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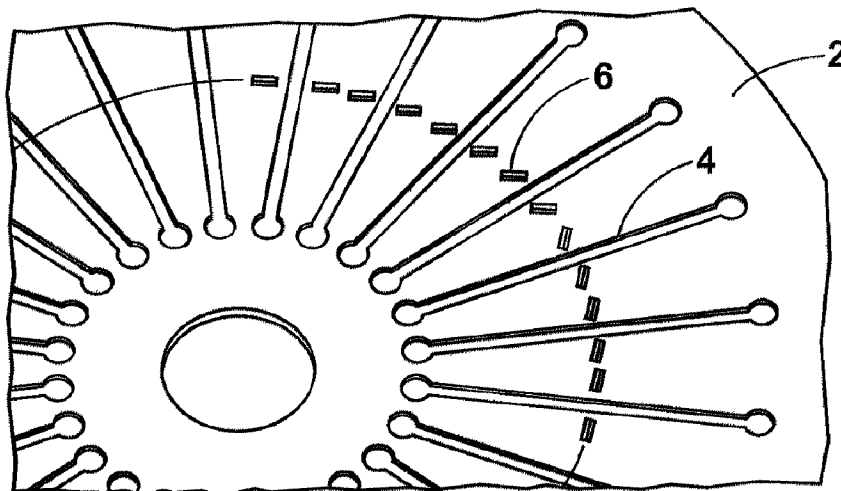
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(54) Titre : DETECTION D'OBJETS MICROSCOPIQUES

(54) Title: DETECTION OF MICROSCOPIC OBJECTS



(57) Abrégé/Abstract:

A substrate for use in manufacture of a production master plate for production of a detection disc for carrying samples in an apparatus for detection of microscopic objects in a fluid, the substrate having a channel and separate focus structure, wherein the focus structure is a groove.

ABSTRACT

5 A substrate for use in manufacture of a production master plate for
production of a detection disc for carrying samples in an apparatus for detection of
microscopic objects in a fluid, the substrate having a channel and separate focus
structure, wherein the focus structure is a groove.

DETECTION OF MICROSCOPIC OBJECTS

The present invention relates to detection of microscopic objects and to related products, apparatuses and methods.

5 It is important in many fields to be able to detect and/or count small objects such as bioparticles, molecules, cells and so on. One particular field where this is of use is in biotechnology, for example in relation to DNA replication/amplification, where it can be important to be able to detect molecular elements such as rolling circle products (RCPs).

10 One known system for detecting microscopic objects is flow cytometry. This is a laser-based, biophysical technology employed in cell counting, cell sorting, biomarker detection and protein engineering. Cells are suspended in a stream of fluid and passed by an electronic detection apparatus. Flow cytometry allows simultaneous multiparametric analysis of the physical and chemical characteristics
15 of up to thousands of particles per second.

 However, flow cytometry is not able to detect individual small objects at a microscopic scale, especially not in a sample of the type obtained by RCP production.

 Flow cytometry is an example of a method that measures the integrated
20 fluorescence within a defined volume, rather than the specific objects that may be contained within the volume. A particular problem arises for these methods when the label used for detection is also present in solution and not only associated with the object of interest. The presence of free labels in solution then easily overwhelms the signal from the object of interest. Another problem arises when the
25 object of interest is very small, such as in single molecule detection methods. A key element to success is to improve signal over background and this is usually performed by reducing the investigated volume by various means. If the volume is small ($< 1\text{fL}$), background can be reduced to a minimum since it scales linearly with volume. The signal however does not change with decreased volume and thus
30 signal over background is increased. This however causes an unreasonably long analysis time if a large volume, such as several microliters or even more several hundred of microliters or even millilitres of sample is to be investigated. Another solution to the problem is to image the fluorescence volume to be analysed with a resolution comparable to the size of objects to be detected. These can then be
35 identified in the image as small areas of locally higher fluorescence intensity.

There is hence a need for new products, methods and apparatuses that would enable the detection and/or counting of single small objects, for example for single molecule detection in the context of RCPs.

5 Viewed from one aspect, the invention provides a substrate for use in manufacture of a production master plate for production of a detection disc for carrying samples in an apparatus for detection of microscopic objects in a fluid, the substrate having a channel and separate focus structure, wherein the focus structure is a groove.

10 To produce a functional structure comprising both a focus structure and a flow structure (also referred to as a channel, a flow channel or a detection channel) using a replication process, first the desired pattern may be transferred to a substrate, which may have a crystallographic structure. The substrate may comprise silicon. The substrate when processed may then be used to serve as a template of opposite polarity compared to a replication master (also referred to as a
15 production master plate, or simply a master) used for production of the flow channel and focus structure. The replication process may for instance be thermoplastic injection moulding or casting. The production master plate may comprise nickel and may be produced by electroplating. The replication master may be used in CD-based thermoplastic injection moulding, to generate CD shaped substrates having
20 the desired flow channel and focus structure. The focus structure is a groove in that it may have a cross-section that is constant along one primary direction of the focus structure, for example its length or width. The channel and groove may be provided in the (100) surface of the silicon. The focus structure is a groove in that it may have a cross-section that is constant along one primary direction of the focus
25 structure, for example its length or width. The focus structure may have sloping {111}-oriented sidewalls. The focus structure may have a V-shaped cross-section. The focus structure may be oriented at right angles (that is, at 90 degrees) to the (110) plane. The channel may have a square or rectangular cross-section. Alternatively, the channel may have a U-shaped cross section, or may otherwise
30 have a curved base such that the channel has an arc-shaped, inverted-arch-shaped or semi-circular shaped cross-section. The depth of the focus structure may be less than the depth of the channel. The depth of the focus structure may have a fixed (that is, predetermined, known in advance, or non-arbitrary) relationship with the depth of the channel. The depth of the channel may be twice the depth of the focus
35 structure. Here, the depth of each of the focus structure and channel is taken to be

the maximum depth, in cases where the depth varies across the cross-section of the focus structure or channel.

There may be multiple detection channels and multiple focus structures. The multiple detection channels may be arranged radially about a disc shape of the substrate, and wherein there may be at least one focus structure associated with each detection channel. In the instances where there are several channels on one disc, the channels may have different geometries and/or depths. The depth of the focus structure may have a predetermined relationship with the depth of the channel such that the same algorithms and optical settings can be used for every channel, independent of the geometry and/or depth of the channel. It is also possible to have independent or fixed offsets between the focal plane of the focus structure and the focal plane in the detection channel.

In another aspect, the invention provides a detection disc for carrying samples in an apparatus for detection of microscopic objects in a fluid, the detection disc having a detection channel and separate focus structure, wherein the focus structure is a groove, and wherein the detection disc is made using a production master plate manufactured using a substrate as described above. The invention also provides a detection disc for carrying samples in an apparatus for detection of microscopic objects in a fluid, the detection disc having a detection channel and separate focus structure, wherein the focus structure is a groove.

The disc may be made from a material with refractive index of greater than 1.22. The disc may be made from an optically transparent material. The disc may be made from a thermoplastic polymer. The disc may be made from a cyclo olefin polymer. The disc may be made from Zeonor® 1060R. The disc may be made from PDMS, UV-grade PMMA, PMMA, PC or a COC polymer-based material.

The focus structure is a groove in that it may have a cross-section that is constant along one primary direction of the focus structure, for example its length or width. The focus structure may have sloping sidewalls. The focus structure may have a V-shaped cross-section. The detection channel may have a square or rectangular cross-section. Alternatively, the channel may have a U-shaped cross section, or may otherwise have a curved base such that the channel has an arc-shaped, inverted-arch-shaped or semi-circular shaped cross-section. The depth of the focus structure may be less than the depth of the channel. The depth of the focus structure may have a fixed (that is, predetermined, known in advance, or non-arbitrary) relationship with the depth of the channel. The depth of the channel may

be twice the depth of the focus structure. Here, the depth of each of the focus structure and channel is taken to be the maximum depth, in cases where the depth varies across the cross-section of the focus structure or channel.

5 The detection disc may have multiple detection channels and multiple focus structures. The multiple detection channels may be arranged radially about the disc, and wherein there may be at least one focus structure associated with each detection channel. In the instances where there are several channels on one disc, the channels may have different geometries and/or depths.

10 Viewed from a further aspect, the invention provides a method of manufacturing a substrate for manufacturing a master for a disc for carrying samples in an apparatus for detection of microscopic objects in a fluid, the substrate having a channel and separate focus structure, wherein the focus structure is a groove, the method comprising: providing the substrate having a crystallographic structure; forming the channel in the substrate with an orientation
15 independent of the orientation of the crystalline planes; and forming the focus structure in the substrate with an orientation aligned with one of the crystalline planes.

The invention further extends to a method of manufacturing a master for a disc for carrying samples in an apparatus for detection of microscopic objects in a fluid, wherein the method of manufacture includes manufacturing a substrate as set
20 out in the aspect described above, and using the substrate to manufacture a master.

The master may be manufactured from the substrate by electroplating. The master may be made of nickel.

25 The invention further extends to a method of manufacturing a disc for carrying samples in an apparatus for detection of microscopic objects in a fluid, wherein the method of manufacture includes manufacturing a substrate as set out in the aspect described above, using the substrate to manufacture a master, and using the master to manufacture the disc.

30 The disc may be manufactured from the master by injection moulding or casting.

The substrate may be silicon. The channel and focus structure may be etched into the (100) plane of the silicon. The focus structure may be etched along the {111} plane of the silicon. The focus structure may have a V-shaped cross-
35 section. The channel may be etched to a depth D, and the focus structure may be

etched to a depth that may be less than D . The depth of the focus structure may have a fixed (that is, predetermined, known in advance, or non-arbitrary) relationship with the depth of the channel. The focus structure may be etched to a depth of $D/2$.

5 Formation of the channel may be achieved using for instance the Bosch process or variants of the Bosch process, for example, DRIE dry etching. The focus structure may be formed using hydroxide etching, for instance using KOH, where, in silicon, the etch rate for the (110) plane is greater than the etch rate for the (100) plane, which is greater than the etch rate for the (111) plane. Therefore, a
10 focus structure aligned with a crystalline axis can be formed.

 Alternatively to KOH, TMAH (Tetramethylammonium hydroxide), EDP (Ethylenediamine Pyrocatechol), CsOH, NaOH or $N_2H_4 \cdot H_2O$ (Hydrazine) may be used. Depending on the desired depth of the focus structures an appropriate mask is chosen. For shallow structures (for example, with depth of less than $50\mu m$) SiO_2
15 is usually the simplest to use. For structures deeper than $50\mu m$ one could choose SiN_x as a mask.

 The use of V-shaped focussing structures can be combined with rectangular shaped flow structures but it is not limited to be combined with this type of geometry for the flow structures. It is also possible to use the V-shaped focussing structures
20 with arc-shaped flow or control channels and this could be beneficial especially if optical registration is to take place in the arch-shaped channel.

 The substrate may be provided with features as described above.

 A still further aspect provides a method of manufacturing a detection disc for carrying samples in an apparatus for detection of microscopic objects in a fluid, the
25 detection disc comprising injection moulding the detection disc using a production master plate produced using the substrate as described above.

 Another aspect provides a method of manufacturing a detection disc for carrying samples in an apparatus for detection of microscopic objects in a fluid, the method comprising: providing a substrate having a crystallographic substrate;
30 forming the channel in the substrate with an orientation independent of the orientation of the crystalline planes; forming the focus structure in the substrate with an orientation aligned with one of the crystalline planes; using the substrate to produce a production plate; and using the production master plate to manufacture the detection disc having a detection channel and a focus structure.

The master may be produced by electroplating using the substrate as a template. The master may be made of nickel. The method may include forming a mould from the master; and injection moulding the detection disc using the mould in order to obtain a detection disc having a detection channel and a focus structure.

5 The focus structure may be formed to have a predetermined depth in relation to the depth of the channel. For example, the focus structure may have a depth that is related to the depth of the channel by a specific ratio.

This method may include providing the detection disc with features as described above.

10 A yet further aspect provides a method of finding a focus plane in an apparatus for detection of microscopic objects in a fluid, wherein the method comprises: providing an optically transparent substrate having a focus structure, the focus structure comprising a groove with sloping sidewalls provided in a first surface of the substrate; illuminating the substrate from a second surface, opposed
15 to the first surface; imaging the focus structure on the first surface using an imaging apparatus, and focussing the imaging apparatus on the focus structure, wherein the angle between each sidewall of the focus structure and the first surface may be greater than the critical angle of the substrate.

 The focus structure is a groove in that it may have a cross-section that is
20 constant along one primary direction of the focus structure, for example its length or width. The focus structure may have a V-shaped cross-section. The method may include using information about the focus plane found by the imaging apparatus to focus a further, separate, imaging apparatus to the same focus plane. The focussing imaging apparatus may be an area detector and the further imaging
25 apparatus may be a line detector. The method may comprise illuminating the detection channel with a line beam, the projection of the line beam being co-linear to the line of the line detector at the imaging plane.

The substrate may be a detection disc as described above and the focus structure may be a focus structure of the disc.

30 A further aspect provides a method of focussing a first imaging apparatus to a focus plane in an apparatus for detection of microscopic objects in a fluid, wherein the method comprises: imaging a focus structure with a second imaging apparatus, focussing the second imaging apparatus on the focus structure to find the focal plane; and focussing the first imaging apparatus to the focal plane to which the
35 second imaging apparatus is focussed. The first imaging apparatus may be a line

detector, and the second imaging apparatus may be an area detector.

Alternatively, an area detector is used as the first imaging apparatus where a selected line or a selected subset of lines can be read out, e.g. a CMOS array.

Alternatively, a single line detector serves the purpose of both the first and second
5 imaging apparatus. Alternatively, a single imaging apparatus where a selected line or a selected subset of lines can be read out, e.g. a CMOS array, serves the purpose of both the first and second imaging apparatus.

The focus structure is a groove in that it may have a cross-section that is constant along one primary direction of the focus structure, for example its length or
10 width. The focus structure may comprise a groove with sloping sidewalls provided in a first surface of the substrate, wherein the angle between each sidewall of the focus structure and the first surface may be greater than the critical angle of the substrate. The method may comprise illuminating the detection channel with a line beam, the projection of the line beam being co-linear to the line of the line detector
15 at the imaging plane. The line of the line detector and the projection of the line beam may both be perpendicular to the channel. The method may comprise using a detection disc as described above, where the focus structure is a focus structure of the disc.

In another aspect, the invention provides an apparatus for detecting and/or
20 counting microscopic objects comprising: a detection disc comprising a detection channel for carrying the microscopic objects in a sample fluid and a separate focus structure associated with the detection channel, a first imaging apparatus for finding a focal plane by focussing on the focus structure; a second imaging apparatus for detecting and/or counting the objects in the detection channel, the second imaging
25 apparatus being focussed to the focal plane using information about the focal plane from the first imaging apparatus. The first imaging apparatus may be a line detector, and the second imaging apparatus may be an area detector.

Alternatively, an area detector is used as the first imaging apparatus where a selected line or a selected subset of lines can be read out, e.g. a CMOS
30 architecture. Alternatively, a single line detector serves the purpose of both the first and second imaging apparatus. Alternatively, a single imaging apparatus where a selected line or a selected subset of lines can be read out, e.g. a CMOS array, serves the purpose of both the first and second imaging apparatus.

The focus structure is a groove in that it may have a cross-section that is
35 constant along one primary direction of the focus structure, for example its length or

width. The focus structure may comprise a groove with sloping sidewalls provided in a first surface of the substrate, and wherein the angle between each sidewall of the focus structure and the first surface may be greater than the critical angle of the substrate. The apparatus may include a light source for illuminating the detection channel with a line beam, the projection of the line beam being co-linear to the line of the line detector at the imaging plane. The line of the line detector and the projection of the line beam may both be perpendicular to the channel. The detection disc may be as described above.

A further aspect of the invention provides an apparatus for detecting and/or counting microscopic objects, the apparatus comprising: a detection disc comprising a detection channel for carrying the microscopic objects in a sample fluid and a separate focus structure associated with the detection channel, a first imaging apparatus for finding a focal plane by focussing on the focus structure; a second imaging apparatus for counting the objects in a detection channel, the second imaging apparatus being focussed to the focal plane using information about the focal plane from the first imaging apparatus, wherein the focus structure comprises a groove with sloping sidewalls provided in a first surface of the detection disc, and wherein the angle between each sidewall of the focus structure and the first surface is greater than the critical angle of the substrate. The focus structure is a groove in that it may have a cross-section that is constant along one primary direction of the focus structure, for example its length or width. The focus structure may have a V-shaped cross-section. The apparatus may comprise a further, separate, imaging apparatus that receives information about the focal plane found by the focussing imaging apparatus and uses this information to focus on the focal plane. The focussing imaging apparatus may be an area detector and the further imaging apparatus may be a line detector. Alternatively, an area detector is used as the first imaging apparatus where a selected line or a selected subset of lines can be read out, e.g. a CMOS architecture. Alternatively, a single line detector serves the purpose of both the first and second imaging apparatus. Alternatively, a single imaging apparatus where a selected line or a selected subset of lines can be read out, e.g. a CMOS array, serves the purpose of both the first and second imaging apparatus.

The apparatus may include a light source for illuminating the detection channel with a line beam, the projection of the line beam being co-linear to the line

of the line detector at the imaging plane. The line of the line detector and the projection of the line beam may both be perpendicular to the channel.

The detection disc may be as described above.

5 A still further aspect of the invention provides a sample processing module for an apparatus for detecting and/or counting microscopic objects, the sample processing module comprising: a carousel for holding a plurality of sample containers; a number of stations for sample processing steps including a filling station for filling the sample containers, a heating station for heating a sample in the sample container; a cooling station for cooling the sample in the sample container;
10 an agitation station for agitating the sample in the sample container; and a purging station for purging the sample from the sample container and passing it to a detection module for analysis of the sample; wherein the heating and cooling stations act on the sample without contact with the sample.

The heating station may use infra-red or heated air to heat the sample.
15 Each of the filling, heating, cooling and purging stations may act without contact between the stations and the sample container held in the carousel.

The filling station may comprise a dispensing nozzle arranged to fill the sample container without contact with the container or carousel. The purging station may comprise a compressed air source arranged to expel the sample
20 through a hole in the container without contact between the compressed air source and the container or carousel.

The sample processing module may be used in conjunction with the apparatus of any of the other aspects described above.

Certain preferred embodiments are described below by way of example only
25 and with reference to the accompanying figures in which:

Figure 1 shows a part of a moulded disc with detection channels and focus structures, the focus structures being shown with exaggerated size for illustrative purposes;

Figure 2 shows a focus structure in close up view;

30 Figure 3a and 3b illustrate a light beam directed at the focus structure and the resultant reflection and refraction of light rays;

Figure 4a and 4b show a schematic and a photograph respectively with, on the left, an out of focus image and, on the right, a focussed image of the focus structure;

Figures 5a and 5b show two schematic cross-sections with the detection channel and focus structures for two example depths of channel;

Figure 6 illustrates images of objects in a detection channel, the images taken with different line rates of a line camera in each of Figures 6a, 6b, 6c and 6d;

5 Figures 7A and 7B illustrate matching the optical sampling volume to the channel geometry;

Figure 8 shows flow rate of the sample fluid across the width of a detection channel;

10 Figure 9 shows an example apparatus using high speed fluorescence detection to detect objects in the detection channels of a disc of the type shown in Figure 1;

Figure 10a and 10b show non-contact heating used in a sample processing module of an example apparatus; and

15 Figure 11 shows purging of a microfluidic reaction container in the sample processing module.

Figure 12 shows the relation between the substrate having a crystallographic structure and the production master.

20 This disclosure concerns an apparatus for detecting of and counting of microscopic objects, such as particles (particularly bio-particles), cells, micro-organisms such as bacteria or molecules including macromolecules. The microscopic objects can include any object that is suitable small in size and can be detected based on optical methods, in particular fluorescence of the object or of a fluorescent composition applied to the object. If the objects are translucent objects without scattering properties, such as RCPs, the objects may be 10µm or less in size, perhaps 5µm in size or smaller. The objects may have a largest dimension of 25 2 µm or less, perhaps 1,5 µm or less, 1 µm or less and in some cases 0.5 µm or less.

30 The example apparatus of the preferred embodiment uses optical elements to detect small objects in a detection channel 4 of a sample substrate 2 in the form of a disc 2. Figure 1 shows a part of the disc 2. The disc 2 has multiple such detection channels 4 and can be rotated to allow repeated testing or different tests during a single test cycle. Figure 1 shows a disc with the same geometry for each channel 4 but the channel geometry could be varied on a single disc to allow different tests to be performed. The disc 2 also includes a focus structure 6, 35 explained in more detail below. The focus structure 6 is used to determine a focus

depth for best detection of small objects in a fluid in the detecting channel 4. Each detection channel has adjacent focus structures 6.

5 The focussing uses a dedicated focussing camera and it is carried out automatically as a part of the test cycle for the apparatus. The focussing camera may be a CCD area imaging chip. It is an area camera, i.e. a camera that captures a two dimensional image. Focussing may be carried out by a suitable algorithm. One algorithm is explained below. The focus information obtained by the focussing camera is used to set the focus depth for a line camera that detects the small objects in the detection channel 4. This line detector may be any suitable line
10 camera, such as a CCD linear sensor and the use of the line detector is discussed in more detail below. The output from the line detector is an image allowing counting of the small objects of interest. Advantageously, by the use of the proposed focus structure and detection channel it becomes possible to automatically focus accurately onto a text volume that is small enough for single
15 small objects, such as single molecules to be detected and counted. The apparatus can hence be used for single molecule detection, for example in relation to rolling circle products (RCPs) generated during DNA replication/amplification. The processes used to generate the samples for the apparatus may be conventional process for production of samples of RCPs, bacteria, cells, macro-
20 molecules or any other microscopic object requiring detecting and/or counting. Possible processes that might be used to provide samples for use with the proposed new apparatus are described in [earlier patent publications., Of special interest are processes generating Rolling Circle Products as a result of detection of nucleic acids, proteins, cellular or other macromolecular targets via the use of so
25 called Padlock probes, Proximity ligation, Proximity Extension or methods for circularization of nucleic acids as described in e.g. US7790388, US8053188, US7320860, RE44265, US7074564, US5871921, US6558928, US8664164, US20140030721 or WO2012160083. One example process that could be used to generate samples in the form of RCPs is described in the applicant's co-pending patent application GB 1321123.0. Fluorescent labelling of bacteria is well known in
30 the art. Examples include the use of non-specific detection protocols that employ signal producing systems that stain the double stranded DNA of bacteria, e.g., via intercalation. Representative detectable molecules that find use in such embodiments include fluorescent nucleic acid stains, such as phenanthridinium
35 dyes, including monomers or homo- or heterodimers thereof, that give an enhanced

fluorescence when complexed with nucleic acids. Examples of phenanthridinium dyes include ethidium homodimer, ethidium bromide, propidium iodide, and other alkyl-substituted phenanthridinium dyes. The nucleic acid stain may be or may incorporate an acridine dye, or a homo- or heterodimer thereof, such as acridine orange, acridine homodimer, ethidium- acridine heterodimer, or 9-amino-6-chloro-2-methoxyacridine. The nucleic acid stain may be an indole or imidazole dye, such as Hoechst 33258, Hoechst 33342, Hoechst 34580 (BIOPROBES 34, Molecular Probes, Inc. Eugene, Oreg., (May 2000)) DAPI (4',6-diamidino-2- phenylindole) or DIPI (4',6-(diimidazolin-2-yl)-2-phenylindole). Other permitted nucleic acid stains include, but are not limited to, 7-aminoactinomycin D, hydroxystilbamidine, LDS 751, selected psoralens (furocoumarins), styryl dyes, metal complexes such as ruthenium complexes, and transition metal complexes (incorporating Tb³⁺ and Eu³⁺, for example). The nucleic acid stain may be a cyanine dye or a homo- or heterodimer of a cyanine dye that gives an enhanced fluorescence when associated with nucleic acids. Any of the dyes described in U.S. Pat. No. 4,883,867 to Lee (1989), U.S. Pat. No. 5,582,977 to Yue et al. (1996), U.S. Pat. No. 5,321,130 to Yue et al. (1994), and U.S. Pat. No. 5,410,030 to Yue et al. (1995) may be used, including nucleic acid stains commercially available under the trademarks TOTO, BOBO, POPO, YOYO, TO-PRO, BO-PRO, PO-PRO and YO-PRO from Molecular Probes, Inc., Eugene, Oreg. Any of the dyes described in U.S. Pat. No. 5,436, 134 to Haugland et al. (1995), U.S. Pat. No. 5,658,751 to Yue et al. (1997), and U.S. Pat. No. 5,863,753 to Haugland et al. (1999) may be used, including nucleic acid stains commercially available under the trademarks SYBR, SYTO, SYTOX, PICOGREEN, OLIGREEN, and RIBOGREEN from Molecular Probes, Inc., Eugene, Oreg. The nucleic acid stain may be a monomeric, homodimeric or heterodimeric cyanine dye that incorporates an aza- or polyazabenzazolum heterocycle, such as an azabenzoxazole, azabenzimidazole, or azabenzothiazole, that gives an enhanced fluorescence when associated with nucleic acids, including nucleic acid stains commercially available under the trademarks SYTO, SYTOX, JOJO, JO-PRO, LOLO, LO-PRO from Molecular Probes, Inc., Eugene, Oreg.

Other methods of staining bacteria utilizes stains that distinguish live from dead bacteria such as is described in e.g. US 5534416 or WO 2012092238.

Advantageously, the samples may be processed automatically using a sample preparation module (SPM) provided as part of the detection apparatus. The SPM may automatically prepare the samples as discussed in more detail below.

Focus Structure

The focus structure 6 is placed just beside the detection channel 4 and is used as a surrogate marker. A dedicated focussing camera focusses on the focus structure 6 instead of focussing directly on the objects to be detected. An
5 advantage of this is that no fluorescence signal is needed for focussing, focussing is not dependent on the objects being in a fixed position, and focussing is always made at a fixed depth, defined by the geometry and depth of the focus structure 6. The depth of the focus marker can be placed at any depth in relation to the depth of the detection channel, as will be seen from the discussion below the focus marker
10 may have a depth that is a half of the depth of the detection channel.

The focus structure consists of a V-shaped groove in the moulded disc. The disc is manufactured by injection moulding using a production master (reference numeral 3 in Figure 12) in e.g. nickel produced from a silicon substrate (a wafer of silicon, reference numeral 1 in Figure 12). This process is similar to the processes
15 used for manufacture of compact discs as used for storage of data and music. In fact a CD moulding machine may be used to create the discs.

To produce a functional structure comprising both a focus structure and a flow structure using a replication process, first the desired pattern is transferred to a substrate having a crystallographic structure. The substrate may comprise silicon.
20 The substrate when processed is then used to serve as a template of opposite polarity as a replication master (production master plate, or master) used for production of the flow and focus structures. The replication process can for instance be thermoplastic injection moulding or casting. The production master plate may comprise nickel and may be produced by electroplating. In one preferred
25 embodiment the replication master is used in CD-based thermoplastic injection moulding, to generate CD shaped substrates having the desired flow and focus structures as described above.

In the silicon substrate, the shape of the flow channels (also referred to as detection channels) are etched independent of the orientation of the crystal axes in
30 the silicon substrate using deep reactive-ion etching (DRIE). The shape of the focus structure (V-groove) is etched along the (111) plane of the crystal wafer. The angle between the surface of the master and the (111) plane is then 54.7 degrees.

The focus structure is etched by wet etching as described above, with e.g. potassium hydroxide (KOH). The depth of the focussing structure is defined by the
35 diameter of opening in the mask used for wet etching, such that it possible to define

structures of different depth and to correlate those with the depth of the flow channels. The depth of the focussing structure is set to be less than the depth of the flow channel. In the examples shown in Figures 5a and 5b, the depth of the flow channel is h , and the depth of the focus structure is $h/2$ (i.e. half the depth of the flow channel). The same focussing strategy can then be used independent of the depth of the flow channel. The focussing camera can obtain good information about the focus depth even if the height and position of the disc is not constant for different discs or for different detection channels on the same disc. There is always a focus reference point adjacent to the detection channel that provides an accurate focus depth for the channel of interest. It should be noted that the relationship between focus structure depth and the detection channel depth can be set at a predetermined off-set to each other.

Since the silicon plane is used to wet-etch the focus structures they can only be oriented in 90 degree angles from the top plane. This is not the case for the DRIE etched flow channels, which are placed radially along the disc. The combination of the two etching techniques generates an optimal system to deliver both a focussing structure defined by the crystal plane and an arbitrarily-oriented but rectangular-shaped flow channel.

The materials used to manufacture the injection-moulded disc may be Zeonor® 1060R with a refractive index of 1.5, or PDMS (refractive index 1.4). The refractive index of the disc material should be greater than about 1.22 ($1/\sin(54.7^\circ)$), for reasons explained below. Other suitable materials can of course also be used for the detection disc such as UV-grade-PMMA, PMMA, PC or other COC polymer based materials. In order to have as sharp focussing structures as possible it is preferable to use thermoplastic materials with excellent form-filling and flow characteristics.

When focussing, the groove is illuminated from below with a light beam, close to parallel, as shown in Figure 3a. As can be seen from Figure 3b, the incident angle of a collimated light beam on the sidewalls of the groove gives rise to total reflection when the refractive index of the disc material is greater than about 1.22 (i.e. $1/\sin(54.7^\circ)$, where 54.7° is the angle between the surface of the master and the (111) planes). In the case of a less than perfectly collimated beam, the reflection may not be total, but sufficient for contrast detection as detailed below.

As a result of the total internal reflection, when viewed from the top, the area of the groove appears dark. If the imaging camera is focussed exactly on the

bottom level of the groove, where the sidewalls of the V-shaped groove meet, a bright line appears. The contrast between this bright line and the dark surrounding changes rapidly with changing focus plane, allowing precise contrast-based focussing. In each of Figures 4a and 4b (which show corresponding view, as a schematic in Figure 4a, and as a photograph in Figure 4b), the figure on the left shows the view when the imaging camera is out of focus, and the figure on the right shows the view when the imaging camera is in focus.

Focussing algorithm

The focus plane of the image used for autofocus is first placed at a known position relative to the position of the bottom of the groove. That is, it is known that the groove will not be in focus, but the position is close enough so the dark area of the entire groove can be identified. Also, it is known whether the actual focus position is on the far or near side of the groove bottom.

First the algorithm finds the dark area of the groove, and masks this. Then, using a pre-defined threshold, the algorithm tries to find a lighter area contained within the dark area. If it succeeds, a contrast value is calculated. The algorithm then moves the focal plane a defined step size closer to the groove bottom, the procedure is repeated, and as long as the contrast value increased, the algorithm moves in the same direction. When a contrast value smaller than the previous is calculated, the direction of movement is reversed, and the step size is halved. This continues until a max focus value using the smallest step size possible is reached. The groove bottom is then focussed to within less than 1 μm , or even within less than 0.5 μm . The invention thus provides a production method and a design of a focus structure enabling more precise focussing using a very simple algorithm than existing other means of producing a substrate to be used for autofocussing of a sample. One of the key features of the invention is the production of the focus structure in the same material as the detection channel and possibly at the same time. No additional work that can affect the positioning of the focus structure in relation to the focus volume in the detection channel needs to be performed afterwards. Such as is the case using e.g. metal markers attached to a surface as is used in existing solutions. The positioning within less than 1 μm of the wanted focus plane of the current invention is superior in precision compared to current methods relying solely on reflection.

Line Detector

A sample volume in the detection channel is imaged with a line detector (a line camera having a single line of pixels).

The different images that would be obtained for a given line rate are shown in Figures 6a to 6d, which show in the top drawings the particles moving through the channel, and in the bottom drawings, the image obtained by the line camera. The image obtained is built up of a large number of lines.

In Figure 6a, the object is stationary, and all lines are identical. When the object moves slowly (as in Figure 6b), the line image will change slowly. When the object moves in sync with the rate of line images taken (as in Figure 6c), the frame accurately reproduces the object. When the object moves faster (as in Figure 6d), the objects appears compressed, in the extreme case to single short lines.

The sample fluid that is pumped through the detection channel has a parabolic flow rate over the channel width (see Figure 8). The line rate of the detector, while variable, is the same over the whole line image. It is therefore not possible to achieve a 1:1 ratio between flow rate and the line rate (as in Figure 6c) over the entire channel width. However this is not necessary when counting the number of microscopic particles (for example, RCPs). By adjusting the flow and line rate it is possible to compress the 2D image of a particle into one single exposure of the line detector. It will then become a line of a certain width. This is done for a certain region of interest (ROI) of the channel, excluding the areas adjacent to the channel sides.

The advantage of compressing the image in this way is that the intensity of the pixels containing then object is strongly elevated over the background compared to 1:1 imaging. Furthermore, the image analysis speed increases. The image size needed to record a given volume of fluid also decreases. Finally, only very large objects falling within the ROI will cover more than 1 line, and can be readily rejected as artefacts.

Optical Sampling Volume

The optical sampling volume should be matched to the channel geometry. Figure 7a illustrates a matched setup, in which the entirety of the optical sampling volume is contained within the sample volume (i.e. within the cross-section of the detection channel). Figure 7b illustrates a mismatch, wherein the optical sampling volume is not entirely contained within the sample volume. This will lead to unwanted reflections and other artefacts relating to the channel top and bottom being included in the optical observation volume.

It is also clear from Figures 7a and 7b that the effect of channel drift in the z-axis is minimized using a deeper channel as smaller drifts will still keep the observation volume within the channel. If a shallow channel is used, any drift will sooner place the observation volume outside the channel.

5 The voxel in which an RCP will be imaged as a point is limited by the optical resolution in the axial direction, and (depending on pixel size, numerical aperture and magnification, and flow rate) either the optical lateral resolution, the pixel size, or the flow rate. First, if the flow rate is disregarded (or set to 0), the lateral resolution using a 40x, MN1.3 objective with an excitation wavelength of 532 nm is
10 0.23 μm , and for a 20x NA 0.8 objective 0.38 μm , according to Equation 1.

$$FWHM_{ill,lateral} = 0.51 \frac{\lambda_{exc}}{NA}$$

Equation 1

Here, NA is the numerical aperture of the objective and λ_{exc} is the wavelength of the excitation light.

15 In the image plane, the projected distance is 9.05 or 7.65 μm for 40 and 20 x objectives, respectively. The pixel width of the line detector is 14 μm . A typical RCP has a diameter of 0.5 μm , but can be around 1 μm , or e.g. 1.5 μm . RCPs can also be of larger size, e.g. 10 μm , 5 μm or 2.5 μm if using additional amplification of the object on the primary RCP. RCPs have the unusual property compared to many
20 other objects of similar size that they do not have scattering properties. This translates in the image plane to 20 μm for a 40x and 10 μm for a 20x objective. Thus, when imaging an RCP the lateral resolution is limited by the pixel size so that a typical RCP covers about 2 pixels using 40x and 1 pixel using a 20x objective.

25 Conversely, the pixel sizes projected onto the object plane are 0.35 and 0.7 μm for the 40 and 20 x objectives.

With regard to axial resolution, this is described by Equation 2

$$FWHM_{det,axial} = \sqrt{\left(\frac{0.88 \cdot \lambda_{em}}{n \cdot \sqrt{n^2 - NA^2}}\right)^2 + \left(\frac{\sqrt{2} \cdot n \cdot PH}{NA}\right)^2}$$

Equation 2

Here, λ_{em} is the emission wavelength, PH is the object-side pinhole diameter (in μm), n is the refractive index of the immersion liquid (the fluid in which the objects to be counted are carried) and NA is the numerical aperture of the objective.

Here, the height of the CCD pixel line, as a virtual slit, is used as pinhole size, 28 μm . It should be noted that this value is higher than the pinhole diameter required for optimum confocality (pinhole size = 1 Airy unit), which is 20 μm for a 40x NA 1.3 objective, and 16 μm for a 20x 0.8 NA objective. This is in line with the main purpose of the design, namely to minimize the influence of fluorescence related to unbound detection fluorophores present throughout the channel. In the present configuration, the 28 μm slit height represents a balance between background rejection and signal loss.

Using eq. 2, the axial FWHM is 2.4 μm and 7.8 μm for 40x NA 1.3 and 20x NA 0.8 μm , respectively. Thus, using a 40x 1.3 NA objective a voxel in the object plane is about $0.35 \times 0.35 \times 2.4 \mu\text{m}$ for the 40x, and $0.7 \times 0.7 \times 7.8 \mu\text{m}$ for the 20x objective.

When using a channel of a fixed height, the choice of these objectives affects the properties of the analysis. With the 40x objective the voxel depth is significantly smaller than for the 20x. Thus, a smaller portion of the channel depth is scanned.

When the focal plane is centred in the mid-channel, (see Figure 8) the flow rate variation over the voxel height and artefacts related to the channel top and bottom are minimal. This translates into a more homogenous volume to be analysed, and in turn a greater counting precision. Using the 20x, on the other hand results in a larger portion of the channel being scanned at the cost of a certain precision loss.

The flow rate in the system is adjustable between 5 and 50 $\mu\text{l}/\text{minute}$. A common value is 25 $\mu\text{l}/\text{min}$. Using a channel with a $60 \times 400 \mu\text{m}$ cross-section, the average flow velocity is then 17400 $\mu\text{m}/\text{second}$. The line rate of the cameras is 5 kHz. The average movement of the fluid per exposure is then about 3.5 μm . The pixel height projected onto the object plane is 1.4 μm if a 20x objective is used. Therefore, a standard RCP of about 0.5 μm diameter will completely pass the detector line in one exposure, giving a 1D representation of the fluorescence intensity profile. In the above calculation, an average speed across the channel is assumed, although this is of course a simplification.

35 High-Fluorescence Detection of RCPs

One use for the apparatus is detection of RCPs. Solutions containing the labelled RCPs are analysed using a dedicated high-speed fluorescence detection instrument as shown in Figure 9. The sample solution 18 is pushed through a flow channel with a cross section of $200 \times 40 \mu\text{m}$ (WxH). The flow channels are aligned radially on a CD-format plastic disc as described above, with appropriate optically clear lid and fluid interfaces, allowing rapid change of channel in case of a malfunction or clog.

Three lasers 20 with wavelengths of 488 nm (Calypso, 100mW, Cobolt AB, Solna, Sweden), 532 nm (Samba 300 mW, Cobolt AB, and 640 nm (Cube 640, 40 mW, Coherent Inc, Santa Clara, CA), are collimated through individually focusable beam expanders 22, bringing the beam diameters up to about 8 mm ($1/e^2$). The beams are made collinear by a system of steerable dichroic and full mirrors. The collinear beams passed through a beam shaping lens 24, designed to produce in conjunction with a high numerical objective 26 (Zeiss Fluor 40x, NA 1.3, Carl Zeiss AB, Stockholm, Sweden), a line illumination profile across the interrogation volume of the flow channel. Finally, just prior to the objective entrance pupil, the laser light passes through a laser-pass dichroic mirror 28 (Semrock Inc, Rochester, NY).

Fluorescent light is emitted by RCPs pumped across the interrogation volume using a syringe pump (Tecan XLP6000, Tecan Nordic AB, Mölndal, Sweden). The emitted wavelength(s) corresponds to the emission spectra of the fluorescent labels bound to RCPs. The emitted light is collected through the objective 26 is reflected or passes through the dichroic mirror 28, and is further collected by CCD line detectors 30 (DALSA Spyder 3, 1024 pixels, line rate 5 kHz, Parameter AB, Stockholm, Sweden). Dichroic mirrors and band-pass filters are used to direct the light from each specific fluorophore to a specific detector. A small portion of the fluorescent light is redirected by a beam sampler onto a CCD area detector 32 ($\mu\text{Eye UI-1545LE-M}$, Parameter AB, Stockholm, Sweden) in order to allow for channel alignment and focussing.

From each detector the results of each sample run are registered as a series of x-t images where each RCP is identified through image analysis. The image analysis consists of background subtraction, pattern recognition, pattern matching across detectors for multiply fluorescent objects discrimination of RCPs and non-specific events, and RCP counting. For each reagent, a threshold for the number of RCPs is set to designate a positive sample.

It will of course be appreciated that this example method can be adapted for use with small objects other than RCPs. Possible small objects that can be detected and counted by the proposed apparatus are discussed above.

Sample Preparation Module

5 The Sample Processing Module (SPM) in this example accepts as a sample either nucleic acid targets to be interrogated, or templated nucleic acid circles created via well-known methods such as immuno-RCA or proximity ligation, indicating target proteins. Of course, other small objects as mentioned above could be used as the sample without departing from the main concepts described herein.

10 The SPM comprises a carousel 40 for holding the samples. The carousel 40 is advanced one step every minute and a new sample can be accepted as rapidly as every 4th minute. If a sample is to be processed, a sample container 42 (microfluidic reaction container, MRC) is inserted into the carousel 40, and stepwise transported and processed through the circle.

15 At certain positions, reagents 41 are dispensed, the MRC 42 is heated, cooled, agitated or purged through a simple, integrated valve 44.

 All reactions such as dispensing, heating, cooling, agitation, and purging are done in a contact-free manner (as shown in Figures 10a, 10b and 11), and the only contact with the processed sample is at the very end of the process where the
20 sample is aspirated to a detection module (DM) for readout.

 The described SPM has the following advantages:

- 1) sample throughput of 100+ samples per day without refill of reagents or consumables;
- 2) rate of analysis up to 1 sample/5 minutes;
- 25 3) random access;
- 4) minimal risk of cross-contamination between samples; and
- 5) small footprint and low weight.

 The process carried out by the SPM can be any known process for preparation of samples for detection. Compared to known devices, the SPM differs
30 in that the reactions and in particular the heating are carried out without contact.

Claims

1. A substrate for use in manufacture of a production master plate for production of a detection disc for carrying samples in an apparatus for detection of microscopic objects in a fluid, the substrate including a channel and a separate focus structure for forming a corresponding channel and a corresponding separate focus structure in the detection disc for focussing an associated imaging apparatus at a predefined imaging plane in the detection disc, wherein the focus structure of the substrate comprises a groove located outside of the channel, said groove comprising sloping sidewalls provided in a first surface of the substrate, wherein an angle between each sidewall of the groove and the first surface of the substrate is greater than a critical angle of the detection disc, and wherein the corresponding focus structure of the detection disc comprises a corresponding groove with sloping sidewalls provided in a first surface of the detection disc with an angle between each sidewall of the corresponding groove and the first surface of the detection disc being greater than the critical angle of the detection disc, such that when the detection disc is illuminated with a light beam that enters the detection disc from a second surface of the detection disc that is opposite the first surface of the detection disc, the light beam is internally reflected by said sidewalls of said corresponding groove and the light beam is transmitted through a bottom of said corresponding groove to a greater extent as compared to said sidewalls of said corresponding groove to allow for contrast-based focussing between the bottom of the corresponding groove and the sidewalls of the corresponding groove.
2. The substrate according to claim 1, wherein the substrate comprises a material having a crystallographic structure.
3. The substrate according to claim 2, wherein the substrate comprises silicon.
4. The substrate according to claim 3, wherein the channel and groove are provided in the (100) surface of the silicon.
5. The substrate according to claim 4 wherein the focus structure has sloping {111}-oriented sidewalls.
6. The substrate according to any one of claims 1 to 5 wherein the focus structure has a V-shaped cross-section.

7. The substrate according to claim 4, 5 or 6 wherein the focus structure is oriented at right angles to the (110) plane.
- 40 8. The substrate according to any one of claims 1 to 7, wherein the channel has a square or rectangular cross-section.
9. The substrate according to any one of claims 1 to 7, wherein the channel has a curved base such that the channel has an arc-shaped, inverted-arch-shaped or semi-circular
45 shaped cross-section.
10. The substrate according to any one of claims 1 to 9, wherein a depth of the focus structure is less than a depth of the channel.
- 50 11. The substrate according to any one of claims 1 to 9, wherein a depth of the focus structure is at a predetermined depth in relation to a channel depth.
12. The substrate according to any one of claims 1 to 9, wherein a depth of the channel is twice a depth of the focus structure.
- 55 13. The substrate according to any one of claims 1 to 12, comprising multiple detection channels and multiple focus structures.
14. The substrate according to claim 13, wherein the multiple detection channels are arranged
60 radially about a disc shape of the substrate, and wherein there is at least one focus structure associated with each detection channel.
15. A detection disc for carrying samples in an apparatus for detection of microscopic objects in a fluid, the detection disc including a detection channel and a separate focus structure,
65 and wherein the focus structure comprises a groove located outside of the detection channel, said groove comprising sloping sidewalls provided in a first surface of the detection disc, wherein an angle between each sidewall of the groove and the first surface is greater than a critical angle of the detection disc such that when the detection disc is illuminated with a light beam that enters the detection disc from a second surface that is
70 opposite the first surface, light from the light beam is internally reflected by said sidewalls of said groove and light from the light beam is transmitted through a bottom of said groove to a greater extent as compared to said sidewalls to allow for contrast-based focussing between the bottom of the groove and the sidewalls of the groove, wherein the detection

disc is made using a production master plate manufactured using the substrate of as defined in any one of claims 1 to 14.

16. A detection disc for carrying samples in an apparatus for detection of microscopic objects in a fluid, the detection disc including a detection channel and a separate focus structure, wherein the focus structure comprises a groove located outside of the detection channel, said groove comprising sloping sidewalls provided in a first surface of the detection disc, wherein an angle between each sidewall of the groove and the first surface is greater than a critical angle of the detection disc such that when the detection disc is illuminated with a light beam that enters the detection disc from a second surface that is opposite the first surface, light from the light beam is internally reflected by said sidewalls of said groove and light from the light beam is transmitted through a bottom of said groove to a greater extent as compared to said sidewalls to allow for contrast-based focussing between the bottom of the groove and the sidewalls of the groove.

17. The detection disc according to claim 15 or 16, wherein the disc is made from a material with refractive index of greater than 1.22.

18. The detection disc according to any one of claims 15 to 17, wherein the disc is made from an optically transparent material.

19. The detection disc according to any one of claims 15 to 18, wherein the disc is made from a thermoplastic polymer.

20. The detection disc according to claim 19, wherein the disc is made from a cyclo olefin polymer.

21. The detection disc according to claim 20, wherein the disc is made from Zeonor® 1060R.

22. The detection disc according to any one of claims 15 to 19 wherein the disc is made from PDMS, UV-grade PMMA, PMMA, PC or a COC polymer-based material.

23. The detection disc according to any one of claims 15 to 22 wherein the focus structure has a V-shaped cross-section.

- 110 24. The detection disc according to any one of claims 15 to 23, wherein the detection channel has a square or rectangular cross-section.
25. The detection disc according to any one of claims 15 to 23, wherein the detection channel has a curved base such that the channel has an arc-shaped, inverted-arch-shaped or semi-circular shaped cross-section.
- 115 26. The detection disc according to any one of claims 15 to 25, wherein a depth of the focus structure is less than a depth of the channel.
- 120 27. The detection disc according to any one of claims 15 to 25, wherein a depth of the channel is twice a depth of the focus structure.
28. The detection disc according to any one of claims 15 to 27, comprising multiple detection channels and multiple focus structures.
29. The detection disc according to claim 28, wherein the multiple detection channels are arranged radially about the disc, and wherein there is at least one focus structure associated with each detection channel.

FIG. 1

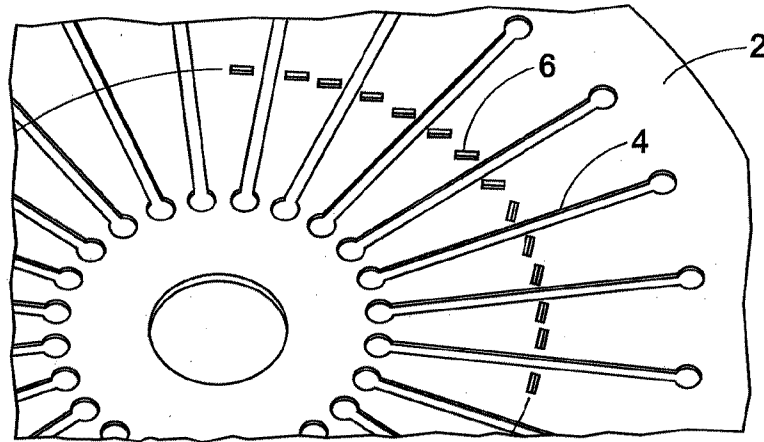


FIG. 2

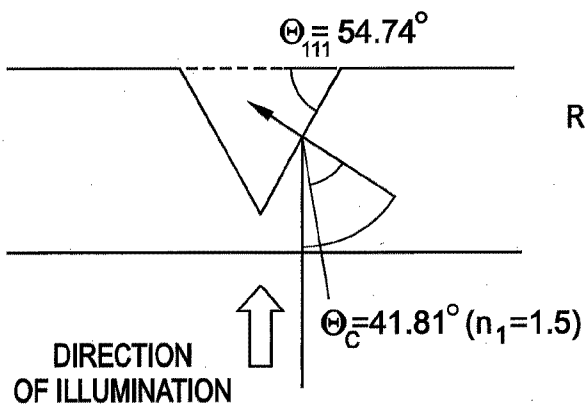
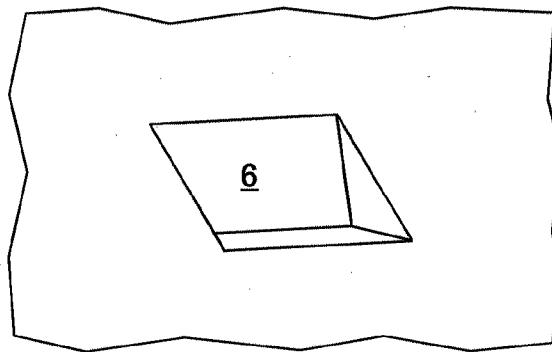


FIG. 3A

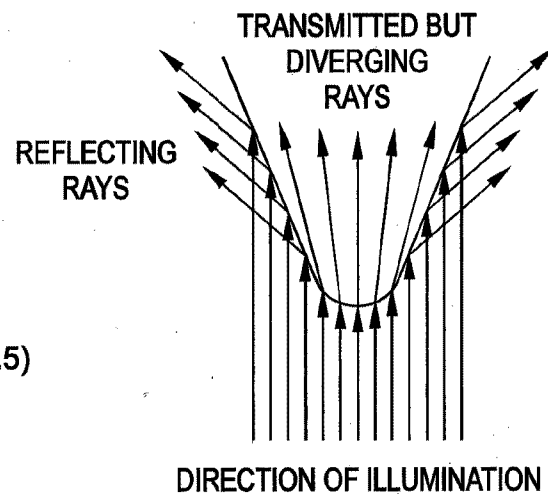


FIG. 3B

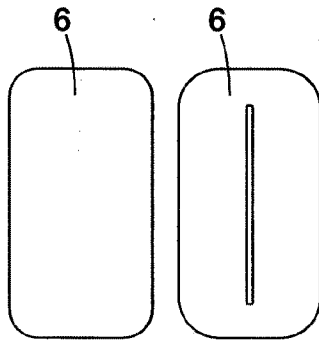


FIG. 4A

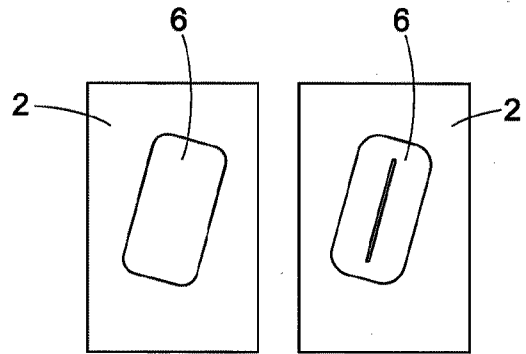


FIG. 4B

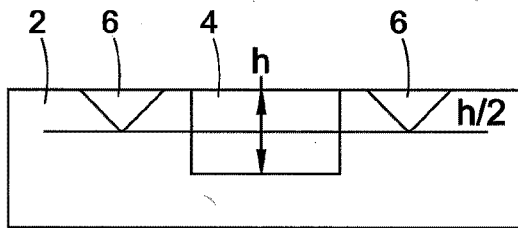


FIG. 5A

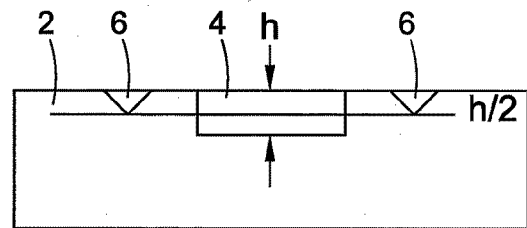


FIG. 5B

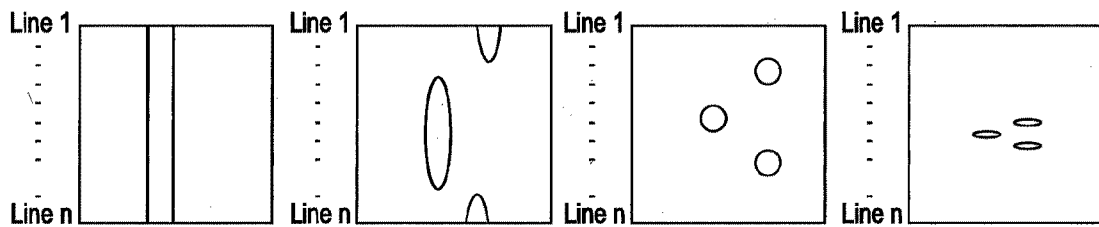
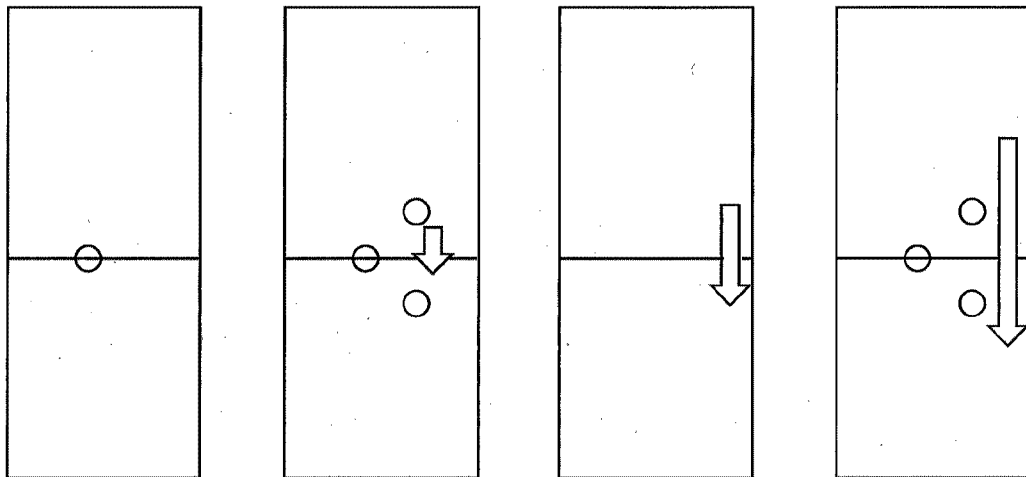


FIG. 6A FIG. 6B FIG. 6C FIG. 6D

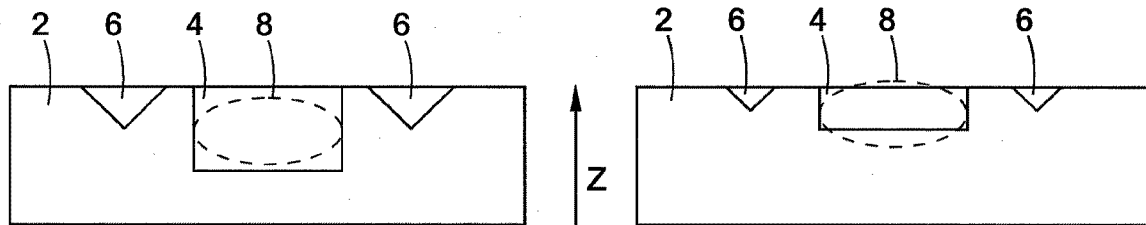


FIG. 7A

FIG. 7B

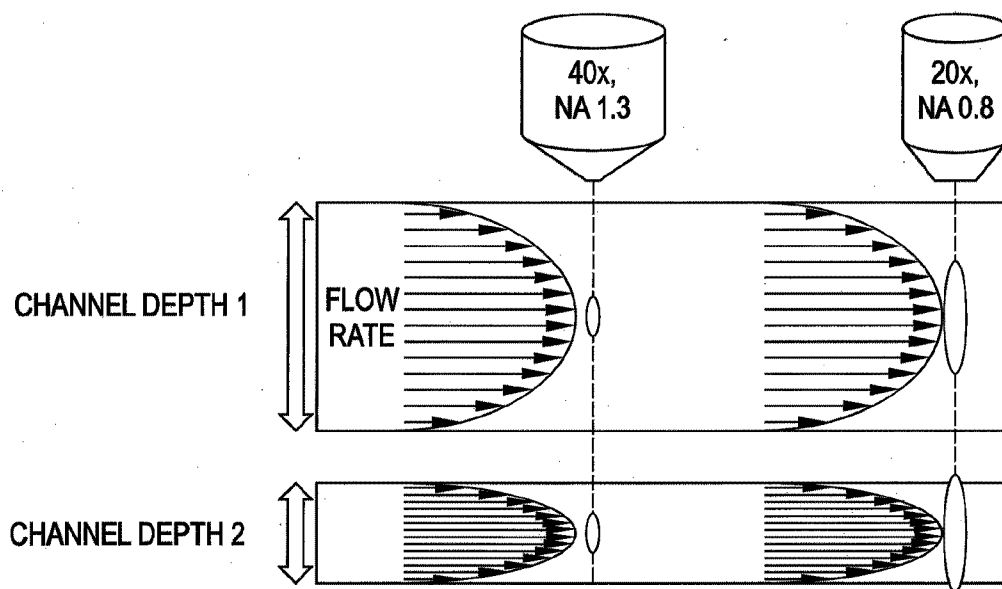


FIG. 8

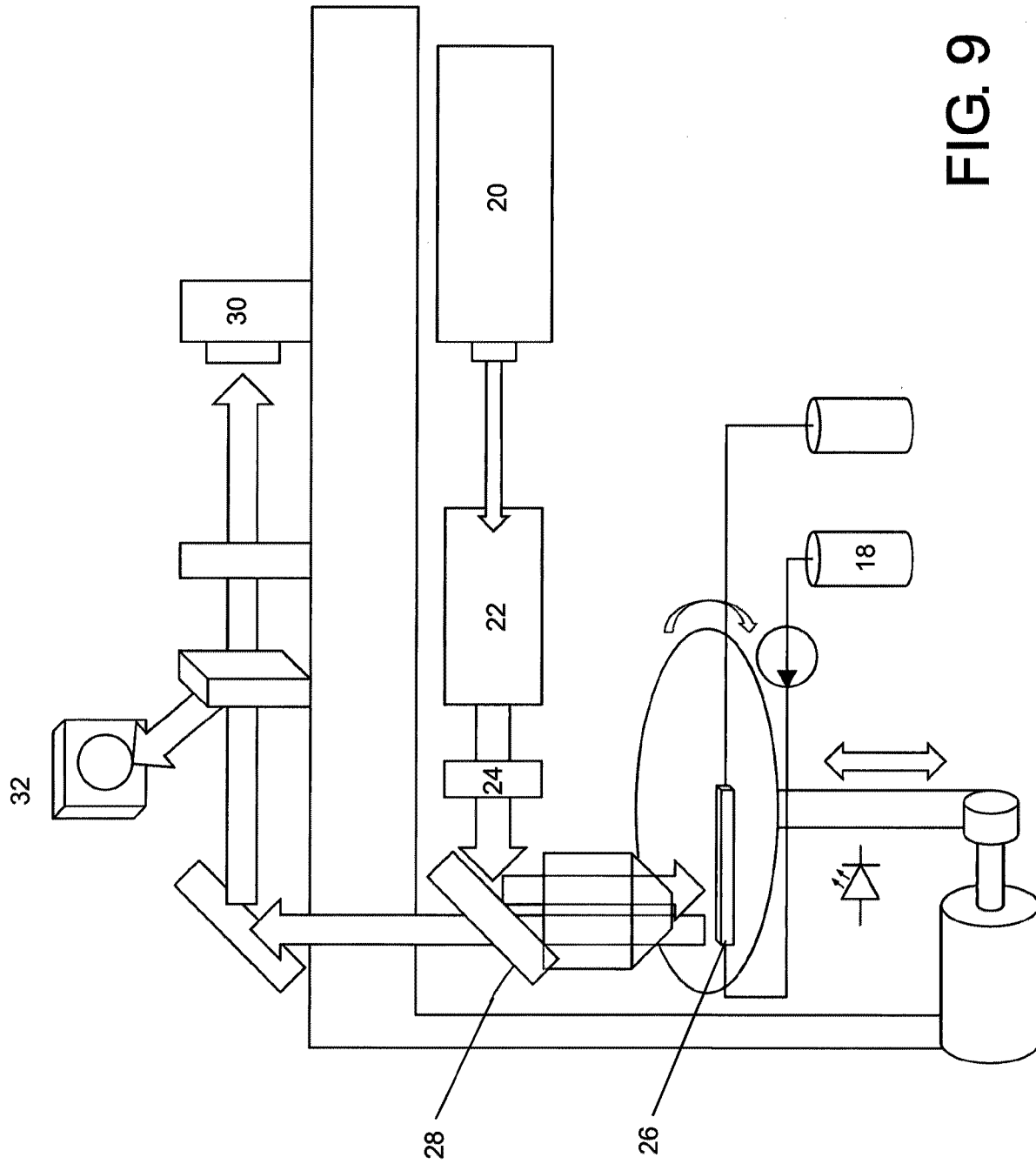


FIG. 9

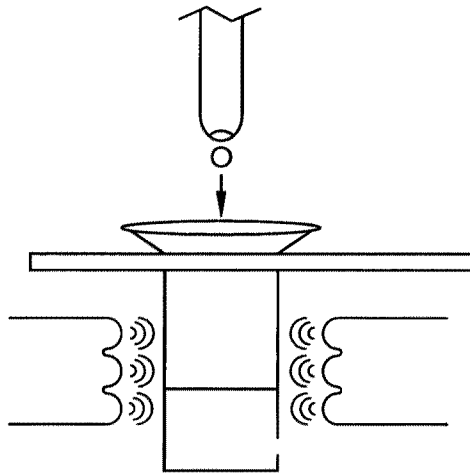


FIG. 10A

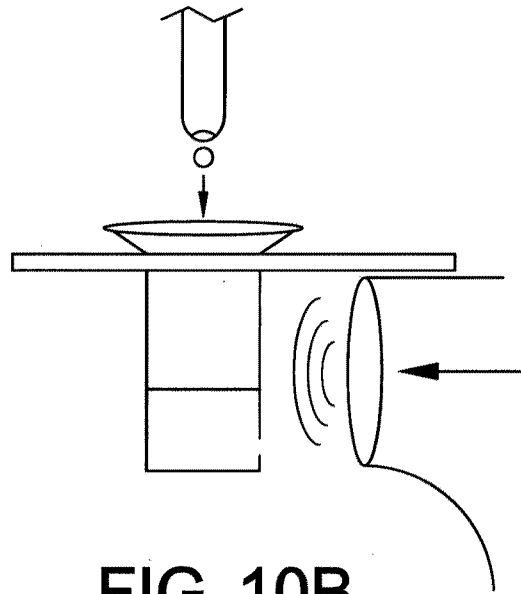


FIG. 10B

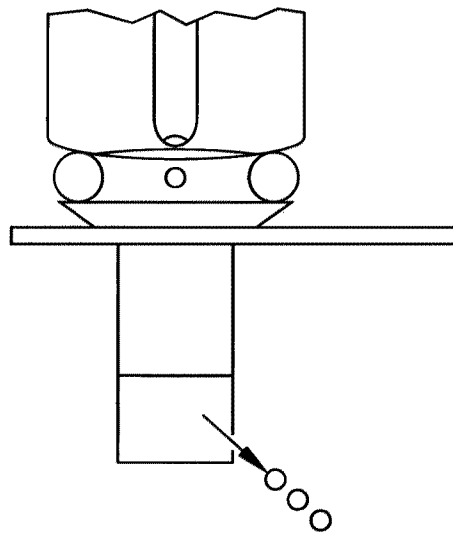


FIG. 11

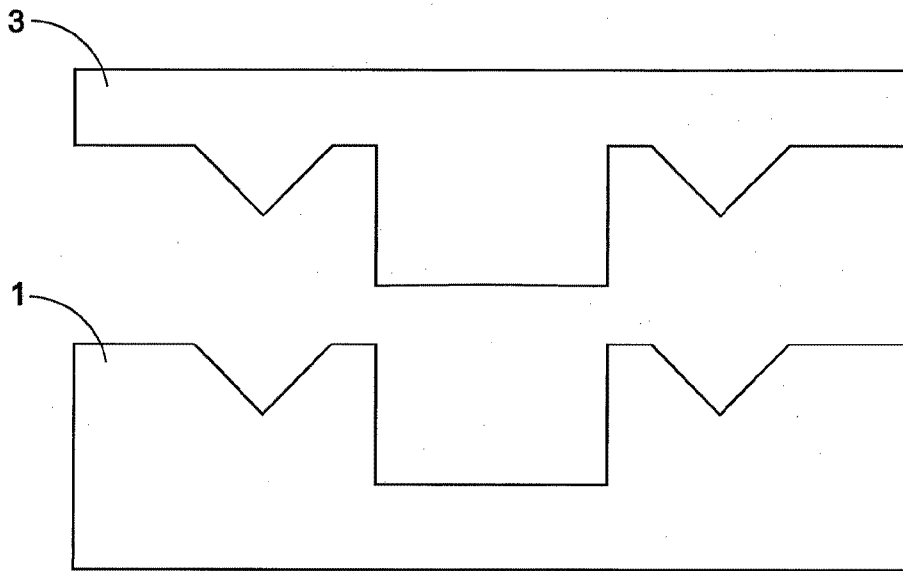


FIG. 12

