sample are directed towards the particle receptors by a positioning liquid which flows into the sample towards the receptors. The volume of positioning liquid used for this purpose can be more than, equal to, or less than, the volume of the sample. The positioning liquid can be one whose only function is to direct the particles towards the receptors, or it can be one which in addition produces some desired change in the particles or the liquid in the sample. It should not of course damage (i.e. cause some unwanted change in) the sample. For example, for most biological samples, appropriate saline conditions should be maintained.

Alternatively or additionally, the particles may have electrical properties such that an electrical or magnetic field is used to direct the particles towards the receptors. The particles

may have different electrical properties and, therefore, respond differently to the electrical or magnetic field, e.g. move in opposite directions.

Techniques for Processing Received Particles

Particles received by the particle receptors can be processed by any appropriate technique. In one embodiment, substantially all the received particles are interrogated simultaneously to provide information about the particles. Such interrogation often includes exposing the particles to electromagnetic radiation, e.g. from a laser, which interacts with the particles to produce a signal, for example a light signal resulting from one or more fluorescent dyes attached to the particles. Alternatively or additionally, the interrogation can be based on the size, shape, morphology, or reflectivity of the particles. It is also possible to apply a voltage to the particles on the receptors, and thus to generate an electrochemical signal or an electrochemically generated optical signal (through chemiluminescence). The signal is generally observed by a detector, but in some cases can be visually observed. The techniques used in cytometry can be used for this purpose. However, in cytometry each particle must be examined separately, whereas in the present invention any number (and preferably all) of the received particles can be examined simultaneously. This makes it possible to use a diffuse source of electromagnetic radiation, e.g. from a laser which is not focused as in cytometry or from an LED. The examination can be carried out very rapidly, e.g. in a few seconds or less, and the resulting signal can be analyzed while one sample is removed from, and a new sample placed into, the processing chamber.

Preferably the cross-sectional area illuminated by the electromagnetic radiation matches the cross-sectional area of the received particles. Because substantially all the particles lie in the same plane, detectors which collect 2D images can be used to monitor how the particles respond to processing. A plurality of lasers or other light sources can be used in parallel. Detectors which that can be used include CCD cameras, CID cameras, diode arrays, and photographic film.

It is also possible for the received particles to be processed so that part or all of the particles (e.g. analyte fragments forming part of the particles) undergoes physical and/or chemical change, for example as a result of a change in the liquid which contacts the particles. Through proper interrogation, it is possible to carry out kinetic measurements by monitoring the particles as a function of time. Thus, it is possible to use the method of the invention for chemical synthesis, using the particles as a solid phase support for the synthesis.

The positioning fluid can be changed sequentially to effect desired chemical and/or physical changes to all or part of the received particles. The sequential changes in the positioning fluid are preferably effected in successive processing chambers, but can be effected in the same processing chamber. For example, a serial four-chamber system can sequentially introduce guanine, cytosine, thymine and adenosine for oligonucleotide synthesis.

It is also possible for the processing chamber to be used for sample preparation, pre-concentration, extraction and clean up. Discrimination for low-concentrations of target analytes and high background analyte concentrations can be achieved by passing a sample over capture particles positioned in the processing chamber.

Pumps

Pumps are generally used to deliver and remove the samples and the positioning liquids from the processing chamber. Preferably the pumps are pneumatic or electroosmotic (also known as electrokinetic) pumps. Such pumps can be precisely controlled and can accurately introduce very small quantities of liquid, e.g. about 100 picoliters to about 100 nanoliters of liquid per cycle. Suitable electroosmotic pumps are described for example in U.S. Patent Nos. 6,277,257, 6,013,164 and 3,923,426. Any other suitable pump can be used, for example a syringe-type, piezo-actuated diaphragm, microbubble, pneumatic, or peristaltic pump. The pumps are preferably resistant to flow when they are in the off state, to prevent liquid leakage from the system.

Methods

The method of the first aspect of the invention involves steps (A)-(D). In many cases, this sequence of steps is repeated a number of times, using one or more fresh samples of the same composition, or one or more samples of one or more different compositions. It is also possible to use in one or more or steps in addition to steps (A)-(D). For example, after the processing step (C) is complete, the direction of flow of the positioning fluid can be reversed before or during step (D) to assist in the removal of the particles from the receptors. A computer-controlled controller can be used to ensure that the various steps are carried out at defined time intervals.

Processing Chambers connected in Parallel

A plurality of processing chambers can be arranged in parallel, preferably in a way such that several, and preferably all, of the particles received in the different processing chambers lie in the same plane and can, therefore, be examined simultaneously. Optionally,

each processing chamber has its own valved sample inlet and outlet. Suitable microvalves are for example piezo-actuated diaphragm valves and the valves described in for U.S. Patent No. 6,019,882. There can be a plurality of barrier members separating different samples, with the positioning fluid flowing sequentially through the different samples (as shown for example in Figure 12), and/or the positioning fluid can be supplied separately to each processing chamber from a manifold (as shown for example in Figure 13). A large number of processing chambers can be arranged in parallel, for example in a straight line or in serpentine form, so that many rows of particles (which can be parallel to each other) can be processed at the same time.

Drawings

The invention is illustrated in the accompanying drawings, in which the same or similar components are denoted by the same reference numerals.

Figure 1 is a cross-section of a processing chamber of the invention. Figure 2 is a cross-section on line I-I of Figure 1. Figure 3 is an enlarged cross-section of part of Figure 2. As shown in Figures 1-3, the processing chamber comprises a bottom member 1 and a top member 2 sealed thereto. The bottom member comprises an elongate central passage 12 having a sample inlet 11 one end and a sample outlet 13 at the other end. The sidewalls 14 and 15 of the passageway 12 are barrier members in the form of weirs having pockets 141 and 151 formed therein. The gaps between the tops of the weirs and the base of the top member 2 are such that liquid can pass through the gaps but the particles cannot. Positioning fluid inlet 16 communicates with diffuser section 161 adjacent to the weir 14. Positioning fluid outlet 17 communicates with diffuser section 171 adjacent to the weir 15.

Figures 4-5, 6-7, and 8-9 are cross-sections showing successive stages in the operation of a processing chamber as shown in Figures 1-3, except that the particle receptors are only on the weir 15. In Figures 4-5, the particle-bearing sample has been introduced into the central passage. In Figures 6-7, introduction of the sample has ceased, and transverse flow of the positioning liquid has caused the particles to be received by the particle receptors. In Figures 8-9, after the particles have been processed, flow of the positioning liquid has ceased, and a fresh sample is being introduced through the sample inlet.

Figures 10 and 11 are cross-sections showing a processing chamber as shown in Figures 1-3, except that the barrier members 14 and 15 are in the form of apertured walls which contact the top member 2 and have conduits 142 and 152 running through them.

Figure 12 is a cross-section of a processing chamber comprising four elongate passages 12a, 12b, 12c and 12d defined by parallel weirs 14, 15a, 15b, 15c and 15d. Mounted on the weirs15a, 15b, 15c and 15d (but not shown in Figure 12) are particle receptors like those shown in Figures 1-3. The composition to be processed is introduced through sample inlet 11 to manifold 111 and samples or the composition not been introduced into the passages, optionally via valves not shown in Figure 12. Positioning fluid is introduced through inlet 16 into diffuser 161, and then passes sequentially through the elongate passages, the diffuser 171 and the inlet 17.

Figure 13 is a cross-section of pass although serpentine assembly comprising a large number of processing chambers connected in parallel. The composition to be processed is introduced into the processing chamber 12 through sample inlet 11 and leaves through sample outlet 13. Positioning fluid is introduced through inlet 16, passes through elongate chamber 12, over weirs 15a, 15b, 15c, 15d 15e and 15f into diffuser 171 and leaves through outlet 17.

Figure 14 shows the processing of retained particles in a processing chamber as shown in Figures 6-7 in order to identify the particles. A laser 52 directs diffuse beam 54 having a selected wavelength through the top member 2 onto the retained particles. Light 55 is reflected or emitted from the particles, and after passing through appropriate optics 56 and filters 58 is detected and analyzed by detector 60.

Figure 15 is similar to Figure 14, except that a different diffused light source such as an LED is used to interrogate the particles, and is directed through the bottom of the processing chamber.

Figure 16 shows a system of the invention. It comprises processing chamber 1, into which samples are introduced and removed by pumps 2 and 3, and positioning fluid is introduced and removed by pumps 4 and 5. Received particles are illuminated by light source 52 and produce signals which are received by detector 60. The various parts of the system are controlled by controller 9.

CLAIMS

1. A method of processing a sample comprising particles dispersed in a liquid, the method comprising

(A) placing the sample in a processing chamber containing a particle receptor;

(B) after step (A), causing at least some of the particles to be received by the particle receptor so that the received particles are all in the same plane;

(C) after step (B), processing at least some of the particles received by the particle receptor; and

(D) after step (C), removing the particles from the processing chamber.

2. A method according to claim 1 wherein each of the particles has a size of 0.1 to 50, preferably 1 to 20, micron.

3. A method according to claim 1 or 2 wherein the processing chamber comprises an elongate passage which includes (i) a sample inlet and (ii) at least one elongate particle receptor comprising an elongate barrier member which permits passage of the liquid but not of the particles.

4. A method according to claim 4 wherein the elongate passage has a width of about 40 to about 100 micron., and a depth of 10 to 50 micron.

5. A method according to claim 3 or 4 wherein the barrier member is a weir and the only way in which liquid can pass the barrier member is over the weir.

6. A method according to claim 3 or 4 wherein the barrier member has a plurality of conduits which traverse the barrier member and the conduits provide the only way in which liquid can pass the barrier member.

7. A method according to any one of claims 3 to 6 wherein the particle receptor includes an elongate member which is adjacent to the elongate barrier member and forms an elongate corner with it.

8. A method according to any one of the preceding claims wherein the particle receptor comprises a plurality of pockets, each of the pockets being capable of receiving one, but only one, of the particles.

9. A method according to any one of the preceding claims wherein step (B) comprises pumping a positioning liquid through the processing chamber, thereby causing particles to be received by the particle receptor.

10. A method according to claim 9 wherein the sample is pumped into and out of the processing chamber by a first pneumatic or electrokinetic pump, and the positioning liquid is pumped into and out of the processing chamber by a second pneumatic or electrokinetic pump.

11. A method according to any one of the preceding claims wherein step (B) comprises applying an electrical field to the particles.

12. A method according to any one of the preceding claims wherein step (C) comprises exposing the particles received by the receptor to electromagnetic radiation which elicits from the received particles a response which provides information about the received particles.

13. A method according to claim 12 wherein substantially all the received particles are simultaneously exposed to the same electromagnetic radiation.

14. A method according to any one of the preceding claims wherein step (C) comprises subjecting the particles received by the receptor to conditions which cause a chemical reaction of the particles.

15. A processing chamber suitable for use in a method according to any one of the preceding claims, the processing chamber comprising

(i) an elongate passage having a first end and a second end; and

(ii) between the first and second ends, an elongate particle receptor comprising an elongate barrier member which permits passage of liquid but not the passage of particles having a selected size in the range of 0.1 to 50 micron.

16. A processing chamber according to claim 15 wherein the barrier member does not permit passage of particles having a selected size in the range of 1 to 20 micron.

17. A processing chamber according to claim 15 or 16 wherein the elongate passage has a transverse dimension of 10 to 100 micron.

18. A processing chamber according to any one of claims 15 to 17 wherein the barrier member comprises a plurality of pockets, each pockets having a maximum depth of about 2 to about 20 micron and a width of about 3 to about 50 micron.

19. Apparatus for carrying out a method as claimed in any one of claims 1 to 14, the apparatus comprising a processing chamber according to any one of claims 15 to 18 and means for applying, simultaneously to all the particles received by the receptor, electromagnetic radiation which elicits from the particles a response which provides information about the particles.

FIG. 1

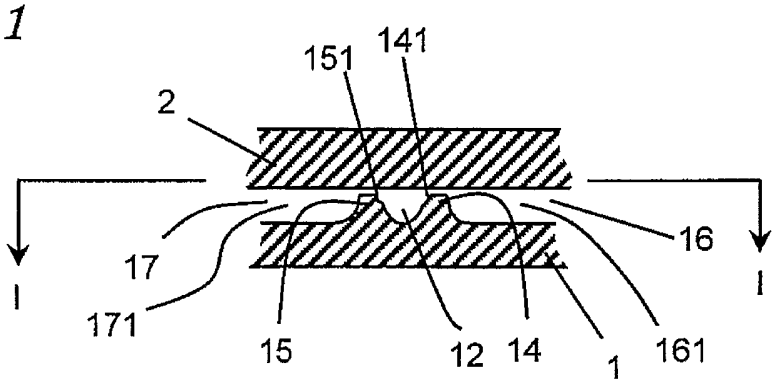


FIG. 2

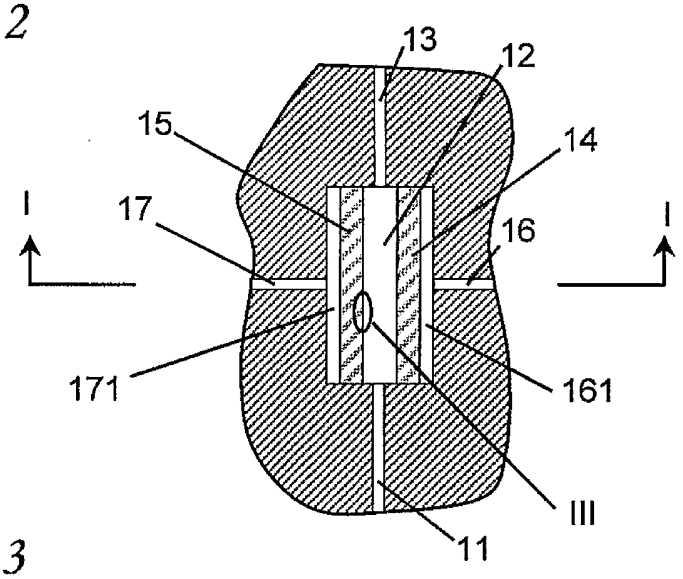


FIG. 3

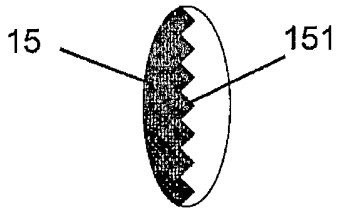


FIG. 4

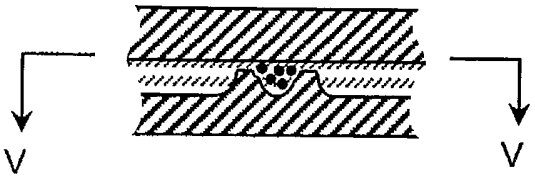


FIG. 6



FIG. 5

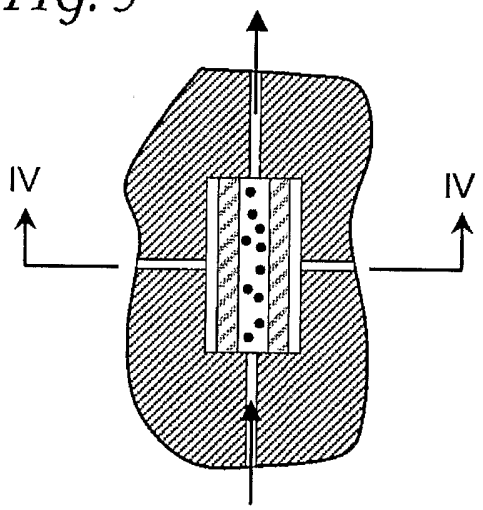


FIG. 7

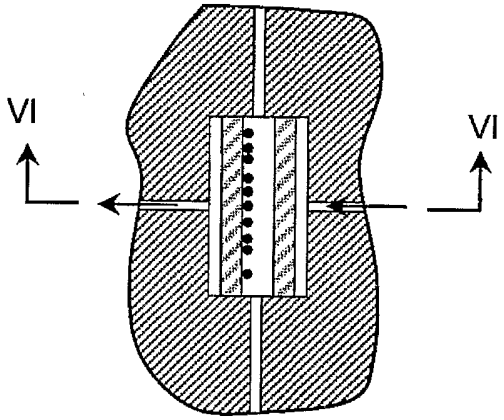


FIG. 8

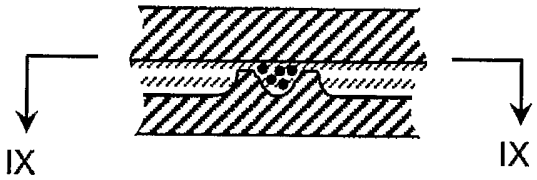


FIG. 9

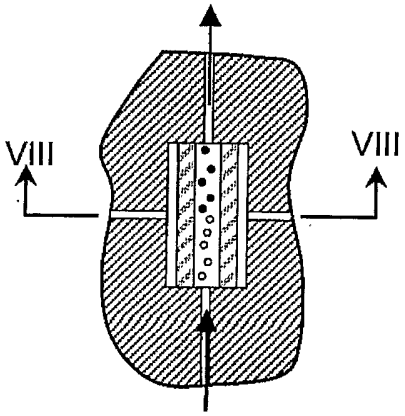


FIG. 10

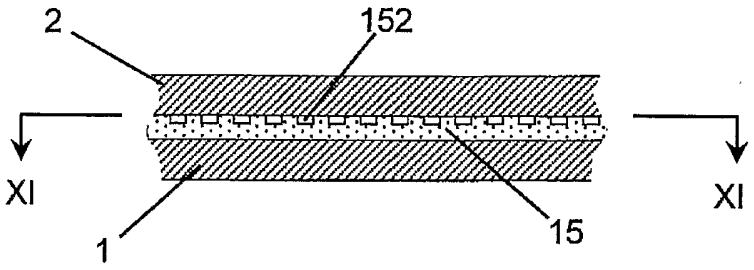


FIG. 11

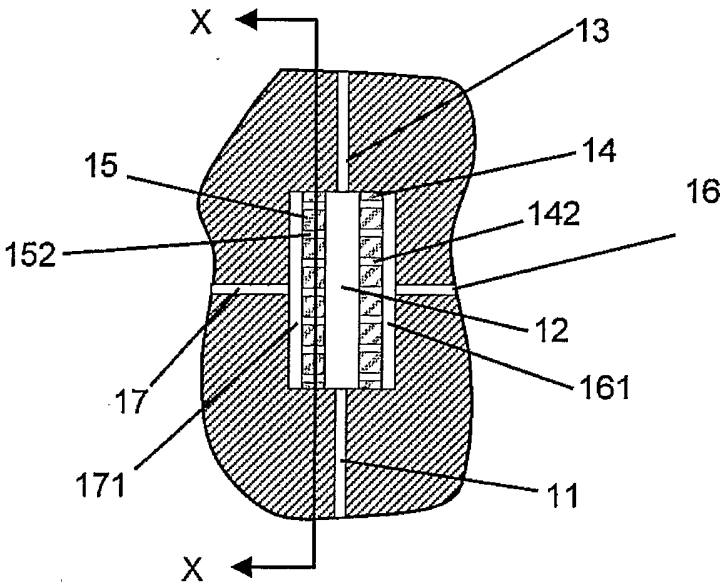


FIG. 12

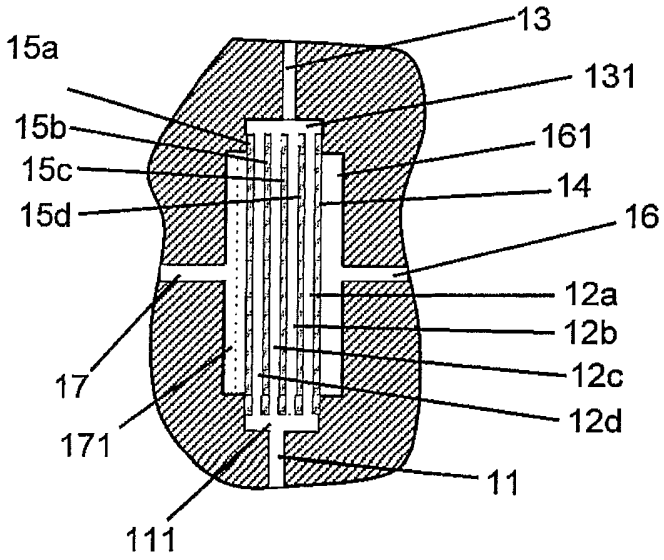


FIG. 13

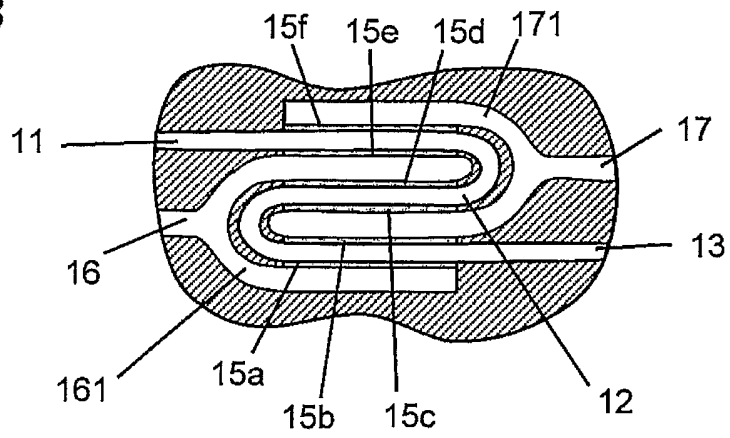


FIG. 14

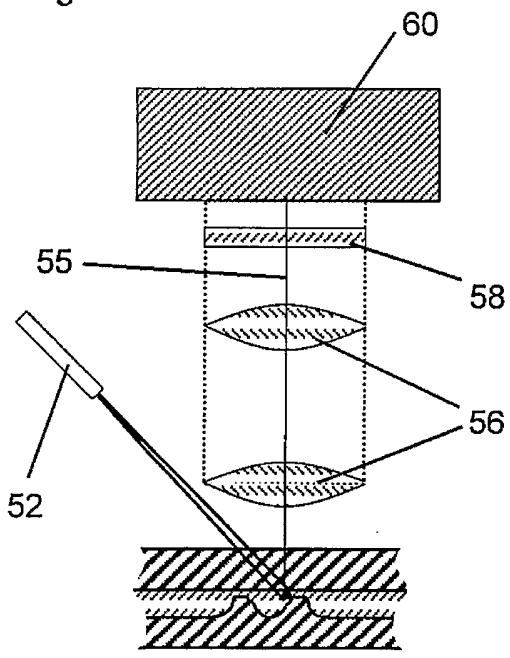


FIG. 15

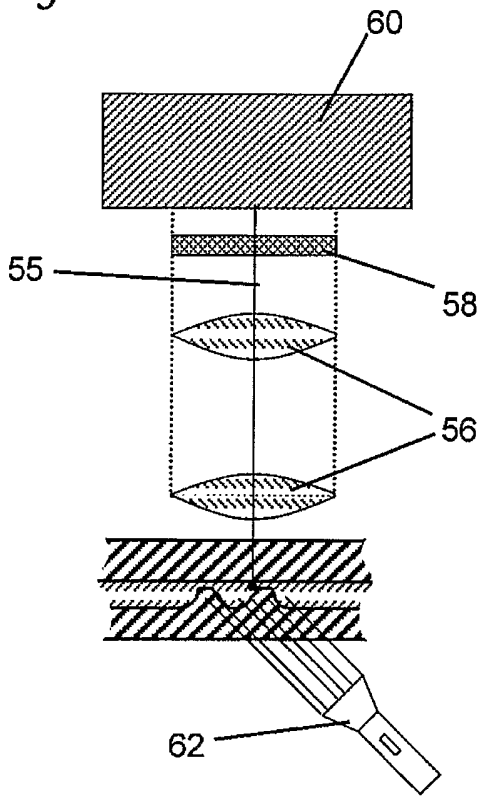


FIG. 16

