

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
29 May 2008 (29.05.2008)

PCT

(10) International Publication Number
WO 2008/063406 A2

(51) International Patent Classification:
G01N 33/53 (2006.01)

(21) International Application Number:
PCT/US2007/023369

(22) International Filing Date:
6 November 2007 (06.11.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/857,423 8 November 2006 (08.11.2006) US

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AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

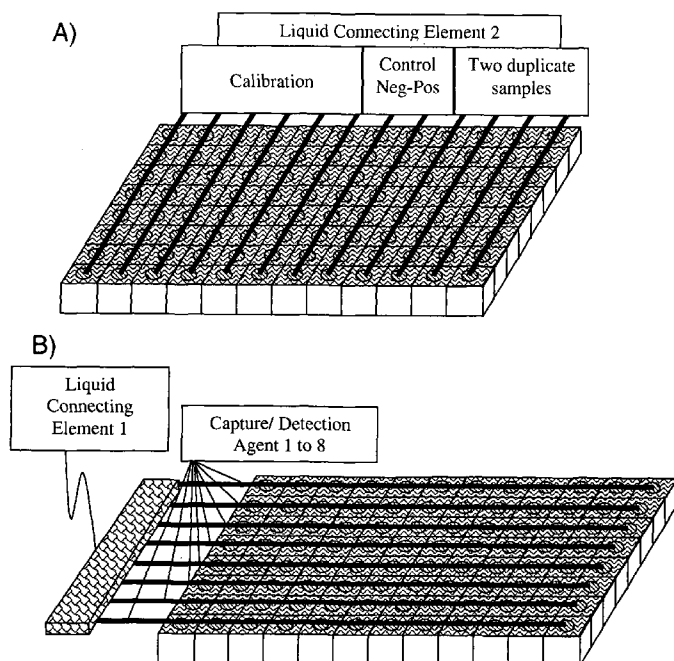
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT,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

Published:

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,

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

(54) Title: A PLATFORM FOR BINDING ASSAYS WITH DUAL MULTIPLEXING CAPABILITY



(57) Abstract: This invention describes a platform and a method for binding assays in a plurality of low volume, light guiding measuring cells into which liquid can be addressed in a dual-multiplexed way. The dual-multiplexing of each individual measuring cell, in which only a single assay is performed, enables the assay optimization for each target under interest while preventing cross-reactivity issues. The measuring cells' dimensions and light guiding capability allow for high sensitivity and high performances assays.

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2. Background of the invention

Binding assays have proven to be a sensitive, efficient and cost effective technique for the detection of one or many target molecules in samples even at low concentration and they are widely used in industry and in academy. To reduce the cost of these assays and to further increase the information content that can be extracted per sample volume, there is a strong need for multiplexed formats that would allow the detection of many targets out of a single low volume sample.

Two of the main challenges in the realization of high sensitivity multiplexed assays are:

1. the dispensing of a specific secondary binding agent (the detection agent) to each immobilized target, in order to reduce cross-reactivity that leads to false positive results;
2. the dispensing of an assay specific diluent for target under interest.

So far, several commercially available platforms provide a way to specifically capture each target, but fail to provide a way to address each bound target with a specific detection agent or assay specific diluent. The reason for this is mainly a fluidic problem since the dispensing of a specific secondary binding agent on each captured target is not compatible with an array format (i.e. due to diffusion and liquid motion, it is practically impossible to dispense an optimized detection agent on each spot of an array without cross-contamination).

We propose a platform based on channels, capillaries or tubing that allows for different multiplexing depending on which end of these channels, capillaries or tubing is used as an input or as an output. It consists of a plate comprising a plurality of measuring cells made by channels, capillaries or tubing of which both ends can be used as input or as output. These measuring cells have their first end connected according to a first multiplexing pattern and their second end connected according to a second pattern. This way, it is possible to dispense fluids (e.g. surface chemistry, capture agent, sample etc.) into the measuring cells in a multiplexed way that corresponds to the patterns in which the ends of the measuring cells are connected. After incubation, rinsing steps can be performed by using either ends of the measuring cells as input, depending on what type of multiplexing is desired. Either ends of the measuring cells can be used as output too, since both ends have input and output capabilities.

Similar steps can be performed with other fluids that need to be dispensed in the measuring cells (e.g. sample, detection agents etc.), using again the end of the measuring cells that corresponds to the desired multiplexing pattern. The advantage of this dual multiplexing is that an optimized rinsing and detection cycle can be performed for each assay, independently of the sample multiplexing.

3. Claims

What is claimed is:

- 5
1. A measuring cell comprising one channel, wherein said channel comprises:
 - a. a first end,
 - b. a second end and
 - c. an inner surface, capable of immobilizing a capture agent that is capable of
 - 10 binding to a target in a liquid sample,
wherein said channel is capable of guiding light;
wherein the first and second ends of said channel can be used interchangeably as an
input and/or an output for the introduction or removal of a liquid into said channel;
 2. The measuring cell of claim 1., wherein said channel is capable of guiding light within a
 - 15 fluid contained in its inner volume;
 3. The measuring cell of claim 2., wherein said liquid is a high refractive index liquid;
 4. The measuring cell of claim 1., wherein said channel comprises at least one reservoir
located at one of its ends;
 5. The measuring cell of claim 1., wherein said channel comprises one reservoir at each of
 - 20 its ends;
 6. The measuring cell of claim 1., wherein said at least one channel has a length comprised
between 1 and 10 centimeters.
 7. A micro-fluidic device comprising:
 - 25 a. A plurality of measuring cell comprising one channel, wherein said channel
comprises:
 - i. a first end,
 - ii. a second end and
 - iii. an inner surface, capable of immobilizing a capture agent that is capable
 - 30 of binding to a target in a liquid sample,
wherein said channel is capable of guiding light;
wherein the first and second ends of said channel can be used
interchangeably as an input and/or an output for the introduction or removal
of a liquid into or from said channel;

- b. a first liquid connecting element (LCE) coupled to either said first end or said second end;
- c. a second LCE coupled to either said first end or said second end;
- 5 wherein said first LCE comprises a plurality of dispensing wells that connect to said measuring cells according to a first multiplexing pattern;
- wherein said second LCE comprises a plurality of dispensing wells that connect to said measuring cells according to a second multiplexing pattern.
8. The micro-fluidic device of claim 7., wherein said first multiplexing pattern is different
- 10 from the second multiplexing pattern;
9. The micro-fluidic device of claim 7., wherein said first LCE connects to the first end of said measuring cells;
10. The micro-fluidic device of claim 7., wherein said second LCE connects to the second end of said measuring cells;
- 15 11. The micro-fluidic device of claim 7., wherein said LCE are removably coupled to either said first end or said second end of said measuring cells.
12. The micro-fluidic device of claim 10., wherein said second LCE is removably coupled to the first end of said measuring cells;
13. The micro-fluidic device of claim 7., wherein at least one of said LCE further comprises
- 20 at least one manifold that connects at least one dispensing well to the plurality of measuring cells;
14. The micro-fluidic device of claim 7., wherein at least one of said LCE also connects light from a light emitting element into said channel of at least one of said plurality of measuring cells;
- 25 15. The micro-fluidic device of claim 7., wherein at least one of said LCE also connects light from said channel of at least one of said plurality of measuring cells to a light detecting element;
16. The micro-fluidic device of claim 7., wherein the plurality of measuring cells is assembled in a one-dimensional array;
- 30 17. The micro-fluidic device of claim 7., wherein the plurality of measuring cells is assembled in a two-dimensional array.
18. The micro-fluidic device of claim 7., wherein the plurality of measuring cells is assembled in a n times m two-dimensional array, with n and m comprised between 2 and 50.

19. A method for combining molecules on the inner surface of the wall of light guiding channels, wherein said molecules are initially contained in different solutions, which method comprises a cycle of four steps comprising:

- 5 a. introducing, according to a first multiplexing pattern, a first set of solutions containing a second set of molecules into the measuring cell of a micro-fluidic device comprising:
- i. a plurality of measuring cells comprising one channel, wherein said channel comprises:
- 10 1. a first end
2. a second end
3. an inner surface on which a first set of molecules have been previously bound,
- wherein said channel is capable of guiding light;
- 15 wherein the first and second ends of said channel can be used interchangeably as an input and/or an output for the introduction or removal of a liquid into or from said channel;
- ii. a first liquid connecting element;
- iii. a second liquid connecting element;
- 20 wherein said first liquid connecting element comprises a plurality of dispensing wells that connect said measuring cells according to a first multiplexing pattern;
- wherein said second liquid connecting element comprises a plurality of dispensing wells that connect said measuring cells according to a second
- 25 multiplexing pattern;
- wherein at least one of said first set of solutions contains molecules capable of binding with said first set of molecules previously immobilized on the inner surface of said channel;
- 30 b. binding the molecules contained in said at least one of said first set of solutions with said first set of molecules previously immobilized on the inner surface of said channel;
- c. consecutively introducing, according to a second multiplexing pattern, a second set of solutions containing a third set of molecules into said measuring cells,

wherein at least one of said second set of solutions contains molecules capable of binding with the molecules previously immobilized on the inner surface of said channel;

- d. binding the molecules contained in said at least one of said second set of solutions with the molecules previously immobilized on the inner surface of said channel.

20. The method of claim 19., further comprising the introduction and binding of additional molecules;

21. The method of claim 19., further comprising at least one washing step;

22. The method of claim 19., wherein at least one solution of one of said sets contains at least one capture agent selected from antibodies, fabs, aptamers, nucleic acids, peptides, and SNIPS.

23. The method of claim 19., wherein at least one solution of one of said sets is a biological sample that contains at least one target selected from antigens, nucleic acids and SNIPS.

24. The method of claim 19., wherein at least one solution of one of said sets contains at least one detection agent selected from antibodies, fabs, aptamers, nucleic acids and SNIPS.

25. The method of claim 19., wherein at least one solution of one of said sets contains at least one optical marker selected from a fluorescent marker, an absorption marker and an enzyme.

4. Brief description of the invention

In one aspect, this invention is directed to a measuring cell which comprises one channel with a first end and a second end. Both ends of this channel can be used as an input or as an output to introduce a liquid into said channel or to remove said liquid from said channel. Additionally, said channel is capable of guiding light and of binding a target from a liquid sample to a capture agent immobilized on its inner wall.

The ability of the channel to be interchangeably filled and/or emptied through one or the other of its ends allows for the dual-multiplexing of the channel. In other words, the channel's first and second end can be connected to other channels according to different multiplexing patterns. The liquid can be introduced into the channel by positive or negative pressure or by capillary forces and it can be removed from it by positive or negative pressure.

Light can be guided within the liquid contained inside of the channel or within the walls of said channels. The ability of the channel to guide light is generated by the material of its wall or by a coating applied on one or both surface of said wall.

The inner wall of said channel has a surface that is capable of immobilizing molecules that are capable of binding a target from a liquid sample. Said molecules may be bound directly to the channel's wall or may be bound to an interstitial layer comprising one or more layers.

In another aspect, this invention is directed to a micro-fluidic device that comprises a plurality of measuring cells, as described in the first aspect, a first liquid connecting element and a second liquid connecting element.

The plurality of measuring cells may be arranged in bundles, in a one dimensional array or in a two dimensional array. Bundles of measuring cells typically comprise between two and fifty of them. A one dimensional array of measuring can typically comprise a row of 2 to 50 cells; a two dimensional array of measuring cells can typically comprise from 2 to 50 rows of 2 to 50 cells.

Both the first and the second liquid connecting elements comprise a plurality of dispensing wells that connect to the measuring cells and they can be coupled to the first or to the second end of the channels. The first liquid connecting element connects said channels according to a first multiplexing pattern and the second liquid connecting element connects said channels according to a second multiplexing pattern. This way, the channels can be filled with a different multiplexing pattern depending on which liquid connecting element is used.

The wells of these liquid connecting elements can be connected directly to the channels, or they can be connected to these channels via some manifolds.

These liquid connecting elements can be coupled to the channels in a fix way or in a removable way. In the case of these liquid connecting elements being coupled in a fix way, dual
5 multiplexing can be achieved by having a first liquid connecting element with a first multiplexing pattern coupled to the first end of the channels and by having a second liquid connecting element with a second multiplexing pattern coupled to the second end of the channels. In the case of the liquid connecting elements being coupled in a removable way, both of them can be coupled interchangeably to the same end of the channels.

10 The liquid connecting elements may also connect light from a light emitting element into the channels and/or from the channels to a light detecting element. This allows optical measurements to be performed inside of the channels by emitting light into said channels, having light interact with the molecules immobilized inside of the channels and by detecting the light coming out of the channels. The change in the amount of light, or the modifications of at
15 least one of its properties, relates to the amount of molecules immobilized inside of the channels.

In a third aspect, this invention is directed to a method for combining molecules initially contained in different solutions onto the inner wall of light guiding channels. This method comprises a cycle of four steps that comprise the successive introduction of two sets of solutions
20 into the channels of the micro-fluidic device of the second aspect of this invention and the binding, onto the inner surface of the channels, of two sets of molecules contained in said two sets of solutions after these solutions have been introduced into the channels.

The first step of the cycle comprises the introduction, according to a first multiplexing pattern, of a first set of solutions into the channels wherein this first set of solutions contain a
25 second set of molecules. The second step of this cycle comprises the binding of this second set of molecules to at least one of a first set of molecules previously immobilized on the inner surface of the channels. The third step of the cycle comprises the introduction, according to a second multiplexing pattern, of a second set of solutions into the channels wherein this second set of solutions contain a third set of molecules. The fourth step of the cycle comprises the
30 binding of this third set of molecules to at least one set of molecules previously immobilized on the inner surface of the channels.

The first set of solutions may be introduced into the channels through a first liquid connecting element that is coupled to the first end of the channels according to a first multiplexing pattern. The second set of solutions may be introduced into the channels through a

second liquid connecting element that is coupled to the second end of the channels according to a second multiplexing pattern. Alternatively, both the first and the second liquid connecting elements are connected interchangeably and removably to the same end of the channels.

5 In one embodiment of the invention, the molecules of the first set of molecules form a layer on the inner surface of the channels. The molecules of the second set of molecules that are contained in a first set of solutions are primary antibodies that bind to this layer. The molecules of the third set of molecules that are contained in the second set of solutions are targets that bind to said primary antibodies.

10 In another embodiment of this invention, the molecules of the first set of molecules are primary antibodies that bind to the inner surface of the channels or to any layer of molecules previously immobilized on it. The molecules of the second set of molecules that are contained in the first set of solutions are targets that bind to the primary antibody that are bound to the inner wall of the channels. The molecules of the third set of molecules that are contained in the second set of solutions are secondary antibodies that bind to the target that are bound to the primary
15 antibodies.

In a further embodiment, the molecules of the first set form a layer on the inner surface of the channels. The molecules of the second set of molecules that are contained in the first set of solutions are nucleic acids that bind to the layer of molecules previously immobilized on the inner wall of the channels. The molecules of the third set of molecules that are contained in the
20 second set of solutions are complementary nucleic acids that bind to the nucleic acids that are immobilized on the inner surface of the channels.

In one embodiment of the invention, at least one set of molecules comprises aptamers, fabs, peptides or SNIP's and in another embodiment of this invention, at least one set of molecules contains a fluorescent marker, an absorption marker or an enzyme.

25 In yet another embodiment of the invention, the cycle comprises additional washing steps to remove any unbound material from the channels. In another embodiment of the invention, the cycle may be preceded or followed by one or more similar cycles; in yet another embodiment, one cycle may overlap with a preceding or a following cycle.

5. List of Brief Description of the Several Views of the Drawings

- Figure 1. Cross-section of a measuring cell showing:

- 5 1. Body of measuring cell
2. Channel
3. Channel wall
4. Immobilized capture agent
5. Chamber
- 10 6. Channel's first end
7. Channel's second end

- Figure 2. Cross-section of a micro-fluidic device comprising four measuring cells and two liquid connecting elements. The measuring cells comprise a channel with a reservoir at each of its ends and are embedded in a matrix. The first liquid connecting element comprises two dispensing wells and the second liquid element comprises three wells that are connected to the measuring cells. The connecting scheme of the wells to the measuring cells provides for the multiplexing pattern of the solutions dispensed into the wells.

- Figure 3. Micro-fluidic device comprising a three times four array of measuring cells. A) shows a view of the first end of the channels: a first liquid connecting element comprising three manifolds connects the first end of the measuring cells channel in a three times 4-plex pattern. B) shows a view of the second end of the channels: a liquid connecting element comprising four manifolds connects the second end of the measuring cells channel in a four times 3-plex pattern.

1. Measuring cell body
2. Channel's first end
3. Three 4-plexed manifolds of the first liquid connecting element
- 25 4. Four 3-plexed manifolds of the second liquid connecting element
- 30 5. Channel's second end
6. Dispensing wells

- Figure 4. Example of micro-fluidic device for dual multiplex assay with 4-plexed sample and 3-plexed secondary binding agent.

A) First End View: The channels' first ends are connected in three groups of four thanks to the three 4-plexed manifolds of the first liquid connecting element. In this example, the first end is used for the dispensing of samples (i.e. three 4-plexed samples). The small letters refer to the measuring cells and the pattern refers to the type of assay performed therein.

B) Second End View: The channels' second ends are connected in four groups of three thanks to the four 3-plexed manifolds of the second liquid connecting element. In this example, the second end is used for the dispensing of the capture agents and of the detection agents (i.e. four 3-plexed solutions). The small letters refer to the measuring cells and the pattern refers to the type of assay performed therein.

1. Body of measuring cell
2. Channel's first end
3. Liquid connecting element comprising three 4-plexed manifolds connecting the channel's first end
4. Liquid connecting element comprising four 3-plexed manifolds connecting the channel's second end
5. Channel's second end

- Figure 5. Schematic view (not to scale) of the micro-fluidic device in its 96-well plate format. 96 measuring cells are assembled in a plate on which two different types of liquid connecting element can be tightly adapted to dispense the solutions required for the performance of eight different assays.

Illustrated is a two (samples) times eight (assays/biomarkers) with a 6 point calibration curve and two controls (positive and negative) multiplex, wherein each assay is performed in duplicate. In this view, both liquid connecting elements connect to the same end of the measuring cells channel.

- Figure 6 Application example: Micro-fluidic device comprising a 3 x 9 array of measuring cells for the performance of three assays (Il-1 β , Il-2, Il-6) on three samples, two controls (one positive and one negative) and four calibration solutions

- Figure 7. Schematic illustration of the proof of concept plate used to demonstrate the feasibility of the invention described in this application. A plate accommodates a

total of 4 x 32 tubes. The plate also serves as Liquid Connecting Element since it has wells that connect the ends of the tubes and the tubes act as measuring cells. This plate allows the performance of a 4-plexed assay on 32 samples.

- 5 - Figure 8. Picture of prototype built to demonstrate reduction to practice of invention. This plate allowed the performance of a 4-plexed assay on 32 samples. The samples were introduced in the grey wells, while the red wells were used to introduce both the capture and the detection antibodies. The channels are embedded inside the plate.

10

6. Detailed description of the invention

A. THE MEASURING CELL

5

I. General Description

1. Structure

10

The measuring cell comprises one channel embedded in a matrix that belongs to the body of the measuring cell. Said channel allows for the performance of a binding assay on its inner wall, while said body provides said channel with the structural features necessary to its assembly with and to its connection to other elements of the platform. The measuring cell is also described in pending US Patent application with serial number 10/670,912, which is incorporated herein by reference in its entirety.

15

a. The channel

20

The channel comprises a first end and a second end and an inner surface where a capture agent is immobilized. It will be described into more detail in section 3.A.II. The channel is a key element of the platform, where many essential functions are carried out: molecules such as capture agent, target and detection agent are immobilized on its inner surface. The channel's inner volume serves as a fluidic cell for the solutions required for the completion of the assay. The walls of the channel, or materials used to coat the inner wall of the channel, or materials used to surround the outer wall of the channel, provide a mean to contain and direct the light necessary to detect the molecules immobilized on its inner most surface.

25

b. The body

30

The channel of the measuring cell is set in a surrounding body that allows for the assembly and connection of said channel to the other elements of the platform. The body may provide fluidic features that make the liquid handling into and out of the channel easier and its material may contribute to enhance the optical properties of the channel. A broader description of the measuring cell's body is provided in section 3.A.III.

2. Functions

The channel of the measuring cell offers a surface and a confined volume for the performance of a specific assay. In one embodiment, multiple measuring cells may be aligned adjacent to each other with a single assay type occurring within each channel. Only one capture agent is immobilized on the inner surface of the channel and each measuring cell is individually addressable with sample and reagents by way of the liquid connecting elements. In this embodiment there is no cross reactivity between the assays in each measuring cell. The addressability of measuring cells through liquid connecting elements having different multiplex patterns allows for the dually-multiplexed introduction of solutions into each measuring cell. See section B.III.1 for a more detailed description of dual-multiplexing of liquid channels.

Optical detection of immobilized molecules through fluorescence or light absorption may be problematic in the longitudinal axis of narrow channels because of cross-talk and light dissipation through scattering or absorption in the channel's walls. This problem is solved by using light guiding channels (capillary waveguides or liquid core waveguides), that allow for high sensitivity detection in the measuring cells. See II.6 for details of capillary waveguides or liquid core waveguides.

3. Mode of use

a. Fluidics

In one embodiment of the invention, both ends of the channel are used as an inlet and as an outlet. In other words, the direction of liquid flowing through the channels can be reversed or, solutions may be introduced into or removed from the channels through either ends. Dual multiplexing is achieved by dispensing a first solution (e.g. in one embodiment, the capture agent containing solution) into the channels through their first end thanks to a first liquid connecting element and according to a first multiplexing pattern. This first solution exits, or is drained, through the first or the second end of the channels and through said first or through a second liquid connecting element that connects the measuring cells according to a second multiplexing pattern. This second liquid connecting element is then used to introduce a second solution (e.g. in one embodiment the liquid containing the sample or targets) into the channels through their second end and according to said second multiplexing pattern.

In another embodiment of this invention, the liquid connecting elements are movable and/or removable and the dual multiplexing is achieved by dispensing the

solutions into the same end of the channels, then swapping the first and second liquid connecting elements with different multiplexing patterns onto said channels' end.

b. Optics

To detect molecules on immobilized on the inner wall of the channels, it is necessary to connect light into said channels and out of them. This may be achieved the first or the second end of the channels and, if necessary, through optical features integrated in the liquid connecting elements. Detecting samples within the measure cells is also described in US patent application serial number 10/670,912.

II. The channel

1. Type of channel

In a one embodiment of the invention, the channel is a section of capillary tubing embedded in the measuring cell's body (see Figure 8). This tubing may be inserted into a hole previously drilled in the body of the measuring cell or a matrix (e.g. rubber or other material) may be cast around it. This provides a low cost solution for which a broad choice of off the shelf materials and dimensions are available.

In an alternative embodiment, the channel is formed directly into the measuring cell's body by milling, etching, embossing or other techniques.

2. Geometry

The channel comprises a first end and a second end.

The cross section of the channel may be circular, ellipsoidal, rectangular, square or of any other convenient shape. The inner section of the channel is typically comprised from 50 times $50\text{ }\mu\text{m}^2$ (or $50\text{ }\mu\text{m}$ inner diameter) to 1 times 1 mm^2 (or 1 mm inner diameter). The length of the channel is typically between one centimeter and one hundred centimeters, depending on the application and the requested assay sensitivity. A person of ordinary skill in the art would know how to best optimize the channel diameter and length for the desired assay based the teachings herein and on their general knowledge of the specific assay. In a one embodiment, the channel is straight; in an alternative embodiment, the channel may be bent to fit into the platform design. In yet another embodiment, a capillary, the inner volume of which defines the channel, may be flexible in order to allow its mounting into a complex platform assembly (see Figure 7).

In one embodiment of the invention, the walls of the channel have sharp edges at their ends, so that they provide a capillary barrier for the liquid inside of the channel.

3. Material

5 In preferred embodiment, the walls of the channel are made out of plastic. Since light should be conducted inside of said channel (in one embodiment, light is conducted within the inner volume of the channel, and not within its wall nor within any material covering its wall) materials having a low refractive index are advantageous because they enable the channel to be a liquid core waveguide by providing a total internal reflection of the light at the interface
10 between the liquid and the channel's wall. The following list provides examples and examples only, of such materials: Teflon AF® (Dupont), FEP (Dupont).

Alternatively, the walls of the channel are made out of fused silica. Such capillaries can be made light waveguides by adequately coating their outer wall.

15 In another embodiment, the walls of the channel are made out of the same material as the body of the measuring cell. Preferably this material would be plastic such as PEEK, HDPE, Rubber, PMMA, Polycarbonate, PP, PTFE or PFA. In yet another embodiment, the walls of the channel and the measuring cell's body would be glass.

4. Fabrication Technique

20 In one embodiment, capillary tubing, the inner volume of which forms the channel, is extruded (BioGeneral Inc. or for Teflon, Zeus Inc for FEP, PolyMicro technologies for fused silica tubing), a well known manufacturing method. To make the handling and assembly of such tubing easier and also to enhance its optical properties, it can be wrapped into a cladding of different thicknesses, materials and colors. For example, a Teflon AF capillary may be
25 extruded with a thick, opaque PEEK cladding (Random Technologies).

Alternatively, the channel may be formed into the measuring cell's body by hot embossing or it can be incorporated in the body by forming both the channel and the measuring cell's body by plastic injection, also a technique well known for people skilled in the art (e.g. Jenoptik).

30 In another embodiment, the channel is etched into the measuring cell body with etching techniques such wet etch (hydrofluoric acid to etch glass, for example).

5. Liquid handling

In a first embodiment, both ends of the channel are used as inlet and as outlet to fill and to empty the channel with the sequence of solutions required to run the desired assay. For example, the capture agent (alone or in a solution comprising the capture agent and at least one solvent and optionally other additives) may be introduced into the channel through its first or its second end and, after incubation, be emptied from the channel through its second end or first end. This fluid may be mechanically pushed or pulled through the channel, or capillary forces may pull the fluid through the channel (see section 5.a below for details). The sample (alone or in a solution comprising the sample and optionally at least one solvent and optionally other additives) may then be introduced through the second end and, after incubation, be emptied from the channel through its second or its first end. Finally, the detection agent (alone or in a solution comprising the detection agent and at least one solvent and optionally other additives) may be introduced into the channel through its first end and, after incubation, be emptied from the channel through its second end or its first end. Alternatively, all solutions may be introduced through one end of the channel and be drained through another one. In a further embodiment, all solution are introduced and drained through the same end of the channel. In yet another embodiment, each of the preceding steps may be preceded by or followed by optionally flowing washing or incubating or other treating fluids through the channel in either the forward or reverse direction.

a. Channel Filling

In a preferred embodiment, the channel is filled by using the capillary force to drive the liquid along its inner surface. To do so, the liquid is brought into contact with one end of the channel, from where it will be pulled inside the channel by the capillary force. Once the liquid reaches the other end of the channel, it will be stopped by the capillary barrier formed by the sharp edge of the channel's wall.

Alternatively, the channel may be filled by applying positive pressure, to push, or negative pressure, to pull, the liquid into or out of the channel.

b. Channel drain

The channel may be emptied by applying a positive or a negative pressure to push or to suck the liquid out its inner volume. If the liquid is drained through the end opposite to the one through which it was introduced, an initial pressure burst may be necessary to break the capillary barrier. Alternatively, the liquid may be removed through the same end through which it was introduced; in this case, the initial pressure

burst may not be needed if the edge of the channel's wall were kept covered with liquid.

c. Liquid flow

5 The liquid flow through the channel may be interrupted to allow for incubation (as required for the capture agent, sample and detection agent) or not as would be the case for rinsing solution (buffer).

6. Light guiding

10 Light is introduced into the channel and detected from the channel perpendicularly to the end's cross section, that is, along the longitudinal axis. In one embodiment, to insure a sensitive optical detection of the molecules immobilized on their inner wall, the channels are capable of guiding light. In the absence of a light guiding property, the light would be dissipated by scattering and absorption in the channels wall or in the bulk of the measuring
15 cell's body. These dissipative mechanisms could prevent accurate measurements. In one embodiment of the invention, the channels are liquid core waveguide tubing. In such waveguides, light is guided inside of a liquid contained inside of the inner volume of the tubing by the total internal reflection of the light at the liquid – wall interface (BioGeneral).

20 Liquid core waveguides increase the performance of optical detection by preventing light from traveling inside of the channels walls. In fluorescent mode, this augments the amount of light available for the excitation of the fluorescent markers immobilized on the inner channels walls. In absorption detection mode, they prevent light from traveling directly from one end of the channels to the other without hitting any absorbent marker.

25 To create a liquid core waveguide, the walls of a capillary must be made out of a material with a lower refractive index than the one of the liquid that they contain in their inner volume. This can be achieved by building tubing with low refractive index material such as Teflon AF ® (n=1.29, Dupont), FEP (n=1.34, Dupont), or by filling the tube with a high refractive index liquid such as glycerol (n=1.47) or immersion oil (Cargill Labs, n up to 1.52). Alternatively, the inner surface of channels or capillary tubing made out of another material
30 may be coated with a low refractive index layer to make them liquid core waveguides (Holger Schmidt, UC Santa cruz).

To prevent light from entering the channels walls at their ends, an optical coating may be applied on the cross section of the tubing. However, this operation may is difficult to realize and not compatible with high volume production. A simple way to solve this problem

is to apply a black coating on the external wall of the capillary tubing (opaque cladding, rubber or paint, for example), or to have them intimately embedded in dark material. This way, any light that enter the walls will be absorb by the external coating.

In another embodiment of this invention, the channels are capillary waveguides, i.e. the light travels both within their inner volume and within their walls. Such channels would typically be used for applications such as evanescent wave sensors.

7. Surface properties

The inner surface of the channels is chosen with well defined physical and chemical properties to insure a proper liquid and light behavior and a controlled immobilization of molecules.

In a one embodiment, the contact angle of standard buffers on the inner wall of the channels is comprised between 10 and 30 deg.; in an alternative embodiment, this contact angle is comprised between 30 and 60 deg. Since the materials that exhibit appropriate optical properties for the fabrication of the liquid core waveguides (low refractive index) usually have a hydrophobic surface, surface treatment is necessary to achieve the contact angles mentioned above. In a first embodiment of this invention, the inner surface of the channels is coated with a layer that makes it hydrophilic. Such coating are commercially available (HydroLast from AST Products). In a second embodiment, the inner surface of the channels is treated to make it hydrophilic. This can be achieved by exposing the inner surface to acids or by breaking chemical bonds of the surface with plasma or UV-Ozone.

Once the inner surface of the channel is made hydrophilic, it is possible to introduce the solution containing the capture agent into the channel by capillary forces and to coat the inner surface of the channels with them.

In a one embodiment of this invention, the inner surface of the channels are coated with PolyVinyl Alcohol (PVA), which provide OH radicals that can be used to further modify the surface to immobilize capture agents such as antibodies, FAB, aptamers or nucleic acids. Additional coating may be needed to prevent non-specific binding of unwanted molecules.

If the walls of the channel are not made out of a low refractive index material, they may need to be coated with a low refractive index layer prior to any other coating to insure proper light guiding.

In another embodiment, the inner surface of the channels is hydrophobic and positive or negative pressure is used to introduce solutions.

III. The Measuring Cell Body

The body of the measuring cell is a mainly passive element in which the channel is embedded. In a preferred embodiment of the invention, the section of the measuring cell's body is square; in another embodiment, this section is hexagonal. In a further embodiment, this section is triangular. In yet a further embodiment, this section is circular.

In a one embodiment of this invention, the length of the measuring cell's body is slightly longer than the length of the channel in order to accommodate some features to facilitate the fluid handling in and out of the channel and to enable the connection with the liquid dispensing elements. Typically, the body is 0.2 mm to 10 mm longer than the channel. In another embodiment, the length of the channel is the same as the one of the body.

In a preferred embodiment of this invention, the measuring cell's body is made out of plastic; examples of plastic includes Black Delrin, HDPE, PEEK and Rubber. The body of the measuring is preferably made out of black material to avoid reflection of light on its surface and to improve light absorption from on the external surface of liquid core waveguide channels.

Alternatively, the body may be made out of glass (Schott Glass AG); its cross section may be hydrogen blackened to avoid light reflection on its surface and to prevent light from entering the body.

In an alternative embodiment of the invention, the measuring cell only comprises a tubing or capillary and in yet another embodiment, it comprises tubing or capillary and a reservoir at one or both of its ends.

IV. Assembly

1. Fabrication

In one embodiment, the channel and the measuring cell's body are extruded together and then cut to pieces of the desired length. As an example, a black PEEK cladding may be extruded around a Teflon AF tubing (Random Technologies) to serve as a body. The channel may also be cast into the measuring cell's body. In a further embodiment, the measuring cell's body maybe bored to accommodate the channel and this latter maybe fixed with glue or rubber.

In yet another embodiment, the body comprises two parts that are bonded together. Prior to this operation, the channel maybe formed directly into one or both parts of the body by hot embossing or etching.

2. Features

5 In a one embodiment of this invention, the measuring cell's body comprises a reservoir at each end of the channel. The function of this reservoir is to prevent the contamination of adjacent channels in the case of overflow of one of the channels (See Figure 1).

10 B. THE MICRO-FLUIDIC DEVICE

I. General Description

15 The micro-fluidic device (MFD) comprises a plurality of measuring cells and two liquid connecting elements that are united to the end of these measuring cells. It permits successive introduction of multiple sets of liquids into said plurality of measuring cells according to two different multiplexing patterns. Typically, a first set of liquids may be a group of capture agents (alone or in a solution comprising the capture agent and at least one solvent and optionally other additives), a second set of liquids may consist of samples (alone
20 or in a solution further comprising and at least one solvent and optionally other additives) and a third set of liquids may be formed by detection agents (alone or in a solution further comprising and at least one solvent and optionally other additives) and the solutions necessary to perform a multiplicity of assays on said samples (alone or in solutions further comprising and at least one solvent and optionally other additives). The advantage of this
25 layout is that, in each measuring cell, an optimized assay can be carried out for the detection of one of the multiple targets that are of interest in each sample. In addition, running identical assays on multiple concentrations of the same calibrators facilitates the establishment of calibrations curves.

30 Figure 2 shows a cross section of a MFD comprising 4 measuring cells (MC) and two liquid connecting elements (LCE). The first LCE comprises two liquid dispensing wells and the second LCE comprises three liquid dispensing wells. The first LCE has a different multiplexing pattern than the second LCE. Figure 3 represents a MFD wherein a first LCE comprising three liquid dispensing wells and three 4-plexed manifolds connects to the first

end of twelve measuring cells and wherein a LCE comprising four liquid dispensing wells and four 3-plexed manifolds connects the second end of these measuring cells.

Figure 4 illustrates the multiplexing patterns of a three times four array of measuring cells. The first ends of said measuring cells are connected with a LCE that comprises three 4-plexed manifolds and that allows the introduction of three 4-plexed samples into the measuring cells. In this example, only one concentration per sample is dispensed. The second ends of the measuring cell are connected to a LCE that comprises four 3-plexed manifolds, allowing for the introduction of four 3-plexed capture agents, detections agents or assay reagents. Thus each sample sub-volume is submitted to a specific assay in the channel of each measuring cell.

In addition to their fluidic functions, the liquid connecting element may also act as light connecting element for the light used for the detection of the molecules immobilized on the inner wall of the measuring cells' channels. They may connect light from a light emitting element into the channels and light from the channels to a light detecting element.

In a preferred embodiment, the MFD exhibits the same dimensions as a standard micro-titer plate, allowing it to be loaded in to a commercial reader for the detection of the immobilized molecules (Tecan GENios). In an alternative embodiment, only the plurality of measuring cells fits into the reader and the liquid connecting elements can be removed to allow the measurements to be performed.

II. Plurality of Measuring Cells

In a first embodiment, the measuring cells of the MFD are assembled in a one dimensional array. For example, the MFD may be a single row of from one to fifty MCS. In another embodiment, the measuring cells are assembled in a two dimensional array. For example the MFD may be from two to fifty rows of one to fifty MCS. In a further embodiment, the MFD dimensions correspond to a micro-titer plate wherein the MCS and the LCES are designed in such a way that the detection of the molecules immobilized on the channels' inner wall may be performed by a commercial reader. In one embodiment, the measuring cells are assembled longitudinally relatively to the micro-titer plate. In another embodiment, the measuring cells are assembled laterally according to these dimensions; in yet another embodiment, the measuring cells are assembled perpendicularly to the micro-titer plate.

In another embodiment, the measuring cells are tubes and their ends are assembled into bundles. These bundles are connected to the dispensing wells of the LCE to achieve the introduction of solutions into these MCS in a multiplexed way (see Figure 7).

The measuring cells may be assembled together by different means. The following methods are provided only as example and are not exhaustive or restrictive: gluing with epoxy, heat bonding, ultrasonic bonding, casting in plastic, casting in rubber, insertion in a pre-drilled framed and fixing with epoxy or rubber.

III. The liquid connecting elements

1. Geometry

a. The multiplexing pattern

In one embodiment of this invention, the LCE connect the first end of the measuring cells to at least one liquid dispensing well according to a defined multiplexing pattern. Said at least one liquid dispensing well is able to receive or deliver the solutions that are necessary to perform the assays and that are dispensed by a liquid dispensing system. In another embodiment of this invention, this at least one liquid dispensing well is connected to the end of the MCS through at least one manifold that distributes said solutions from said well into the MCS channels according to a defined multiplexing pattern. Examples of a liquid dispensing system are a robot with a multiple pipetting capability or an operator with a manual pipet.

The dual multiplexing of the solutions into the measuring cells is achieved using two LCE that have a different connecting scheme and that are used alternatively to introduce said solutions into the channels of the MCS. In one embodiment, one LCE connects to one end of the MCS and the other LCE connects to the other end of the MCS. In another embodiment, the LCE are movable and can be removed from the MFD once all the steps of the assays have been completed. In this embodiment, the LCE may both be connected in a successive way to the same end of the measuring cells.

In yet a further embodiment wherein the LCE may be disconnected from the MCS, the MCS are assembled into a plate that matches the dimensions of a micro-titer plate once the LCE have been removed. Figure 5 shows a plate with 96 MCS assembled in an eight times twelve array and comprising one LCE with twelve 8-plexed manifold and another LCE with height 12-plexed manifolds. In this example, both LCE are successively connected to

the same end of the MCS, whereas in the embodiment showed in Figure 4, one LCE connects to one end of the MCS and the other LCE connects to the other end of the MCS.

In yet a further embodiment, the LCE only comprise a plurality of dispensing wells, to which the MCS are connected. The multiplexing of the solutions dispensed into these wells is achieved according to the connection scheme of the MCS to these wells. This LCE embodiment is well adapted to the MCS embodiment, in which the MCS only consist of tubing (with or without reservoir at the ends of the channel). Figure 2 represents a cross section of a MFD with such LCE and MCS, wherein the MCS are embedded in an opaque matrix.

b. Assembly to the measuring cells

In a first embodiment of the invention, the LCE are assembled to the measuring cells in a fixed manner. Ways to achieve such an assembly include, but are not limited to, heat or ultrasonic bonding, epoxy, adhesive tape or laser welding. In another embodiment of this invention, the assembly of the LCE to the MCS is non permanent. Ways to achieve such an assembly include, but are not limited to, insertion fitting or clamping, either with or without gasket or O-rings.

c. Optical features

In addition to its fluidic functions, the LCE may include features that allow for the introduction of light from a light emitting element into the MCS channel or for the connection of light from said channels to at least one light detecting element. Such features include, but are not limited to, lenses, micro-lenses, micro-lenses arrays, diffraction grids, optical fiber or Brewster windows. In addition to these features, the LCE may comprise filters that allow for a selection of the light wavelengths that are introduced into and connected outside from the channels in order to achieve the optical detection of the molecules immobilized on the inner wall of said channels.

In one embodiment of this invention, the dimensions of the MCS array and of the LCE are such that the respective locations of the optical features match the well location of commercial micro-titer plates and allow for the use of commercial readers for the optical detection of immobilized molecules. In the embodiment wherein the LCE are removable, the ends of the measuring cells match the respective location of a micro-titer plate, so that the introduction of light into the channel and its detection from said channels can be performed by a commercial reader.

2. Fabrication

a. Material

In a preferred embodiment, the LCE are made out of plastic. The following materials are provided as examples and are not restrictive: PP, PC, PTFE, HDPE, FEP and PFA. The dispensing wells, the manifolds and the optical features may be made out of the same material or out of different materials. In another embodiment, the LCE are made out of glass, and in a third embodiment, they are made out of a combination of plastic and glass.

b. Manufacturing

In a first embodiment, the LCE are made by plastic injection; alternatively, they may be made by hot embossing or CNC machining. If they comprise more than one parts, these parts may be assembled together with epoxy, by heat bonding, ultrasonic bonding or laser welding. These techniques, which are well-known in the arte, are cited as example only and are by no way limiting or restrictive.

3. Liquid handling

Liquid handling inside and through the LCE may be achieved by pressure, positive or negative, by capillary forces or by a combination of both. The surfaces of the LCE maybe treated or coated to adjust the contact angle of liquids on these surfaces. Examples of such treatment include, but are not limited to, oxygen plasma or UV ozone activation and acid etch; examples of coating of plastic or glass surfaces include, but are not limited to, PLL, silanes, SAMs, polymers. Providers for such coatings are for example (ArrayOn, AST Products).

C. THE METHOD

I. Description

The embodiments described in the following section are presented as examples only and are not restrictive or limiting.

1. Combination of molecules by successive immobilization on the inner wall of light guiding channels

Bio-assays require the successive combination of multiple kinds of molecules. Such combinations may be achieved by mixing solution containing said molecules (Olink Biosensors, Inc.), or by successively immobilizing the different kinds of molecules on a surface (ELISA). The following sequence provides an example of such immobilization process:

1. a capture agent (e.g. an antibody) is bound to the inner surface of the tube
2. a target (e.g. antigen) is captured by said capture agent
3. a biotinylated detection antibody is bound to said captured target
4. streptavidin tagged with a marker (e.g. fluorescent marker) is attached to said biotinylated detection antibody.

In addition, rinsing or washing steps are required between the immobilization steps described above, which makes such a process labor intensive by requiring many dispensing steps. Furthermore, it might be desirable to immobilize such molecules on the inner surface of light guiding or liquid core waveguides in order to improve assay performances such as sensitivity, incubation time or cross reactivity.

Thus, the use of a micro-fluidic device comprising a plurality of measuring cells with a light guiding channel and a dual-multiplex capability such as described in this application, may help reduce the labor amount, and thus the time and cost required to achieve said combination of molecules on the inner wall of light guiding channels.

2. The disclosed method

The method disclosed in this invention refers to a cycle comprising the successive introduction of two sets of molecules into the light guiding channels of the micro-fluidic device described in this invention and to the immobilization of these two sets of molecules on the inner wall of said channels. Said cycle is preceded by the immobilization of a first set of molecules on the inner wall of said channels. The immobilization of this first set may be part of another cycle or may be an independent step. Said cycle comprises:

1. The introduction into said channels of a first set of solutions containing a second set of molecules. Said first set of solutions is introduced into said channels thanks to a first liquid dispensing element that distributes said solutions into said channels according to a first multiplexing pattern. At least

one of this first set of solutions contains molecules capable of binding to the molecules of the first set of molecules previously immobilized on the inner wall of the channels.

2. The binding of the molecules of said second set of molecules contained in said at least one solution of said first set of solutions with the molecules of said first set of molecules previously immobilized on said inner wall of said channels.

3. The introduction into said channels of a second set of solutions containing a third set of molecules. Said second set of solutions is introduced into said channels thanks to a second liquid dispensing element that distributes said solutions into said channels according to a second multiplexing pattern. At least one of this second set of solutions contains molecules capable of binding to the molecules of the second set of molecules previously immobilized on the inner wall of the channels.

4. The binding of the molecules of said third set of molecules contained in said at least one solution of said second set of solutions with the molecules of said second set of molecules previously immobilized on said inner wall of said channels.

II. Application examples

The application described below is provided as an example and is by no mean restrictive or limiting. Figure 6 illustrates a micro-fluidic device with a 3 x 9 array of measuring cells for the performance of three assays (Il-1 β , Il-2, Il-6 from BD BioSciences Pharmingen,) on three samples, two control solutions (one positive and one negative) and four calibration solutions.

The channels of the measuring cells are made out of low refractive index plastic (FEP, Dupont.) and their inner surface is first coated with PVA (Mitsubishi International Corp.), to make it hydrophilic and to facilitate the introduction of solutions. The inner surface of the channel is then coated with an additional layer of poly-lysine (Poly-L Lysine, Electron Microscopy Sciences) to allow the binding of the capture agent. In this example, the PLL is the first set of molecules of the cycle. These initial layers are applied in a bulk manner to the channels and don't require any specific multiplexing.

Then, in the first step of the cycle comprised in the method described in this invention, the primary antibodies (capture agents) of the three assays (anti- Il-1 β , anti- Il-2, anti- Il-6) are introduced into the channels using a first multiplexing pattern. These primary antibodies are thus

the second set of molecules of this cycle and the solutions in which they are contained belong to the first set of solutions of this cycle. Said primary antibodies bind to the PLL layer (the first set of molecules) during the second step of said cycle. A washing solution may or may not be run into the channels to remove any un-bound molecules. A blocking solution may or may not be run into the channels to reduce non-specific binding (e.g. 1% BSA).

Next, in the third step of said cycle, the samples containing the targets (antigens Il-1 β , Il-2, Il-6), the control solutions and the calibration solutions are introduced into the channel according to a second multiplexing pattern. These samples, control solutions and calibration solutions are forming the second set of solutions that contain the third set of molecules of this cycle. In a fourth step of said cycle, these targets (the third set of molecules) are bound to the primary antibodies (the second set of molecules). An additional washing step may or may not be performed to remove any un-bound molecules.

These third and fourth steps of this first cycle are also the first and second steps of a second cycle, which third step comprises the introduction of the secondary, or detection antibodies (biotinylated anti- Il-1 β , biotinylated anti- Il-2, biotinylated anti- Il-6), according to the same first multiplexing pattern used for the first step of the first cycle. In a fourth step of this second cycle, these detection antibodies are bound to the targets that were previously bound to the capture antibodies. Again, a washing solution may be introduced into the channels to remove any un-bound molecules

A solution containing streptavidin-bound PBXL (Columbia BioSciences) is then introduced in all channels and bound to the biotin of the secondary antibodies. It may be followed by a washing step aiming at removing any un-bound material and finally, (50%) glycerol is introduced into all channels to make them liquid core waveguides.

III. Reduction to practice

1. Experimental set-up and procedure

In order to show the feasibility of the invention described in this application, a demonstration prototype was built (see Figure 8). The aim of this prototype was to show that the fluidic handling described in this invention was possible and to compare the fluorescent signal intensity of the channels at the end of the assay with standard ELISA.

In a standard black micro-titer plate, a total of thirty-six wells formed two groups of two columns of eight wells with a diameter of three millimeters and one group of four wells with

a diameter of five millimeters located between the two groups of two columns. These wells had a depth of approximately 3.5 millimeters.

The ends of 128 FEP tubing (Zeus Inc.) with a length of seven centimeters, an inner diameter of 250 microns and an outside diameter of 500 microns were embedded into the bottom said wells with rubber (Tap Plastic) according to the pattern illustrated in Figure 7. This way, a 4-plexed assay was performed on 32 samples. The ends of the tubing protruded about 1 millimeter from the bottom of the wells.

The tubes were embedded in black rubber (Tap Plastic) to fix them to the plate, to avoid any liquid leak and to absorb the light that may enter the tubes wall through their cross section. In this embodiment, the plate acts as the liquid connecting element (wells connecting a plurality of measuring cells), the tubes are the measuring cells and the channels.

In this experiment, the inner wall of the FEP tubes was coated with PVA and PLL to make them hydrophilic and to allow the immobilization of a first set of molecules (capture agent). This was done by inserting one end of the tubes and the needle of a syringe into each end of a C-Flex tubing (Cole-Parmer) and by pushing the solutions with the syringe into the PFE tubing. An overnight incubation was then performed. This step was performed prior to the assembly of the FEP tubes to the plate.

After mounting the tubes on the plate, the capture agent was loaded into them by capillary forces. Thus, a different kind of capture agent could have been introduced in each group of 32 tubes connected to each of the four larger well. Since the goal of this experiment was mainly to demonstrate the feasibility of the fluid handling, only one kind of capture agent was dispensed (anti-IL-6, at a concentration 144 micrograms per ml). After 2 hours of incubation the tubes were incubated with BSA for one hour then washed with PBST (50 microliters per channel) and dried.

Next, the channels were filled with the sample solution (IL6 in PBST, concentration range 2.5 to 10,000 picograms per ml) from the smaller wells by again by capillary effect. Again, it would have been possible to dispense 32 different samples. After 2 hours of incubation the tubes were washed with PBST (50 microliters per channel) and dried.

In the next step, the secondary antibody was introduced in the tubes through the larger wells, in the same way as the capture agent. This secondary antibody was a biotinylated anti-IL6 at a concentration of 1 microgram per ml. After an incubation time of 2 hours, the un bound molecules were washed with PBST buffer. Finally the detection agent (SA-PBXL-1, Coubia BioSciences, at a concentration of 1 microgram per ml) was introduced, also through the larger wells and incubated during 20 minutes. As a last step, the detection agent was rinsed and

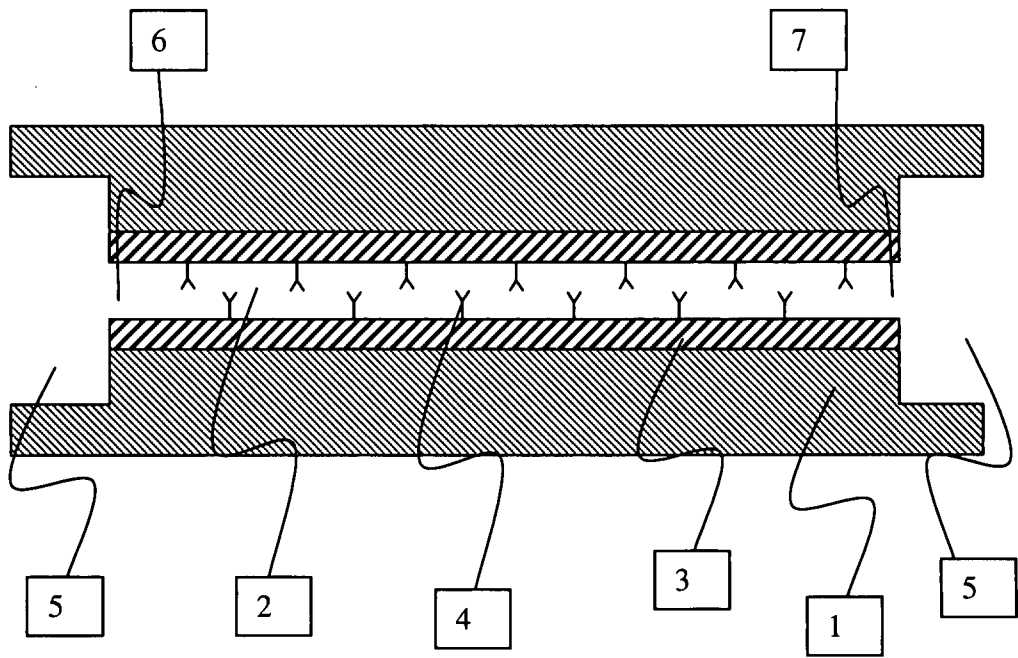
glycerol at 50% was introduced into the channel (also though the larger wells) to insure a proper light guiding.

2. Results

5

The plate was loaded into a Tecan GENios reader to measure the intensity of the fluorescent signal coming out of each channel.

10 The position of the tubes ends was identified as a critical parameter, since it affects both the input of the excitation signal and the output of the fluorescent signal. A more reproducible method to manufacture the plate, such as plastic injection of the plate and cladding of the tubes, may reduce the variation by a considerable amount.



5

Figure 1

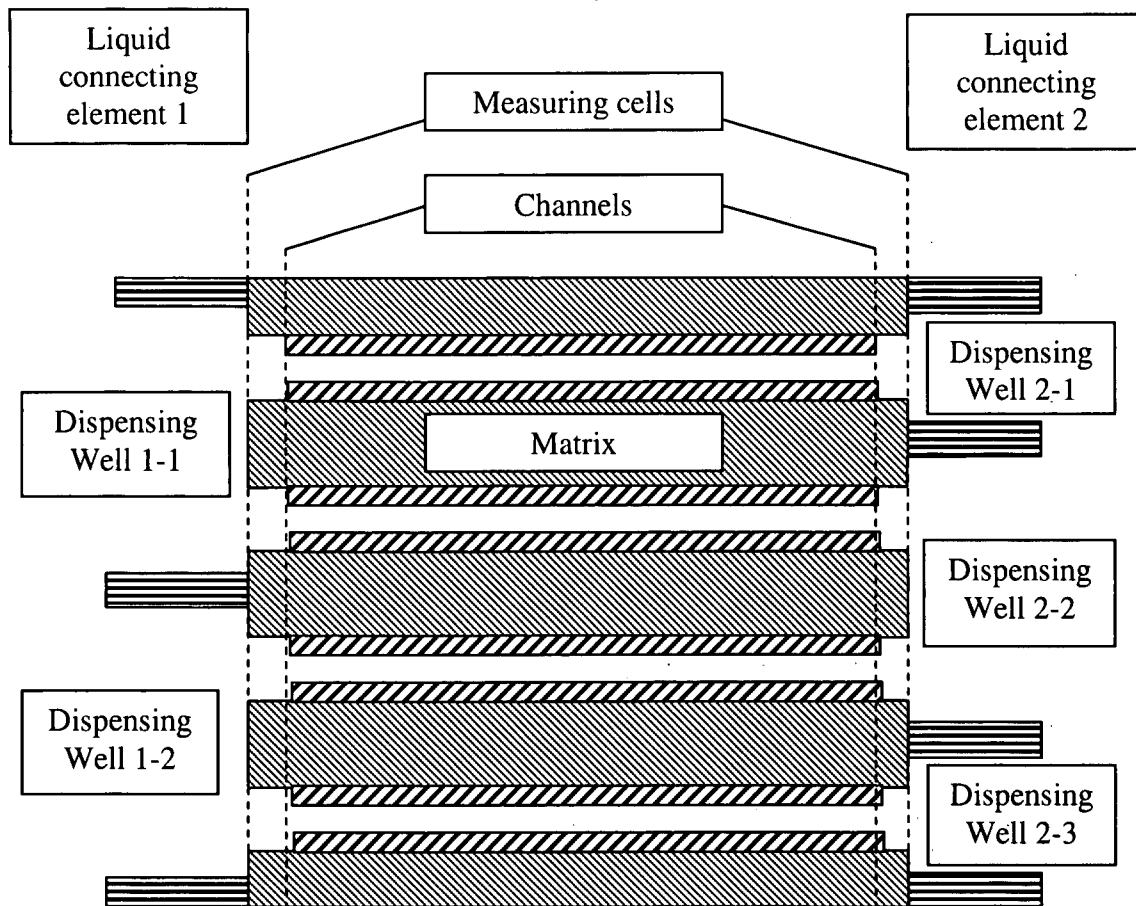


Figure 2

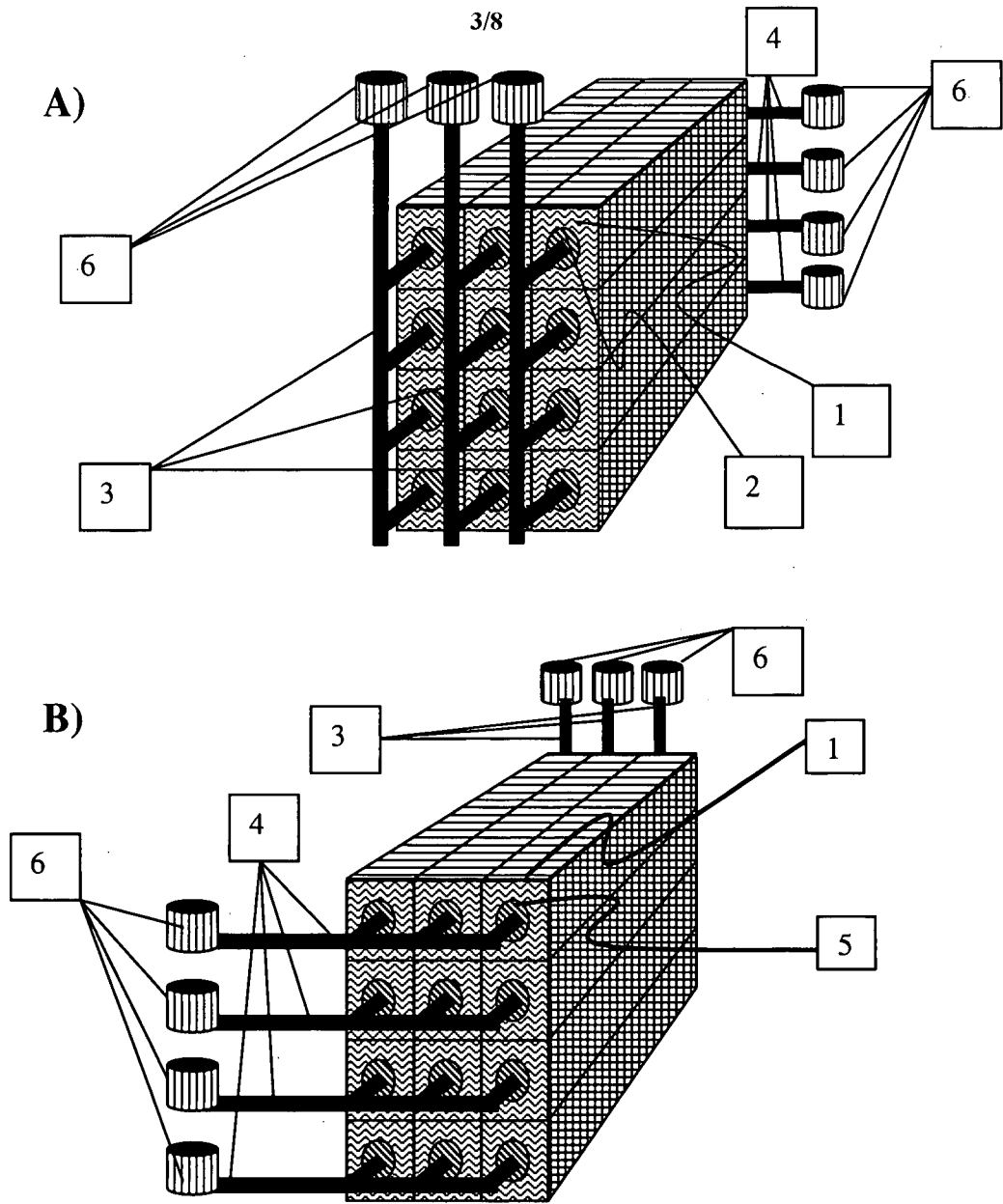


Figure 3

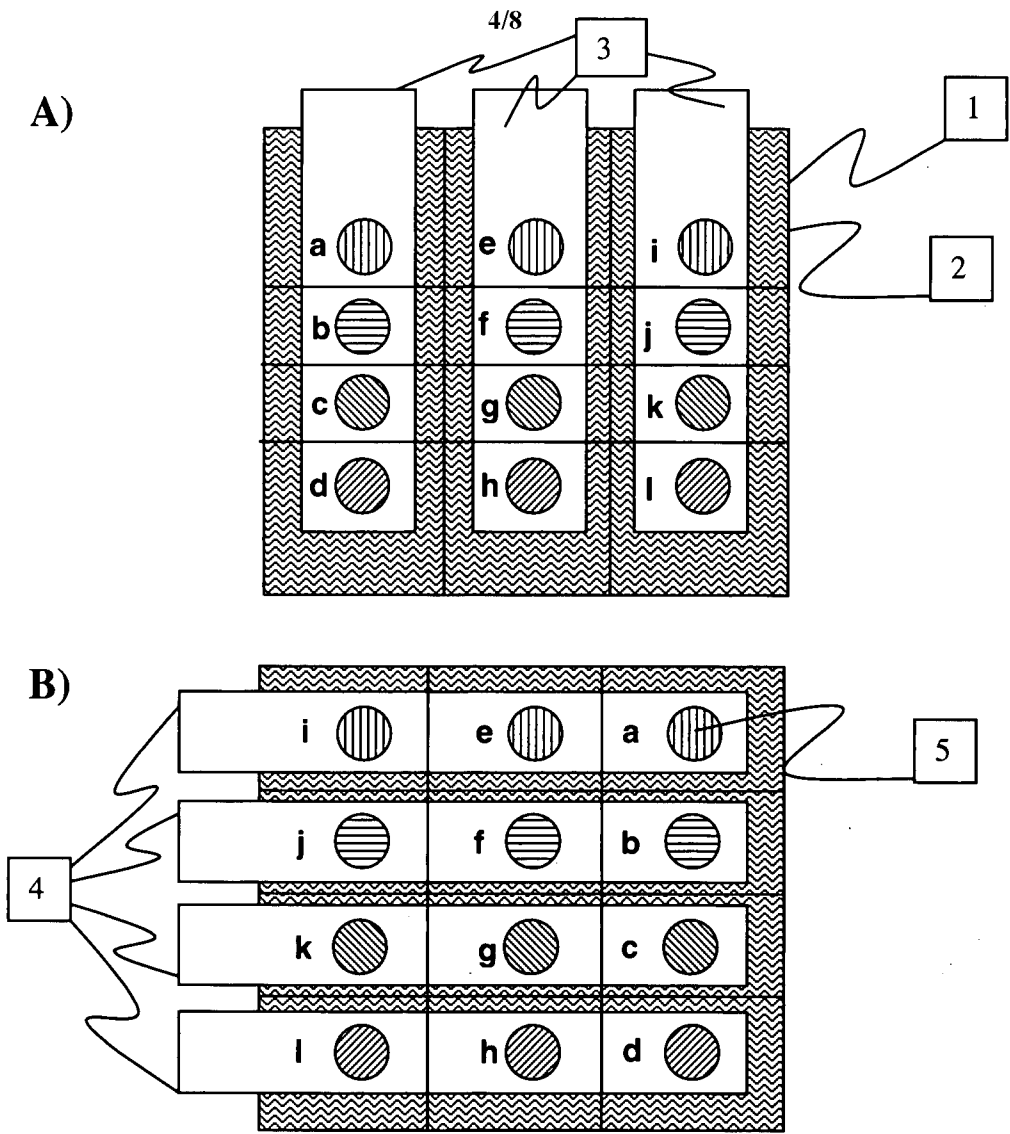


Figure 4.

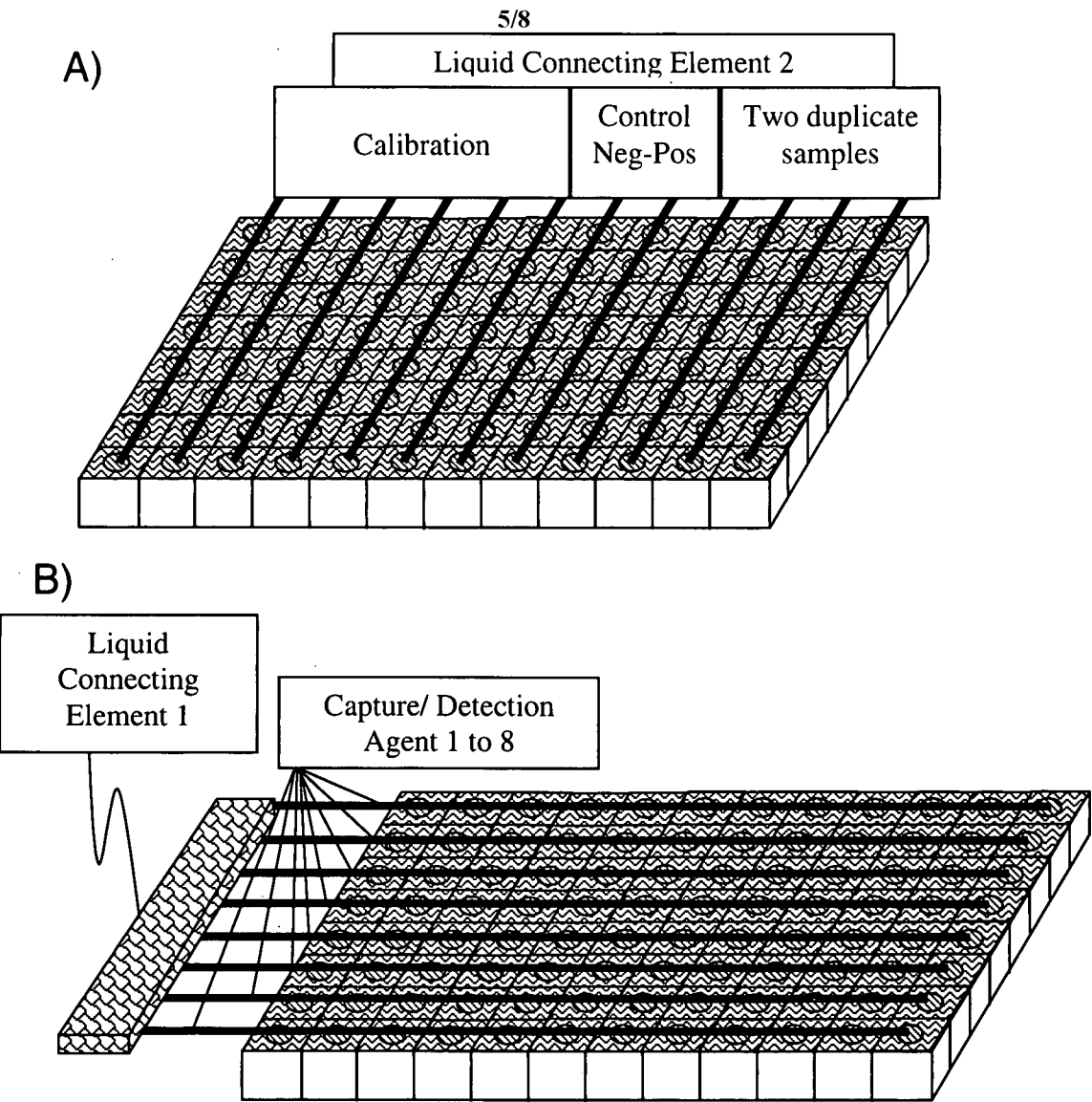


Figure 5

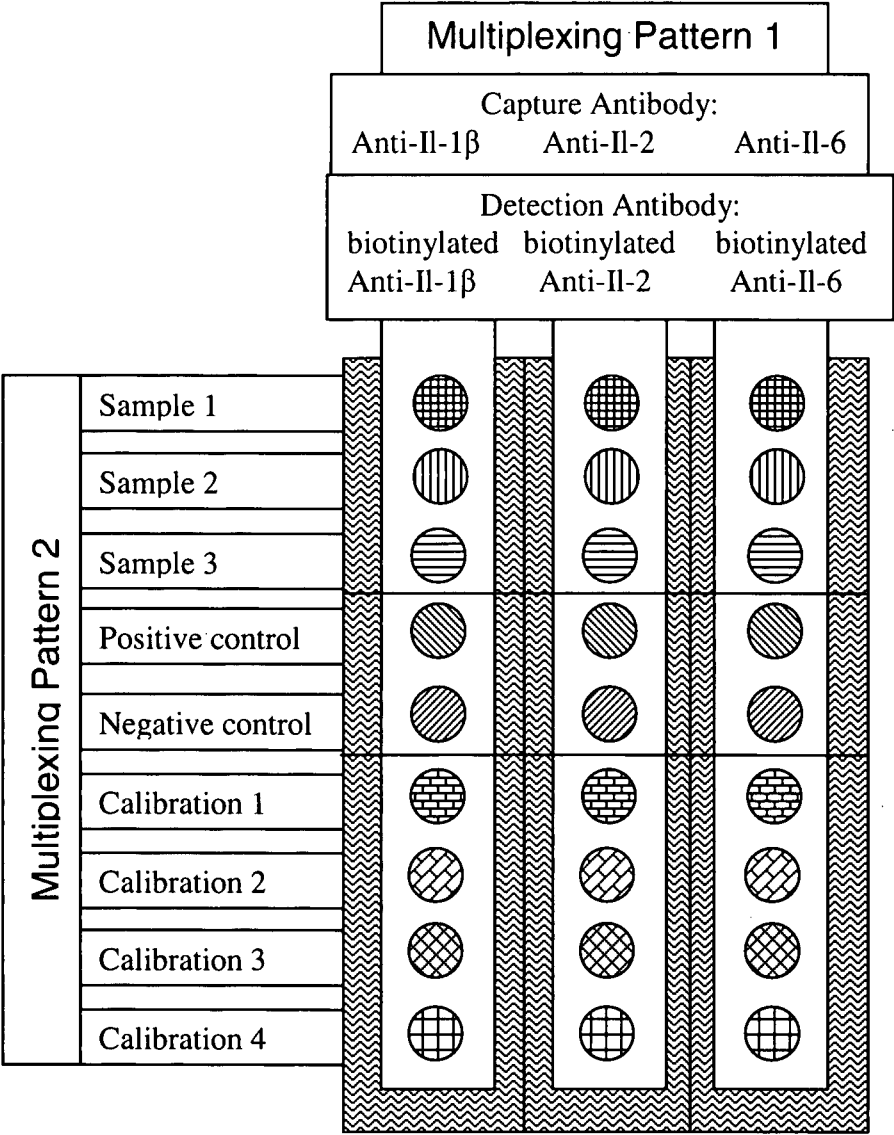


Figure 6

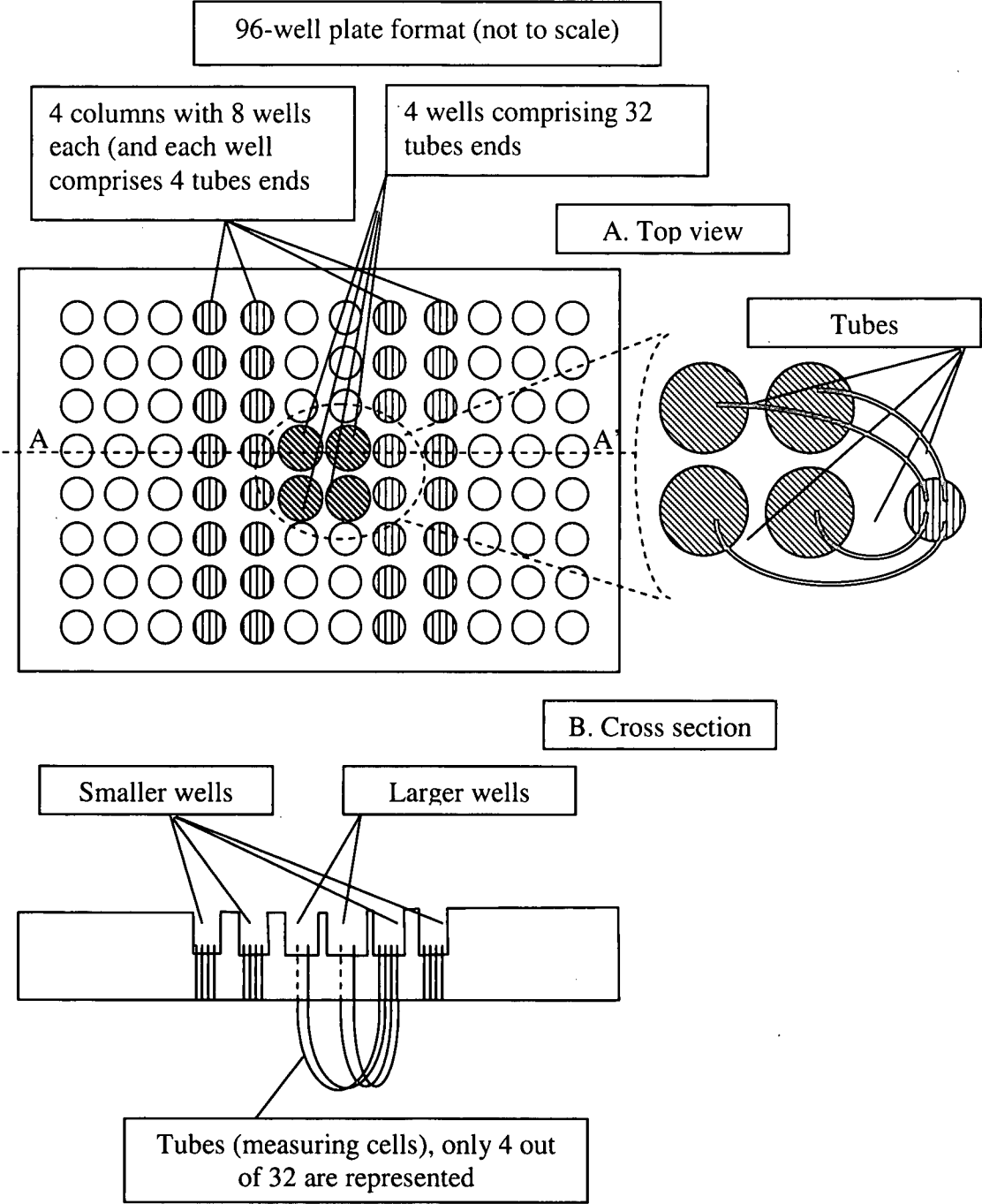


Figure 7

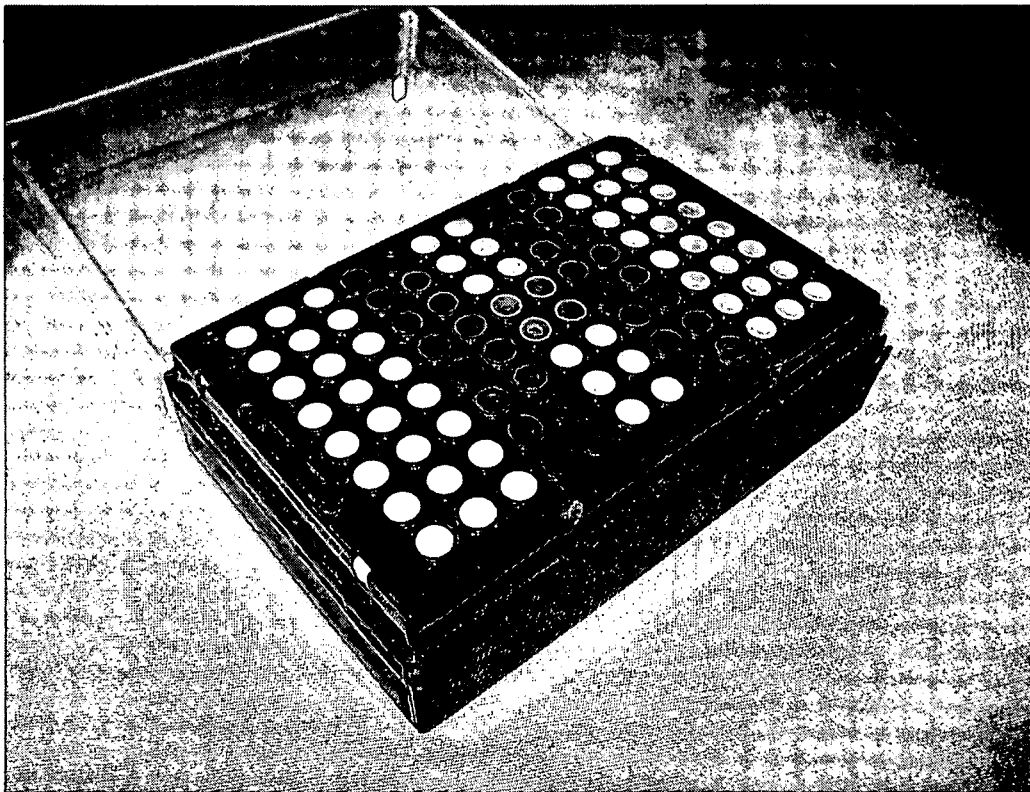


Figure 8