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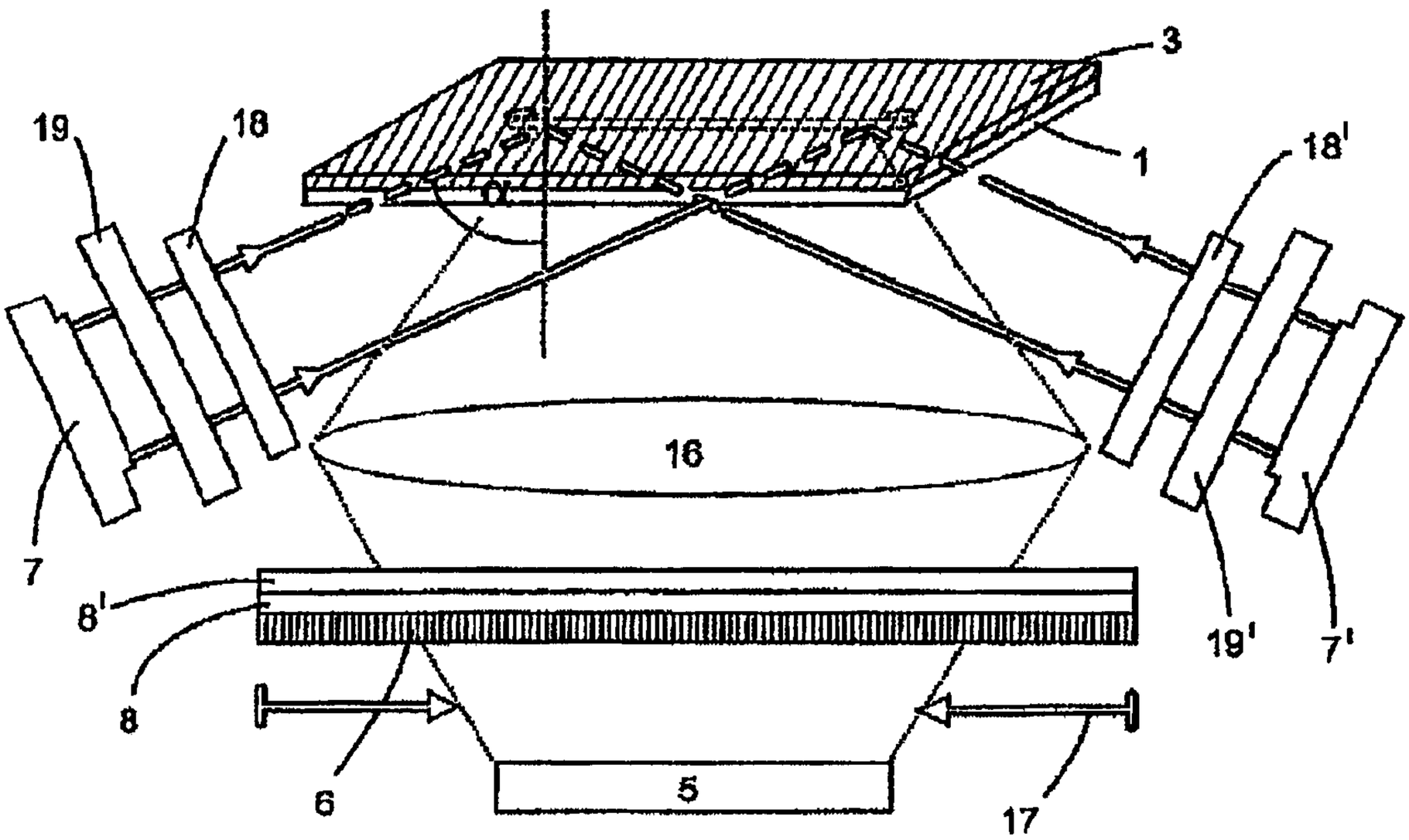


(57) Abrégé/Abstract:
The invention relates to a device and a method for carrying out quantitative fluorescence immunoassays by means of evanescent field excitation. Various known biochemical assays of general receptor-ligand systems can be used. For this purpose, light of at least one light source is directed at an angle α onto the boundary surface of two media which have different refractive indices. A light source is selected which emits substantially monochromatic light with a wavelength which is suitable for exciting a marking substance. The light is directed onto the boundary surface which is disposed between an optically transparent base plate made of a material where the refractive index n_1 is greater than the refractive index n_2 of the material above the boundary surface, and a cuvette-shaped receiving region for the sample. The receiving region is covered with a covering plate on the side disposed opposite the base plate. Arranged between base plate and covering plate is at least one functional layer and a detector for detecting the fluorescent light being disposed on the same side of the base plate as the light source.

Abstract

The invention relates to a device and a method for carrying out quantitative fluorescence immunoassays by means of evanescent field excitation. Various known biochemical assays of general receptor-ligand systems can be used. For this purpose, light of at least one light source is directed at an angle α onto the boundary surface of two media which have different refractive indices. A light source is selected which emits substantially monochromatic light with a wavelength which is suitable for exciting a marking substance. The light is directed onto the boundary surface which is disposed between an optically transparent base plate made of a material where the refractive index n_1 is greater than the refractive index n_2 of the material above the boundary surface, and a cuvette-shaped receiving region for the sample. The receiving region is covered with a covering plate on the side disposed opposite the base plate. Arranged between base plate and covering plate is at least one functional layer and a detector for detecting the fluorescent light being disposed on the same side of the base plate as the light source.

Device and method for carrying out fluorescence immonoassays

The invention relates to a device for carrying out quantitative fluorescence immunoassays by means of evanescent field excitation. Various known biochemical assays of general receptor-ligand systems, such an antigen-antibody, lectin-carbohydrate, DNA or RNA-complementary nucleic acid, DNA or RNA-protein, hormone receptor, enzyme-enzyme cofactors, Protein G or Protein A-immunoglobin or avidin-biotin can be used as a basis. However, antigen-antibody systems are preferred. In particular, high-molecular and low-molecular compounds (e.g. haptens) can be detected according to the invention.

Fluorescence immunoassays or even fluorescence immunosensors have already been generally used for a long time, and they serve, mainly in a liquid sample matrix, to quantify an unknown amount of a specific chemical or biochemical substance. Antibodies are selectively bound to the substance to be determined. The substance to be determined is also referred to by the expert as an antigen. In the fluorescence immunoassays, the analyte-specific antibodies are marked with a marking substance which is optically excited at a certain substance-specific wavelength λ_{ex} and the fluorescent light with a different wave length, which is generally greater, is used with a suitable detector with evaluation of the intensity of the fluorescent light. The exploitation of the evanescent field excitation in carrying out such fluorescence immunoassays, or respectively in the fluorescence immunosensors, is known in the prior art. Thus different solutions have already been described in WO 94/27137, by R.A. Badlay, R.A.L. Drake, I.A. Shanks, F.R.S., A.M. Smith and P.R. Stephenson in "Optical biosensors for

immunoassays; fluorescence capillary-fill device", Phil. Trans. R. soc. Lund. B 316, 143 to 160 (1987) and D. Christensen, S. Dyer, D. Fowers and J. Herron, "Analysis of Excitation and Collection Geometries for Planar Waveguide Immunosensors", Proc. SPIE-Int. Soc. Opt. Eng. Vol. 1986, Fiber Optic Sensors in Medical Diagnostics, 2 to 8 (1993).

In addition, in WO 90/05295 A1, an optical biosensor system is described. In this system, one or more samples are guided, with the use of pumps and valves, through ducts to one or more flow-through measuring cells. These flow-through measuring cells are open upwards and biomolecules can be quantitatately detected by an optical structure disposed above them. For measuring successive new samples, considerable purification outlay is consequently required, in order to avoid measuring errors. Necessary preparation of such a sample generally has to be carried out externally of this system, before the actual measuring, since no elements or measures suitable for this purpose are provided.

In WO 90/06503, a sensor is described in which the excitation light is directed at an appropriate angle through an optically transparent substrate onto a boundary surface to an optically transparent buffer layer. An additional waveguide layer is provided to which the analytes to be determined can be bound.

The refractive index of the buffer layer is smaller than that of the substrate and of the waveguide. At the boundary layer substrate/buffer, total reflection comes about through appropriate choice of the angle of the excitation light, and via the evanescent field product here, the excitation light is coupled into the waveguide situated above the buffer

layer. The light coupled into the waveguide is guided via total reflection in the waveguide and the evanescent field formed during this process is used for fluorescence excitation.

The sample can be received in one or more cavities. The dimensions of such a cavity are only restricted to the extent that its size permits the transport of the samples in the cavities by means of capillary force. After the sample has been received in the cavities, no further flow or movement of the sample takes place.

The known solutions have, however, in general the disadvantage that they are only suitable for specific assay formats and an expensive structure with corresponding process management is necessary.

It is therefore a feature of an embodiment of the invention to create a means to carry out, with a very simply constructed device, quantitative fluorescence immunoassays with different biochemical assays.

In accordance with one embodiment of the present invention there is provided a device for carrying out fluorescence immunoassays by evanescent field excitation comprising: at least one light source, emitting substantially monochromatic light; an optically transparent base plate made of material having a refractive index n_1; a cuvette-shaped receiving region for a sample; a covering plate covering the receiving region on a side disposed opposite the base plate; at least one functional layer between the base plate and covering plate or in an inflow region of

the sample in the receiving region, the functional layer permitting lateral or transverse flow through suction, pressure or capillary force; a detector on the same side of the base plate as the light source to detect the fluorescent light; wherein the at least one light source directs light rays having a wavelength causing fluorescence of a marking substance bound to a chemical or biochemical partner of a general receptor-ligand system, at an angle α, onto the boundary surface; and wherein the refractive index n_1 of the material of the base plate is greater than a refractive index n_2 of a material above the boundary surface.

In accordance with another embodiment of the present invention there is provided a method of carrying out fluorescence immunoassays by evanescent field excitation, the method comprising the steps of: providing a sample volume; guiding the sample volume through at least one functional layer; guiding the sample volume through a cuvette-shaped receiving region; binding a marked chemical or biochemical component to a surface in the receiving region; emitting light with a wavelength causing fluorescence of the marked chemical or biochemical component bound to the surface of the receiving region; measuring, through evanescent field excitation, the fluorescent light.

In the device light of at least one light source is directed at an angle α on the boundary surface of two media with differing refractive indices. Here a light source is selected which emits practically monochromatic light with a wavelength which is suitable for exciting the marking substance, in this case the fluorophore. Particularly suitable as the light source are laser diodes, since they have a suitable beam profile and sufficient luminous

efficiency, with small constructional size and low energy consumption.

However, other light sources which emit monochromatic light can also be used.

The angle α, at which the emitted light is sent to the boundary surface, determines, besides the refractive index of the material disposed in the beam path before the boundary surface, and the material adjoining same, together with the wavelength of the light, the penetration depth d for the evanescent field. The refractive index n_1 of the material which is disposed in the beam path before the boundary surface must render possible total reflection at the boundary surface and should therefore be greater than the refractive index n_2 of the other material disposed thereafter. The angle α is preferably chosen such that the following is true: $\sin(\alpha) > n_2/n_1$. If this condition is met, all the light is reflected at the boundary surface and thus total reflection is achieved. However, when this condition is met, a relatively small portion of the light penetrates through the boundary surface into the material, which is disposed in the beam path after the boundary surface, and the evanescent field is produced. Through the evanescent field, only those marking substances which are located in the immediate proximity of the boundary surface are optically excited. For carrying out the fluorescence immunoassays, the result of this is that only the marking substances of the antibodies or antigens which are bound to the surface of the boundary surface are excited. The fluorescence intensity of the light emitted by these fluorophores is thus directly proportional to the concentration of the marked antibodies or antigens bound to

the surface, and, according to the biochemical assay used, proportional or inversely proportional to the antigen concentration.

Now the device noted above uses at least one light source, which emits practically monochromatic light and directs this at an angle providing the penetration depth d for the evanescent field, onto a base plate which is transparent to this light. The refractive index n_1 of the base plate should be greater than 1.33. On the other side of the base plate, a cuvette-shaped receiving region is formed between a covering plate. Between the base plate and the cuvette-shaped receiving region is formed said boundary surface. The evanescent field acts, with the given penetration depth d, within the cuvette-shaped receiving region on marked chemical or biochemical partners, binds to the surface of a general receptor-ligand system and excites the fluorophores used as the marking substance.

The fluorescence caused is measured at the corresponding intensity with a detector. The detector is disposed on the same side of the base plate as the light source.

A single light-sensitive detector, or a linear or a surface arrangement of a plurality of light-sensitive detectors can be used as the detector.

In the above-described arrangement it is advantageous to direct polarised light onto the sample to be determined. For this purpose, a polarizer can be arranged in the beam path of the light, following the light source.

The spacer and possibly the separating layers to be used are

0.001 to 10 mm thick, preferably 50µm. A recess in the spacer forms the receiving region for the sample. Spacer and separating layers are preferably a biocompatible adhesive film, which is designed to adhere on both sides.

The method is based essentially on the fact that a defined sample volume is guided through the cuvette-shaped receiving region and is subjected to an evanescent field excitation, as has been described already. The sample volume can be guided through the cuvette-shaped receiving region and the functional layer(s) by way of suction, pressure or capillary forces.

In an advantageous embodiment, there is provided at least one inlet aperture in a covering plate, into which a sample container can be inserted or disposed. The aperture is disposed in the covering plate such that a connection may be produced between inlet aperture or sample container and receiving region. In addition, there is a second aperture which represents an outflow and which is also connected with the cuvette-shaped receiving region.

The second aperture can also be provided in the covering plate. An external pump can be connected to this second aperture, or an internal pump may be inserted.

The invention is characterised by the fact that a relatively simply constructed basic device according to the invention can be altered or used in various forms. Thus the essential elements, base plate, covering plate and spacer with cuvette-shaped receiving region, can be combined in the various ways. They can be combined through functional layers, the separating layers disposed if necessary in

between yet allowing the sample to flow through. One or more of such functional layers can, however, also be arranged in the inflow region for the sample into the receiving region. An inlet aperture or a connection between a sample container which may be inserted into the inlet aperture, or the connection of inlet aperture and receiving region forms part of this inflow region.

With the invention, the different assay formats may be carried out and thereby high- and low-molecular compounds can be equally detected. All known assay formats, such as sandwich-titration/competition and displacement formats, can be carried out.

Where separating layers are used between functional layers, or enclosing the functional layers, the separating layers must have corresponding openings, such that the sample volume can flow through the entire device. As separating layers, adhesive films having openings therein through stamping can be used.

The invention will be described in more detail below, by way of example.

The Figures show:

Fig. 1 illustrates a portion of the device according to the invention for receiving a sample;

Fig. 2 is a schematic representation of an embodiment of a device configured according to the invention, with two light sources;

Fig. 3 illustrates a device with sample container;

Fig. 4 illustrates a device with cylindrical hollow body;

Fig. 5 illustrates a device with additional functional layers, with lateral flow;

Fig. 6 illustrates a device with a plurality of functional layers and transverse flow;

Figs. 7+8 illustrate time-dependent fluorescence intensity patterns;

Fig. 9 illustrates a sandwich assay format;

Fig. 10 illustrates another sandwich assay format;

Fig. 11 illustrates a titration or competition assay format;

Figs. 12+

13 illustrate a competition or displacement assay format with directly proportional ratio of analyte concentration and signal intensity;

Fig. 14 illustrates an assay format using an additional solid phase;

Fig. 15 illustrates a displacement assay with additional solid phase;

Fig. 16 illustrates a further displacement assay; and

Fig. 17 illustrates a general key to the assay formats shown in Figs. 9 to 16.

Fig. 1 shows the basic structure of a portion of the device according to the invention. The three parts shown, i.e., the base plate 1, the spacer 4 and the covering plate 3, can be connected to one another before the fluorescence immunoassay is carried out. Alternately, the three parts can be in the form of an already completely finished unit and resemble in their structure a flow-through cell and a measuring cuvette.

The base plate 1 consists of a highly refractive transparent material, such as, for example, glass or a plastics

material, such as a polymer (PMMA or PC) with a refractive index $n_1 > 1.33$. The thickness of the base plate can be within a range of 0.01 to 10 mm, preferably between 0.5 and 1 mm.

The spacer 4 is preferably a thin foil, which is provided on both sides with an adhesive film. Alternately, a thin adhesive film may be applied firstly to the base plate 1 and secondly to the covering plate 3. The total thickness of the spacer including the adhesive used should be in a range between 0.001 and 10 mm, preferably between 0.01 and 0.2 mm. A thickness of 50 µm is most particularly preferred. An opening is worked into the spacer 4 and forms a cuvette-shaped receiving region 2.

As noted in Fig. 1, the covering plate 3 can also have continuous apertures 9 and 11 formed therein. The function of these apertures or bores will be described hereinafter. Apertures 9 and 11 are disposed to at least partially overlap the area of the receiving region 2 of the spacer 4. The spacer 4 can preferably also consist of a biocompatible adhesive film, which is preferably provided on both sides with a detachable commercially available protective layer.

In the example represented in Fig. 2 the device according to the invention uses two light sources 7, 7', filters 19,19' and polarizers. The light source 7' emits light of a wavelength which is different from the first light source 7. In this example, polarised light is preferably used. The device shown in Fig. 2 can be advantageously used when differing marking substances, which can be excited at different wavelengths, are used. Examples of these are the fluorophores Cy5 and Cy7. To excite the fluorophore Cy5, a

laser diode is used with light having awavelength between 635 and 655 nm. To excite the fluorophore Cy7, a laser diode is used which emits light having a wavelength between 730 and 780 nm.

In this embodiment, measuring takes place by way of the diodes 7,7′ being either switched in alternating manner or, for example, correspondingly synchronised choppers can be used, to ensure that only light from one light source 7 or 7′ can reach the sample to excite it and thus no falsifications occur.

However, since in this arrangement two different fluorescence signals have to pass the same filter, a wideband filter 8 can no longer be used. Therefore, two filters 8, 8′ should be disposed in succession, which selectively block the wavelengths of the exciting light sources 7, 7′. Notch filters can, for example, be used for this purpose.

With this arrangement, a reference signal can first be obtained which renders an internal calibration of the measuring signal possible. For reference measurement, a reference antibody is used which is not directed against an antigen from the sample. The reference antibody is first quantified and made distinguishable, with a different marking substance, from the analyte-specific antibody Ak to be determined. The amount of reference antibody actually bound to the surface can be determined with a second light source 7′, which causes light of a fluorescence of the different marking substance, a second scattered light filter 8′ and the detector 5. With this determination, the losses of the marked analyte-specific antibodies Ak or antigens Ag,

not bound to the surface, can be taken into account.

Besides obtaining a reference signal, however, two immunoassays, running independently of one another, can be carried out, the difference coming about with the aid of the different fluorophores.

Fig. 3 shows how a sample container 10 is disposed towards aperture 9 in the covering plate 3 thus forming a connection between sample container 10 and the receiving region 2 via aperture 9. Here the sample container 10 forms the container in which the known amount of biocomponent marked with the marking substance fluorophore is mixed in the sample to be determined. The sample container 10 can clearly define the sample volume and thus, with a fixed and known sample volume, a quantitative statement about the antigen concentration can be obtained. The sample container 10 must, therefore, always be filled with the same amount in order to be able to obtain reproducible results. Advantageously it should always be filled to the maximum. In some assay formats which may be carried out, the specific biocomponent is respectively on the surface of the sample container 10, and through contact with the liquid sample, it detaches itself from the surface and gets into the sample. Moreover the biocomponents can also be found on additional solid phases in the sample container 10. A simple and already known method consists in applying lyophilized antibodies to the surface of the sample container 10. In this way, it becomes possible to store the whole for a relatively long time before the immunoassays are actually carried out. The receiving region 2 defines the surface on the base plate on which, according to the assay format, the respectively corresponding chemical or biochemical

substances are immobilised.

As illustrated in Fig. 4 a preferably cylindrical hollow body 12, in which a piston 13 or some other suitable covering is received serve together as a pump. If the piston 13 moves out of the cylindrical hollow body 12, a negative pressure is produced which sucks the sample material out of the sample container 10 through the receiving region 2 in a direction towards the cylindrical hollow body 12. The flow is maintained by capillary forces in the receiving region 2 and by an absorbent fleece, until the entire sample volume is conveyed through the receiving region 2. The cylindrical hollow body 12 is configured and positioned such that a connection to the receiving region 2 is present. This connection can be provided through the second aperture 11 in the covering plate 3. If no covering plate 3 is used, the connection can also be configured in another manner. The hollow body 12 may also have a hole in its base to facilitate connection.

An external pump can also be connected to aperture 11.

After application of the sample (with the sample container 10), there is a wait time so that the desired binding between the partners of a general receptor-ligand system can take place completely. Thereafter, the pump 12, 13 is activated and one waits until all the liquid has been pumped through the receiving region 2. After excitation with light source 7 or light sources 7 and 7', the antigen concentration can then be determined. This structure according to the invention, as represented in Fig. 2, is to be used for determination of the antigen concentration.

The structure, as previously shown and described, can be used for the most varied biochemical assays, and further examples will be returned to.

As can be seen especially from Figs. 1, 5 and 6, the essential part of the device according to the invention can be designed in various ways. Thus the different elements (plates, layers) can be composed of a kit in variable configurations and can be made available for different assay formats in situ, according to desired requirements.

Fig. 5 shows an example of a device according to the invention, in which additional functional layers with lateral flow are represented. In this arrangement, functional layers 26 and 27 and separating layers 25, 25' are incorporated in the structure as explained as explained in the description of Fig. 1. In this example, two functional layers 26 and 27 are disposed one above the other and are enclosed on all sides by separating layers 25 and 25'. The separating layers can preferably be configured as adhesive films, in which openings are formed, as previously described. These openings serve to make a connection possible between inlet aperture 9, the functional layers 26, 27, the receiving region 2 and the outflow aperture 11. The arrows drawn in Fig. 5 show the direction of flow.

Adaptation to different assay formats can be achieved by variation of the arrangement and/or selection of the functional layers 26, 27. Thus the functional layers 26 and 27 can be, for example, a reagent reservoir or a pure reaction layer.

There also exists the possibility of arranging at least two different functional layers in one plane, such that they can be flowed through in succession.

The structure shown in Fig. 6 of a portion of a device according to the invention differs from the example shown in Fig. 5 in that a transverse flow can be achieved. In this example, three functional layers 28, 28' and 29 are disposed, one directly above the other, i.e. without separating layers, directly on the base plate. Within the stack of layers so formed from functional layers 28, 28' and 29, the spacer 4 with the cuvette-shaped receiving region 2 is disposed underneath the covering plate 3. The arrows indicate the direction of flow.

Other arrangements to that shown in Fig. 6, which ensure transverse flow can, of course, also be constructed. As already noted the functional layers can vary in their number, arrangement and choice of function. In an opposite manner to the example shown, the arrangement can also be designed above the spacer 4.

Separating layers can be used in this example too, however, the transverse flow must not be hindered. The functional layers can again serve as reagent reservoir or reaction layer. The functional layers to be used according to the invention have the advantage that a complete, integrated measuring system is produced and only the sample has to be led through the structure.

Combinations of transverse and lateral flow are also possible. As an example, the combination of the arrangements of Figs. 5 and 6 can be utilized.

The functional layers 26, 27, 28, 28' and 29 can be used for the tasks of preparing the samples (buffering, filtration, separation, elimination of interferences, amongst other things), can be used as reagent carrier layer (e.g. for conjugate release) or as a reaction layer (e.g. for derivatization, for immobilisation of biocomponents or for the course of chemical and/or immunochemical reactions).

Suitable material for the sample preparation are e.g. membranes made of fibrous material to separate plasma and red blood corpuscles, which are available, for example, from the company Pall Biosupport as "Hemadyne-Membran". However, filter papers made of cellulose or regenerated cellulose can also be used for this function.

Suitable materials for the reagent carrier layer are paper made from 100% cellulose or activated nylon 66. It is possible for the surfaces of the materials to be activated or modified in order to alter the flow properties (commercially available from the company Pall Biosupport under the trade name "Prodyne oder ACCUWIK-Membrane"). For lateral flow systems, polyester carriers with a modified surface and in which the flow properties may be controlled are particularly preferred.

Suitable materials for the reaction layers are nitroflow membranes made of nitrocellulose, PVDF (polyvinyl difluoride) membrane (commercially available from the company Millipor with the trade name "Immobilon"). Here too, if desired, the surface can be modified.

In general, fibrous materials, cellulose, nitrocellulose, polypropylene, polycarbonate, polyvinyl difluoride,

hydrogels (e.g. dextran, acrylamide, agar-agar, carrageenan, alginic acid) polyelectrolytes (e.g. acrylic acid, poly-L-lysine, poly-L-glutamic acid) or nuclear track membranes or glass-fibre membranes can be used.

Methods of evaluating the measurement signals are represented in Figs. 7 and 8.

In Fig. 7, the intensity of the measured fluorescence signal is shown dependent on time. With the linear rise in the intensity of the fluorescence signal, it is sufficient to determine the signal rise by differentiation, since the rise can be correlated with the temporal alteration in the amount of fluorophore, which can be measured with the device according to the invention. In this way, the measuring time can be kept very short, since the rise in the intensity of the fluorescence only has to be determined over a short period of time, independently of whether this takes place at the beginning or a later point in time, in carrying out the chemical or biochemical assay. Only the saturation range has to be borne in mind, and care taken that the measurement is only carried out in a time domain in which a temporal alteration of the fluorescence intensity signal can be detected.

Differing from this, another possibility is represented in principle in Fig. 8. Here the difference between an initial and a final value is formed and used for evaluation. A basic signal S_1 is first received before the addition of the analyte to be determined at time t_1 and, following the addition of the analyte, at a point of time t_2, which can be predetermined, a final value S_2 of the measured fluorescence

intensity is determined. The analyte concentration can then be determined through the difference of the values S_2 and S_1. The difference between the time t_2 and t_1, must be so great that an equilibrium has formed.

Possible assay formats are represented in Figs. 9 to 16 which can be carried out with the invention.

Fig. 9 shows a sandwich assay format which is suitable for high-molecular compounds (proteins, amongst other things).

This sandwich format can be carried out in principle in a device, such as represented in Fig. 5 or Fig. 6, in which at least one functional layer is to be used.

The analyte is incubated with the marked antibody first and then led into the detection region of the base plate 3 for evanescent field excitation and corresponding fluorescence.

Another possible way of carrying out a sandwich assay format in sequential form, is to form the sandwich step by step utilizing the analyte and then the marked antibody.

Further possible ways of immobilising the antibody in the base plate region are:

1. adsorption
2. covalent bonding
3. affinity bonding (e.g. A-protein A/G or after biotinylation to avidin)
4. by hybridisation of a nucleic acid marker located on the antibody (single-strand RNA or DNA) to an immobilised single-strand nucleic acid (RNA or DNA)

with complementary sequence.

Coating the base plate region, for the evanescent field excitation, with protein A/G, avidin, amongst other things, offers the possibility of producing a universal element (for the most varied of analytes).

A particularly advantageous embodiment provides the pre-incubation of the analyte with a biotinylised (collector) antibody and a fluorescence-marked (detector) antibody. The two antibodies can, for example, be released simultaneously or in sequence from functionalised layers. The whole immunocomplex is then bound by binding to a sensor surface coated with avidin (alternatively streptavidin or neutravidin). Critical for signal formation is the very high affinity between biotin and avidin; this leads to an improvement in the sensitivity of the assay.

In this embodiment, a device according to Fig. 2 can also be used in conjunction with two different marking substances. Thus the determination of concentration for two different analytes can be carried out quasi simultaneously also independently of the respective binding sites in the receiving region, such that the binding of the marked biocomponents does not have to take place locally selectively.

However, an assay format can also be carried out in which an antibody and a marked antibody fragment (e.g. a Fc-part or an ScFv-fragment) are incubated simultaneously with the analyte, as is shown in Fig. 10. In this arrangement, only the complete antibody binds (to protein A/G or, after biotinylation, also to avidin), and thus the necessity for

an incubation disappears. With this format there is the basic possibility of regenerating the structure used. However, this is not possible with avidin/biotin.

In the simultaneous incubation of analyte, antibody and marked antibody fragment in a sandwich assay, as is shown in Fig. 10, the marked antibody fragment and the antibody can be contained, for example, in function layer 27, of the example shown in Fig. 5.

Instead of immobilising a collector antibody, other biocomponents, binding the analyte, can also be immobilised (e.g. protein A/G in the case of a sandwich assay for determining antibodies).

In all the sandwich assay formats, a directly proportional correlation between the signal and the concentration of the analyte occurs.

One or more components of the immunochemical reaction can, moreover, be prepared on functional layers, such as is the case for conjugate release.

In Fig. 11 possibilities for titration/competition formats are represented which differ from one another through sequential or simultaneous incubation of the immuno-components. These two assay formats are suitable in particular for determining low-molecular compounds which cannot form a sandwich.

Moreover, the assay formats shown in Fig. 11 have no directly proportional correlation between the analyte

concentration and the intensity of the measured fluorescence signal. There is thus an inversely proportional correlation.

Thus, in the upper example, shown in Fig. 11, the marked antibody can be present for example in the functional layer 27, in the example shown in Fig. 5.

The middle example of Fig. 11 can be configured such that a marked analyte can be contained, e.g. also in the functional layer. The lower representation of Fig. 11 can be so implemented that a marked analyte is contained, for example, in functional layer 26 and an antibody in layer 27 of the example shown in Fig. 5. However, the implementation of the lower example, which is shown in Fig. 11, can also be carried out in such a way that an antibody is contained in functional layer 26 and the marked analyte in layer 27 as in the example shown in Fig. 5.

From this it follows that, in the assay formats shown in Fig. 11, either the analyte or the antibody can be immobilised (cf. upper and middle examples of Fig. 11). Therefore the methods described for the sandwich assay formats can also be used, at least partially. Thus a generic antibody (cf. lower example in Fig. 11) or, however, also protein A/G (after biotinylation of the specific antibody also avidin) can be immobilised. In this case, the immobilised biocomponent serves exclusively to enrich the added specific antibody and can therefore be immobilised in excess.

Further assay formats having directly proportional correlation between analyte concentration and fluorescence

signal intensity will be described below.

For this there are basically two possibilities. It is possible to carry out the respective assay with an additional solid phase or in solution.

For example, all the components can be incubated in solution. The assay format shown in Fig. 12 provides for a pre-incubation of the reactants and the forming of a binding equilibrium. A free analyte competes with the marked analyte for binding to the antibody, the same body being immobilised on the base plate 3 in the detection region also contained in the solution.

The immobilisation can be carried out as in sandwich assay formats.

Since only free, i.e. not antibody-bound, marked analyte is determined, a directly proportional correlation between the analyte concentration and the fluorescence signal results.

Moreover, the immobilised biocomponent serves exclusively to enrich the hapten-fluorophore conjugate and can thus be immobilised in excess. Through immobilisation of a specific antibody, a corresponding structure of the device according to the invention can, however, only be used for respectively one analyte.

The assay format shown in Fig. 12 can be carried out with a device such as is shown in Fig. 5, if a marked analyte is contained in functional layer 26 and antibody in functional layer 27.

For the case where, instead of the marked analyte, a marked analyte analogue is used, which has a clearly reduced affinity to the antibody, a replacement assay, already described, can be carried out. This is shown in Fig. 13. A marked analyte or an analyte analogue can be contained for example in functional layer 28, of the example shown in Fig. 6.

However, an additional solid phase can also be exploited, which can be accommodated either in a separate reaction space or as a functional layer directly on the detection region of the base plate 3.

The additional solid phase can in principle exercise the same functions as the functional layers.

The use of a separate reaction space (e.g. an incubation test tube) such as the sample container 10, which is shown in Figs. 3 and 4, has the advantage that generic structures, i.e. structures utilizable for all the analytes, can be used.

On this universal structure, not a specific but a generic anti-antibody or protein A/G, avidin (after biotinylation of the antibody), amongst other things is immobilised. Since only one biocomponent above the base plate 3 is enriched, the immobilised components can be applied in excess.

The procedure can, in general, be such that one of the immunocomponents (the marked antibody or marked analyte) is kept back on a solid phase, for example a functional layer with hapten-protein conjugate. Only in the presence of free analytes is a portion of the marked components not bound to

the solid phase and can then be measured above the base plate 3 in the detection region. These circumstances are represented schematically in the example shown in Fig. 14.

Here free analyte and analyte immobilised on the solid phase complete for binding to the specific antibody, as is shown in a first step in Fig. 14, at the top.

The solid phase is only passed by antibodies which have bound beforehand to analyte, as is represented in the lower part of Fig. 14. Consequently, only analyte-bound antibody can be detected, for example by a generic anti-antibody. Here, too, there is a directly proportional correlation between the analyte concentration and the intensity of the measured fluorescence.

If, in the concrete case of Fig. 14, a membrane is used as the additional solid phase, which is integrated into the structure, functional layer 26 (reservoir for the marked antibody) and the solid phase layer 27, of the example shown in Fig. 5, can be used in the example shown in Fig. 14.

Various material can serve as solid phases, and of these, membranes can be easily integrated as functional layers. Such membranes can be nitrocellulose, immuno-dyne, conjugate release membranes, regenerated cellulose, amongst other things. Here the respective biocomponent can be immobilised by adsorption, covalent bonding or by affinity bonding. Haptens can be immobilised as hapten-protein conjugate.

As opposed to membranes with transverse flow, membranes with lateral flow and packed columns offer advantages through repeated establishment of equilibrium and render a

quantitative binding of the biocomponents possible. Suitable materials for packed columns are: sepharose, porous media, amongst other things.

The wall of a suitable vessel, for example the wall of the sample container 10, or the supply pipes can also serve as the solid phase and be, for example, polystyrene vessels or glass capillaries. Particle suspensions, in which the sample can be a suspension with solid particles (magnetic particles, latex, amongst other things) can also be utilized. These particles can be separated through the application of a magnetic field or through subsequent filtration.

With the invention it is also possible to carry out so-called displacement assay formats, two variations of this are possible. The displacement can take place on an additional solid phase in a functional layer or externally, i.e. not in an integrated functional layer, or directly on the base plate 3 in the detection region.

In Fig. 15, an example of a displacement assay with additional solid phase is represented. The solid phase can be either the sample container 10, a supply pipe or a functional layer. A marked antibody or analyte is bound through specific ligand/receptor action. Through the addition of a free analyte, the displacement of the biocomponents can be achieved.

The solid phase can be, for example, functional layer 26, in the example according to Fig. 5.

In the example shown in Fig. 15, the marked antibody is bound on the base plate 3 in the detection region by a generic anti-antibody or, protein A/G, avidin, amongst other things.

If however, the opposite procedure is carried out and a marked analyte is bound to an immobilised antibody and then displaced, in the detection region on the base plate 3, a specific antibody, directed against the analyte, is immobilised. Since in every case the displaced component is always detected, there is a direct proportional correlation between the concentration of the respective analyte and the fluorescence signal intensity.

However, the displacement can be carried out directly in the detection region on the base plate 3 as a very simple assay configuration, since only one sample is guided through the element.

No pre-incubations or similar steps take place. Conditional of the sample can be achieved through integration of corresponding functional layers. Here, two different possible ways of immobilising the analyte or the specific antibody present themselves, as is shown in Fig. 16.

The decrease in the fluorescence intensity signal is measured, such that an inversely proportional correlation between the analyte concentration and the fluorescence signal intensity occurs. The absolute value of the rise in the fluorescence intensity signal is directly proportional to the analyte concentration and can be evaluated in the form previously described.

Fig. 17 serves as a general key for the different assay formats shown in Figs. 9 to 16.

CLAIMS:

1. A device for carrying out fluorescence immunoassays by evanescent field excitation comprising:

at least one light source, emitting substantially monochromatic light;

an optically transparent base plate made of material having a refractive index n_1;

a cuvette-shaped receiving region for a sample;

a covering plate covering the receiving region on a side disposed opposite the base plate;

at least one functional layer between the base plate and covering plate or in an inflow region of the sample in the receiving region, said functional layer permitting lateral or transverse flow through suction, pressure or capillary force;

a detector on the same side of the base plate as the light source to detect the fluorescent light;

wherein said at least one light source directs light rays having a wavelength causing fluorescence of a marking substance bound to a chemical or biochemical partner of a general receptor-ligand system, at an angle α, onto the boundary surface; and

wherein the refractive index n_1 of the material of the base plate is greater than a refractive index n_2 of a material above the boundary surface.

2. The device according to claim 1, wherein a spacer is disposed between the base plate and the covering plate, said spacer forming the cuvette-shaped receiving region.

3. The device according to claim 1 or 2, wherein the covering plate has an inlet aperture and an outlet aperture.

4. The device according to any one of claims 1 to 3, wherein said at least one functional layer comprises a material selected from the group consisting of a fibrous material, cellulose, nitrocellulose, polypropylene, polycarbonate, polyvinyl difluoride, hydrogel, polyelectrolytes and nuclear track and glass fibre membranes.

5. The device according to any one of claims 1 to 4, wherein the at least one functional layer is configured as a packed column.

6. The device according to any one of claims 1 to 5, wherein at least one functional layer in the receiving region is in direct contact with the base plate.

7. The device according to claim 4, wherein a plurality of functional layers are provided, said plurality of functional layers are separated by separating layers and are disposed one above the other, and wherein the inlet and outlet apertures of the covering plate are connected via the receiving region by openings provided therein.

8. The device according to claim 6, wherein at least two different functional layers are provided and are disposed beside one another in a plane.

9. The device according to any one of claims 1 to 6, wherein a plurality of functional layers are disposed one directly above the other.

10. The device according to any one of claims 1 to 9, further comprising a sample container disposed such that a

connection is formed between the cuvette-shaped receiving region and the sample container, said sample container defining a predetermined sample volume.

11. The device according to claim 10, further comprising a solid phase formed in the sample container in an inflow duct in the receiving region or as a functional layer.

12. A method of carrying out fluorescence immunoassays by evanescent field excitation, said method comprising the steps of:

providing a sample volume;

guiding said sample volume through at least one functional layer;

guiding the sample volume through a cuvette-shaped receiving region;

binding a marked chemical or biochemical component to a surface in the receiving region;

emitting light with a wavelength causing fluorescence of the marked chemical or biochemical component bound to the surface of the receiving region; and

measuring, through evanescent field excitation, the fluorescent light.

13. The method according to claim 12, wherein the at least one functional layer provides for filtration, separation, elimination of interfering substances and the release of reagents or chemical reactions.

14. The method according to claim 12, comprising the further step of determining analyte concentration through a rise in the measured fluorescent light intensity.

15. The method according to claim 12 or 13, further comprising the step of:

determining analyte concentration by measuring a difference between two fluorescence intensity signals at predetermined intervals.

16. The method according to claim 12 or 13, further comprising the step of

carrying out biochemical assays of general receptor-ligand systems selected from the group consisting of antigen-antibody, lectin-carbohydrate, DNA or RNA-complementary nucleic acid, DNA or RNA protein, hormone receptor, enzyme-enzyme cofactors, protein G or protein A-immunoglobin and avidin-biotin.

17. The method of any one of claims 12 to 16, wherein at least one of sandwich, titration, competition and displacement assays are carried out.

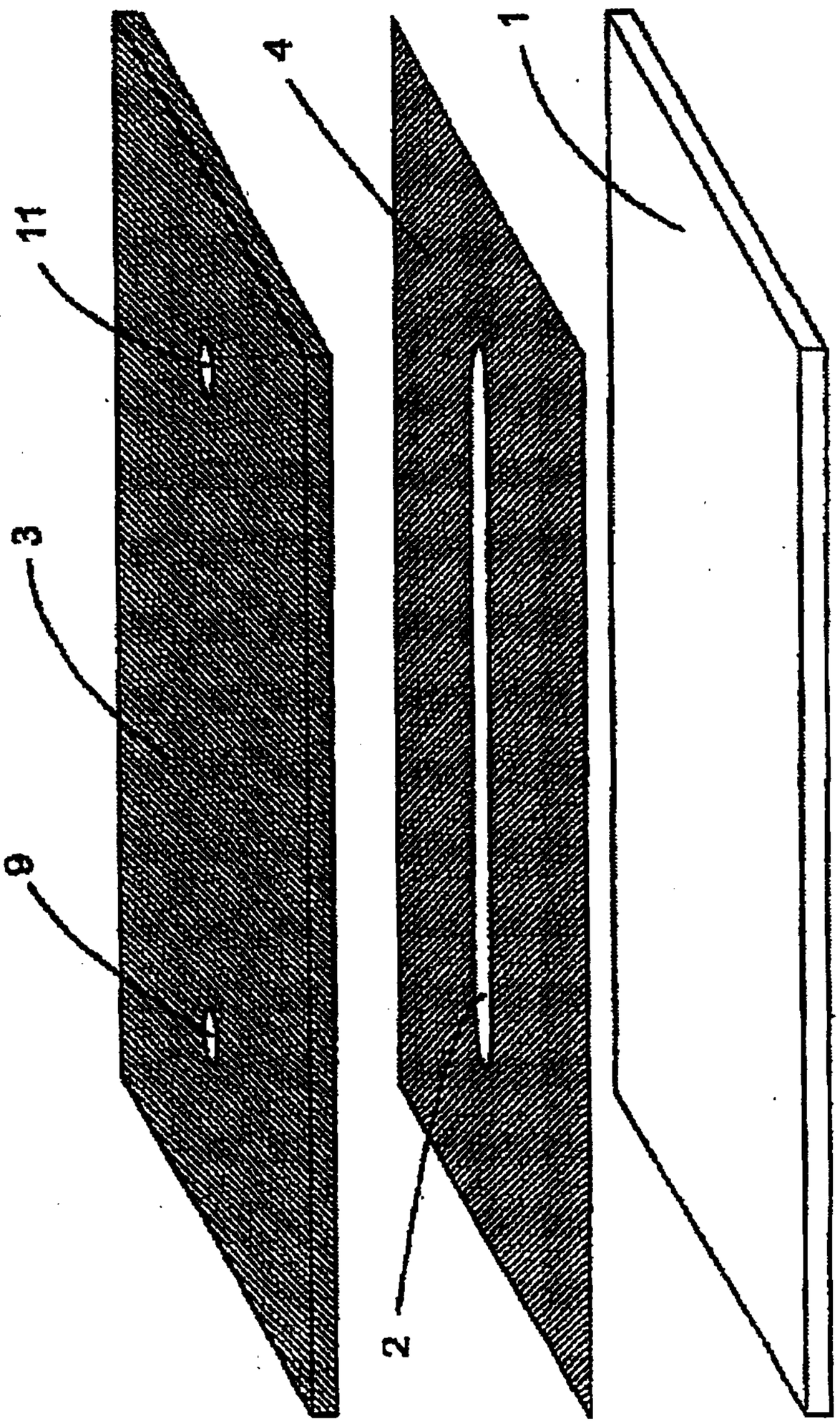


FIG. 1

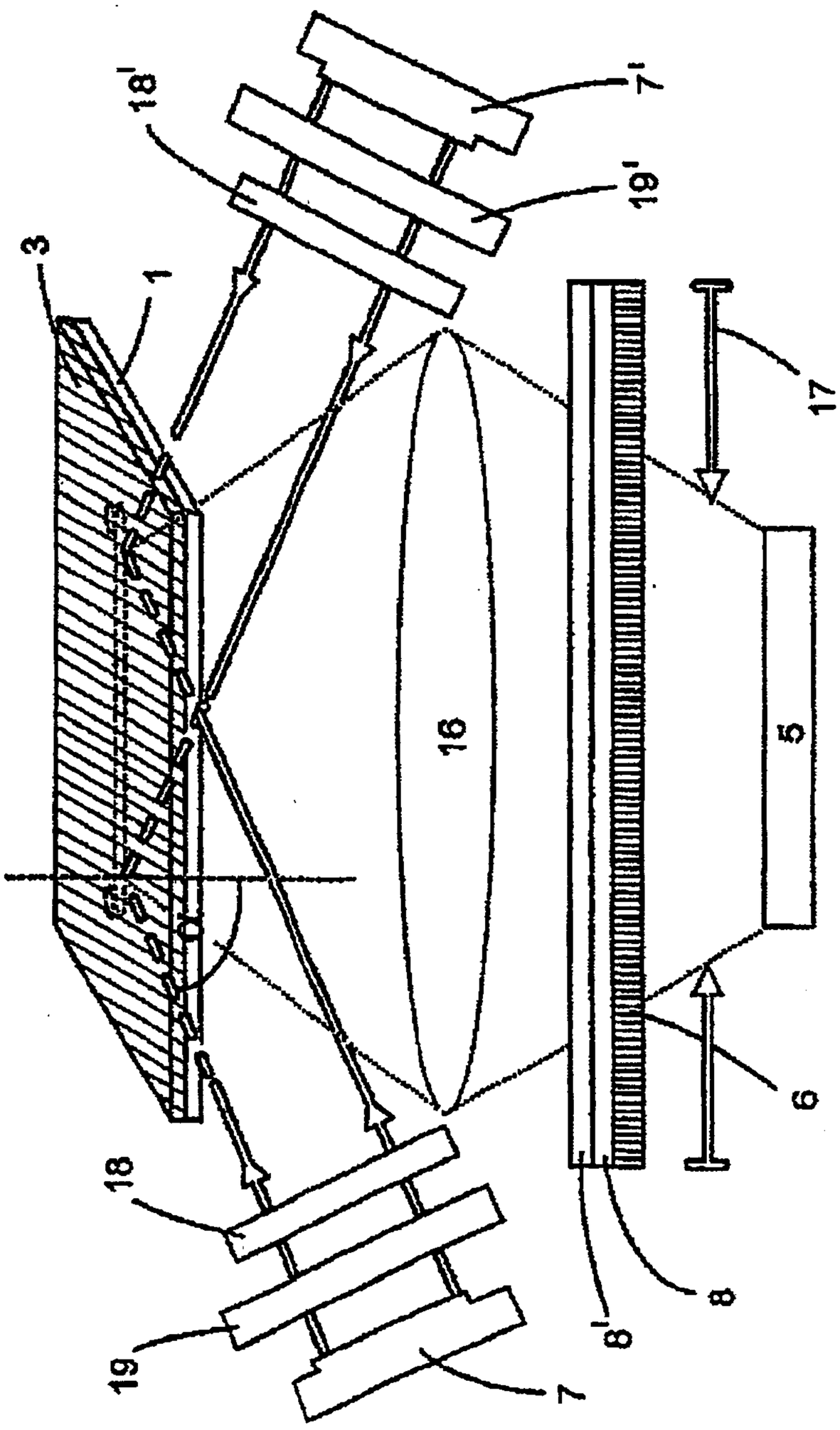


FIG. 2

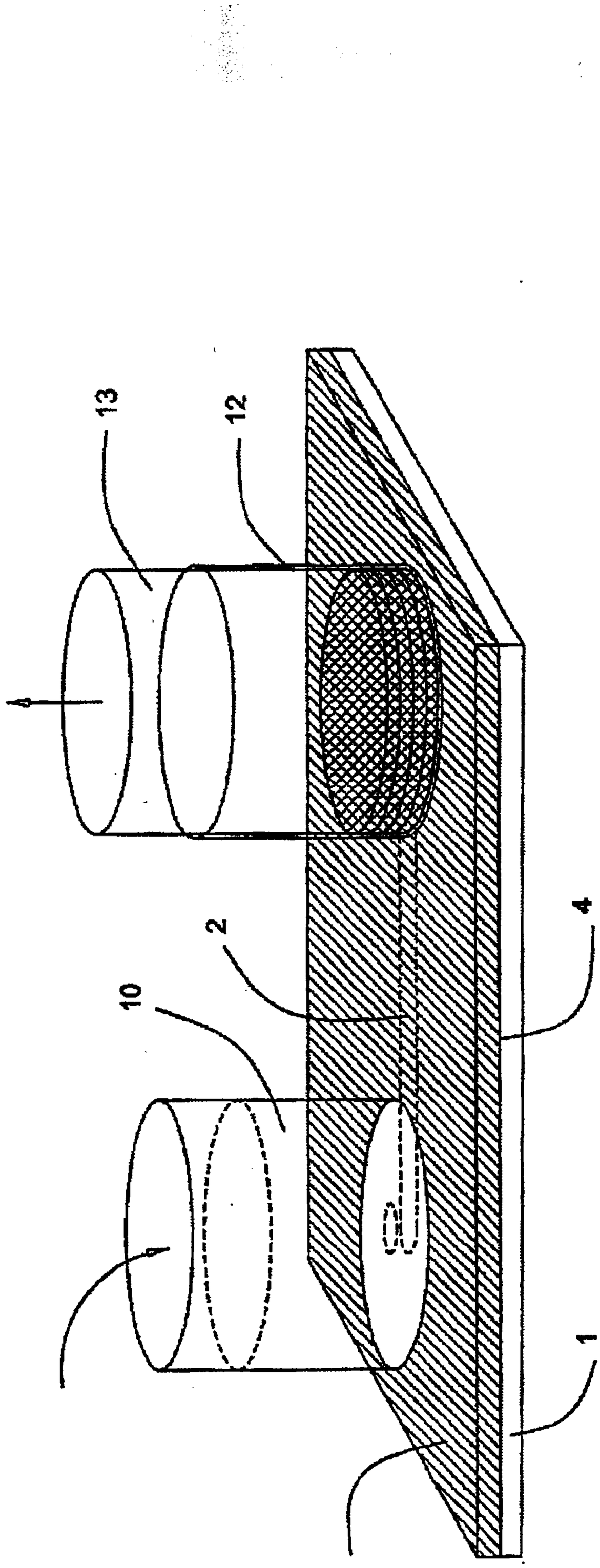


FIG. 3

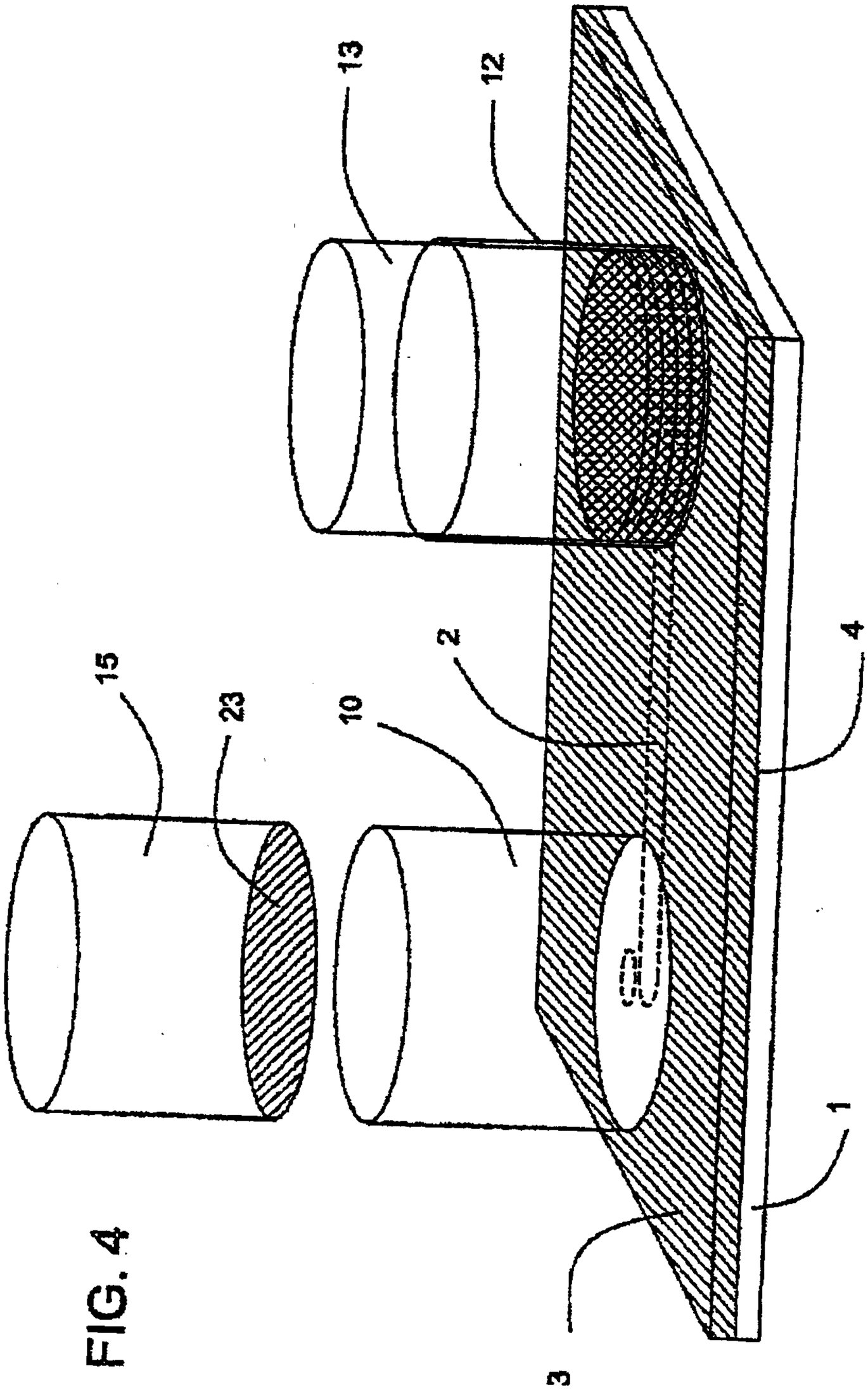


FIG. 4

FIG. 5

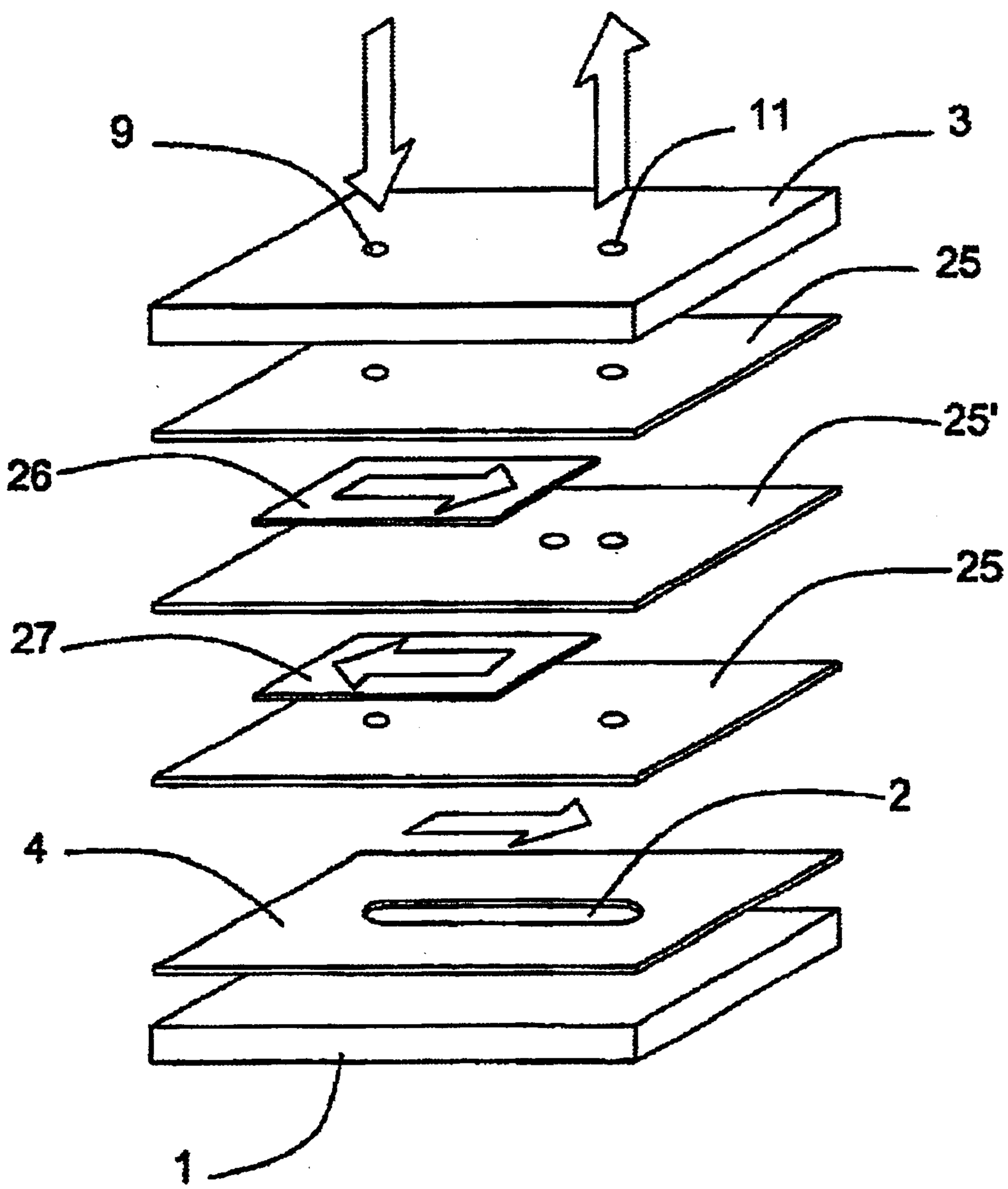


FIG. 6

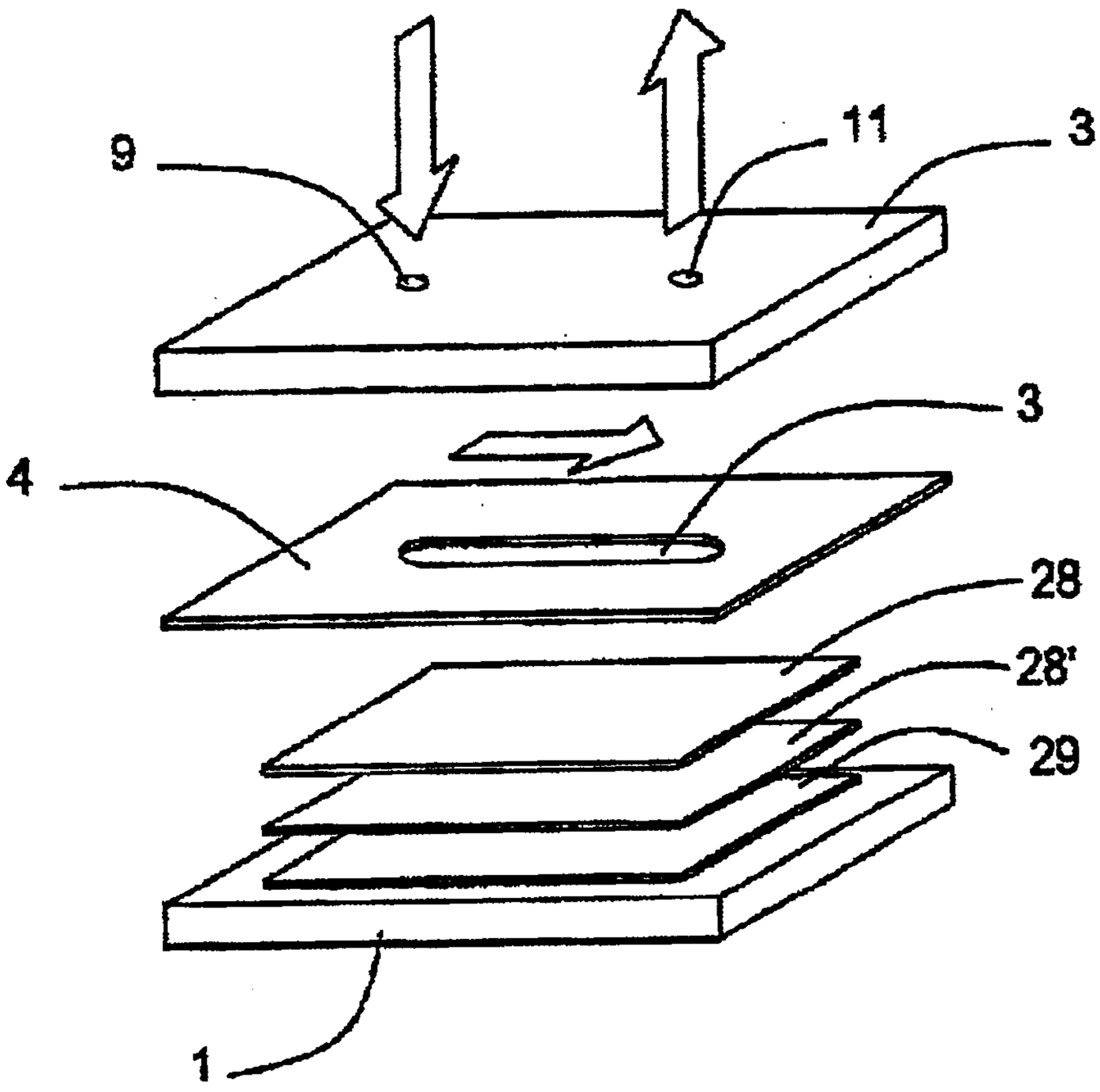


FIG. 7

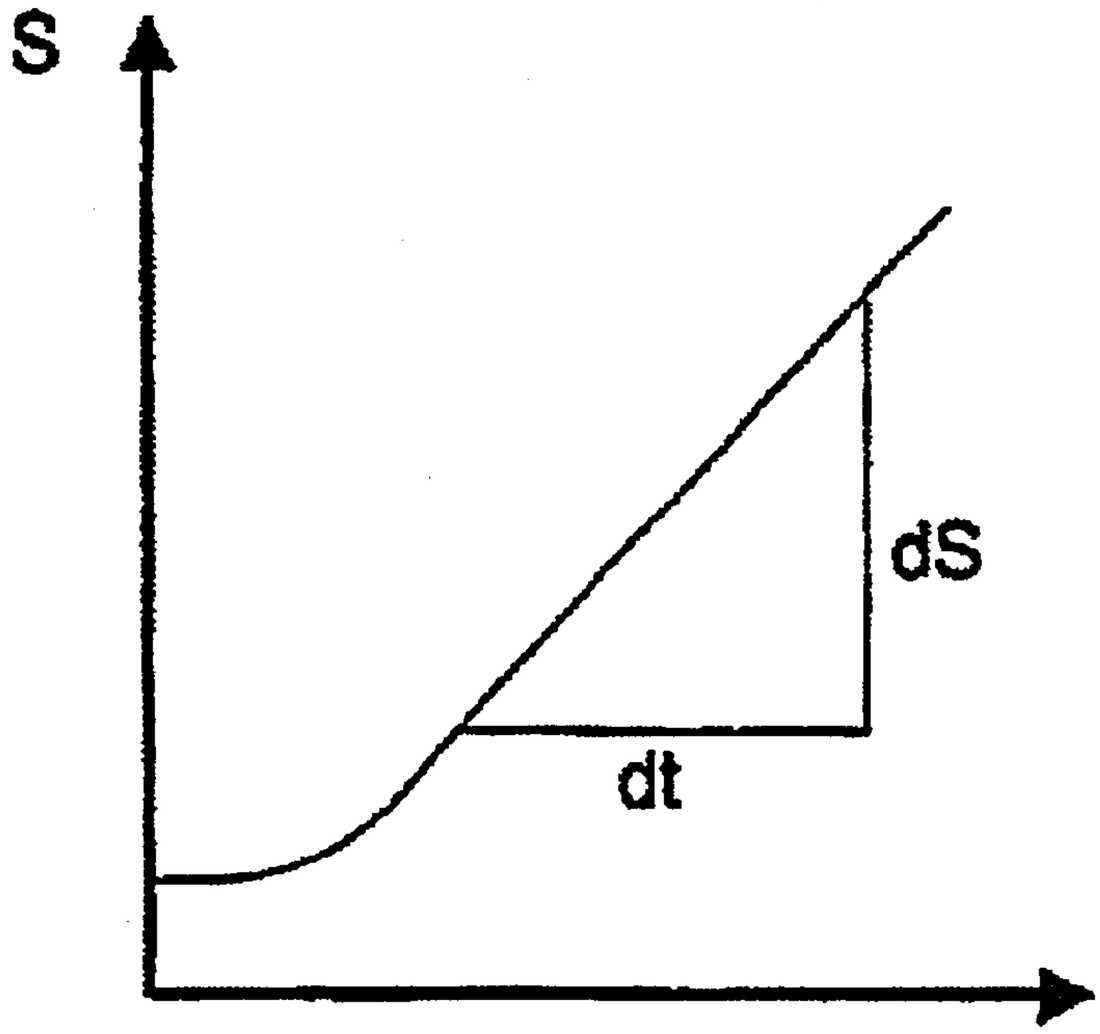


FIG. 8

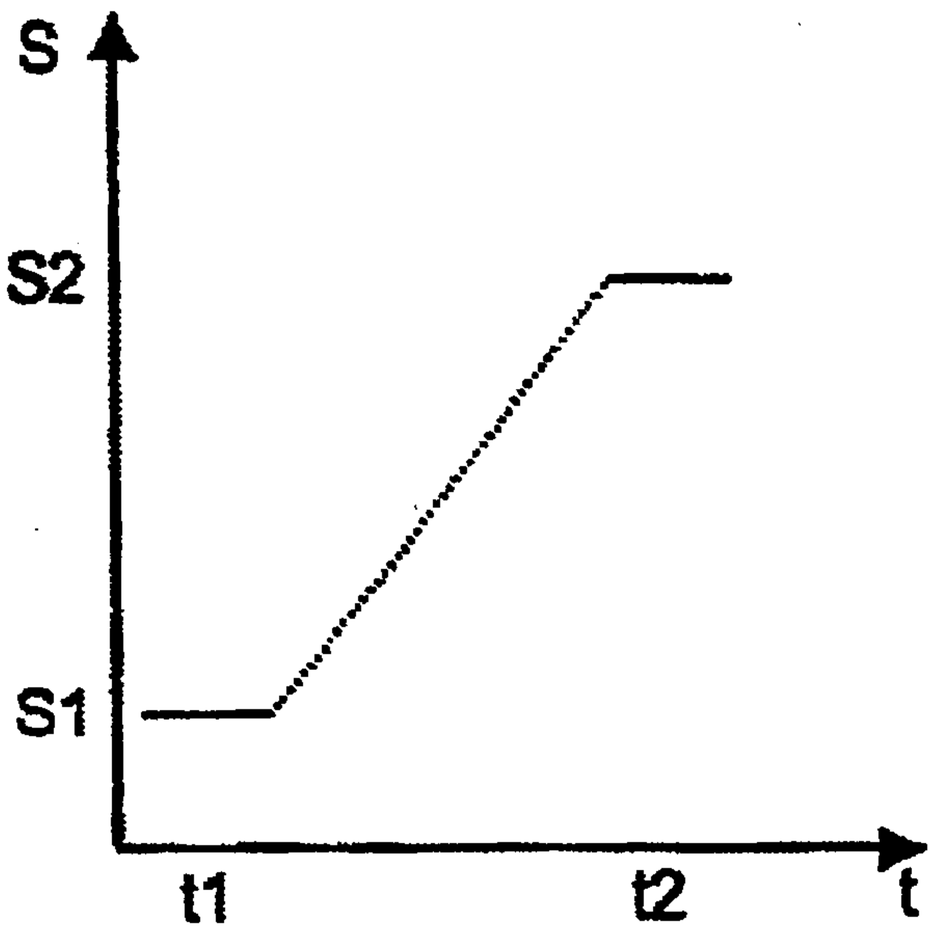


FIG. 9

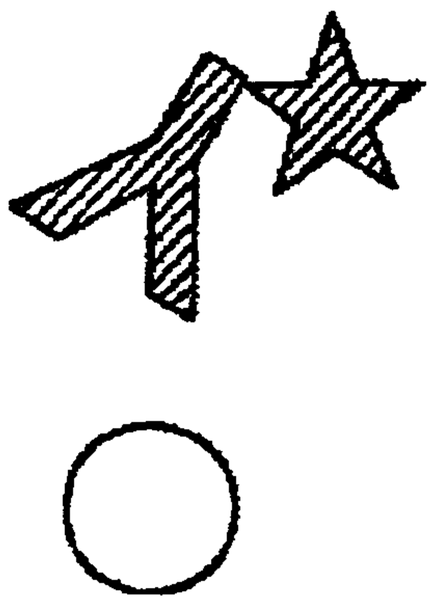


FIG. 10

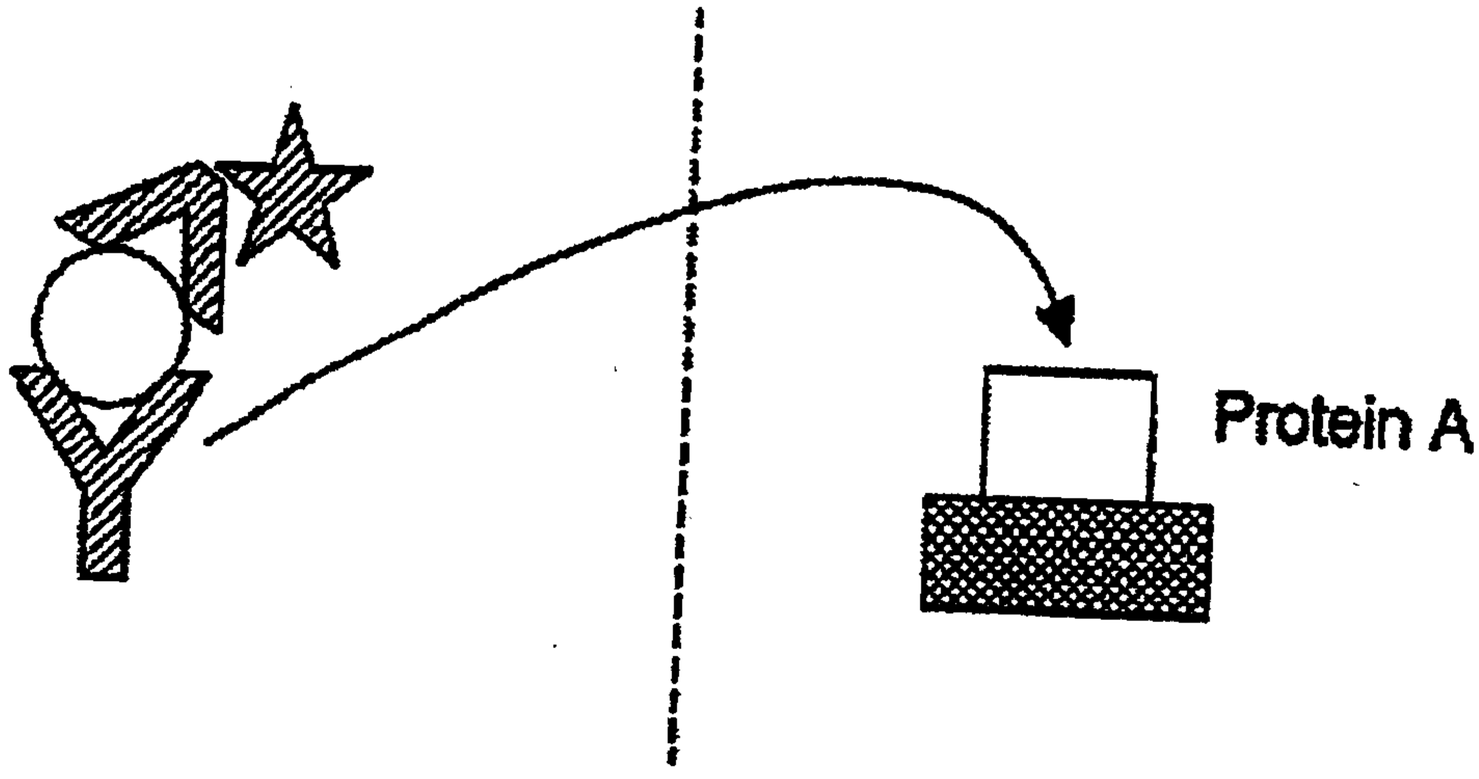


FIG. 11

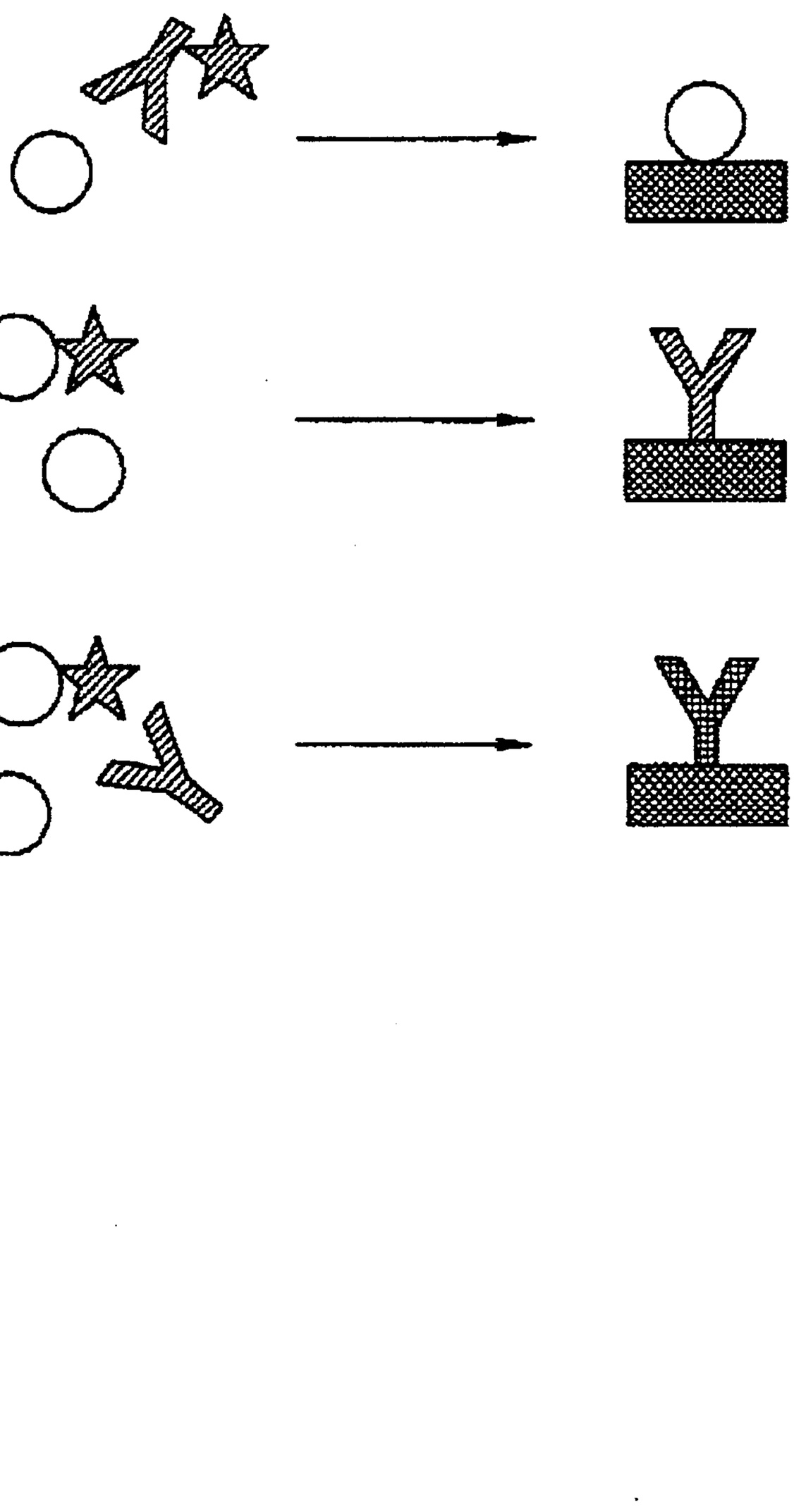


FIG. 12

FIG. 13

FIG. 14

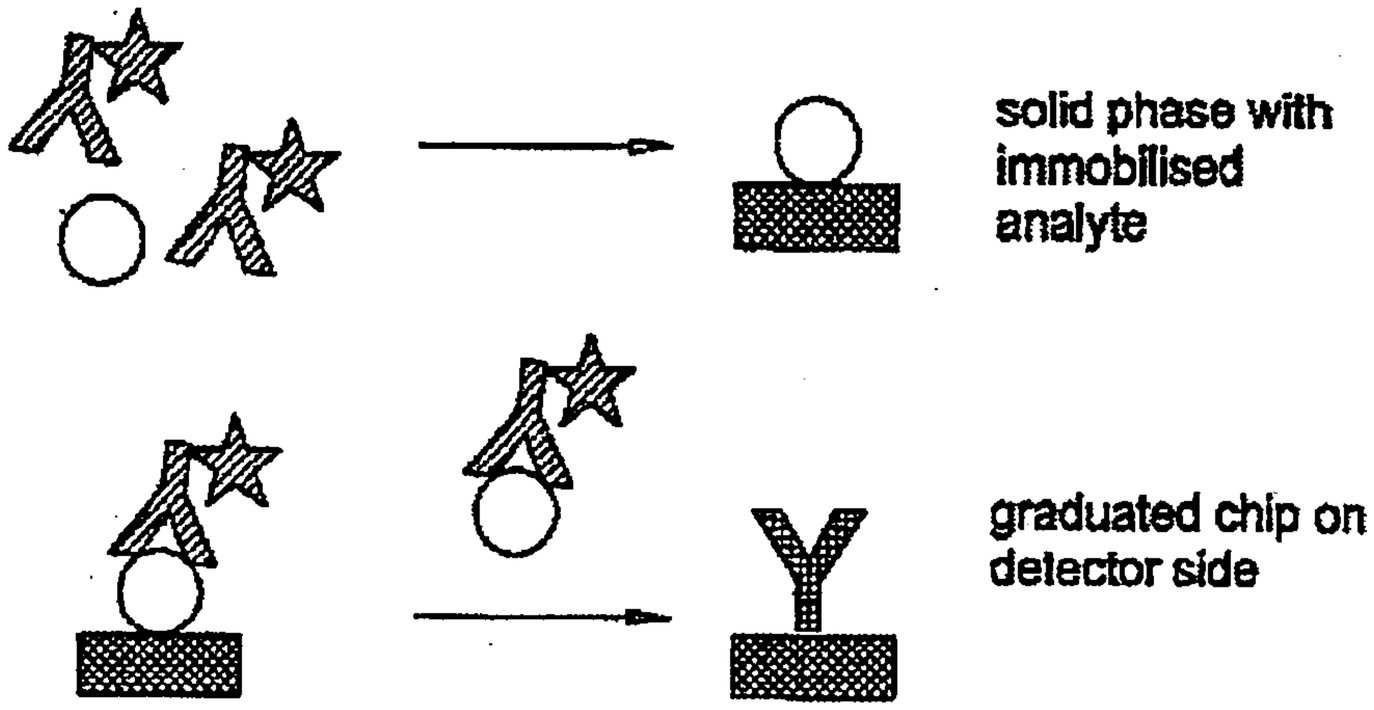

solid phase with
immobilised
analyte
graduated chip on
detector side

FIG. 15

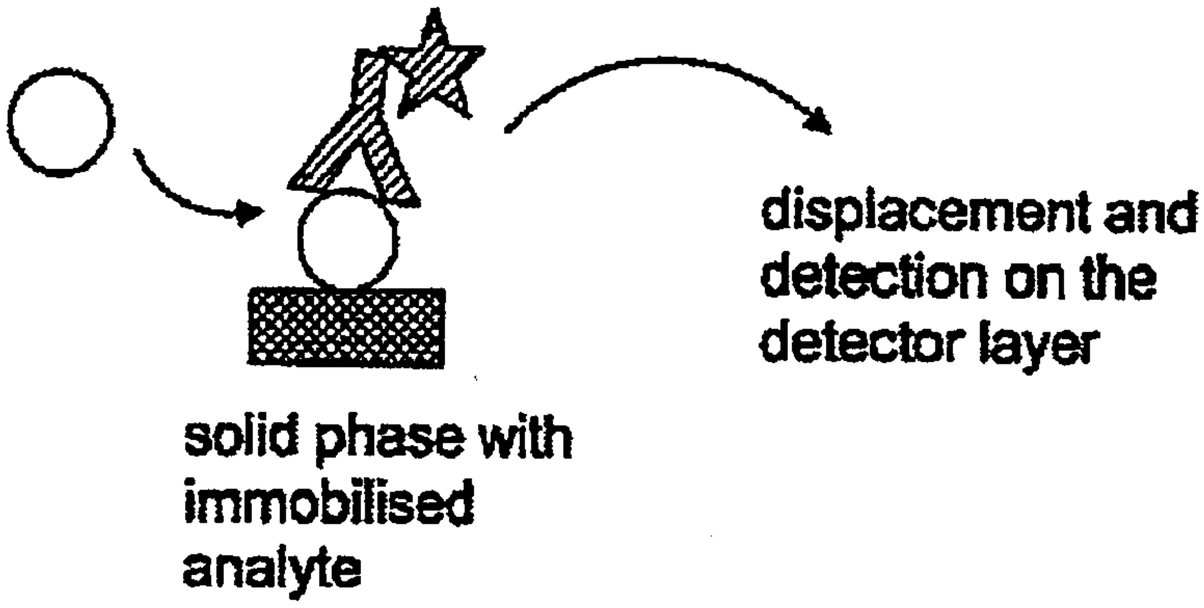

displacement and
detection on the
detector layer
solid phase with
immobilised
analyte

FIG. 16

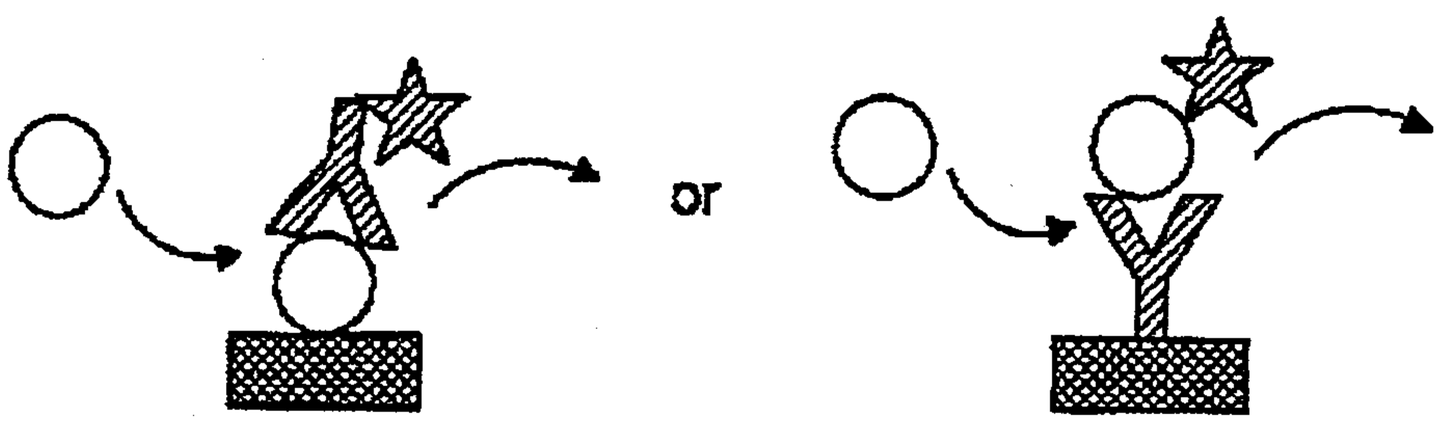
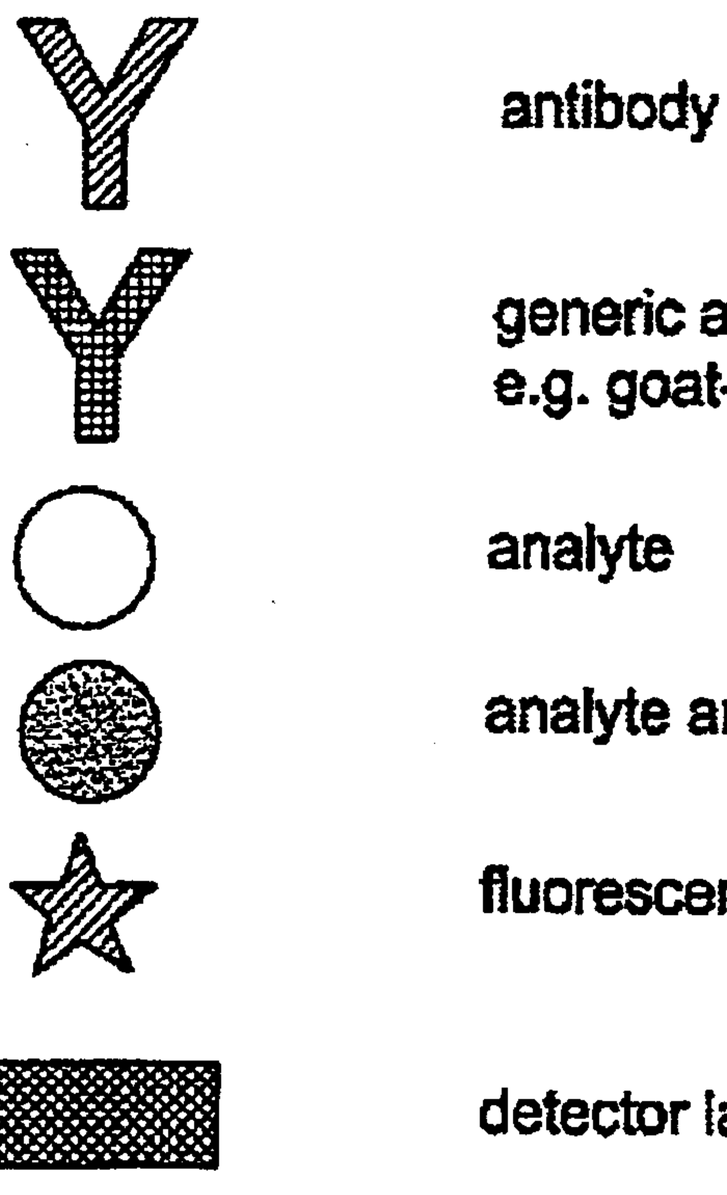
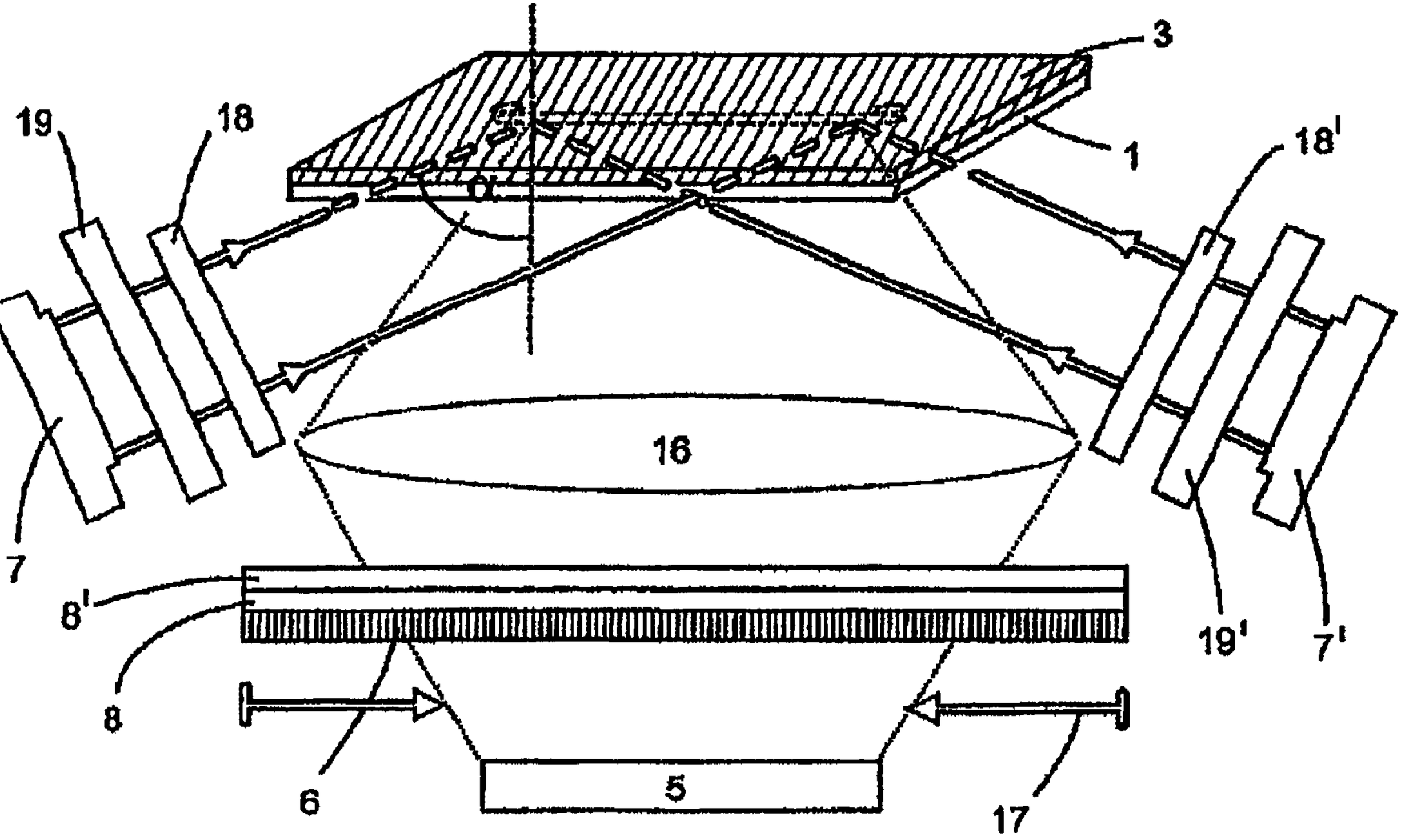


FIG. 17


antibody
generic antibody
e.g. goat-anti-mouse
analyte
analyte analogue
fluorescence marker
detector layer


3
19
18
1
18'
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