

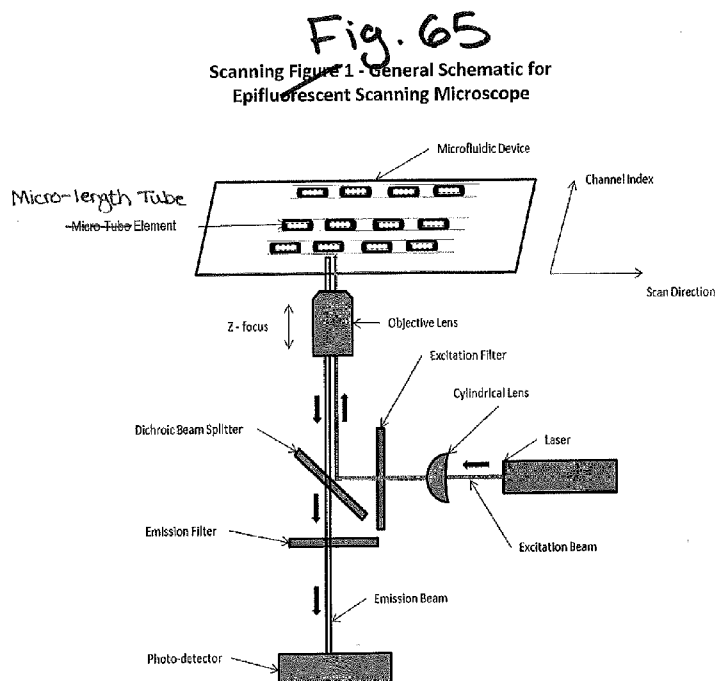


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(54) **Title:** METHODS AND SYSTEMS FOR MANUFACTURE OF MICROARRAY ASSAY SYSTEMS, CONDUCTING MICROFLUIDIC ASSAYS, AND MONITORING AND SCANNING TO OBTAIN MICROFLUIDIC ASSAY RESULTS

(57) **Abstract:** A method of flowing a fluid with a tracer in a microfluidic channel of an assay device and detecting the tracer for determining the channel location or condition of the channel.





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Methods and Systems for Manufacture of Microarray Assay
Systems, Conducting Microfluidic Assays, and Monitoring and
Scanning to Obtain Microfluidic Assay Results

TECHNICAL FIELD

The invention concerns assays in microfluidic systems, including systems that employ portable microfluidic devices. Some versions of microfluidic devices are in the form of microfluidic cartridges (cassettes) that are actuated and read by an associated apparatus such as a bench-top instrument that both conducts the assay protocol within the cartridge and reads the results.

The invention also concerns multiplex microfluidic assays in which multiple assays performed in a microfluidic system are read or scanned with epi-fluorescence.

The invention in particular relates to monitoring assays performed within microfluidic systems, to detecting assay results after microfluidic assays have been run (performed), and to determining the precise relative location of a microfluidic system to a precise detection system, for conducting monitoring or detection with precision.

The invention has broad aspects that are applicable to microfluidic assay systems, in general, and more specific aspects that concern the assays conducted within portable microfluidic cartridges, and particularly cartridges in which the relative position of the cartridge and a precise outside scanning system is not precisely determined. Particularly important applications of the invention concern microfluidic assay cartridges that are inserted into a multi-function apparatus that both causes the assay to be performed within the cartridge and the results detected.

BACKGROUND

As is well understood, with any microfluidic assay system there is the potential for failure, and with complex systems, typically, there are numerous potential failure modes. Examples of failure modes for microfluidic assay systems relate to flows in microfluidic channels and to valves and pistons that control the flows according to a pre-determined assay protocol. The failure modes occur with any microfluidic system, but can be of particular concern when the assay is performed within a microfluidic cartridge. Blockage of a microfluidic channel and inability of a valve to open or close are examples

of failure. If a valve does not open, flow is prevented; if it does not close completely, valve leakage may occur at an inopportune time. There is also the possibility of a contaminant in the microfluidic channels.

There are also potential for human errors. For example, most samples for
5 immunoassays, e.g., human plasma or human serum samples, are diluted with a diluent at a prescribed ratio, for example one-to-one (one part sample to one part diluent) or one to five. It is important for the proper ratio to be supplied, but personnel may improperly prepare the samples.

With microfluidic assays in general, and especially automated microfluidic
10 immunoassays performed within portable cartridges, there are many steps in the assay protocol that need to occur with specific timing and specific reagents. For instance it is necessary to know when a buffer liquid or reagent is being flowed through a microfluidic channel and when a sample is being flowed, and whether, in each case, it is flowed at the proper rate and/or for the proper duration. It is also necessary to know whether there is
15 leakage or flow into regions where no flow can be permitted. Further, when a liquid volume is displaced in a pulsed flow type microfluidic system, for instance as a reciprocating piston pump pushes small slug (portions) of liquid sequentially through a channel, it is important to have a precisely determined quantity of fluid in each slug.

Problems in attempting to obtain this information arise. For instance, because
20 liquids flow in microfluidic channels are very tiny (e.g., 100 - 200 microns cross section width and depth, only millimeters in length) flows are difficult to visualize. The channels are so small that it is difficult for the human eye to observe the fact that liquid is not flowing where it is desired. The assay reagents are typically transparent, compounding the difficulty of visual or optical observation.

The problems are especially acute when seeking highly accurate quantification in
25 a microfluidic assay. In quantifying assays it is desired that a given amount of immobilized capture agent be exposed to a given amount of various fluids to enable reactions over defined times so that results can be compared to a standard to enable the quantification. Results need to be determined with an overall coefficient of variation of
30 less than 10% (accuracy within 10%), preferably much less.

Thus, to be quantitative, assays require consistent run-to-run performance. For example in an assay employing a fluorescent dye conjugated with immobilized, captured moieties, the concentration and the volume of the buffer or wash liquid, of secondary reagents such as antibodies, and of fluorescent dye all need to be the same from run to run if one is to compare the result to a standard calibrating curve precisely generated from previous calibrating runs. This is particularly true in blood testing in which a patient human plasma or serum sample is measured for the presences or the quantity of specific health-related analytes, for instance, antibodies such as interleukins (a class of antibodies called cytokines), e.g., IL5 or IL6. There are many other classes of antibodies to be measured in plasma or serum.

For these reasons it is important to verify that at the end of an assay when a result is generated, that the result has been produced precisely according to the desired protocol.

The result of an assay is typically measured by detecting an emanation, e.g., a fluorescence intensity, from a reaction site. The emanation may come from a bead, a micro particle or an immobilized spot. As presently preferred, it comes from an immobilized glass nano-reactor (GNR) in the form of a small hollow tube or micro-tube, of length no more than 1000 micron, typically less than 500 micron, with capture agent, e.g., antibody, immobilized on its inside surface.

The fluorescence intensity from the region of the capture agent is essentially all that is measured at the completion of many assays. That fluorescence intensity is compared to a calibration curve. From the calibration curve the unknown concentration of the analyte is determined. For the calibration curve to be valid to a particular run, it is necessary that all of the conditions for that assay are repeated specifically and reproducibly from run to run. Improved means to measure such conditions are to be greatly desired.

For the following description of novel techniques for monitoring microfluidic assays, it is important that the exact location of features on a microfluid cassette be known. Novel techniques for doing this are described later herein.

SUMMARY

In a first aspect, the invention features a method of flowing a fluid with a tracer in a microfluidic channel of an assay device and detecting the tracer for determining the channel location or condition of the channel.

5 Preferred implementations of this aspect of the invention may incorporate one or more of the following:

An assay in which an assay fluid having a desired property may be present within a microfluidic channel at a given phase of the assay protocol, including the step of providing the assay fluid with a detectable tracer that is benign (e.g. inert) to the
10 respective phase of the assay, and, during conduct of that phase of the assay, under required assay conditions, monitoring a selected region of the microfluidic channel with a detection system to detect the tracer, and comparing the detected results with a standard of acceptable results. In some implementations, a method may be conducted to determine the precise location of a portion of a microfluidic channel relative to a detection system,
15 including the step of providing a fluid with a detectable tracer, and performing a detection operation that locates the microfluidic channel or a portion of it by a detection system that detects the tracer. In other implementations, this method may be conducted during a step of an assay in which the tracer is inert with respect to an assay fluid in which it is carried. A detectable property of the tracer may be used to verify the assay
20 phase. Different values of the detectable property of the tracer may be used to confirm the phase of an assay, e.g. different and unique concentrations of the a tracer are used for each phase of an assay. The property of the tracer may be fluorescence intensity. The property of the tracer may be optical density. The detection steps may be performed by an epi-fluorescent system employing a light to excite fluorescence within a microfluidic
25 channel, and to translate the beam with relation to the channel over a set of channels or along the channel for detecting position, monitoring, or assay-reading purposes. The light source may be a laser. The laser beam may have an aspect ratio of at least 2:1. Relative movements along a channel may be used to index between monitoring locations, and to progressively read assay results, e.g. from one or a set of immobilized detection
30 elements, such as micro-length tubes (or glass nano-reaction vessels). Relative movements along a channel may be used to determine the locations of immobilized

detection elements, such as micro-length tubes (or glass nano-reaction vessels). The microfluidic flow channel may have a flow axis, along which a series of discrete, axially-spaced apart, transparent hollow flow elements may be secured in fixed position, each hollow flow element may have at least one axially-extending flow passage through its interior, the elements may have interior and exterior surfaces extending in parallel in the direction of the channel axis, and end surfaces may extend transversely to the axis, the surfaces of the elements may be exposed to liquid in the channel, and assay capture agent may be fixed to the interior surface of the elements for capture of an analyte in liquid flowing through the interior of the hollow flow elements, the device may be constructed to enable light to be transmitted into and out of the elements transversely to the flow axis for excitation and reading of fluorescence from captured analyte.

In another aspect, the invention features a device for performing any method recited herein.

In a one aspect, the assay method or device associated with a positive-displacement pump may be arranged to introduce a segment of liquid sample, and may cause the sample to move back and forth with respect to a hollow flow element to produce capture of analyte only in the interior surface of the element and to repeat this action for successive segments of liquid sample. Capture agent on the interior surface of a hollow element in the channel may be configured to define a code. The code may be a bar code.

In another aspect, the invention may feature an assay method or a device for performing a method in which the active capture agent is an antibody, antigen, or oligomer.

In a second aspect, the invention features a micro-length tube element for use in an assay device comprising a hollow body with a through passage for liquid flow, wherein active capture agent for a given analyte resides on at least a portion of the interior surface of the hollow body for interaction with fluid sample, the capture agent on the interior surface of the element being configured to define a code.

In a third aspect, the invention features a micro-length tube element for use in an assay method or device comprising a hollow body with a through-passage for liquid flow, wherein active capture agent for a given analyte resides only on a portion of the interior

surface of the hollow body for interaction with fluid sample; the exterior surfaces of the micro-tube element being free of active capture agent, and un-reactive with the analyte in the sample and in which the capture agent on the interior surface of the element is configured to define a code.

5 Preferred implementations of this aspect of the invention may incorporate one or more of the following:

The code may be a bar code. The active capture agent is an antibody. The active capture agent may be an antigen. The active capture agent may be an oligomer.

10 In a fourth aspect, the invention features reading code on a discrete hollow flow element, according to the following steps: (A) provide hollow flow element with code pattern written in capture agent on inside surface of the element; preferably provide as a micro-length tube element; preferably the code represents the identity and/or concentration of capture agent on the element; (B) Pick and place the hollow element into the flow channel; (C) Conduct analyte capture step by sample flow through channel, attaching analyte molecules to capture agent in the code pattern on inside surface of
15 hollow element; (D) Attach fluorophore tag to captured analyte molecules by flow through the channel; complete the fluid assay; (E) Stimulate and detect pattern of fluorescent emission from tagged analyte through the wall of the hollow element; (F) Conduct pattern analysis, identify pattern of light emission from captured analyte and match the read pattern to stored code table; (G) Use the coded information associated
20 with the specific code pattern of the hollow element; present e.g. by print-out or associate the data with stored or transmitted assay data; (H) And, or, use the detected information in data form in computer logic as part of an algorithm determining analyte concentration.

25 Preferred implementations of this aspect of the invention may incorporate one or more of the following:

Step A may be provided according to the steps of (A) Provide micro-length tube element uniformly coated with capture agent on inside tubular surface with no active capture agent on outside cylindrical surface; (B) With laser beam, deactivate or remove the capture agent according to the blank spaces of a pre-determined code pattern,
30 preferably a bar-code pattern; (C) Place thus-coded micro-length tube element in flow

channel constructed to enable sample flow both through the interior and past the exterior of the element, capturing analyte.

In a fifth aspect, the invention features reading code on a discrete hollow flow element, and quantifying assay data, according to the following steps: (A) Provide
5 hollow carrier element with code pattern written in capture agent on inside surface of the element; preferably provide as a micro-tube element; preferably the code represents the identity and/or concentration of capture agent on the element; (B) Pick and place hollow element into flow channel; (C) Conduct analyte capture step by sample flow through channel, attaching analyte molecules to capture agent in the code pattern on inside
10 surface of hollow element; (D) Attach fluorophore tag to captured analyte molecules by flow through the channel; complete the fluid assay; (E) Stimulate and detect pattern of fluorescent emission from tagged analyte; (F) Substantially simultaneously with step E, read intensity of emission from discrete hollow carrier element (preferably, a micro-length tube element), quantify the magnitude of detected emission; determine the
15 concentration of analyte in the sample as a function of this quantification; (G) Conduct pattern analysis, identify pattern of light emission from captured analyte and match the read pattern to stored code table; (H) Use the coded information associated with the specific code pattern of the hollow element; present e.g. by print-out or associate the data with stored or transmitted assay data; (I) And, or, use the detected information in data
20 form in computer logic as part of an algorithm determining analyte concentration.

Preferred implementations of this aspect of the invention may incorporate one or more of the following:

The tracer may comprise a substance not employed in the conduct of the assay. The tracer may be a substance employed in the assay. The tracer may be a fluorescent
25 tag employed to be captured at the site of analyte to enable reading of the assay. The fluorescent tag may be capable of fluorescent emission under stimulation, the method may include exciting the fluorescence. The fluorescent tag may be capable of fluorescent emission by light stimulation, and the step may include reading the fluorescence by an epi-fluorescence detection system. Any methods discussed herein may include an
30 apparatus for performing the method of any of the foregoing claims.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

Fig. 1 is an illustration of assembly steps for an assay cassette having flow channels in which discrete micro-length tube flow elements are fixed;

Fig. 2 is a diagrammatic plane view, on enlarged scale, depicting 4 micro-length tube elements fixed in series in a flow channel, Fig. 2A illustrating substantial liquid flow both through the flow elements and as by-pass flow through by-pass passages on the outside of the elements, while Fig. 2B illustrates the continued flow condition in the case in which one micro-length tube becomes blocked and Fig. 2C is across-section, on enlarged scale, depicting flow conditions in which a micro-length tube element becomes plugged (i.e. blocked);

Fig. 3 is a much enlarged perspective view of a portion of the cassette, denoting 4 parallel channels, in each of which are fixed six micro-length tube flow elements;

Fig. 4 is a flow diagram of steps A through K in the manufacture and use of the cassette (i.e. "flow chip") constructed according to the foregoing Figures;

Fig. 6 depicts a flow element and a micro-length tube element example, illustrating the percentage reduction of active capture agent under differing conditions of the surfaces of the element;

Fig. 7 depicts a device employed to aggressively agitate a suspension of micro-length tube elements in a capture agent, e.g. antibody, antigen, or oligomer-containing liquid;

Fig. 8 illustrates diagrammatically, by vectors, sheer forces T to which the outside and inside surfaces of the micro-tubular element are exposed;

Fig. 9 illustrates a step in the manufacture of micro-tubular elements from micro-bore drawn filament;

5 Fig. 10 is a plane view of portion of an alignment plate for micro-tubular elements;

Fig. 10 A

Fig. 10 B

Fig. 10 C

10 Fig. 10 C'

Fig. 10 D

Fig. 10 E

Fig. 11 illustrates a centrifugal dryer cooperating with the alignment plate to dry the micro-length tube elements;

15 Fig. 12 is, on enlarged scale, a diagrammatic representation of laser beams ablating (removing or rendering in-active) selected regions of capture agent on end and inside surfaces of a micro-length tube element;

Fig. 13 is a view similar to Fig 12, depicting the formation of a code of capture agent on the inside surface of a micro-length tube flow element;

20 Fig. 14 depicts a photo mask exposure scheme for forming a large laser beam into beam-lets that perform the steps of Fig. 12 or 13;

Fig. 15 is a diagrammatic top view of a system for placing discrete micro-length tube elements into open channels of a microfluidic assay device;

Fig. 16 is a side view of a pick and place apparatus employing a tweezer instrument for engaging end surfaces of the micro-length tube elements;

Fig. 17 depicts the ends of tweezer tines approaching the oppositely directed end surfaces of a micro-length tube element positioned in the alignment plate;

5

Fig. 19 depicts the ends of tweezer tines leaving the oppositely directed end surfaces of a micro-length tube element positioned in a flow channel of the microfluidic device;

10

Figs. 21 A-E represent, in large scale end cross-section views of a sequence of steps involved in placing and fixing the position of micro-elements in an assay device and completing the enclosure of a flow channel in the device;

Figs. 22 A and B illustrate the flow cross-sections of a flow channel with micro-length tube element in place.

Fig. 23 A

15

Fig. 23 B

Fig. 23 C

Fig. 23 D

Fig. 23 E

Fig. 23 F

20

Figure 24 – A plane view of the fluidic sub-assembly on an enlarged scale;

Figure 25 – A perspective view of parts of the pneumatic sub-assembly as they come together;

Figure 26 -- A plane view, looking up at the underside of the pneumatic sub-assembly through its transparent membrane;

Figure 27 – A plane view, again of the underside of the pneumatic sub-assembly and the mating upper surface of the fluidic sub-assembly;

Figure 28 – A perspective view diagrammatically illustrating the mating action of the two sub-assemblies;

5 Figure 28a – A side view illustrating the mating surface of the two subassemblies being pressed together with slight pressure;

Figure 28b – A magnified view of a portion of Fig. 28a;

Figure 28c – A perspective view of the completed assembly viewed from above;

Figure 28d – A perspective view of the completed assembly, viewed from below;

10 Figure 29 – A top view of the completed assembly;

Figure 30 -- A schematic diagram in perspective of assembly steps for another microfluidic assay device;

Figure 30a – An exploded perspective view of the device of Figure 24;

Figure 31a -- A perspective view of a fluidic channel of Figs 24 and 25;

15 Figure 31 b – A magnified view of a portion of Fig. 25a showing flow channels, hollow flow elements, valve seats and pump chambers;

Figure.31c – An even more greatly magnified view of as single extremely small hollow flow element disposed in a channel of Figures 25 a and b;

20 Figure 32 – A greatly magnified plane view of a portion of the channel structure, showing two channels, with four hollow flow elements disposed in each; Figure 33 – A plane view of a single channel, with schematic illustration of on-board pump and vale, and showing flow paths through and alongside hollow flow elements;

Figure 33a -- A view of the valve of Figure 12;

Fig. 33A

25 Fig. 33B

Fig. 33C

Fig. 33D

Fig. 33E

Figure 34 -- A diagrammatic cross section, with parts broken away of channels of a device, and depicting lines of flow through and outside the flow element;

Figure 34a -- A view in which two layers have been fused by covalent bonding to close the channels and secure the hollow flow elements;

5 Figure 41 -- A diagram of steps in the assembly process for the device of Figs. 24-34;

Figures 41 a, b, c, and d--Illustrate steps in employing covalent bonding to form the liquid-tight channels and secure the extremely small hollow flow elements in place in the channels;

10 Figure 42 -- A diagram of a pick-and-place instrument positioned above an X,Y translation table, a delivery plate for discrete, extremely small hollow flow elements and a receiving channel of multiplex micro-fluidic assay devices of the preceding Figures;

Figures 43, 44, respectively, diagrammatic front and side views of a tweezer type pick and place device, and its support tower;

15 Figure 45 -- A sequence of positions during placing of a flow element, ns diagrammatically illustrating the use of close-space electrostatic attraction between the channel wall and the element being delivered; 45 is a pick and place front view of the system

20 Figures 46 and 47 -- Respectively, picking, and placing views of a vacuum pick up device;

Figures 48, 49 and 49A -- A sequence of positions during placing of a flow element with the vacuum device, NS diagrammatically illustrating the use of close-space electrostatic attraction between the channel wall and the element being delivered;

25 Figures 49B and 49C -- illustrate element-securing and channel-sealing actions occurring during assembly of the device of Figs. 24, et seq.

Fig. 50A, C, E, F, & D show

Fig. 50 B

Fig. 50 H

Fig. 50 G

30 Fig. 51 is a diagrammatic view showing the repeated cycling of a diaphragm valve formed by an overlying portion of a PDMS layer, which is bonded to the opposed

structure at each side, the valve, repeatedly closed with 3 psi positive pressure and opened with negative 8 psi pressure (vacuum), is found to overcome the molecular bonds being formed between diaphragm and valve seat, thus over time neutralizing the tendency for permanent co-valent bonds to form between contacting surface-activated surfaces, thus enabling the thus-formed valve to properly operate;

Fig. 51 A-1 is a diagrammatic showing of two layers opposed layers of PDMS showing them in the natural state of the PDMS which is a hydrophobic state with methyl group endings exposed.

Fig. 51 A-2 is a similar Figure following plasma oxygen plasma treatment showing the separated layers are terminated in OH groups.

Fig. 51 A-3 is a Figure illustrating permanent bonds between the hydroxyl groups producing bridging oxygen covalent bridging oxygens.

Fig. 51 A-4A is similar to Fig. 51A(4') except that a valve seat is shown rather than both surfaces being deflected away from each other. A single deflection showing also a valve seat opposing that surface.

Fig. 51 A-4B

Fig. 51 A-6

Fig. 51 C are views similar to Fig. 51 illustrating three stages of the system applied to multiple valves simultaneously.

Fig. 51 D-1 and D-2 are graphs illustrating properties during make and break process

Fig. 51 B(i) shows a tool to which a fluidic is to be brought into contact and joined diagrammatically the system of applying a pressure or vacuum to the pneumatic tool.

Fig. 51 B (ii) shows the pneumatic tool and plane view and the connections to supply ports for application of vacuum or pressure to the pneumatic tool.

Fig. 51 A-4' is a Figure illustrating two regions of PDMS following plasma activation and contact in region A and activation or separation or what is referred to as the valve region illustrating permanent or the initiation of permanent bonding through the with hydroxyls and the condensation reaction resulting in bridging oxygen in the region A which is the region of contact and the region of noncontact or the region where contact

had occurred only temporarily and then removed resulting the surface having an increasing number of methyl groups or nonbonding or low energy state species.

Fig. 51 A-5

Fig. 51 A-4” is a figure illustrating a single surface deflected opposing a planar surface.

Fig. 51 A-7

Fig. 51 A-9

Fig. 51 A-10

Fig. 51 A-11

Fig. 51 A-12

Fig. 52 A1

Fig. 52 A1M

Fig. 53 pictures diagrammatically a pumping and valve state sequence by which liquid flow can be drawn into the piston from the left and expelled to the right to produce a desired directional, pulsating flow.

Figs. 54 and 54 A. illustrate, respectively, two positions of a microfluidic cartridge relative to a carrier plate upon which it is intended that the cassette be fixed, while the plate is moved on a precision X, Y stage, relative to a fixed, finely focused optical detection system.

Fig. 55 is a cross-section view of the cartridge of Figs. 6 and 6A now fixed to the carrier plate on the movable stage, to move over a fixed heater plate and optical detection system, the objective of which is exposed to the cartridge through a hole in the heater plate. Fig. 55A is a magnified view of a portion of Fig. 55.

Fig. 56 is an exploded view of the assembly of a bench top operating and scanning unit for scanning the microfluidic cartridge of Figs. 54 and 54A.

Figs. 57A-57C are plane views of the microfluidic and pneumatic channel architecture of the cartridge of Figs. 54 and 54A.

Fig. 58 outlines the fluidic architecture of a single microfluidic subunit of the cartridge of Figs. 54 and 54A, and, in tabular form, presents the steps of an immunoassay conducted within the cassette.

Figs. 53A-K

5 Fig. 61 diagrammatically illustrates the procedure of precisely determining the location of channels of a microfluidic cartridge, for instance when fixed within the precise X, Y stage system of Figs 54, 54A, 55 and 56.

10 Fig. 62 illustrates the fact that the precise position of channels, for monitoring, and the location of detection elements in the channel, for later reading of results, can be accomplished in the same system.

Figs. 63 and 64 are representations repeated in the later Scanning drawings, illustrating signals obtained during position determination in the absence of tracer.

Fig. 65 - General Schematic for Epi-fluorescent Scanning Microscope

Fig. 66 - Laser Beam Shape Isometric View

15 Fig. 67 - Laser Beam Shape Layout View

Fig. 68 – Micro-length tube element scan schematic

Fig. 69 - Acquisition Time Series

Fig. 70 - Overall Scan Sequence

Fig. 71 - Scan Sequence – Imaging

20 Fig. 72 - Scan Sequence – Discrete photo detector

Fig. 73 – Reading Code from Micro-length tube flow element

Fig. 74 - Preferred Implementation of previous Figures first block

Fig. 75 - Reading code and analyte quantity from micro-length tube flow element simultaneously

Fig. 76 – Reading Code Consolidated

Fig. 77 - Bar Code in Micro-length tube Element

5 Fig. 78 - Scan Data File Snippet

Fig. 79 - Chip Layout

Fig. 80 - Find Channels ROI

Fig. 81 - Find Channels Scan Plot

Fig. 82 - Find Channels Data Segment Plot

10 Fig. 83 - Find Channels Processing Flowchart

Fig. 84 - Find Elements ROI

Fig. 85 - Find Elements Scan Plot

Fig. 86 - Find Elements Processing Flowchart

Fig. 87 - Auto Focus Scan Plot

15 Fig. 88 - Auto-Focus Processing Flowchart

Fig. 89 - Auto-Expose Schematic

Fig. 90 - Auto-Exposure Procedure Flowchart

Fig. 91 - Laser/ROI Alignment

Fig. 92 - Fluorescence Scan ROI, bright field

20 Fig. 93 - Fluorescence Scan ROI, laser on, LED off

Fig. 94 - Fluorescence Scan Data, Full San

Fig. 95 - Fluorescence Scan, One Channel

Fig. 96 - Fluorescence Scan, One Element

Fig. 97 - Fluorescence scan data processing

Fig. 98 depicts a microfluidic system having microfluidic channels and monitor locations;

Fig. 98A depicts signals obtained in three phases at a set of monitoring positions
5 under three different conditions, illustrating a properly running assay.

Figs. 98B and 98C, similar to Fig. 98A, depict signals obtained during improperly running assays.

Fig. 99 illustrates monitoring a microfluidic channel at a single location, over a brief period of time over which flow changes.

10 Fig. 100 is similar to Fig. 99, but illustrates monitoring a fluid to detect operation of a pump over many cycles of producing oscillating flow.

Fig. 101 illustrates, diagrammatically, the relation of a scanning system to a microfluidic device, shown aligned with a channel at a region that does not contain a detection element;

15 Fig. 102 illustrates the cross section of the region of interest (ROI) of the optical system of Fig. 101 in relation to the microfluidic channel and the cross-section profile of a fluorescence-exciting laser beam.

Fig. 103 outlines the fluidic architecture of a single microfluidic subunit of the cartridge of Figs. 54 and 54A, and, in tabular form, presents the steps of an immunoassay
20 conducted within the cassette.

DETAILED DESCRIPTION

DESCRIPTION

One of the problems addressed concerns the surface area associated with a micro-length tube element, i.e., an element having length less than 700 micron and a micro-bore diameter between about 75 +/- 50 micron that is fixed in a flow channel and exposed to flow of liquid sample, e.g., a glass nano reactor “GNR”. Such devices are typically made of endlessly drawn micro-bore filament such as used to form capillary tubes, but in this case, the filament is finely chopped in length to form discrete, shorter micro-flow elements. It is realized that capture agent immobilized on the surface of such a device, applied by immersion techniques, can raise a significant depletion problem. This occurs, for instance, when attempting to characterize concentrations of an analyte at low levels such as a few pico-grams per milliliter, as is desired. The phenomenon referred to as “depletion” occurs in which the concentration of analyte in the sample being measured can be disadvantageously depleted volumetrically as a result of binding to a large active area of the flow element. This results in reduction of sensitivity of the assay, and therefore its usefulness. To explain further, any analyte in an ELISA or sandwich type of amino assay on antigen will bind to a capture antibody in a way that is governed by a kinetic reaction, a dynamic process. While analyte such as an antigen binds to capture agent such as an antibody, the reverse also occurs, the bound analyte molecules unbind from the capture agent. The kinetics concern an “on” rate and an “off” rate --analyte being captured and analyte being released. The capture reaction will continue, depleting the analyte in the ambient volume, and reducing its net rate of capture, until the system reaches equilibrium in which the rate of binding is equal to the rate of unbinding. The gradual action occurs according to a substantially exponential curve.

The absolute value of the equilibrium condition depends on the original concentration of the analyte in the volume of sample being assayed. Increase in concentration results in a higher signal, decrease in concentration results in a lower signal. In cases in which assay depletion occurs, the concentration of the analyte in the sample is detrimentally decreased over time. It is realized that micro-length tubes fixed in flow channel may present an excess of capture agent in the volume of liquid sample to which the element is exposed, decreasing the effective concentration of the analyte. The

concentration decreases at an excessive rate, relative to initial, starting point concentration sought to be measured. While efforts to calibrate for this are helpful, such depletion ultimately lowers the sensitivity of the assay because, as the signal goes down, it approaches the noise level, and results in a lower signal-to-noise ratio, i.e. an inherent reduction of effectiveness of the assay. (Already there are significant contributors to noise i.e., background, nonspecific binding of capture antibody, fluorescence noise, electronic noise, etc.). Therefore, especially for detecting small concentrations, it is desired not to deplete the initial volume of the analyte in manner that does not contribute positively to the assay measurement. Efficient ways to do that, as by somehow limiting the amount of exposed surface have not been apparent. This may be seen as an inherent problem with use of micro-flow elements of various descriptions that are coated by immersion or the like and used in an immunoassay or sandwich assay or even a molecular diagnostic type of assay. One typically wishes to immerse the elements in capture agent, e.g. an antibody or some type of moiety that is a capture molecule for the analyte to be sensed or detected, to uniformly coat all surfaces of the element. One object of invention is to overcome this problem with respect to micro-length tube elements characterized by an inside surface and an outside surface, or often also with two end surfaces. Adding up all surface area over which a density of capture molecules is coated can add up to a surface area on the order of over 100,000 square microns. This is the case for a preferred form of micro-length tube, having on the order of about: a length of 200 microns, an external diameter or width of 125 microns, and an internal diameter or width of 70 microns. A particular problem addressed here is to find practical approaches for accurately reducing active surface area of immersion-coated flow assay elements in general, and in particular, micro-flow elements, and in particular micro-length tube elements.

A further problem being addressed here concerns treated micro-flow-elements that are to be in fixed positions in channels for exposure to flow of sample. It is desirable to expose the elements in batch, in free state to an immobilization process for applying the capture agent or antibody to the element surface, and then transfer each element mechanically to its fixed position in a channel, for instance in a channel of a multiplex micro-fluidic "chip" (or "cassette"). It is desired to use a quick and accurate placement

process, for instance a pick and place device mounted on an accurate X, Y stage. For such purpose, it is desirable to physically contact the tiny element for picking it up from a surface and placing it in an open channel, which is then closed to form a micro-fluidic passage. It is desirable to employ grippers, e.g. a tweezer instrument that contacts the outer surface of the device. The pick and place action is made possible by pre-aligning open channels to receive the micro-flow elements and the surface on which the free elements are supplied with the automated pick-and-place instrument. This enables the grippers to pick up and place the micro-flow elements precisely in desired flow channel positions in which they are to be fixed. We recognize a problem arises with having an active capture agent, e.g. antibody, immobilized on outer surfaces of an element. Such a coating is susceptible to mechanical damage as a result of the mechanical manipulation process. Outside surfaces of micro-flow elements come in contact with (a) a supply surface, e.g. an aligning pocket or groove, (b) the transferring grippers, and (c) surfaces of the channel in which it is being deposited. All of these contacts opportunities give rise to possible damage to the fragile coated capture agent, which typically is a very thin layer of antibody or the like adsorbed to the surface of the flow element. This coating is often only a few molecules thick, thickness of the order of nanometers or tens of nanometers, and is quite fragile. The net result of damaging a capture surface of the placed micro-element is seen during read out of the assay. If the surface has been scratched or perturbed in any way, that can give rise to an irregular concentration or presentation of captured analyte, the signal can be irregular, and contribute to irreproducibility or poor performance of the assay.

We thus realize it is desirable not to have immobilized active capture agent on the outside surface of a micro-flow element, and especially micro-length tube element, where it is susceptible to damage and where it contributes to increasing the total surface area of the capture agent or antibody that contributes to depletion.

The features described in the claims and hereafter address these and other important problems.

Discrete micro-flow elements are immersed in liquid containing capture agent, such as antibodies or antigens, and, after coating by the liquid, are picked, and placed into channels for flow-through assays. The micro-flow elements are in preferred form of

discrete micro-length tubes, defined as micro-flow elements of length less than about 700 micron, and bore diameter of 70 +/- 50 micron. The flow elements are surface-treated so active capture agent, e.g. capture antibody, is not on the outside, or is of limited outside area. For this effect, micro-flow elements, or in particular, micro-length tubes, are
5 disposed in a bath of active agent and violently agitated, resulting in coating of protected inside surface, but due to extreme shear forces, a clean area on the outside surface, for instance the entire outside cylindrical surface of a round cross-section discrete micro-length tube. In lieu of or in addition to this shear procedure, a special filament-manufacturing process is conceived that results in preventing coating an exterior
10 surface of flow elements with a predetermined capture agent. Capture agent on selected coated areas are ablated or deactivated with precisely positioned laser beam, such as can be produced by a mask for simultaneous treatment of a large number of elements, leaving residual active agent of defined area on the inside surface of micro-flow elements. Residual capture agent, itself, on the inside of the elements, usefully defines a readable
15 code related to the desired assay. Flow channel shape is sized relative to flow elements fixed in the channel to allow (a) bypass channel flow along the exposed outside of a micro-flow element to reach and flow through later elements in the channel in case of clogging of the first element, along with (b) sample and assay liquid flow through the micro-flow element to expose the surface to capture agent and other assay liquids. Lacking the need to attempt to seal the outside, the element can simply be gripped, as by
20 an elastomeric sheet pressed against the element. Electrostatic attraction between flow element and channel wall is employed to fix the element in position, overcoming any disturbing force of the placing instrument as it is drawn away after delivery of the element. After assay, fluorescence is excited and read by special scanning confined to
25 micro-flow element geometry. Locators are seeded in the recorded data, and used to locate the regions of interest in detected fluorescence data, e.g. from micro-length tubes. Code, written with the capture agent substance inside the micro-flow element is read through a transparent wall of the element. Efficient assembly and tooling features are disclosed. All features are applicable to micro-length tubes, enabling their efficient use. A number of the features are or will be found to be useful with other hollow elements, for
30 example, longer micro-flow elements.

In respect of scanning, the purpose of this invention to deliver a method for performing a fluorescence measurement of multiple immobilized elements contained in a microfluidic chip. This method provides for determining the paths to be followed during the scanning, as well as the proper focus, and camera exposure. The method is based on a known general chip layout. The method provided results in the ability to place the chip to be measured into the scanner and then start the scan without any additional manual settings required. The method does the rest, and produces the desired fluorescence measurements as the results.

Certain aspects of invention involve eliminating or preventing the occurrence of active capture agent on outside surfaces of micro-flow elements, e.g. extended outside cylindrical surface, and/or end surfaces, while leaving active capture agent on the inside surface unperturbed, or of a desirable area or pattern. Features addressing this aspect include techniques to selectively limit the capture agent on the interior surface and steps that act in combination with outside and inside surfaces to achieve the desired result.

For the specific advantage of reducing the overall capture surface area, two aspects of invention will first be described, and the effect of their combination. A first technique is employed to eliminate or prevent capture agent, e.g. antibody, from immobilizing to the outside surface of hollow flow elements, especially, micro-length tube elements. That is done during a batch coating process, and involves suspending discrete hollow elements, especially micro-length tube elements, in an Eppendorff tube or other tube with the capture agent of interest and aggressively agitating fluid to impart disrupting shear forces to the exterior surface of the elements. Preferably, this is achieved by vortexing the fluid at high speed, for instance employing an instrument that orbits the container at approximately 2000 rpm of the orbiter, about an orbital path of the supporting shaft of diameter of about 25 mm.

The micro-tub elements are placed with a volume, e.g. a milliliter of capture agent, e.g. antibody. The appropriate vortexing speed, is dependent e.g. on the nature of the suspension, e.g. the viscosity of the liquid chosen, and can be easily determined experimentally. It is set by observing whether the capture agent is effectively non-existent on the outside, long surface of the micro-length tube elements, e.g. the outside cylindrical surface in the case of the body being of circular cross-section.

The physical principle involved concerns shearing force on the outside surface of the micro-length tube element that acts to prevent binding of the capture agent to the surface through an adsorption process. One can observe whether the vigorous agitation is sufficient to shear off any capture agent, e.g. antibody that has already been bound to that surface. At the same time, the inside surface is environmentally shielded from this shearing by virtue of the geometry which is tubular, and the micro-size of the bore of the tube. This prevents vortexing from causing any turbulence to occur within the element. Only laminar flow conditions exist. With micro bore elements the Reynolds number is always low enough to ensure that that laminar flow condition exists on the inside surface. Under these conditions, the velocity of fluid traversing in the micro-length tube element at the interior wall interface is by definition zero. So there is no shear force involved there, whereas the outside is in a highly turbulent, high shear force environment.

The observed result of aggressive agitation, e.g. vortexing, is that fluorescence which is observed by performing a sandwich assay is completely absent from the outer cylindrical surface of a micro-length tube element, whereas it is present in an observable way on the inside surface. In the case of square-end micro-length tube elements, fluorescence is also present on the end faces of elements.

Vortexing is the presently preferred technique for producing the shear forces. The case showed here employs orbitally rotating the micro tube in a very rapid manner back and forth in small circles at a rate of approximately a couple thousand rotations per minute, and an excursion of about 25 mm.

However, any type of rapid oscillation that creates a high degree of turbulence can be employed, so a back and forth motion, a circular rotation, anything that would very rapidly mix the fluids and create high shear forces will suffice.

In summary, micro-length tube elements in the presence of aggressive agitation leads to removal of capture agent, e.g. antibodies, from outside surface of the elements, and prevention of their coating with the agent, but leaves the inside surface of the micro-length tube element in condition to immobilize capture agent, e.g. capture antibodies, for subsequent interaction with analyte of the sample.

As an alternative to the high shear technique, we conceive an alternate process in which, during the original drawing of the small bore tube, and prior to the point along the

draw path that the usual removable protective polymer coating is applied to the filament, that a non-stick coating, e.g. sputtered gold, silver or graphite, is applied to the filament, e.g. by passing through a sputtering chamber. Silane or similar coating must be applied to receiving surfaces before capture agent, e.g. antibodies will attach. However, due to
5 the properties of the sputter coating, or the like, the surface will not receive the silane or equivalent, then likewise, the active capture agent.

Another feature of invention concerns realizing the desirability and technique of removing coated capture agent from selected end surfaces of the flow elements and a margin portion or other portion of the interior surface. Preferably, following the
10 aggressive agitation process, the micro-length tube elements are further processed using a laser elimination process that removes or de-activates capture agent, e.g. antibodies, from surface from which the agent was not removed by the high shear process. Those surfaces include transverse end surfaces and a selected portion of the inside surface, leaving only an annular stripe on the inside surface sized sufficient to process the assay, but small
15 enough to reduce depletion of the analyte from the sample.

In a preferred form an ablating laser is arranged transversely to the axis of elongation of the micro-length tube elements with the effect that the energy arrives though parallel to the end faces has a neutralizing or removal effect on the capture agent that is on those end faces, as a result of incidence of substantially parallel radiation, but
20 also of internal reflection scattering of the radiation by the transparent substance that defines those end faces.

The net effect of two novel processes described, if used in novel combination, is to leave only a band of selected dimension, which can be small, of capture agent immobilized on the inside surface of the micro-length tube element. This can be done in
25 a way that leaves one or more bands separated by a space of no capture agent. Thus one can generate a single band in the center or a single band closer to one end or multiple bands distributed along the length of the micro-length tube element. These bands can be of different widths, can have different spacing, and can be of the form of a code, e.g. a bar code, which is useful to encode the particular flow element, e.g. micro-length tube
30 element.

Further is a description of manufacturing techniques that have important novel features.

The micro-length tube elements are first cut, i.e. chopped, from previously supplied continuous small-bore filament into the short, discrete micro-length tube elements. They are then treated in batch manner.

A bulk of the micro-length tube elements is then exposed in an Eppendorff tube to wash buffer. After washing processing is performed, the buffer is removed, and replaced with a silane. By use of this simple, low-cost immersion step, the silane is allowed to bind to all of the surfaces of the micro-length tube elements. Excess silane after a period of time is washed away with water in a buffer. Then a capture agent, e.g. antibody, in solution is added to the Eppendorff tube with the bulk of micro-length tube elements and allowed to incubate overnight. The incubation is performed on the orbital vortexer for approximately 16 hours at 2000 rotations per minute, with of the order of one-centimeter diameter orbital motion. The orbital plate that contains the numerous Eppendorff tubes is approximately 6 inches in diameter, but the orbital motion is a circular pattern counterclockwise and then clockwise motion in a circular pattern of diameter of approximately 2 centimeters.

After the vortexing process is completed, the net result is that the capture agent has been immobilized on the inside surface of the micro-length tube element and also on the end faces but it is not present on the outside cylindrical surface of the tubular element. The capture agent solution is removed from the Eppendorff tube, which is replaced with a wash buffer, a wash buffer solution, and the wash buffer solution is then further replaced with a stabilizing buffer, what we call a blocking buffer. In the preferred embodiment, a commercial material called StabilCoat® solution is used.

StabilCoat® blocking solution is introduced to the Eppendorff tube along with micro-length tube elements, then a portion of those elements is aspirated in a pipette along with some of the StabilCoat®, and dispensed onto an alignment plate. The alignment plate contains a series of rectangular shaped pockets, each designed to accommodate and position a single micro- tube element within a small space, preferably with clearance tolerance sized in microns, a space of 10 to 50 microns between the micro-length tube element and the walls of the pocket. After the elements are allowed to roam

on the plate, they fall into these pockets still in the presence of the solution of the buffer solution. The excess buffer solution is removed from the alignment plate containing the micro-length tube elements by placing their plates with elements into a centrifuge or centrifuge holder and centrifuging at approximately two thousand rpm, for 30 seconds, thereby removing all excess StabilCoat® solution from the plate and the micro-length tube elements. This process is facilitated by the novel design of the plate shown, in which drain channels extend radially from the pockets.

The process of creating immobilized antibodies or other active biological species to serve as assay capture agent on micro-particles, and particularly the inside of hollow glass micro-particle elements, and then transferring the functionalized micro-particles to the channel of a microfluidic device, according to the invention, involves a number of critical steps. Long spools of continuous capillary tubing of approximately 125 micron OD, 70 micron ID, with a few microns thick polyamide protective coating on the exterior are obtained from a manufacturer. Typically these are drawn, fiber-like filaments, with highly polished and accurately dimensioned inner and outer surfaces. The spools are rewound onto a mandril in a tight mono-layer wrapping fashion such that each turn or strand, wound around the mandril is in contact with adjacent strands. The wrapped series of strands consists of for instance a hundred strands. It is then wrapped with an adhesive tape by which the strands are captured in an assembled unit. The tape is then slit parallel to the mandrill axis and the tape removed bringing the strands with them. This produces a thin linear array of monofilaments in close contact with one another. The relatively long array of monofilaments is then presented to a wafer dicing saw(as used in the semiconductor industry for dicing thin ceramic wafers), the filaments are diced at repeat distances of approximately 250 microns, producing cylindrical tubular micro-particles of that length.

After the dicing process, the individual micro-particle elements still retained on the tape are liberated using a hot aqueous detergent solution which liberates the individual elements from the tape. They are allowed to settle into the bottom of a beaker, the tape is removed from the solution, then the aqueous solution is removed and replaced with a hot sulfuric acid and peroxide solution, used to dissolve the polyamide coating from the outside surface of the glass elements. This is followed by a substantial washing

cycle, a number of flushings with de-ionized water to remove the residual sulfuric acid solution. After the hollow glass elements have been thoroughly washed, they are silanized using a silane reagent such as APTES which stands for aminopropyltriethoxysilane (“silane”). The micro-particles are allowed to incubate in the silane solution for approximately an hour after which they are rinsed and cured in an oven for another hour after which they’re then stored in an ethanol solution. To ensure the silane reaches the inside surface of the tubular micro-particles, vigorous vortexing is used to uniformly distribute the silane throughout the interior of the hollow elements. The micro-particles are then transferred to a reagent solution containing the capture molecule of interest, for example a capture antibody.

For this purpose the silanized micro-particles are transferred to a vial containing the capture agent for example a capture antibody. The capture antibody is allowed to bind to the active silane surface for a period of 16 to 24 hours after which a rinsing cycle is performed to remove any loosely bound capture agent and then finally the functionalized micro-particle elements are transferred to another vial containing a stabilizing compound such as SurModics’ brand StabilCoat®. They remain in the StabilCoat® solution until it is desired for them to be transferred to a microfluidic device, such as a microfluidic cartridge. They are stored in the StabilCoat solution in a refrigerator until ready to be used.

Thus, after the immobilization process is complete wherein the surface of the micro-particle, or inside surface of the hollow glass micro-particle elements have the active species immobilized to the surface, the particles are re-suspended in a stabilizing compound such as StabilCoat (trademark of SurModics, Inc.) for storage until needed. The purpose of the stabilizing compound is to protect the activity of the immobilized species when the micro-particles are taken out of the reagent and exposed to atmosphere. We have discovered that the antibodies immobilized to a surface without a protective coating, during storage, have a tendency to degrade in their functionality (become partially denatured) with the result of a higher coefficient of variation of assay execution, severely affecting the precision (repeatability) and sensitivity of the assay, and thus preventing an accurate quantification assay..

The stabilizing reagent consists of high concentrations of sugars and proprietary compounds. We have found that when the water component is allowed to evaporate, thick residue of this sugary compound is left behind, which under low humidity conditions has a tendency to crystallize and become a fairly rigid structure which can cause particles in this compound to become almost irreversibly stuck to any surface that it comes in contact with and has been allowed to dry in.

According to a preferred step in the process of moving the micro-particles from the liquid state to the dry state involves dispensing the micro-particles in a solution of the protective coating liquid, e.g., StabilCoat, onto an alignment plate such as a precisely micro-machined grooved pocketed plate such as a silicon micro-machined plate with pockets configured to accept the micro-particles in an array pattern. The shaped pockets are, e.g., rectangular in the case of short segments of capillary tubing forming hollow micro-particle elements. The excess liquid compound is either spun off in a centrifuge or wicked away using an absorbent pad, leaving behind a small residue of protective compound about the micro-particle, e.g., both inside the hollow glass micro-particle element and around the outside of the element in the pocket capturing the element.

We have discovered that allowing the stabilizing compound to dry in an environment of relative humidity less than approximately 60% relative humidity has a deleterious effect of retaining the micro-particles through a sugar crystalline structure that bonds the elements into the pocket. It has been found maintaining the humidity of 55 to 60% relative humidity or higher softens (hydrates) the stabilizing compound to the point at which the viscosity approaches nears that of water enabling a pick and place process to proceed for placing the micro-particles in channels of a microfluidic device. Under these conditions, one may use automated tweezers or a vacuum picking head to grab the micro-particles out of the capturing pockets and place them into their final destination, a microfluidic channel of a microfluidic device.

The steps of assembly of the micro-particles is summarized as follows.

When ready to assemble the pick and place technique previously described in U.S. Application Nos. 61/608,570 and 13/427,857 is employed in which the micro-particles are placed in a groove locator plate.

There is, however, an alternative way that this could be done. This involves distributing the micro-particles in random fashion onto a flat surface not having grooves or alignment pockets. The advantage of this process is not requiring a micro machined component for the manufacturing process. The disadvantage is that the micro-
5 particles are randomly distributed in a pile. The tendency is for them to come to rest in a monolayer on the surface, but with random orientation. Further, as a result of removing the excess StabilCoat by centrifuge or wicking away the excess stabilizing solution, it has been observed that the micro-particles have a tendency to agglomerate into dense pack of the randomly oriented elements.

10 We realize a solution exists to this problem. Individual micro-particles can be picked from this dense pack by use of a placement tool, e.g., a vacuum tip, that engages the top surface of the elements, in combination with a vision system used to identify an individual element and a motion system responsive to the vision system, which orients the relative relation of the vacuum pickup tip and the micro-particles in both X and Y
15 coordinates, and angular orientation. The placement tool can be moved in minute movement, slightly laterally within channel, to bring GNR against one side wall. Preferably a table carrying the fluidic layer, preferably channel side up, moves in computer-controlled X, Y and Z, and the placement tool is stationary, with only the grippers (e.g. tweezers) moving under computer control.

20 CyVek temporarily secures the micro-particles in open channels prior to this fluidic component with open-sided channels being turned upside down for bonding to the flexible membrane. The membrane is carried by the pneumatic component of the cartridge, to complete the assembly.

Currently this is done in over-width, under-depth open micro channels.
25 Electrostatic attraction draws the GNRs from the placement tool (enabling tool withdrawal) and holds them against one side of the over-sized channel with sufficient certainty that the assembly can be overturned for bonding against the membrane. Compressive force of the elastically compressible membrane permanently then fixes the GNRs permanently in position.)

30 An alternative technique is contemplated that would avoid need for the placement tool to move laterally against one side of the over-size channel. In this case the open

channel in a resilient PDMS (silicone rubber) channel-defining layer is slightly undersize in width and may be oversize in depth relative to the GNR.

In this case the placement tool thrusts the GNR down with force-fit into the channel, and the sides of the channel are slightly, resiliently deformed to accept the GNRs. The sides then grip the micro-particles tightly. The GNRs may even be thrust so deep into the channels that they are submerged below the face plane of the fluidic layer.

Final assembly then proceeds: the fluidic layer is turned upside down and bonded to this up-facing membrane.

In a typical system the GNRs are short segments of fine capillary tubing, e.g., outside diameter, e.g., 125 micron, inside diameter 70 micron and length 250 micron).

The accepting microfluidic channels in one instance are (uniquely) wider than the micro-particles, and shallower. Successful placing depends upon electrostatic force associated with silicone rubber (PDMS, an electric insulator material) to attract the micro-particles from the placing instrument, and retain them in position during the completion of the assembly process, which even involves turning the over-size channels upside down. In this case elastic deformation of the covering membrane at the sites of the raised top surface of the micro-particles permanently fixes the location of the micro-particles.

In alternative technique, the microfluidic channels are undersize, widthwise, relative to the micro-particle width, and the placing tool forces the micro-particles into the channels, to obtain a mechanical grip by elastomeric material forming the sides of the channel. Again, the microfluidic channels can have depth less than the micro-particles, so that the membrane stretches over them to further fix their location. In another instance, the channel depth may exceed the depth of the micro-particles, and the placement submerges the particles such that the overlying membrane is not locally disturbed by the presence of the micro-particles.

The novel process of creating immobilized antibodies or other active assay capture agents on micro-particles, and particularly on the inside of hollow micro-particle elements (micro-length tube elements) will now be described by reference to a specific example. This will be followed by examples of novel transfer of functionalized micro-

particles to an operative position within a microfluidic device, for instance to a microfluidic channel.

In the preferred case of micro-length glass tube elements, long spools of continuous glass capillary tubing of approximately 125 micron OD, 70 micron ID, with a few microns thick polyamide protective coating on the exterior are obtained from a manufacturer. Typically these are drawn, fiber-like filaments, with highly polished and accurately dimensioned inner and outer surfaces. The spools are rewound onto a mandrel in a tight mono-layer wrapping fashion such that turns or strands wound around the mandrel are in contact with adjacent strands. The wrapped series of strands comprises, for instance, one hundred strands. The strands are then wrapped on their outside with an adhesive tape by which the strands are captured in an assembled unit. The tape is then slit at one point parallel to the mandrel axis and the tape with the strands is removed. This produces a thin linear array of monofilaments in close contact with one another. The relatively long array of monofilaments is then presented to a wafer dicing saw (as used in the semiconductor industry for dicing thin ceramic wafers). The filaments are diced at repeat distances of the order of 1000 micron or less, to produce micro-length tube elements. Preferably, the repeat distances are less than 700 micron, and in preferred instances, of the order of 250 microns, producing cylindrical micro-length glass tube particles of that length. Those tubes having internal volume of the order of a nanoliter and are referred to as “glass nano reactors”, or “GNR”s).

After the dicing process, the individual micro-length particles or elements, still retained on the tape, are liberated from the tape using a hot aqueous detergent solution. They are allowed to settle into the bottom of a beaker and the tape is removed from the solution. Then the aqueous solution is removed and replaced with a hot sulfuric acid and peroxide solution, to dissolve the polyamide coating from the outside surface of the glass elements. This is followed by a substantial washing cycle, employing a number of flushings with de-ionized water to remove the residual sulfuric acid solution. After the hollow glass elements have been thoroughly washed, they are silanized using a silane reagent such as APTES (aminopropyltriethoxysilane, “silane”). The micro-particles are allowed to incubate in the silane solution for approximately an hour. To ensure the silane reaches the inside surface of the tubular micro-particles, vigorous vortexing is used to

uniformly distribute the silane throughout their interior to form a silane coating. The micro-length tubes are then rinsed and cured in an oven for another hour after which they are stored in an ethanol solution, ready for being functionalized, (i.e., treated to surface-immobilize a capture molecule of interest, for example a capture antibody).

5 For functionalizing, the silanized micro-particles are transferred to a vial containing the capture agent, for example a capture antibody. Again, to ensure the capture agent reaches the inside surface of the tubular micro-particles, vigorous vortexing is used, which is found to be capable of substantially uniformly distributing the capture agent throughout the tubular interior of the micro-length particles to produce substantially
10 uniform immobilization of the capture agent over the length of the interior surface. The capture antibody under these conditions is allowed to bind to the active silane surface for a period of 16 to 24 hours. Advantageously, as herein further described, it is found, that the vortexing conditions of the process, prevent immobilization of the capture agent to occur to longitudinally-extending outer surfaces of the violently agitated micro-length
15 particles.

After the immobilization process is complete, with inside surfaces of the hollow elements carrying a substantially uniform coating of immobilized active species, the functionalized micro-particle elements are re-suspended by transfer to another vial. The vial contains a stabilizing compound such as SurModics brand StabilCoattm. We have
20 discovered that antibodies or other biological capture agents immobilized to a surface without a protective coating, during storage have a tendency to degrade in their functionality (become partially denatured) with the result of a higher coefficient of variation of assay execution. This can severely affect the precision (repeatability) and sensitivity of many assays, and thus prevent quantification to the desired degree. Thus the
25 purpose of the stabilizing compound is to protect the activity of the immobilized species when the micro-particles are taken out of the reagent and exposed to ambient conditions.

The functionalized micro-length tube elements remain in the stabilizing solution stored in a refrigerator until ready to be used, i.e. until it is desired for them to be
30 transferred to a dry state within a microfluidic device, such as a microfluidic cartridge.

A preferred step in the process of moving the micro-particles from the liquid state to the dry state involves dispensing the micro-length particles in a solution of the protective coating liquid, e.g., StabilCoat, onto a so called "pick-up plate", for presenting the micro-particles for subsequent picking and placing in microfluidic channels. A preferred example of a pick-up plate is a precisely micro-machined grooved locator plate, preferably a pocketed locator plate such as a silicon micro-machined plate, with pockets configured to capture individual micro-particles in an array pattern. The shaped pockets are, for example, rectangular in the case of short segments of capillary tubing forming hollow micro-particle elements (micro-length tubes). The excess liquid compound is either spun off in a centrifuge or wicked away using an absorbent pad, as described herein. Either technique leaves behind a small residue of protective compound about the micro-particle, e.g., both inside the hollow glass micro-particle element and around the outside of the element, i.e. in the pocket capturing the element. In cases later described in using a planar pick up plate, a small residue remains with the elements in a puddle on the plane surface on which the elements are displayed. In mass manufacture, it is typically desired, after applying the micro-particles to a pick up plate, to store the plate and particles prior to installation into the microfluidic device.

We have discovered that allowing the stabilizing compound to dry in an environment of relative humidity less than approximately 55% has a deleterious effect of retaining the micro-particles through a sugar crystalline structure that bonds the elements to their supporting surface. As previously noted, the stabilizing reagent consists of high concentrations of sugars and proprietary compounds. We have found that when the water component is allowed to evaporate, thick residue of this sugary compound is left behind, which under low humidity conditions has a tendency to crystallize and become a fairly rigid structure which can cause micro-particles in this compound to become almost irreversibly adhered to any surface with which it comes in contact and allowed to dry.

It has been found maintaining relative humidity of a minimum of about 55%, preferably about 60% or higher, softens (hydrates) the stabilizing compound to the point at which the viscosity approaches that of water, and enables a pick and place process to proceed for placing the micro-particles in a microfluidic device. Under these conditions, one may use automated tweezers or a vacuum picking head to grasp the micro-particles,

e.g. withdrawing them from the capturing pockets or the supporting surface of the pick-up plate, and placing them in their final destination, e.g. a microfluidic channel of a microfluidic device.

When ready to assemble, the pick and place technique elsewhere described herein is employed in which the micro-particles are first disposed in a groove or pocket of a locator plate, from which they are picked by tweezer or vacuum tool.

An alternative, novel technique involves distributing the micro-particles in random fashion onto a flat surface not having grooves or alignment pockets. The advantage of this process is not requiring a micro machined plate component for the manufacturing process. The disadvantage is that the micro-particles are randomly distributed in a pile. The tendency is for them to come to rest in a monolayer on the surface, but with random orientation. Further, as a result of removing the excess StabilCoat by centrifuge or wicking away the excess stabilizing solution, it has been observed that the micro-particles have a tendency to agglomerate into a dense monolayer concentration of randomly oriented elements. However we realize that there are solutions to this problem. Individual micro-particles can be picked from this dense concentration by use of an automated vacuum tip that engages the top surface of the elements, such as previously described herein, in combination with a computer-control vision system used to identify an individual element and its orientation from a detected image, and a motion system responsive to the vision system, which orients the relative relation of the vacuum pickup tip and the plate supporting the micro-particles in both X and Y coordinates and angular orientation. Similarly, automated tweezers may be operated with controlled motions in X and Y coordinates, and angular orientation, as described herein.

The GNRs immediately after immobilization are stabilized in a reagent that is capable of protecting or stabilizing the catcher antibody on the surface of the GNRs, e.g., StabilCoat®. GNRs are transferred to an alignment plate and dispensed on to the alignment plate in a puddle with the stabilizing reagent and the liquid form of the stabilizing reagent is used to aid in the assembly or the self-assembly of the GNRs into certain alignment pockets. Because the stabilizing compound has a very high sugar and salt content it is desirable to remove as much of the mass of the liquid from the surface or around the surface of the GNRs prior to allowing any residual reagent to evaporate. As

evaporation causes a concentration of sugar content and salt content nearby a crystallization process occurs which acts to hold or cement the GNRs into or onto whatever surface they are presented to. It is important therefore to remove as much of the residual reagent as possible and a technique involving centrifugation was previously described wherein the excess material is spun in a centrifuge and spun off of the plate and away from the GNRs as shown in Fig. 10 D. An alternative process that is considerably easier to implement a manufacturing process involves using an absorbent pad placed at the edge of the channels associated with the pockets holding the GNRs wherein a capillary working process is used to soak up residual StabilCoat® into the absorbent pad and draw out of the channels through the channels and out of the channels and away from the GNRs.

Typical dimensions for the channels are shown in Fig. 10 A-C'. The plane view of the channels retaining the GNRs. The GNRs are located in pockets approximately 125 microns wide by 400 microns in length separated from each other by a narrower channel approximately 75-80 microns in width by 200 microns in length. The Figure is broken away, showing microtubes 1, 2 and n, where n may be as large as about 50. The narrow channel is provided to aid in the flow of the excess reagent through the channels and away from the GNRs to the wicking pad and narrower to prevent GNRs from migrating out of the retaining pocket that they are intended to fall into. The length of the channels can range typically from 5 millimeters to 25 millimeters depending on the or even 75 millimeters depending on the size or the scale of the manufacturing process involved. The number of GNRs ranges from a few hundred to a few thousand depending on the cross-section area. Per channel, the number of GNRs is on the order of 50.

Fig. 2 is a side view of the GNRs on a surface or in a channel with a puddle StabilCoat® surrounding the channels and contained on the plate around the plate. It depicts a wicking pad in the process of being moved toward the end of the channels full of the stabilizing solution. The GNRs are completely submerged under the material.

Fig. 3 illustrates a wicking pad shown on the left top surface of the plate and having drawn the excess StabilCoat® away from the GNRs through the channels.

The micro-length tube elements while still in the plate can be further processed with a laser, preferably an ultraviolet laser, which could be an excimer laser,

fluoride or krypton fluoride laser, with two beams that are spaced such that the ends and an end margin portion or section of the micro-length tube element are exposed perpendicular to the element axis by a laser beam in a way that either ablates or denatures the capture agent, e.g. antibody, from the ends of the element as well as a section of the inside surface of the element. It is a feature of the laser configuration that the two laser beams are separated by a fixed distance that defining the desirable width of the remaining band of capture antibody surface. The micro-length tube elements within their pockets of the alignment plate can be allowed to move back and forth with a degree of liberty, while still the laser processes substantially the ends of the elements and leaves a fixed width pattern near the center of the element, plus or minus a reasonable some tolerance window.

It is possible instead to define a series of three or more laser beams, with gaps, such that the pattern produced by the various widths of laser beams in the various gaps between the laser beams defines a pattern of exposure in the tubular element that looks like and is useful as a bar code.

Further, it is realized as useful to have significant by-pass flow in a channel outside of the micro-flow element as well as through the element. One advantage is simplicity of manufacture as the element can be held but without being sealed and with no attempt to use cumbersome adhesive to adhere the element to the channel walls. Another advantage is the avoidance of the risk of totally spoiling an assay because a chance particle obstructs internal flow of one of the micro-flow elements when arranged in series in a liquid flow path. Having significant by-pass flow on the outside, e.g. a flow greater than the flow through an element, thus “short circuiting” the element, ensures that despite one element being plugged or obstructed and flow stopped, the other elements will receive flow and the assay will only be partially affected by the obstructing particle. It is realized further that with concepts presented here, enabling the avoidance of having active capture agent on either the cylindrical exterior of the micro-length tube element or on its end faces, does not result in a depletion problem. The techniques previously described, of avoiding active capture agent from adhering to the exterior cylindrical surface of the micro-length tube elements and laser treating the ends, thus contribute to the practicality of employing the by-pass flow described.

Referring to Fig. 6, in general the advantage of so treating the surfaces of the micro-length tube element, in reducing depletion, and in particular, with regard to the considerations of by-pass flow, may be understood by considering the total surface area exposed to the coating solution as represented by the sum $A+2B+C$ where A is the internal cylindrical surface, B is the end face, and C is the external cylindrical surface area. It is pointed out that according to the concepts articulated that it is possible to reduce the depletion area to A only, that being the only area that carries meaningful information. Indeed the reduction can be extended by suitably sizing the laser beams to treat the margins of the inner cylindrical surface from which the active agent has been removed or has been deactivated can be of preselected length. For instance, in a dilute solution, it may be desirable to have more area than in a less dilute solution of the analyte. Indeed, as previously mentioned, it is possible to have the laser act upon capture agent in one or more mid regions of the area of the inner cylindrical surface, even form a code. Such patterns are not visible when only in the state of an active capture agent, but made visible or photo detectable by the captured fluorescently labeled analyte.

As previously discussed, it is very important having no capture agent on the outside surface of the micro tube elements, avoiding the potential for damaging the functional surface and negatively influencing the results of the assay. As a result of transferring the micro-length tube elements from an alignment plate to the microfluidic device or other channel it is quite possible for the outside surface to encounter contact with other hard surfaces, leading to damage of a functionalized surface. So it is beneficial to prevent binding of capture antibody to the outside surface therefore, excluding signal that might arise from the capture agent coming from the outside surface. As an example of A, B, and C, where C is the exterior surface area of the micro-length tube element having a length dimension of approximately 250 microns, interior diameter of 75 microns and an outside diameter of 125 microns, C being the exterior surface area is approximately 98,000 square microns. B which is the two-times the end face surface area is approximately 15,600 square microns, and A being the inside surface area approximately 58,000 square microns. The total of A, 2B, and C is approximately 171,000 square microns.

By cooperation of the processes, it is easy to eliminate the surface area associated with the exterior surface area of the micro-length tube element and the end face of the micro-length tube element, thereby reducing the total surface area by 66%. It is possible to further reduce the surface area of the capture antibody even on the interior surface to an arbitrarily small surface area. An example would be a narrow stripe on the inside of a 75 micron diameter tube, resulting in a total surface area of approximately 10, 000 square microns.

Assays run in the microfluidic device can use various types of micro-length tube elements with different capture agent present on the inside surface of the elements. For example, one type of micro-length tube element would contain a capture antibody associated with the antibody interleukin-6. Another one could be interleukin-2, and yet a third would be interleukin-12. Each of the micro-length tube elements of respective types can be placed into different channels and or locations within that channel, thereby determining at the time of performing the assay what type or what particular antibody was used in that particular location. That is a method for identifying the type micro-length tube element for what has bound onto that surface. In addition to using location as a means for identifying a particular type of micro-length tube element, another means is using the striped code pattern created by selectively ablating or selectively denaturing antibody functionality along the length of the micro-length tube element for providing for a code, a bar code, which is then used to identify that particular type.

There are various possible ways of doing the ablation or creating the ablated pattern, within the micro-length tube element using a laser beam.

A wavelength in the range of about 193 nanometers to 250 nanometers is presently preferred. One possible way is to establish a single laser beam of a particular width and then translating micro-length tube element, then dispensing a fluence of laser to the element, turning the laser off, translating to a new position then dispensing another amount of fluence thereby eliminating or denaturing only portions of a particular tubular element. Another method, as mentioned previously, is to use an opaque mask to establish a particular pattern, and illuminate simultaneously the entire micro-length tube area with the ultraviolet light. Yet another method is to scan the micro-length tube element using a synchronized system of X, Y galvanometers.

Besides writing the code, one could scan simply the ends of the micro-length tube elements to ablate capture agent. It is anticipated that a feature size as small as 30-40 microns is possible with an ultraviolet laser. For example, a 250-micron long micro-length tube element having a feature size of 30 microns would result in 8 possible stripe zones and with 8 possible stripe zones, one could produce a number of patterns. For example, a pattern using a binary coding system would lead to a total number of combinations of 28, which are 256 possible combinations. The method:

Step (1): provide micro-length tube elements.

Step (2): apply coating, the manufacturing process, provide a coating particular of a capture antibody,

Step 3 a manufacturing process that coats only the inside surface of the micro-length tube element. Two novel ways of achieving this: One is providing a capture agent, e.g. antibody, in a solution that contain the micro-length tube elements and agitating the liquid in an aggressive manner, generating high shear force on the exterior of the elements, whereby binding occurs only on protected inside surface of the micro-length tube elements. The second means of coating only the inside of the micro-length tube element. The agitation process would prevent coating of the antibody on the outside surface only, but not the end faces. So further reducing surface coating on the end faces would involve using a laser process. The second means of preventing coating on the outside surface (but not chopped end faces) concerns the manufacturing process at the stage of drawing and coating endless micro-bore tubing, prior to chopping to form discrete micro-flow elements or micro-length tube elements. By adding a bond-preventing coating prior to the usual polymer coating prevents silanization of the exterior surface, preventing silane from adhering to the outside surface, and therefore defeating the ability for many capture agents, for instance antibodies and antigens, from adhering to the surface.. This would further prevent antibodies from being bound to the outside surface. In either case, the polymer coating is added to the glass filament during its manufacturing process to maintain the intrinsic strength of the glass filament. The process for providing or manufacturing the micro-length tube elements, the raw elements without a coating, involve chopping the micro-bore tubing into the particular length element, which for the preferred configuration is approximately 250 microns, then

removing the polymer coating using a sequence of acid and basic baths. Subsequent to the manufacturing process that coats only the inside surface of the micro-length tube element, the micro-length tube elements are then secured into a channel of a microfluidic device.

5 The channel of the microfluidic device is configured such that a portion of the flow is allowed to bypass the outside of the micro-length tube element. Micro-length tube elements are placed into a channel in a way that, preferably, approximately two times the flow volume proceeds around the outside of the tubular element as compared to the volume proceeding through the inside area. So the ratio of the cross-sectional areas
10 of the by-pass flow versus the inside diameter is approximately 2:1. Micro-length tube elements are then secured into the channel whereby the channel wall is an elastomer, which allows the micro-length tube element to be placed in the channel, and the grippers used to place the element into the channel can be released because the adhesion of the elastomer is sufficient to secure the element into the channel. Any residual adhesion
15 between the micro-length tube element and the tweezers is overcome by the larger adhesive force between the micro-length tube element and the channel. Subsequent to that step, a top is then secured over the open channel containing the micro-length tube element. The top also includes an elastomeric material. That elastomeric material is used to compress and thus secure the element in the channel because the open channel duct is
20 of smaller depth than the outside diameter of the tubular element. The elastomeric "roof" or top thus provides a means of securing the micro-length tube elements in their locations in that channel.

Referring to Figures 9 et seq., another useful technique, with considerable advantage, of completing the assembly between two pre-constructed subassemblies, in
25 contrast to using a non-permanent bonding process, is to form permanent bonds.

It is found that low pressure operation permitted by the general organization and design just described, can so-diminish the driving pressure on the air to permeate the membrane, that the bubble problem is lessened to the extent that an elastomeric membrane, such as PDMS, is employable with significantly reduced risk of air
30 penetration and bubbles than designs of the prior art. This has the advantages of low cost

and simplicity of manufacture, and enables achieving extremely consistent and sensitive assays.

A difference in the implementations now to be described is that the membrane or the flexible layer that is actuated by vacuum or pressure to operate the valves and the
5 pistons is made from elastomeric material and different, advantageous techniques are used to fabricate the device.

One of the problems addressed concerns the surface area associated with a hollow flow element as has been de0icted and as has been described above, i.e., an element having length less than 700 micron, preferably less than 500 micron, and in many cases
10 about 200 micron, and a bore diameter between about 75 +/- 50 micron, that is fixed in a flow channel and exposed to flow of liquid sample. (Such hollow flow elements and assay devices based on them are available from CyVek, Inc., Wallingford, CT, under the trademarks "Micro-length tube TM, u-Tube TM, and Mu-Tube TM). Such devices are efficiently made of endlessly drawn micro-bore filament such as used to form capillary
15 tubes, but in this case, the filament is finely chopped in length to form discrete, extremely short hollow flow elements, rather than capillary tubes. It is realized that capture agent immobilized on the surface of such a device, applied by immersion techniques, can raise a significant depletion problem. This occurs, for instance, when attempting to characterize concentrations of an analyte at low levels such as a few pico-grams per
20 milliliter, s is desired. The phenomenon referred to as "depletion" occurs in which the concentration of analyte in the sample being measured can be disadvantageously depleted volumetrically as a result of binding to a large active area of the flow element. This results in reduction of sensitivity of the assay, and therefore its usefulness. To explain further, any analyte in an ELISA or sandwich type of amino assay on antigen will bind to
25 a capture antibody in a way that is governed by a kinetic reaction, a dynamic process. While analyte such as an antigen binds to capture agent such as an antibody, the reverse also occurs, the bound analyte molecules unbind from the capture agent. The kinetics concern an "on" rate and an "off" rate analyte being captured and analyte being released. The capture reaction will continue, depleting the analyte in the ambient volume, and
30 reducing its net rate of capture, until the system reaches equilibrium in which the rate of

binding is equal to the rate of unbinding. The gradual action occurs according to a substantially exponential curve.

The absolute value of the equilibrium condition depends on the original concentration of the analyte in the volume of sample being assayed. Increase in
5 concentration results in a higher signal, decrease in concentration results in a lower signal. In cases in which assay depletion occurs, the concentration of the analyte in the sample is detrimentally decreased over time. It is realized that hollow flow elements fixed in flow channel may present an excess of capture agent in the volume of liquid sample to which the element is exposed, decreasing the effective concentration of the
10 analyte. The concentration decreases at an excessive rate, relative to initial, starting point concentration sought to be measured. While efforts to calibrate for this are helpful, such depletion ultimately lowers the sensitivity of the assay because, as the signal goes down, it approaches the noise level, and results in a lower signal-to-noise ratio, i.e. an inherent reduction of effectiveness of the assay. (Already there are significant contributors to
15 noise i.e., background, nonspecific binding of capture antibody, fluorescence noise, electronic noise, etc.). Therefore, especially for detecting small concentrations, it is desired not to deplete the initial volume of the analyte in manner that does not contribute positively to the assay measurement. Efficient ways to do that, as by somehow limiting the amount of exposed surface have not been apparent. This may be seen as an inherent
20 problem with use of small detection elements of various descriptions that are coated by immersion or the like and used in an immunoassay or sandwich assay or even a molecular diagnostic type of assay. One typically wishes to immerse the elements in capture agent, e.g. an antibody or some type of moiety that is a capture molecule for the analyte to be sensed or detected, to uniformly coat all surfaces of the element. One object
25 of invention is to overcome this problem with respect to hollow flow elements characterized by an inside surface and an outside surface, or often also with two end surfaces. Adding up all surface area over which a density of capture molecules is coated can add up to a surface area on the order of over 100,000 square microns. This is the case for a preferred form of hollow flow element formed of small-bore filament, the element
30 having on the order of about: a length of less than 700 micron, preferably about 500 micron or less, and in presently preferred implementations, 200 microns. Likewise, the

inner bore is found desirable to have within a range of 50 micron +/- 25 micron, for achieving uniform coating by immersion and agitation. In one preferred case, an element has an external diameter or width of 125 microns, and an internal diameter or width of 70 microns. A particular problem addressed here is to find practical approaches for accurately reducing active surface area of immersion-coated flow assay elements in general, and in particular, hollow flow elements, and in particular elements of the dimensions mentioned.

A further problem being addressed here concerns treated hollow flow elements that are to be in fixed positions in channels for exposure to flow of sample. It is desirable to expose the elements in batch, in free state to an immobilization process for applying the capture agent or antibody to the element surface, and then transfer each element mechanically to its fixed position in a channel, for instance in a channel of a multiplex micro-fluidic “chip” (or “cassette”). It is desired to use a quick and accurate placement process, for instance a pick and place device mounted on an accurate X, Y stage. For such purpose, it is desirable to physically contact the tiny element for picking it up from a surface and placing it in an open channel, which is then closed to form a micro-fluidic passage. It is desirable to employ grippers, e.g. a tweezer instrument, or a vacuum pickup that contacts the outer surface of the device. The pick and place action is made possible by pre-aligning open channels to receive the hollow flow elements and the surface on which the free elements are supplied with the automated pick-and-place instrument. This enables the grippers to pick up and place the hollow flow elements precisely from supply pockets to desired flow channel positions in which they are to be fixed. With a vacuum pick up, it is possible to serve the hollow element in end to end abutting relationship in supply grooves, and engage the outer cylindrical surface with the vacuum pick up. We recognize a problem arises with having an active capture agent, e.g. antibody, immobilized on outer surfaces of an element. Such a coating is susceptible to mechanical damage as a result of the manipulation process. Outside surfaces of micro-flow elements come in contact with (a) a supply surface, e.g. an aligning pocket or groove, (b) the transferring grippers or vacuum pickup device, and (c) surfaces of the channel in which it is being deposited. All of these contact opportunities give rise to possible damage to the fragile coated capture agent, which typically is a very thin layer of antibody or the like adsorbed to the

surface of the flow element. This coating is often only a few molecules thick, thickness of the order of nanometers or tens of nanometers, and is quite fragile. The net result of damaging a capture surface of the placed hollow flow element is seen during read out of the assay. If the surface has been scratched or perturbed in any way, that can give rise to an irregular concentration or presentation of captured analyte, the signal can be irregular, and contribute to irreproducibility or poor performance of the assay.

We thus realize it is desirable not to have immobilized active capture agent on the outside surface of a hollow detection element, and especially the fine bore elements formed of micro-bore filaments, where it is susceptible to damage, and where it contributes to increasing the total surface area of the capture agent or antibody that contributes to depletion.

The features described in the claims and hereafter address these and other important problems.

Discrete hollow flow elements are immersed in liquid containing capture agent, such as antibodies or antigens, and, after coating by the liquid, are picked, and placed into channels for flow-through assays. The hollow flow elements are in preferred form of discrete elements of length less than about 700 micron, and bore diameter of 70 +/- 50 micron, preferably 50 +/- 25 micron. The flow elements are surface-treated so active capture agent, e.g. capture antibody, is not on the outside, or is of limited outside area. For this effect, the hollow flow elements are disposed in a bath of active agent and violently agitated, resulting in coating of protected inside surface, but due to extreme shear forces, a clean area on the outside surface, for instance the entire outside cylindrical surface of a round cross-section discrete element. In lieu of or in addition to this shear procedure, a special filament-manufacturing process is conceived that results in preventing coating an exterior surface of flow elements with a predetermined capture agent. Capture agent on selected coated areas are ablated or deactivated with precisely positioned laser beam, such as can be produced by a mask for simultaneous treatment of a large number of elements, leaving residual active agent of defined area on the inside surface of hollow flow elements. Residual capture agent, itself, on the inside of the elements, usefully defines a readable code related to the desired assay. Flow channel shape is sized relative to flow elements fixed in the channel to allow (a) bypass channel

flow along the exposed outside of a hollow flow element to reach and flow through later elements in the channel in case of clogging of the first element, along with (b) sample and assay liquid flow through the hollow flow element to expose the surface to capture agent and other assay liquids. Lacking the need to attempt to seal the outside, the element can simply be gripped, as by an elastomeric sheet pressed against the element. Electrostatic attraction between flow element and channel wall is employed to fix the element in position, overcoming any disturbing force of the placing instrument as it is drawn away after delivery of the element. After assay, in the case of use of epi-fluorescent detection, fluorescence is excited and read by special scanning confined to the hollow flow element geometry. Locators are seeded in the recorded data, and used to locate the regions of interest in detected fluorescence data, e.g. from the elements. Code, written with the capture agent substance inside the hollow element is read through a transparent wall of the element. A number of the features are or will be found to be useful with other hollow elements, for example, longer elements.

In respect of scanning, the purpose of this invention to deliver a method for performing a fluorescence measurement of multiple immobilized elements contained in a microfluidic chip. This method provides for determining the paths to be followed during the scanning, as well as the proper focus, and camera exposure. The method is based on a known general chip layout. The method provided results in the ability to place the chip to be measured into the scanner and then start the scan without any additional manual settings required. The method does the rest, and produces the desired fluorescence measurements as the results.

Certain aspects of invention involve eliminating or preventing the occurrence of active capture agent on outside surfaces of the hollow flow elements, e.g. extended outside cylindrical surface, and/or end surfaces, while leaving active capture agent on the inside surface unperturbed, or of a desirable area or pattern. Features addressing this aspect include techniques to selectively limit the capture agent on the interior surface and steps that act in combination with outside and inside surfaces to achieve the desired result.

For the specific advantage of reducing the overall capture surface area, two aspects of invention will be described, and the effect of their combination. A first

technique is employed to eliminate or prevent capture agent, e.g. antibody, from immobilizing to the outside surface of hollow flow elements. That is done during a batch coating process, and involves suspending discrete hollow elements in an Eppendorff tube or other laboratory tube with the capture agent of interest and aggressively agitating fluid to impart disrupting shear forces to the exterior surface of the elements. Preferably this is achieved by vortexing the fluid at high speed, for instance employing an instrument that orbits the container at approximately 2000 rpm of the orbiter, about an orbital path with total lateral excursion of the supporting table of the order about 0.5 cm, measured across the center of rotation of the orbiter.

The hollow flow elements are placed with a volume, e.g. a milliliter of capture agent, e.g. antibody. The appropriate vortexing speed is dependent e.g. on the nature of the suspension, e.g. the viscosity of the liquid chosen, and can be easily determined experimentally. It is set by observing whether the capture agent is effectively non-existent on the outside, long surface of the hollow flow elements, e.g. the outside cylindrical surface in the case of the body being of circular cross-section.

The physical principle involved concerns shearing force on the outside surface of the element that acts to prevent binding of the capture agent to the surface through an adsorption process. One can observe whether the vigorous agitation is sufficient to shear off any capture agent, e.g. antibody that has already been bound to that surface. At the same time, the inside surface is environmentally shielded from this shearing by virtue of the geometry which is tubular, and the micro-bore of the tube. This prevents vortexing from causing any turbulence to occur within the element. Only laminate flow conditions exist. With micro bore elements the Reynolds number is always low enough to ensure that that laminar flow condition exists on the inside surface. Under these conditions, the velocity of fluid traversing in the hollow element at the interior wall interface is by definition zero. So there is no shear force involved there, whereas the outside is in a highly turbulent, high shear force environment. The shortness of the length of the elements enables uniform coating of the inner surface, whereas longer elements, coated by immersion, are susceptible to detrimental non-uniform coating.

The observed result of aggressive agitation, e.g. vortexing, is that fluorescence which is observed by performing a sandwich assay is completely absent from the outer

cylindrical surface, or other shape of a hollow element, whereas it is present in an observable way on the inside surface. In the case of square-end hollow flow elements, fluorescence is also present on the end faces of elements.

Vortexing is the presently preferred technique for producing the shear forces. The case showed here employs orbitally rotating the coated element in a very rapid manner back and forth in small circles at a rate of approximately a couple thousand rotations per minute, and an excursion of about 25 mm.

However, any type of rapid oscillation that creates a high degree of turbulence can be employed, so a back and forth motion, a circular rotation, anything that would very rapidly mix the fluids and create high shear forces will suffice.

In summary, hollow flow elements in the presence of aggressive agitation leads to removal of capture agent, e.g. antibodies, from outside surface of the elements, and prevention of their coating with the agent, but leaves the inside surface of the element in condition to immobilize capture agent, e.g. capture antibodies, for subsequent interaction with analyte of the sample.

As an alternative to the high shear technique, we conceive an alternate process in which, during the original drawing of the small bore tube, and prior to the point along the draw path that the usual removable protective polymer coating is applied to the filament, that a non-stick coating, e.g. sputtered gold, silver or graphite, is applied to the filament, e.g. by passing through a sputtering chamber. Silane or similar coating must be applied to receiving surfaces before capture agent, e.g. antibodies will attach. However, due to the properties of the sputter coating, or the like, the surface will not receive the silane or equivalent, then likewise, the active capture agent.

Another feature of invention concerns realizing the desirability and technique of removing coated capture agent from selected end surfaces of the flow elements and a margin portion or other portion of the interior surface. Preferably, following the aggressive agitation process, the elements are further processed using a laser elimination process that removes or de-activates capture agent, e.g. antibodies, from surface from which the agent was not removed by the high shear process. Those surfaces include transverse end surfaces and a selected portion of the inside surface, leaving only an

annular stripe on the inside surface sized sufficient to process the assay, but small enough to reduce depletion of the analyte from the sample.

In a preferred form an ablating laser is arranged transversely to the axis of elongation of the hollow elements with the effect that the energy arrives though parallel to the end faces has a neutralizing or removal effect on the capture agent that is on those end faces, as a result of incidence of substantially parallel radiation, but also of internal reflection scattering of the radiation by the transparent substance that defines those end faces.

The net effect of two novel processes described, if used in novel combination, is to leave only a band of selected dimension, which can be small, of capture agent immobilized on the inside surface of the hollow element. This can be done in a way that leaves one or more bands separated by a space of no capture agent. Thus one can generate a single band in the center or a single band closer to one end or multiple bands distributed along the length of the element. These bands can be of different widths, can have different spacing, and can be of the form of a code, e.g. a bar code, which is useful to encode the particular flow element.

Further is a description of manufacturing techniques that have important novel features.

The short, hollow elements are first formed i.e. chopped, from previously supplied continuous small-bore filament into the short, discrete elements. They are then treated in batch manner.

A bulk of the hollow elements is then exposed in an Eppendorff tube to wash buffer. After washing processing is performed, the buffer is removed, and replaced with a silane. By use of this simple, low-cost immersion step, the silane is allowed to bind to all of the surfaces of the elements. Excess silane after a period of time is washed away with water in a buffer. Then a capture agent, e.g. antibody, in solution is added to the Eppendorff tube with the bulk of hollow elements and allowed to incubate overnight. The incubation is performed on the orbital vortexer for approximately 16 hours at 2000 rotations per minute. The order of 0.5-centimeter diametric displacement by the orbital motion. The orbital plate that contains the numerous Eppendorff tubes is approximately 6 inches in diameter, but the orbital motions is a circular pattern counterclockwise and

then clockwise motion the orbiting causing the displacement of approximately 0.5 centimeters from side to side, for instance.

After the vortexing process is completed, the net result is that the capture agent has been immobilized on the inside surface of the hollow element and also on the end faces but it is not present on the outside cylindrical surface of the hollow element. The capture agent solution is removed from the Eppendorff tube, which is replaced with a wash buffer, a wash buffer solution, and the wash buffer solution is then further replaced with a stabilizing buffer, what we call a blocking buffer. In the preferred embodiment, a commercial material called StabilCoat® solution is used.

StabilCoat® blocking solution is introduced to the Eppendorff tube along with hollow flow elements, and then a portion of those elements is aspirated in a pipette along with some of the StabilCoat®, and dispensed onto an alignment plate. For tweezer pick up the alignment plate contains a series of rectangular shaped pockets, each designed to accommodate and position a single element within a small space, preferably with clearance tolerance sized in microns, a space of 10 to 50 microns between the element and the walls of the pocket. After the elements are allowed to roam on the plate, they fall into these pockets still in the presence of the buffer solution. The excess buffer solution is removed from the alignment plate containing the elements by placing their plates with elements into a centrifuge or centrifuge holder and centrifuging at approximately two thousand rpm, for 30 seconds, thereby removing all excess StabilCoat® solution from the plate and the elements. This process is facilitated by the novel design of the plate, in which drain channels extend radially from the pockets.

(In the case of vacuum pickup continuous grooves are employed to receive the treated elements, in greater density than enabled by the pockets, as the elements can be close together, end to end, since pickup will be by engaging the cylindrical surface, not the end surfaces as is the case with tweezers.)

The hollow elements while still in the plate are further processed with a laser, preferably an ultraviolet laser, which could be an excimer laser, fluoride or krypton fluoride laser, with two beams that are spaced such that the ends and an end margin portion or section of the element are exposed perpendicular to the element axis by a laser beam in a way that either ablates or denatures the capture agent, e.g. antibody, from the

ends of the element as well as a section of the inside surface of the element. It is a feature of the laser configuration that the two laser beams are separated by a fixed distance that defines the desirable width of the remaining band of capture antibody surface. The hollow elements within their pockets of the alignment plate can be allowed
5 to move back and forth with a degree of liberty, while still the laser processes substantially the ends of the elements and leaves a fixed width pattern near the center of the element, plus or minus a reasonable tolerance window.

It is possible instead to define a series of three or more laser beams, with gaps, such that the pattern produced by the various widths of laser beams in the various gaps
10 between the laser beams defines a pattern of exposure in the hollow element that looks like and is useful as a bar code.

Further, it is realized as useful to have significant by-pass flow in a channel outside of the hollow element as well as through the element. One advantage is simplicity of manufacture as the element can be held but without being sealed and with
15 no attempt to use cumbersome adhesive to adhere the element to the channel walls. Another advantage is the avoidance of the risk of totally spoiling an assay because a chance particle obstructs internal flow of one of the hollow -flow elements when arranged in series in a liquid flow path. Having significant by-pass flow on the outside, at least as great as 50%, in many cases 75% or larger, and in certain preferred instances
20 100% or more is highly useful. As least to some extent, this enables "short circuiting" the element, ensuring that despite one element being plugged or obstructed and flow stopped, the other elements will receive flow and the assay will only be partially affected by the obstructing particle. It is realized further that with concepts presented here, enabling the avoidance of having active capture agent on the exterior, i.e. for cylindrical elements, on
25 either the cylindrical exterior of the hollow element or on its end faces, does not result in a depletion problem. The techniques previously described, of avoiding active capture agent from adhering to the exterior cylindrical surface of the hollow elements and laser treating the ends, thus contribute to the practicality of employing the by-pass flow described.

30 Sizing of Hollow Flow Elements

It has long been accepted knowledge that the smaller the surface area of the capture agent, e.g. antibody, the more sensitive the assay is from a theoretical point of view. The desire has always therefore been to keep the inside diameter of a hollow element as small as possible to minimize that surface area. But it has now been
5 determined empirically that, within limits, the performance of the assay is improved as that diameter is increased to an extent. It is believed this is a direct result of non-uniform coating by the batch process desired to be employed, as well as probably some effects that occur during the assay in that it is possible that there are perturbations in the amount of volume, total volume, that actually flows through the hollow elements in cases where
10 the tube element diameter is small compared to an element of i.e. of 75 microns. We have found that the internal diameter should be about 75 +/- 50, and in preferred cases, 50 + 25.

It is preferable that the exterior diameter have a diameter or width within the range of 1.2 and 4 times the internal diameter or width

15 For length of the hollow flow elements, best results are obtained with lengths of less than about 700 micron, and in many cases, less than 500 micron. In a presently preferred form, the length is 250 um.

In some embodiments, it is preferable that the interior diameter have a diameter or width have a length to inner diameter of 20:1.

20 It has been discovered that the shorter hollow elements lead to greater uniformity of the coating of capture agent when coated by the batch process described herein, and as well, shorter hollow elements are found to be more amenable to withstanding axial tweezing forces during pick and place motions.

As previously mentioned there are significant advantages in providing two
25 subassemblies that are each fabricated on their respective solid substrates or carriers, which are dimensionally stable, though permissibly flexible. The extremely small hollow flow elements (or other detection elements to be fixed in position within the cassette) are placed into open locations on the mating face of one of the subassemblies, prior to aligning. Once the subassemblies are aligned, the two subassemblies are brought
30 together under bonding conditions to form one completed assembly, and fixing the embedded location of the elements. Then the two subassemblies are brought together to

complete the fluidic channels. Ringing them together completes the valve and piston devices as well as embedding the detection elements. These features occur with the non-permanently bonded implementation.

Another implementation of the broad assembling concept will now be described,
 5 employing permanent bonding features. We will refer now to the Figures beginning with Figure 30. The following is a list of components called out in Figures 30 et seq.

- 20. Completed Cartridge
- 22. Sample Inlet wells
- 24. Buffer Inlet Wells
- 10 26. Waste Well Reservoir
- 28. Reservoir Well – Detect Antibody Reagent
 - Preferred Embodiment – Dried
- 30. Microfluidic Channels
- 32. Extremely Small Hollow Flow Elements (“Elements”)
- 15 34. Microfluidic Valve Seats
- 35. Microfluidic Valve Pneumatic Chamber
- 36. Piston Fluidic Chamber
- 37. Piston Pneumatic Chamber
- 38. Elastomer Membrane
- 20 39. Plasma Bonded Interface
- 40. Arrows Depicting Flow
- 41. Bypass Flow Path
- 42. Glass Substrate
- 43. Bulk Material
- 25 44. Microfluidic Channel Walls
- 46. Control Reservoir Layer
- 48. Fluidic Layer Sub Assembly – No Elements
- 50. Fluidic Layer Sub Assembly – With Elements
- 52. Single Sample Four Analyte Microfluidic Network
- 30 54. Microfluidic Valve – Full Assembly
- 55. Piston – Full Assembly

- 56. Reservoir/Control Plastic Member
- 58. Pneumatic Interface Ports
- 60. Piston Control Lines
- 62. Valve Control Lines
- 5 64. End of arm tooling (tweezer or vacuum probe)
- 66. Pick and Place Arm (moves up and down)
- 68. Source / Target X, Y table (moves in X and Y coordinates)
- 70. Source of Hollow Flow Elements (groove or well plate)
- 72. Target Microfluidic device
- 10 74. End of arm tooling - vacuum
- 76. End of arm tooling - tweezer
- 78. Activated Surface

In Figure 30, starting from the upper side, the subassembly 46, i.e. the controls/reservoir layer 46, is comprised of two elements, the upper injection molded or machined plastic component 56 with a PDMS membrane sheet 38 bonded to its lower surface.

The bottom fluidic layer or subassembly 50 has detection elements, e.g. hollow short cylindrical flow elements 32. The fluidic subassembly consists of a thin glass sheet 42 with a PDMS gasket or sheet 38 permanently bonded face-wise to its upper surface, the sheet 38 having cut-outs defining fluidic channels between channel walls 44, the channel bottomed on the glass sheet 42, Fig. 31C. Before those two subassemblies are brought together, the detection elements are dispensed, in the embodiment shown, by pick and place action, into fixed positions in the channels of the fluidic layer 48. The two subassemblies 46 and 50 are brought together and bonded in a way that provides fluid-tight and leak-free operation, but also enables the actuation of valves and pistons by portions of membrane 38. One novel feature of this construction is that the two subassemblies as described, using a PDMS gasket, enables capture or embedding detection elements, here extremely short hollow flow elements, (Micro-length tube TM elements) into channels. Combining those two subassemblies into a single assembly provides the functionality of having microfluidic channels that contain the hollow flow elements as well as functioning valves and pistons. In a fluidically robust and leak-free

microfluidic structure, using the plasma-bonding process, known per se, to perform the numerous functions described, securing the detection elements in place and forming the valves and pump diaphragms in a way that completely seals the channels, together with a process to be described that defeats plasma bonding at the exposed valve seat contacted by the PDMS membrane.

The fluidic subassembly is assembled by covalently bonding PDMS to glass, and then upper assembly, the reservoir assembly is formed by covalently bonding PDMS to plastic. The dominant advantage is the placing the discrete, small detection elements, the hollow flow elements, into open channels prior to assembling.

The importance of the technique also relates to enabling the immobilization of capture agent, e.g. antibody, onto a solid substrate in an efficient batch process, thereby allowing many thousands of these elements to be fabricated in one very simple batch process, which is cost effective and highly reproducible. The process itself is absent of process parameters that would cause damage to biological content, and can be a room temperature process.

Thus features of the concept include bringing together subassemblies to capture elements in a fixed position, the capture (or detection) elements having been pre-prepared in batch process, with the final assembly, which employing a bonding process, especially the permanent plasma bonding process to join the subassemblies, and doing it in a selective way at the valve seats by repeatedly locally deflecting and bringing in contact the valving surfaces, which will now be described.

Valve Break-In Process

- Connect pneumatic control input ports to externally controlled pneumatic line/s
- Actuate all valves using vacuum (5-14 psi) to draw membrane up into pneumatic valve chambers.
- Bring surface-activated (e.g. plasma activated) Reservoir/Control layer into conformal contact with Fluidic Layer.
- Momentarily apply pressure (1-10 psi) to valve control lines to force PDMS membrane into intimate contact with the PDMS surface of the Fluidic layer.

Allow contact for approximately 1-3 seconds before switching back to vacuum pressure in control lines.

- Perform initial break-in of valves by rapid performing a sequence of actuations between vacuum and pressure for approximately 20 cycles, over a time period of 1-2 minutes.

- Continue to cycle valves with vacuum and pressure over a period of 5-20 minutes, depending on the surface activation and thermal history of the PDMS surfaces. Once the initial break-in cycles are performed, a slower and more protracted actuation sequence is preferably used to prevent the slow inexorable bonding of the PDMS surfaces, until all inclination for bonding is prevented, which can be achieved by actuating the valve with pressure for up to 1 minute followed by intermittent actuations with vacuum so as to break any newly formed bonds. Continuing this process for up to 20 minutes has been shown to completely prevent future permanent bonding between the valve membrane and the valve seat.

- Other materials which have molecular bonding capabilities when like surfaces are brought together may also be employed, and the molecular bonds destroyed at valve seats in similar manner,

Description of Valve Break-In Process

Native PDMS, comprised mainly of repeating groups of $\text{-O-Si(CH}_3\text{)}_2\text{-}$ is hydrophobic in nature, and, without special treatment, has a tendency to adhere to, but not permanently bond to other like surfaces such as PDMS, glass and silicon. However, upon treatment with oxygen plasma or the like the methyl groups (CH_3) are replaced with silanol groups (SiOH), thus forming a high surface energy, hydrophilic surface capable of bonding irreversibly with other like surfaces containing high densities of silanol groups. This irreversible bonding process occurs via condensation reaction between OH groups on each surface resulting in covalent Si-O-Si bonds with the concomitant liberation of water (H_2O).

Oxygen plasma and similar techniques have control parameters such as pressure, power, and time all of which determine the concentration of surface OH groups. Higher concentrations of OH groups lead to more covalent bonds between the two surface and

therefore higher mechanical bonds. Left exposed to atmosphere after oxygen plasma or similar treatment, the hydrophilic surface will undergo “recovery” back to its native hydrophobic state via migration of short, mobile polymer chains from the bulk to the surface. Full “recovery” occurs over a period of hours at room temperature and can be
5 accelerated with increased temperature and retarded by storage in vacuum and/or low temperatures. This is accommodated by storing activated substrates at -50C in vacuum bags for several days to lock-in the hydrophilic surface treatment prior to bonding.

Since the bonding mechanism follows a fairly slow condensation reaction, which involves the liberation of, water over a period of several minutes to a few hours before
10 completely consuming the available OH sites, it is possible to interrupt this process before completion. Once completed, the bond strength between the interfaces is comparable to the bulk tear strength leading to an irreversible attachment of the two materials. Attempts to separate the layers at this stage will lead to bulk damage of one or both of the layers. However, interruption of the bonding process by mechanically
15 separating the surfaces during the early stages of the bonding cycle is found to irreparably damage only the small number of formed bonds between the two surfaces. The tear strength of the bulk is considerably higher than the interface bond, therefore separation produces no irreparable damage to the bulk. Also, if the bonding process is interrupted early enough (typically in first few seconds), then the force required to separate the layers
20 is little more than the adhesion force required to separate untreated layers. Bringing the layers back into contact for a short duration (typically a few more seconds), will initiate, and interrupt bonding again. Each time this cycle is repeated, potential bonds are incrementally eliminated until all such bond sites are consumed and the material reverts back to having the properties of the untreated material.

25 In a preferred novel technique, microvalves are formed between layers of PDMS by surface activating, e.g. plasma activating, the PDMS or similar surfaces, bringing them into contact and then activating the valves to open and close in such a manner that permanently disrupts bonding between the flexible membrane and the valve seat, but results in complete and robust bonding elsewhere over broad surfaces to hold the device
30 together.

Device Manufacture

Referring to Figure 30, a product employing the concepts described is a consumable microfluidic cartridge for the purpose of quantifying antibody concentrations in human plasma samples. The cartridge, such as shown in Fig. 30, contains on board provisions for sample inlets, in other words, a reservoir that will receive a sample to be analyzed, e.g. a blood plasma or serum sample.

A completed cartridge 20 contains sample inlet wells 22 for receiving patient plasma or serum sample or other type of bodily fluid, including cerebral spinal fluid, or urine. It will also contain a buffer inlet well 24, buffer being a reagent used during the processing of the assay, a waste reservoir well 26 designed to contain all of the reagents and sample that flow through the microfluidic channels and that are no longer needed all self-contained on the microfluidic cartridge, also containing a reservoir well 28 which has contained in it a detection antibody with a fluorescent label. The preferred embodiment, the detection antibody will be dried down in the channel or in the reservoir and rehydrated during operation using the buffered contained in buffer well 24.

Referring now to Figs. 31, 32 and 33, Fig. 31 shows the microfluidic channels containing 4 independent microfluidic channel groups containing the extremely small hollow fluidic flow elements, referred to hereafter as elements. Fig. 31 shows those four channel groups each containing six channels 30. There are extremely small hollow flow elements 32, microfluidic valve seats 34, and pistons 36. The extremely small hollow flow elements are formed in a batch process with a capture antibody provided on the inside surface of the elements and those elements are placed into channels 32.

Example of dimensions of the hollow elements: The length of the preferred embodiment is approximately 250 microns, the inner diameter approximately 75 microns, and an outer diameter of approximately 125 microns. Fig. 32 is a blown up schematic of the hollow elements shown in two example channels parallel example channels.

In presently preferred practice, the channels are wider than the elements, and the elements are attracted by near electrostatic force to adhere to one channel wall, defining by-pass flow paths on the other side.

Fig. 34 shows a cross-sectional view of a hollow flow element in channel 30 with space surrounding hollow element on the outside of the element. Fig. 34 depicts hollow

element 32 in microfluidic channel 30 with flow arrows 40 depicted, the hollow element as captured by the top surface elastomer membrane 38 and on the bottom surface by glass substrate element 42.

Typical dimensions for the glass substrate layer 42 are 200 microns thick of borosilicate glass and the elastomer membrane layer element 38 has typical thickness of 100-200 microns. Also providing the channels are an elastomer, PDMS material typical 100-150 microns tall thus forming the microfluidic channel. Also shown in Fig. 34 the elastomer membrane layer continues both to the left and to the right as well as the glass substrate continuing to the left and to the right and on either side containing one or more parallel microfluidic channels also containing hollow glass elements, glass layer element 42 is bonded to elastomer wall, a micro-fluidic channel wall 44, previously formed in a subassembly process using a covalent bonding technique involving plasma activation of the PDMS surface and subsequent contacting and therefore bonding to the glass layer, the hollow element is inserted into that channel.

There are additional channels 30 in parallel. The purpose of parallel channels is to isolate different antibodies from each other for preventing cross-reactivity.

Channel depth is less the diameter of hollow element that are picked and placed against one of channel walls such that electrostatic forces between the element and channel walls release the placing device, e.g. tweezers or vacuum pickup, from the element. In this process, by moving in an "L" shaped motion, laterally at the end, increases the electrostatic attraction and allows the tweezer to be released from engagement with the element and tweezers to be removed. Channel 30 enclosed by bringing into contact both ends of elastic membrane 38 of control/reservoir 46. Elements are retained in channel 30 between elastomeric 38 and glass 42.

Fig. 32 shows schematically two example channels containing a series of four spaced apart elements 32 and by-pass flow space 41.

Fig. 35 is a top view of the fluidic layer sub-assembly 48 with elements 32 in channels 30. The assembly 50 contains the elements.

In Fig. 35 four sets of microfluidic single sample, i.e., four analyte networks 52 are shown, each network is designed to perform an assay with its own respective sample.

Fig. 33 is a blowup schematic of a single channel 30 containing four elements 32 and microfluidic piston chamber 36, and valve 54 having seat 34, Fig. 31.

Fig. 33 depicts by arrowheads, flow through the bypass flow path 41 around the hollow element 32 as well as through the element.

Referring to Figs. 30 and 35, the channels 30 are formed by glass substrate 42 and micro-fluid channel walls formed by knife cutting sheet of PDMS of 110-micron thickness 32.

Fig. 30 shows forming the fluidic area 48 by bringing together glass sheet 42 and the unique cut-patterned PDMS sheet 42 using known techniques.

Reservoir/control plastic member 56 (containing fluidic reservoirs for sample, 22, assay buffer 24 and reagent waste 26) is bonded to PDMS membrane 38 to form control/reservoir layer 46.

Fig. 24 is a top view of the fluidic layer sub-assembly 48 with elements 32 in channels 30. The assembly 50 contains the elements.

In Fig. 24 four sets of microfluidic single sample, i.e., four analyte networks 52 are shown, each network is designed to perform an assay with its own respective sample.

Referring to Fig. 24, the channels 30 are formed by glass substrate 42 and micro-fluid channel walls formed by knife cutting sheet of PDMS of 110-micron thickness 32

Referring to perspective of Fig. 36, layer 46 and membrane 38 are ready to be assembled by plasma-activated molecular bonding. Fig. 37 is a top view depicting final assembly 46. Pneumatic interface ports 58 are adapted to match with computer-controlled pneumatic control lines that provide pressure and vacuum actuation to valves 54 (formed by membrane 38 and microfluidic valve seat 34) and pistons 55 (the pistons being formed by elastomer membrane 38 lying over piston fluidic chamber 36 and piston pneumatic chamber) piston control lines 60 and valve control lines 62. The piston pump formed by membrane 38 sandwiched between 37 and 36 is activated by vacuum in one direction and pressure in the other.

For pneumatically controlled microfluidic systems, there is need for forming a fine, closely-spaced pneumatic channel network with high fidelity over an extensive area. One important use of such networks is for actuating a distribution of microfluidic valves or systems of microfluidic pistons and valves that constitute microfluidic pumps. As is

well known to those in the microfluidic field, there are significant problems associated with doing this economically and reliably. The problems become more acute as the extensiveness, complexity and greater miniaturization of the microfluidic network increases. Many of the problems relate to materials, material handling and manufacturing techniques.

In the case of using high precision molds to form three-sided micro-pneumatic channels integral with a body that may perform other functions, there is extreme cost and inflexibility involved in creating the precision molds and in making mold changes required over the life of the mold. For many molded parts, there can be difficulty in achieving sufficiently intimate bonding with another material to close open channels and achieve air-tightness under both positive and negative air pressures.

In the case of forming a composite channel with walls of one material and top and bottom closures of different materials, it is difficult to find the right materials and manufacturing techniques that meet all needs. Form instability as well as difficulty in achieving intimate, air-tight, long-lasting bonds under positive and negative air pressure are among the problems. During the production of such a device, when formed of parts that need to be joined to complete the fine features of the pneumatic system, there is a particular need for the finely formed parts to have dimensional stability in handling to achieve registration and proper mating with cooperating parts. Without precise registration, desired pneumatic pressure levels may not reach microfluidic valves or pistons to operate them in proper sequence.

These considerations apply especially to microfluidic assay devices that perform multiplex assays, e.g. ELISA, in which in one device, multiple assays of bodily fluids are performed. In many cases, for instance in drug development, it is desired that multiple isolated samples be simultaneously subject to multiple assays.

There is particular need to find a combination of materials and manufacturing techniques that meet all of the many requirements of micro-pneumatic structures described here and otherwise well known in the field of microfluidics.

As a specific example, it is desired to provide a highly quantitative immunoassay device capable of receiving multiple samples, for instance approximately sixteen to sixty four samples on a single portable microfluidic cartridge, and provide, for each sample, a

microfluidic network capable of quantitative determination of a number of analytes simultaneously, for instance conduct four to eight different assays, the assay cartridge having macro sized features for containing patient sample (e.g. blood serum, urine), reagents such as buffers and secondary antibodies, and waste.

5 Especially, it is desired to find an efficient approach to manufacturing such a portable microfluidic assay device of overall (“footprint”) dimensions of, e.g., 5 inches by 3 inches. In such device it is important to have the microfluidic channels closely packed with other channels and features, of a distance of the order of four to eight times the width of a pneumatic micro-channel, with, for instance, channel sizes on the order of
10 100 microns or less, for instance, channel widths approximately 100-150 microns and depths of 100-150 microns, with precision in features of 10-20 microns of tolerance. In many cases, macro-size features such as sample wells and reagent reservoirs are desired to be incorporated in the portable device, typically of several millimeters in dimension, i.e. several millimeters cross-wise and several millimeters deep.

15 To be of practical utility, it is necessary to find a way to provide the entire assembly reliably and inexpensively despite the optimum qualities of the materials for various features being different, and the need to join them in a reliable manner while retaining full functionality.

The invention is especially useful in microfluidic assay devices which have a
20 pneumatic channel component, a fluidic channel component, and a flexible membrane joining the two. For this type of construction, as well as more generally, we have solved the foregoing problems, in particular with respect to the pneumatic component of such system, as follows.

We found an excellent starting material for forming the pneumatic channels
25 comprises a double sided pressure sensitive adhesive sheet having a non-fluorescent central layer formed of rigid material, non-fluorescent adhesive on each side, and peelable liner layers protecting the adhesive and we found a process for forming the pneumatic channels and features by processing this sheet by using a CO2 laser, followed by very simple bonding process. The laser is used to ablate pneumatic micro-channels
30 and other structures by cutting entirely through the core, the adhesive layers and the liners to form the sidewalls of the desired channels and other features. Thus a pneumatic

channel has a side wall formed partly of the inner core layer of the adhesive sheet, and partly by the adhesive itself, at both sides of the core layer. This technique is found to form fine precise features having sizes on the order of 100 microns or less, with channel widths approximately 100-150 microns, and depths of 100-150 microns, with precision in features of 10-20 microns of tolerance.

The dual-sided pressure sensitive adhesive sheet with suitable low fluorescence is available commercially with size up to 27 inches, for instance. It is important that the material have very low tendency to fluoresce when exposed to an excitation laser such as green laser or red laser used to excite fluorescence in the conduct of epi-fluorescent reading of fluorescent-tagged analyte at the capture sites of an assay. Mylar™ (polyester), a material often used in the manufacturing of pressure sensitive adhesive sheets as a structural component upon which the adhesive is applied, has a high degree of auto-fluorescence which interferes with the process of taking a measurement in the cartridge, and is inappropriate for use with assay cartridges intended to be ready by an epi-fluorescence or other stimulated fluorescent emission process.

It has been found that a core layer of polypropylene is excellent for this purpose. A polypropylene layer of approximately 2 mils (50 micron) thickness with the adhesive on each side of 1.8 mils or 45 microns thickness is found to perform very well with silicone adhesive layers. A suitable product is sold by Adhesive Research under the product designation AR 90880 having layers of silicon based adhesive, known as SR26 silicon adhesive.

An example of a product formed by the techniques described is a portable consumable immunoassay cartridge (cassette) constructed of several layers, the pressure sensitive adhesive with channels formed by through-laser cutting being one of the layers integrated into the cartridge. That layer, with its peel strip removed, is attached to the bottom flat surface of a rigid reservoir layer that defines the macro features previously mentioned, i.e. sample wells and buffer and reagent reservoirs. Laminated to the bottom surface of the pneumatic channel layer by the second pressure sensitive adhesive sheet, with peel strip removed, is a membrane layer which is formed on a 100 micron thick PDMS membrane containing fluidic vias that are aligned with vias in the reservoir layer and the adhesive sheet layer. This assembly is bonded to the fluidic layer, elsewhere

described, that bonding being effective to capture discrete detection elements that have been introduced to the micro-fluidic channels and connect the microfluidic channels of the cartridge, though the vias, to the sample wells and reservoirs elsewhere described.

The rigid reservoir cartridge layer is either a machined plastic body approximately 6 to 14 millimeters thick or an injected molded plastic body approximately 6 to 14 millimeters thick having reservoir macro-features located on its top surface, the features approximately 3 to 6 millimeters in dimensions, and with fluidic vias from the reservoirs penetrating through the bottom of the reservoir layer, aligned with vias laser-cut in the pressure sensitive adhesive layer. The adhesive sheet with laser-through-cut pneumatic channels, vias and other features is laminated to the bottom surface of the rigid reservoir layer in alignment with the vias in the reservoir layer so that the fluidic vias are arranged to transport sample and reagents from the reservoir layer to the fluidic layer through the vias in the reservoir layer through vias in the pressure sensitive pneumatic layer and vias in the following membrane layer. To then enter into the fluidic layer. Also contained in the pneumatic layer are features associated with valves and pistons in addition to the fluidic vias. These features can be ovals approximately 800 um long and 500 um wide in the case of valves, or 3000 um long and 800 wide in the case of pistons with long channels connecting these features along the entire surface of the substrate and terminating at the pneumatic actuation ports located at the end of the device in a series of pneumatic vias.

One of the challenges in creating a highly functional disposable immunoassay cartridge is in constructing one that has a large number of features which therefore provides a high degree of functionality. For example, one which is capable of running multiple samples preferably 16 to 48 different samples and for each sample the ability to precisely quantify several analytes, 4-8, on one cartridge. Such requirements often drive the complexity and the need for a high density of fluidic features, including valves, vias and piston pumps.

For each sample there is on the cartridge an independent fluidic circuit having the ability to perform measurements of up to 8 unique analytes. The independent fluidic circuit is fluidically isolated from all other fluidic circuits on the cartridge and is used to perform the same measurements in parallel on different samples. The current design squeezes as many as 20-30 different fluidic features, such as valves, piston pumps and

vias into an area of approximately 200 square millimeters (10 x 20 mm), as well as the pneumatic channels that connect the features.

A cartridge that measures 16 individual samples would have 16 independent fluidic circuits. However the functionality of each circuit is identical which means that every circuit is architecturally identical. So across the cartridge there would be 16 buffer inlet valves (one for each circuit) as well as 16 valve banks associated with each of the detect reagents, waste outlets and pistons. Since the circuits are identical copies it's possible to share pneumatic control lines across all circuits and use a small set of independently controlled pneumatic channels, limiting the complexity of the instrument that runs the cartridge. So for example, a cartridge with 16 samples running up to 8 different analytes, could for example have as few as 7 pneumatic channels where each of those pneumatic channels connects the same set of functional features located in each of the independent fluidic circuits. The functional sets would include banks of valves for example a bank of valves that allow the detect reagents to flow at a particular time or a bank of valves designed to close off and isolate a set of fluidic channels from one another in the manifold region of the fluidic circuit, or a bank of valves located at the output or a bank of pistons. Sets of functionality are connected to each other through a single contiguous pneumatic channel which terminates at one end at the pneumatic interface and the other end at the last feature in the string of connected features. Pneumatic channels in an effort to intersect with the sets of active features at every circuit are required to serpentine back and forth across a microfluidic cartridge, never overlapping one another, in an effort to cover all of the features located on the surface of the cartridge. Long contiguous channels, as long as 10 to 20 inches in length. And also as a result of the high density of pneumatic channels located on the devices it is necessary to keep channels, pneumatic channels as tightly packed as possible in order to accommodate the high degree of functionality required to run such an assay. As a result of these long channels tightly packed and located on a cartridge, and having a serpentine like path nature it was discovered that laser cutting a PSA based film for the purposes of creating these pneumatic channels had the deleterious effect of being structurally unsound. During the manufacturing process or immediately following the laser cutting process it

was discovered that the substrate with such formed channels was unable to structurally support itself and retain the required necessary dimensional tolerances.

As a result, features intended to have very tight physical tolerances for the purposes of forming the precision actuation of valve and pump piston were lost as a result of having this physical instability due to the formation of the long serpentine like channels back and forth across the substrate.

The solution to the problem involved interrupting the channel formation, so rather than forming long contiguous uninterrupted channels the channel were broken into segments approximately 20 to 30 millimeters in length, and depending on the nature of the channel path if it involved turning a corner for example or channels were closely packed to one another then the segment lengths could vary from 10 millimeters to 30 millimeters. These channel interruptions were formed by making the channel path with short, un-cut gaps approximately 150 microns in length, forming bridges between the channel segments approximately 150 micron. The result is a structure that is entirely self-supporting, which can be handled without the concern of losing the registration or the intended tight dimensional tolerances. The channels now having interruptions in them and not having the continuity of air flow from the pneumatic input to the final terminal structure at the end of the channel as a result of the bridges is made functional again by deploying shunts either directly underneath the bridges in the membrane layer or directly above the bridges in the reservoir layer, as shown in figure 53i and 53j.

In the case of shunts being formed in the membrane layer shown in figure 53i a hole or a via is cut in the membrane similar to those used for the fluidic vias. In the case of the shunt being formed in the reservoir layer as shown in figure 53j a small pocket is machined into the bottom surface of the reservoir layer or it can be formed in the process of injection molding a piece of plastic involving the formation of the reservoir layer.

Once combined, the channel network of pneumatic channels and the shunts whose alignments overlap with the bridges form a contiguous pneumatic channel capable of actuating all of the features, such as pistons and valves, located throughout the area of the cartridge. Such channels have cross sectional dimensions of approximately 150 microns by 150 microns.

The benefits of the approach include lower cost of manufacturing and higher precision in feature locations. Because the raw material for the pressure sensitive adhesive is relatively inexpensive per cartridge (<\$1/ cartridge) and because the relatively high speed of manufacturing these channels also results in a relatively low cost yet high precision structure necessary to implement the precision actuated pistons and valves in a pneumatically actuated microfluidic device.

The overall cost impact as compared to injection molding or machining or other methods used for forming similarly such pneumatic channels is significant.

Although one could injection mold such a piece, there are high costs and long development times associated with developing an injection molding process as the inflexibility of an injection molding process to adapt to changing design concepts. It is anticipated that a number of variations or configurations of cartridges will be supplied resulting in the need for some flexibility in the formation of the pneumatic circuits, and with injection molding each component requires a unique mold. Whereas with respect to using the present invention, the same equipment, the laser set up can be used with a different program to execute drawings that have been made.

One of the other important benefits is, the implementation of the membrane layer, which is an integral part of the fluidic cartridge as it is responsible for a number of functions. Its responsible for closing off the channels and making them closed fluidic channels, for containing elements placed into such channels for the flexibility associated with forming microvalves and pistons. A PDMS membrane is necessary as an integral part of the microfluidic cartridge and needs to be permanently adhered to the pneumatic channel surface whatever that pneumatic channel surface is formed in. In the case of an injection molded plastic, the bonding process between the PDMS membrane and the PDMS is difficult and costly as it involves multiple steps typically, and also is limited to a small subset of plastics such as polystyrene, polycarbonate, COC and COP. Some of those plastics are unsuitable for the formation of a reservoir layer that is formed in a thick such as 6-12 millimeter thick having both macro features on one side and micro features on the other side. In the case of COC and COP, the cost of such plastics makes the formation of a device such as that prohibitively expensive. Which then relegates one to a

very few number of available plastics such as polystyrene.

Although some plastics such as polystyrene make good candidates for such a process, most others, such as polycarbonate and PMMA are not good candidates . So
5 developing a cartridge around a more flexible machine process then transferring it to injection molding would be difficult because the material choices aren't well suited for both processes.

Typically one starts with a machining process to wring out the design and then transfers it to injection molding once the design has been stabilized and locked down
10 essentially. Which is really a direct consequence of the high nonrecurring expenses associated with injection molding.

But you're bringing working towards the fact that the material for instance with pressure adhesive on both sides can be easily bonded to any

Exactly, that is sort of the point, is that the pressure sensitive adhesive because it
15 is naturally adherent or naturally adheres to nearly any clean smooth surface, the available range of plastics and processing processes are opened up widely. One can then employ polycarbonate or polystyrene or any number of plastics without regard to the bondability of the PDMS membrane to that surface of plastic.

And just as an aside, if you were to make the pneumatic layer as a part of
20 injection molded or machined part, what is done to make it bondable to a PDMS membrane?

Well, there's a process that involves plasma activating the surface of that plastic surface and then exposing it to an intermediate bondable layer such as a silane or an organosilane type component which readily adheres to both the plastic surface and the
25 PDMS membrane surface.

The examples of Figs. 51A i-iv illustrate PDMS bonding to PDMS. For a region R_1 in which bonding of the bondable surfaces is not desired, Fig. 5A iv shows deflection of the two PDMS layers in opposite directions by elastic deformation, and dashed lines indicate the layers in region R_1 relaxed, un-deflected. Adjacent regions R_2
30 of the layers are retained in contact, either solely by initial surface-to-surface bonding, or with the added benefit of outside confinement or compression, indicated by arrows P.

Thus the Figure illustrates the two conditions achieved cyclically during exercise of the make and break protocol previously described, enabling bonding at contiguous regions, but bond prevention in a selected region of the potentially bondable surfaces.

For instance, for ease of tooling and product design, instead of employing
5 physical engagement and mechanical movement to produce the make and break conditions at region R_1 , it is frequently desirable that the make and break contacts be caused by application of fluid pressure differential evenly across the region R_1 of each layer. (The term “fluid” is here employed in its generic sense, to cover all “fluids”, i.e. liquids or gases). Use of fluid pressure assures even loading and prevention of
10 disrupting distortion of a layer during the cyclical make and break protocol. To achieve the deflected condition, a deflection cavity at the backside of region R_1 provides space for the deflection. The adjacent regions R_2 of the layers are retained in contact, either solely by surface-to surface bonding, or with the added benefit of confinement or compression.

For reasons of convenience and economics in many situations, it is found
15 desirable to employ differential gas pressure to produce the deflections. For instance, thus can be avoided the need for design of special mechanical moving devices or the need for drying associated with the use of liquid pressure. The gas pressure differential can be achieved by application of positive gas pressure to the interstitial space, as by a special channel, with the benefit of being able to use high values of pressure to speed the
20 operation or for use where bonds are formed rapidly or are of high strength.

In many cases however, it is found desirable that gas pressure differential be produced by application of vacuum to the outside of a layer to produce its deflection. One advantage is that the manufacturer can employ channels and cavities of the device itself, such as those associated with pneumatic operation of the device during its normal use.
25 By this the manufacturer can reduce the need for special, costly tooling and extra manufacturing steps. An example is the manufacture of microfluidic cartridge device described herein.

Referring to Fig. 51 A v, in the case of applying vacuum on the backside (outside surface) of a PDMS layer to produce gas pressure differential, the cavity is a closed
30 vacuum chamber engaged upon the backside of the PDMS layer. Fig. 51A v illustrates such a deflection chamber on each side of the pair of contacting membranes surfaces.

The vacuum-actuated deflected state of region R_1 is shown in solid lines while the dotted lines illustrate the natural relaxed and undeflected state when there is no vacuum is applied to the vacuum chambers. Preferably, indeed, positive pressure is applied to both chambers, having the effect of enhancing the momentary contact, and therefore lessening the time needed before another break phase of the cyclical is performed, thus speeding the neutralization of the layers in regions R_1 .

Fig. 51A vi illustrates a deflection chamber on only one side of the assembly, deflecting a single membrane. The opposing membrane is shown rigidly backed against a planar surface to which it may previously have been adhered, to ensure that it remain stationary when region R_1 pulls away.

Fig. 51A vii illustrates a single deflection chamber and an opposing valve seat on planar surface in construction similar otherwise to that of Fig. 51 A vi.

In all the cases of Figs. 51 A iv-vii, the deflection chamber may be formed by manufacturing tooling constructed only for that purpose and then removed. In other cases as has been shown in the examples of the microfluidic assay cartridge shown in this application, the deflection chamber is in fact part of the final microfluidic product, with numerous obvious advantages with respect to tooling cost and economy of manufacturing steps.

Fig. 51A viii diagrammatically illustrates a microfluidic cartridge having a complex microfluidic (liquid) channel network, a large number of microfluidic valves formed by the make and break protocol, and other pneumatically controlled features that are all actuated simultaneously with the valves.

There are many obvious technological advantages and beneficial effects of perform this make and break operation simultaneously on many different valves, or on many other features, such as those described below, or on combinations of many valves and many other features. For instance, from a manufacturing point of view there can be great ease with which a complicated structure can be made by applying only a simple pneumatic force in the form of a vacuum or pressure and actuating all of features at once. This of course includes the benefits of low cost and low complexity of the manufacturing process.

As has been indicated, there are numerous advantages to using cycles of pneumatic vacuum and positive pressure applied on the exterior or the nonbonding side of the flexible sheet. While such vacuum deflection of the make and break protocol in manufacture of portable microfluidic assay cartridges has great advantages, more broadly viewed the essence of the activating fluid aspects of the invention has to do with deflection of a region of a membrane layer by application of differential fluid pressure across the layer no matter what medium is employed or on which side the greater pressure is applied. In particular, there are important circumstances in which advantage is obtained in creating differential gas pressure across the layer by pneumatic forces applied between the sheet-form layers, i.e. applying positive pressure to cause the deflection of one or both layers and negative pressure between the sheet-form layers to cause collapse and contact.

One of the advantages of employing positive pressure relates to the fact that in employing vacuum to deflect the membrane, the maximum pressure that can be applied is 1 atmosphere, approximately -14 psi, which limits the deflection forces applied to a membrane whereas a positive pressure applied between the sheets is nearly unlimited in magnitude to cause separation to occur between the sheets.

In respect of applying positive pressure to produce the outward membrane deflection, two alternatives for the deflection cavity will be described, that of a deflection slot defined by walls that engage the outer surface of the layer, but that is open and un-limiting with respect to the deflection distance for the layer, and that of a chamber that also has the walls, but is closed with a ceiling. The walls of an open or closed cavity for pressurized deflection define the physical perimeter of the area of the deflection, thus defining regions R_1 and R_2 , and hence the area over which the make or break process occurs. In the case of use of a simple open slot, the limit of deflection is a function of the elastic properties of the membrane and the pressure applied, and may be most useful in the use of moderate positive pressures, or where there is ample margin for error regarding the strength of the membrane. The use chamber which, in addition, has a ceiling creates an additional physical limit for outward deflection, which can potentially protect the membrane from deleterious effects of tearing or bursting the membrane,

useful e.g. where particularly thin membranes or particularly high outward forces are to be employed.

In the discussion so far, the flexible, deflectable sheet has been monolithic, and in the examples of Figs. 51 A i to viii, it has been PDMS. A composite sheet may be used
5 instead, comprised of multiple layers, the basic requirements being that the overall sheet is flexible to be capable of elastic deflection, and that the inner surface be bondable.

While not necessary according to broad aspects of the invention, it is advantageous that the bondable surface of the composite still be surface-activated PDMS, either pre-formed as a separate sheet or as a coating on a carrier sheet which itself may be monolithic or a

10 composite. In the example of Figs. 51 A ix the composite comprises a bondable pre-formed layer joined to a layer of another substance. For instance the bondable layer may be surface-activated PDMS and the back layer of a pre-formed layer of a material other than PDMS, for example a sheet of PDMS laminated to a second pre-formed flexible sheet of a compatible substance, or one that can be rendered compatible by use of a
15 treatment, such as flame or plasma treatment or by use of an intervening layer that can be bonded to both. For instance the second layer may be a pre-formed sheet of Mylar TM (PET), Polycarbonate or Polyurethane produced as blown or cast film sheet, as appropriate for the resin and the circumstances. Figure 51A x illustrates a flexible pre-formed back sheet coated with a thin coating of PDMS material typically ranging from
20 approximately one to three microns in thickness, having its surface activated in preparation for a bonding process. The back layer for instance may be Mylar TM (PET) of thickness approximately 5 to 15 microns.

In still another implementation, not shown, an essentially monolithic layer of PDMS may carry an exterior coating of another substance, for purposes such as
25 improving the gas-barrier properties of the composite.

Fig 51A xi illustrates a laminate structure similar to that of Fig. 51 A x, but with a flexibility-increasing feature. It is useful for the condition in which a flexible outer sheet bonded to PDMS sheet (or it could be a flexible sheet having a PDMS surface coating) wherein the flexible sheet is less flexible than PDMS and it is desired that the composite
30 exhibit increased flexibility, e.g. to increase the deflection capability and thus the flow capacity of a valve or pump formed by the deflectable membrane. The principle being

illustrated is use of an interruption or reduction in thickness of the back layer. The interruption can be a single moat in the back layer, extending around the perimeter of the defined area R_1 , or, as shown, a series of concentric moats that allow a greater displacement to be generated as a result of allowing the flexibility of the PDMS to perform the majority of the stretching during an activation of either a vacuum deployed or pressure deployed activation protocol.

By way of summarizing certain technological advantages and beneficial effects of features regarding materials selection for the flexible membrane:

PDMS has the advantages of being a low cost material that is easily bonded, flexible and easily machined or otherwise easily formed into channels surfaces to cooperate with the deflectable membrane features described. In the specific case of laminated structures, including that of Fig. 51A xi is the ability of a material other than PDMS to block gas passage or reduce the overall gas permeability coefficient of the structure. Whereas PDMS is known to have a high degree of gas permeability other plastic material such as polyester, polycarbonate and polyethylene exhibit extremely low permeability relative to PDMS. This can be of great benefit in microfluidic type devices in which positive gas pressures are used for actuating valves, preventing gas permeation through the membrane into the fluidic channels thereby creating bubbles that can have a deleterious effect. For instance, under many assay flow conditions, air bubbles that enter the reagent stream can attach to the capture agent and prevent binding; air bubbles can prevent complete wetting of surfaces, and thus inhibit the capture agent from capturing an antigen of interest; and air bubbles can also displace fluid in the microfluidic channels causing the microfluidic system to become less stiff from a fluidic point of view and also increasing the variability of flow rate, producing uncertainty of flow rate that can impair quantification assays.

With specific reference to the technological advantages and beneficial effects of the last Figure, Fig. 51A xi. It employs a series of narrowly defined moats or channels that are cut into the more rigid yet somewhat flexible air-impermeable backing sheet part of the flexible sheet/ PDMS laminate. The moats allow the stiffer component, e.g. Mylar TM (polyester) or polyethylene to deflect using the underlying flexible PDMS to act as an expansible spring, therefore achieving greater deflection. Thus, decreased

permeability of the relatively stiff backing layer, to decrease air permeability within a microfluidic valve can be obtained, while achieving enhanced membrane deflection away from a valve seat to allow adequate or improved flow across the cooperating valve seat when the membrane is deflected to open position. This is a novel feature in its own right, as such benefits can be obtained even when different approach is employed to join materials of the device.

Referring again to Fig. 51 A vi, the single deflectable membrane implementation, a fixed bondable surface opposite to a flexible membrane PDMS bondable face is not limited to the same material, and there are circumstances in which advantages are obtained by using a different bondable surface. Referring to Fig 51A xiv, the deflectable membrane has a PDMS bondable surface, but the opposite bondable surface is provided by a rigid silicon based material including crystalline or amorphous silicon, amorphous silica, silicates and ceramics. Benefits of different thermal conductivity, electrical conductivity, or the ability to add electrical contacts, as in the case of silicon or having the beneficial properties of silica in the form of optical clarity low autofluorescence optical smoothness or the special hardness properties and insulating properties of ceramics can be of advantage.

Figure 51A xv illustrates the accommodation of a flexible membrane with a surface-activated PDMS bondable surface to a synthetic resin or metal based device employing an intermediate bifunctional layer. For example surfaces of well-known plastics including COC (cyclical olefin polymer), COP (cyclical olefin copolymer), polycarbonate, polysulfone, polystyrene can be surface-activated or metals such as aluminum or iron that either readily form oxide layers can be employed. Such surfaces can be modified with an intermediate bifunctional layer such as an organol, to create an oxide layer.

In a preferred implementation, a portable microfluidic cartridge 2 is placed into an operating and scanning instrument by the user. It enters in a receptacle or reception area 6 at which the cartridge is retained for conducting the assay while scanning.

A suitable receptacle is shown in Figs. 54 and 54a, and the relationship of the receptacle and cassette when in assay/reading position is shown in Fig. 55 and the detail of Fig. 55a. An implementation of the overall system is shown in exploded view,

Fig. 56. Fig. 56 includes x, y precisely movable stage 13 that moves the cartridge on its carrier relative to the stationary objective lens.

Referring to Fig. 54 and 54a, cartridge 2 and the cartridge receptacle 6 are shown with a clamping mechanism 12 and pneumatic interface 8. A series of computer-operated solenoid valves 9 that move on the stage with the cassette apply positive and negative air pressure to ports that interface with the positioned cassette.

Fig. 57 illustrates a microfluidic configuration within a cartridge, illustrating both a series of fluidic networks and a pneumatic channel network to actuate on-board membrane micro-valves and micro-pistons in the fluidic network. The fluidic network in Fig. 57A comprises eight discrete microfluidic circuits closed by an over-lying elastic membrane, e.g., a continuous layer of PDMS. Each of those circuits has a number of microfluidic channels, valve locations, and piston locations. Portions of this membrane are located at formations in the channel that define valve and pump cavities. The corresponding portions of the membrane define movable elements of the valves and the piston.

The pneumatic channel network Fig. 57B is shown as an overlay in in Fig. 57C. It matches the fluidic network with respect to the various features that need to be actuated.

Fig. 58 is a magnified view of one of the circuits of Fig. 57, illustrating a number of the micro-features including valves, pistons, and the various reagent or reservoir inputs including the sample, the buffer (wash), the assay reading dyes, the secondary antibodies and the waste. The four elements GNR shown in black in each of the four individual (isolatable) channels represent glass nano-reactors (GNRs) embedded in those channels. This illustrates the basic micro fluidic unit replicated a number of times in the cartridge depending on the number of samples that the cartridge is designed to accommodate. The assay protocol flow sequence shown at the left of Fig. 58 starts with the prime flow step, and is followed by sample step, wash step, secondary antibody step, another wash step, a dye step for reacting to attach reading dye to the captured moiety, and finally another wash step. This illustrates an example assay sequence capable of being performed in this microfluidic structure. Each one of the fluids: sample, secondary (e.g., secondary antibody), wash, and reactive dye is caused to flow from its respective inlet well by

activation of the pumps formed by each piston and upstream and downstream valves, with the end result of captured moieties at the detection elements that are labeled with the reactive dye, ready for reading to quantify the result of the assay. Examples of volumes employed on this device include the sample at 20 microliters, a buffer of 150 microliters (as shown in the table)— the total volume of the microfluidic circuit is approximately 1.8 microliters.

In Fig. 98, four micro channels on a portable microfluidic cartridge are illustrated, each having two monitor positions. The further discussion relates to the first set of monitor positions 1, 2, 3, 4 in respective channels. In Fig. 98a, three different operations of an illustrative assay with discrete phases are represented by times t1, t2, and t3. In phase 1 at time t1 at four different locations on a cartridge to sample four channels, the tracer signal is detected to be at the expected nominal value within the acceptance rate. It is therefore considered a successful phase 1 disposition. Phase 2, at time t2 the nominal level is near zero, which might indicate that a buffer or some fluid that intentionally had no tracer was properly flowing in the channels at that particular phase. In phase 3, at time t3 third reagent or fluid in the channel has a tracer level that is different from that of phase one but is detected to occur at its nominal value within its acceptable and expected range. So the entire operation considering phases 1, 2, and 3 would be considered successful. This represents proper operation with no failures.

Fig. 98b illustrates another run of the same assay. In phase 1, time t1, a failure has occurred wherein the detected tracer signal occurs outside (here, below) the acceptance range. In channel 1, the tracer signal is shown present, but lower than the acceptance range, whereas in channel 4 the detected tracer value is shown as not present. In Fig. 98c at time t1 of the assay run, all four channels are shown as having a detectable tracer signal below acceptable range. But note that all four signals are equal and uniform. This indicates that there is not an independent failure mode within that cartridge but probably indicates that an improper dilution had been used to create the reagent that was used.

There are other failure modes such as the improper interface of the pneumatic seat which could lead to valves not opening fully or not closing fully or pistons not operating in full extension or lift so that their fluid volumes might not be what was anticipated. A

hierarchy of signal modes can be constructed, e.g., in the simplest case a signal versus no signal, a simple digital response, and in other cases where the quantitative value of the signal does not meet expectations.

In digital response, there is no quantification. The signal is either present or not.
5 A further level of complexity involves quantifying the level and comparing that quantity to an acceptable level where there is range of acceptable levels (acceptance range) not just on or off. That quantification technique might be used to determine whether a proper dilution or proper concentration or proper reagent was used in the proper location. Another advantageous level of sophistication is in monitoring and analyzing the signal
10 structure over time, to obtain the temporal response of the signal relative to an expected temporal response. That requires a more detailed explanation.

Referring to Fig. 99, the evolution of a detected tracer signal is shown while monitoring over time a fixed location in a microfluidic channel, for example a channel approximately 100 microns wide by 100 microns deep in a length section of
15 approximately 20-50 microns long. Thus a very specific isolated location within the channel is monitored over three different phases. The first phase shown depicts the condition of no flow occurring within the channel but the channel has present in it a reagent laced with a tracer of a certain concentration that provides a detected signal of any type, e.g., detected fluorescence. Phase 2 in Fig. 99 follows the evolution of the
20 signal, showing that it decreases over time as the reagent with the tracer is displaced by a reagent without a tracer, for example a buffer reagent or wash liquid that has no tracer in it.

The signal evolution decays very rapidly in phase 2 as the new reagent displaces the old reagent with tracer, and the signal goes down. Then in phase 3 of Fig. 99 the
25 displacement process stops and the signal is monitored with no net flow during phase 3. This represents successful washing of a channel.

The benefit of thus staring at one location is not only to watch the real time evolution of what is occurring at the location but also in the case of using a fluorescent dye that is photo bleachable, to look at the fine structure not shown in this trace but
30 shown in subsequent traces that reflect details of exactly what is occurring in the microfluidic device.

Another benefit of staring at a specific location is to acquire information in the development of an optimized protocol. In the development phase for an ELISA or any other assay that involves multiple sequences of reagents and flushing followed by new reagents, it is important to know whether the displacement of the prior reagent is complete and how many cycles or how long or what type of flow rate for example is required to ensure that the next phase of the assay process is firmly established. What is called “open protocol” refers to assays that are not monitored. For such assays, it is vitally important to characterize the microfluidic system beforehand. The technique being described may be used as a tool to be used during test runs of an assay, to characterize the system and optimize the protocol. So for example if insufficient flushing or wash steps were applied, then residual reagent would be present at a phase that could be harmful to the performance of the assay. The presence of such errant reagent may be detected by presence of its respective tracer. This therefore is an advantageous technique for evaluating performance and optimizing an assay protocol.

One implementation is to provide a cassette in development on an x, y movable table. The table is indexed to any selected position relative to the detection systems, e.g., optical system, and the assay can be run while detecting signal from that position. A commercial instrument constructed to run and read an assay has substantially all of the functionality required to generate development data that is fed back into developing an optimized protocol.

Fig. 100 illustrates the time response of a tracer signal while staring at a single location in a microfluidic device, referring back to Fig. 99, location 2, for example. The tracer dye present in the liquid in the channel is selected to be photo bleachable progressively over time. In an example, a red laser diode is employed to excite red-excitabile fluorescent tracer material. When the fluid is stationary, a photo bleaching process is observed, the detected signal decaying as a function of time. The decay rate is dependent upon the laser power and type of the dye and the concentration.

Flow may be introduced to the channel in an oscillatory fashion. The purpose of oscillating the flow in normal operation of an assay is to enhance the interaction of the analyte present in the unknown concentrations sample with a capture moiety, e.g., an antigen in the sample with an immobilized capture antibody. A typical defined volume

(“slug”) of liquid is used, of fixed volume that is much larger than the volume exposed at the detection point. When portions of this oscillating slug of liquid move away, this allows time for diffusion to take place in those portions to bring the material into equilibrium before it comes back for exposure to the capture site, and back and forth.

5 Whenever it comes back, the analyte in closest proximity to the capture moiety, e.g., some percentage of an antigen is captured and drawn out of that sub-volume of the sample – depleting that sub-volume. Then as it is flowed away, and diffusion allows that sub-volume to approximately re-equilibrate, to reach substantially equilibrium concentration – in time, it replenishes the sub-volume in the vicinity of the capture agent.

10 And so the flow is oscillated back and forth to give maximum opportunity for all the sub-volumes to interact with the capture moiety, to substantially optimize the reaction by optimization condition with the capture moiety.

Referring back to Fig. 100, the time response of a detected tracer signal is shown from a given monitoring location in a scenario with oscillatory flow. Fluorescent dye is

15 used that is subject to photo bleaching. One observes a peak-like nature, or an up and down signal structure with peaks and valleys at a given time sequence and periodicity, due to the oscillation frequency of the fluid back and forth. So there is no net flow of fluid away from the location, but with oscillation, there is an opportunity to replenish the photo-bleached portions of the reagent with fresh reagent. In those cases where the flow

20 rate is maximum, passing by the excitation beam quickly, the photo bleaching decay rate is offset by replenishment of new reagent. That is shown in the peaks labeled “max flow rate.” At the turnaround points where the flow rate of the oscillating flow goes to zero, the photo bleaching decay is maximum that is indicated by the valleys, labeled “min flow rate” on this graph. From detail shown in this particular graph, in addition to the

25 frequency and the peak-like nature, one is able to see that there are two types of peaks, taller peaks and shorter peaks. The taller peaks are associated with the flow that is being driven by the piston moving fastest, in this particular fluidic device – when the piston is under vacuum actuation. The smaller peaks are generated by the piston displacement when the piston is moving slower, being actuated a positive pressure. The negative

30 actuating pressure value is greater than the positive pressure value and therefore induces a greater rate of displacement of the piston. For example, the negative pressure for

actuating the membrane diaphragm of the pump is about -8 or -10 psi, while the positive pressure actuation of the piston is under about 4 psi.

The signature shown in Fig. 100 is indicative of normal operation. In the case where a pneumatic interface was improperly sealed or seated, then these peaks heights
5 would occur at different levels, outside of normal acceptance range, so this is a type of failure mode that could be detected. Another factor involved here is the flow rate, which depends not only on displacement volume of the piston, but also on the impedance of the fluid in the microfluidic channel. If the impedance is increased by the addition of blockage from a contamination source or some other problem, such as a detection
10 element being misplaced in the channel, then the nature of the signature structure would be different from what is expected and shown in this graph.

The trace shown in Fig. 100 and the detected values in Figs. 98a-99, are acquired by capturing the fluorescence intensity during steps of the assay by an imaging system shown diagrammatically in Fig 26. It has an objective lens and a series of optical
15 elements. An excitation beam from a laser is introduced to the monitoring location at a microfluidic channel, Fig. 102. The optics transform the stimulated fluorescent object, see Fig. 102, to an image plane shown as a photo detector (but in a preferred implementation, a CCD camera). The intensity of the pixels within a region of interest (ROI) captured on the camera are summed (integrated) to produce a single intensity point
20 for that frame for that moment in time.

In Fig. 102 the laser beam is shown in cross-section as an oval while a rectangular box circumscribing that oval illustrates the region of interest (ROI) over which the pixel intensities are integrated to produce a single resultant signal point. That value at this point in time is plotted as a point on the graph in Fig. 100.

The scanning system is adapted, during reading of assay results, to interrogate a
25 detection element on which assay capture agent is immobilized, see the Scanning Figures described later herein. But in monitoring mode, as depicted in Fig. 102, by relative movement between optics and microfluidic system, the system is focused on a selected monitoring point on a fluid-carrying channel at a point in which the detection element is
30 not present.

The optical arrangement of Figs. 101 and 102 is used to generate the signal trace in Fig. 100 or the “snap shot” at a monitoring point in Figs. 98a-98c, or the measurements over time of Fig. 99.

Another important use of the novel tracer detection technique is identifying the
5 location of microfluidic channels with high fidelity within a portable cartridge (cassette), relative to a cartridge position that is subject to some variation in position on a scale relevant to the small features of the microfluidic cassette. This is described further on, after describing a preferred implementation of a scanning/assay conducting instrument.

In a preferred implementation, a portable microfluidic cartridge 2 is placed into an
10 operating and scanning instrument by the user. It enters in a receptacle or reception area 6 at which the cartridge is retained for conducting the assay while scanning.

A suitable receptacle is shown in Figs. 54 and 54a, and the relationship of the receptacle and cassette when in assay/reading position is shown in Fig. 55 and the detail of Fig. 29a. An implementation of the overall system is shown in exploded view, Fig. 56.
15 Fig. 56 includes x, y precisely movable stage 13 that moves the cartridge on its carrier relative to the stationary objective lens.

Referring to Fig. 54 and 54a, cartridge 2 and the cartridge receptacle 6 are shown with a clamping mechanism 12 and pneumatic interface 8. A series of computer-operated solenoid valves 9 that move on the stage with the cassette apply positive and negative air
20 pressure to ports that interface with the positioned cassette.

Referring again to Fig. 102, for a suitable instantaneous image size, it is appropriate to use an excitation beam imaged through the objective lens to a spot size of approximately 12 microns wide by 250 microns long. The region of interest (ROI) of the camera that includes that spot has an area of approximately 35 microns width by 250
25 microns length. The microfluidic channel of the cassette that will be monitored has a channel width of approximately 180 microns.

Three scenarios for monitoring the tracer dye with such a beam are: (1) Continuous Scanning Modality. The microfluidic channels are scanned e.g., with substantially constant velocity across all channels of a microfluidic system, such as on a
30 cartridge. In that case, the beam crosses over the channels and measures the fluorescence intensity as a function of position or as a function of time, as the channels are crossed.

(2) A Rapidly Shift-Momentary Dwell Modality. In this case the detection axis is moved rapidly relative to the microfluidic system, to a selected location, followed by a momentary dwell, e.g., of a few seconds, in which the scanning system stops, and the optical detection system stares and collects tracer information or tracer signals as a function of time at the fixed location. The sage then moves on to another location to repeat the stare and this is done for a large number of locations over the micro-fluidic system in a short period of time. (3) Long Stare Modality. In this case a single location is selected at which the system stops and starts for an extended period of time, e.g., many seconds or even minutes. It can characterize a specific pumping or fluidic operational step. It is especially useful if there is suspicion about that particular location, determined as a result of one of the prior two scanning modalities.

The system and method have aspects useful for monitoring during operation of every assay and others that are useful as a diagnostic tool for design.

In respect of monitoring every assay, the Scanning Modality is especially useful. It is used to scan across all channels of a microfluidic system, e.g., on a cartridge, repeating this during each phase of execution of the assay. It can also stop at various locations for a short period of time to collect a trace at that location, and stop long term for staring to characterize the flow over time even in the case of monitoring usual assay function.

For a presently preferred implementation of a microfluidic cassette and how scanning can be accomplished, refer to Figs. 57A, B, C Fluidic /Pneumatic Architecture and Fig. 58 Fluidic Architecture and Protocol.

Fig. 57 illustrates a microfluidic configuration within a cartridge, illustrating both a series of fluidic networks and a pneumatic channel network to actuate on-board membrane micro-valves and micro-pistons in the fluidic network. The fluidic network in Fig. 57A comprises eight discrete microfluidic circuits closed by an over-lying elastic membrane, e.g., a continuous layer of PDMS. Each of those circuits has a number of microfluidic channels, valve locations, and piston locations. Portions of this membrane are located at formations in the channel that define valve and pump cavities. The corresponding portions of the membrane define movable elements of the valves and the piston.

The pneumatic channel network Fig. 57B is shown as an overlay in in Fig. 57C. It matches the fluidic network with respect to the various features that need to be actuated.

Fig. 58 is a magnified view of one of the circuits of Fig. 58, illustrating a number of the micro-features including valves, pistons, and the various reagent or reservoir inputs including the sample, the buffer (wash), the assay reading dyes, the secondary antibodies and the waste. The four elements GNR shown in black in each of the four individual (isolatable) channels represent glass nano-reactors (GNRs) embedded in those channels. This illustrates the basic micro fluidic unit replicated a number of times in the cartridge depending on the number of samples that the cartridge is designed to accommodate. The assay protocol flow sequence shown at the left of Fig. 58 starts with the prime flow step, and is followed by sample step, wash step, secondary antibody step, another wash step, a dye step for reacting to attach reading dye to the captured moiety, and finally another wash step. This illustrates an example assay sequence capable of being performed in this microfluidic structure. Each one of the fluids: sample, secondary (e.g., secondary antibody), wash, and reactive dye is caused to flow from its respective inlet well by activation of the pumps formed by each piston and upstream and downstream valves, with the end result of captured moieties at the detection elements that are labeled with the reactive dye, ready for reading to quantify the result of the assay. Examples of volumes employed on this device include the sample at 20 microliters, a buffer of 150 microliters (as shown in the table)—the total volume of the microfluidic circuit is approximately 1.8 microliters.

Fig. 61 illustrates the same microfluidic unit in a schematic fashion, showing four independent fluidically isolated microfluidic channels in each of which the respective piston and valves define a pump dedicated to that channel. The Figure illustrates controlled pneumatic control lines overlaid, terminating at a number of circles containing x's that represent valves. The horizontal lines with arrows labeled "Find channel scan 1" and "Find channel scan 2" illustrate the path that a scanner or a stage carrying the microfluidic system moves in order to identify the fluorescence intensity, and thus identify the location, of each of the fluidic channels. As the relative scan motion moves along that path, it will produce a trace shown adjacent to the schematic. The upper and

lower black and white traces shown in the center illustrate the fluorescence intensity being high in channels 1, 2, 3, and 4 based on the peaks shown on the traces. This illustrates that the scanner while scanning across that path encounters high fluorescence because of benign tracer in liquid in each of the channels. The locations of the channels are then determined very precisely by taking the encoder information superimposed on that trace. The precise location of those channels is thus determined relative to the absolute coordinate frame of the scanning system. It is possible now to produce high resolution and highly aligned scans because the precise locations of the scans are now determined with respect to the x, y stage. Thus, while a holding system firmly fixes the position of cassette within the operating scanning position, its exact location at the level of micron accuracy need not be precise because of the trace-determined high accuracy detection. An example of a system that performs these operations is described below in relation to the scanning Figures.

The primary benefit of the approach described, of precisely identifying the location of the channels, is to relax the requirement that the cartridge be precisely aligned on the stage by the user.

It has to be fixed relative to the stage but only within a fairly course acceptance range. A benefit of the technique is that it does not require precise absolute (and costly) positioning by the user. It permits the presently preferred clamping implementation about to be described.

The apparatus for conducting the assay and simultaneously scanning for benign tracer presence will now be described with reference to Figs. 54-56. In Fig. 54, assay cartridge (cassette) 2 is shown above carrier plate 4, in preparation for being placed into receptacle area 6. When placed, the cartridge will make intimate contact with pneumatic interface 8, so that pneumatic controls (solenoid valves 9) can actuate appropriately to apply air pressure and vacuum via interface 8, to actuate the micro-valves and pistons on board the cassette and thus perform the assay. The cartridge is retained in the receptacle interface 6 by retaining clamp 12. In Fig. 55, clamp 12 is shown with the cartridge in place in receptacle area 6. In Fig. 54A, the retaining clamp is shown in the process of being closed. Figs. 55, 55A, and the exploded view of Fig. 56 illustrate the relationship between the carrier plate 4 and cartridge 2 in it and the rest of the mechanical assembly of

the instrument in an exploded view. The precision x, y stage 12, chassis 16, heat plate 14, and optic subassembly 18 are shown. Not shown is an enclosure for the system that excludes ambient light from the cartridge or other microfluidic assay system, and from the optical system, such that ambient light does not interference with fluorescent
5 excitation and detection during performance of the assay and during the reading of assay results.

Referring especially to Fig. 55A, a further magnified view, the pneumatic interface and the clamping pneumatic interface 8 are shown with the cartridge 2 in intimate contact with pneumatic interface 8 while the clamping anvil 26 is resiliently
10 compressed, providing a force compressing the cartridge against the pneumatic interface. The clamp is held in its down position by a latch.

Previously described in earlier provisional application regarding the finding of the channels algorithm (as Scanning Figs. 79, 80 and 81, below), we have Figs. 62, 63 and 64. Fig. 62 again illustrates four isolation channels and the path of the scanning sweep
15 performed to identify the precise location of each of the channels. Fig. 63 shows a trace obtained by such a scan using white light illumination as opposed to using fluorescence with benign tracer, by laser-based epi-fluorescence process. The signal shown in Fig. 63 illustrates a high level of signal followed by fairly small dropouts or spikes illustrating where the edge of the channel or shadow is formed as a result of white light illumination
20 as it impinges upon a channel. One can see in the trace that the signal change is a fairly small percentage of the overall background signal, the signal dropping from approximately 3500 counts to just under 2300 counts. This low signal makes the signal processing potentially challenging in that small aberrations or perturbations of the signal caused by other means could interfere with true identification of the channel. Fig. 64
25 shows a magnified view of two of those spikes illustrating both the left and right edge of a channel as the scanner proceeds across the channel. The description of the present invention utilizing a benign tracer fluorescent dye provides for much greater signal to noise level for this particular trace, and therefore is a significant improvement that can be substituted in the system. The signal level outside of the channel where there is no
30 fluorescent dye present is only the material use in the construction of the cartridge. The fluorescence of those materials is exceedingly low whereas the fluorescence found when

crossing over a channel containing liquid with fluorescence dye is exceedingly high, giving a much higher signal to noise level, and therefore greater accuracy and robustness.

In summary, a number of points of great value of the invention will be reviewed. An assay is performed under strictly controlled assay conditions, e.g., heated uniformly, and the controls (e.g., pneumatic valves and pistons) are controlled precisely. This is done while some or all of the assembly is detected, e.g., translated in x, y coordinates relative to an optical axis of a detector (e.g., camera), such that the cassette is simultaneously detected, and tracer condition determined within the various channels while the assay is run. In advantageous implementations, the very same detection instrumentation is later used to detect assay results from capture agent.

A simple technique to implement, having significant value, uses a scanner during the assay protocol to simply detect the presence or absence of the tracer dyes at the various phases of performing the assay protocol. The scanning process generates signal patterns that are compared to predetermined anticipated levels associated with the normal performance of the assay on the cartridge.

This may be readily performed by computer computations, or empirically, based on acceptance levels defined from a number of experimental runs to determine normal levels are and some acceptable range of those levels.

This tracer-based process provides great value in determining whether the assay or cartridge did what it was intended to do during the assay run.

The invention provides an entire system of monitoring methods that can be employed in coordinated fashion to address the previously described failure modes, and others. For example, another failure mode not previously discussed is in the controls based in the bench top operating and scanning instrument itself. If a control fails, e.g., a pneumatic pressure controlling solenoid valve, this failure is also detected. Thus the invention provides a generic means of detecting a host of potential failure modes during a microfluidic assay system run and especially determining whether a reagent is present or not in that channel, and if it is the proper reagent in that channel.

Similarly, the invention enables simply scanning across channels with benign fluorescent dye for the purpose of precisely locating the microfluidic channels, for setting up scan parameters, e.g. for the purpose of identifying optimal focus location, another

important feature of significant value. This is in the set up process during the execution of the assay protocol that is described further within, in relation to the Scanning Figures. While the cartridge is running the assay protocol, e.g., under pneumatic protocol, the scanner system can simultaneously be used to perform a number of measurements useful to the later detection phase when the assay is completed. Those measurements include locating the channels based on the fluorescence properties of the channels containing liquid with the tracer dye. Also for determining the optimal focus location – scanning of the z-axis or the focus axis to determine the optimal location of the focus based on the fluorescence intensity profile as well. These are for use in the final scanning performed after the fluidic phase of the assay to make the quantification.

The benefits of the concept primarily pertain to making a robust operational system for making quantitative immunoassay measurements. The reproducibility and the enhanced validity of the data quality provides value for this approach.

Process controls are routinely used in the art in measurements, for example, in ELISA plates, controls are used as individual wells on an ELISA plate. Researchers when running any type of instrument always want to know or have positive verification that the measurement that they made is believable, and it is performed the way it is expected to be performed. The present invention is a means to producing that confidence in a microfluidic system and the data that the system produces.

Using the concepts herein, one may provide reliability scanners or monitoring scanners, independent of any particular type of microfluidic system. Such scanners can be used by anyone running a microfluidic assay to monitor how it is performing.

But the invention is particular beneficial to a microfluidic cartridge in which a series of reagents are flowed in different volumes and different timings, to positively identify that each one was performed properly.

The fluorescent dyes used for benign tracers inherently should not interact with the components of the assay. However if chemical interaction were found, it would be routine to chemically modify the dye to make it more inert or more benign with respect to interferences in the system. There are known conjugations that can be performed on the dye mark for such purpose.

The invention has special utility in respect of complex microfluidic based systems that run a sequence of reagents, assays where quantification is the primary outcome of the measurement

Many preferred embodiments leverage what is already available in a system for making quantitative analytical measurement, e.g., existing systems in which analytical measurements are being made over a number of locations and therefore require a moving system to put the cartridge in those locations or move the detector to those locations on the microfluidic system, e.g. cartridge. Techniques following the invention can be readily retrofit into such systems. The invention is particularly applicable to situations in which the fluidic component and the optical components can perform simultaneously and the assay environmental requirements can be met. Temperature is an important parameter to be controlled during the assay protocol. The temperature is typically controlled for immunoassay systems at least to plus or minus one degree and preferably plus or minus ½ degree C. In addition, absence of ambient light or stray light is beneficial, so the assay is performed in a dark environment, as provided by an enclosure. The techniques of the preset invention are compatible with these requirements.

Some of the techniques described have accuracy beyond what ordinary blood laboratories can accomplish. For instance, the GNR's may be useful in stationary, non-portable microfluidic systems, to make very accurate measurements. Techniques of monitoring described have applicability in such instances, e.g. in high throughput blood testing.

This the inventors envision the invention applied to large, less portable higher throughput machines having a high degree of accuracy in a diagnostic type of environment. The main point is that the diagnostic measurement must come with a certain degree of certainty in its accuracy and the novel method of enhancing the degree of certainty in the outcome provided here has wide utility.

A unique assay system will now be described, which involves pneumatically actuated valves and pistons for delivering precise volumes of reagents throughout a microfluidic disposable cartridge.

It is desired to have a bench top operating and reading instrument that can operate the assay cartridge to conduct an assay through timed operation of pneumatic

valves on the cartridge that cause flows of fluids that have been placed on the cartridge prior to the assay, and to thereafter read the results.

The instrument needs to have significant robustness in terms of the useful life of the instrument.

5 It is advantageous to provide the cartridge instrument interface on a movable stage capable of transporting the cartridge in X, Y scanning motions relative to a fixed axis of the optical system that determines location of the cartridge and fluidic channels in it, and monitors progress of the assay and reads the results.

10 A key requirement is that the cartridge must interface with a pneumatic control component on the instrument in reliable fashion so that no pneumatic leaks occur between the cartridge and the pneumatic actuation system. The valves and pistons on the cartridge are controlled by pressure and vacuum provided by the instrument, and if a leak were to occur at the interface between the cartridge and the reader, those valves would not actuate precisely and reliably. The result would be imprecise control of the flow of
15 reagents on the cartridge and uncertain results with regard to the assay. This is because the assay depends upon precisely timed actuation and metering of reagents, precise volumes and precise times for exposure of the unknown sample to the capture agent, the subsequent flushing and washing of the sample prior to mixing, and exposure with secondary capture agent and then followed by the subsequent washing of that component
20 followed by exposure to a fluorescent dye, or regarding the last two steps, alternatively, exposure to a fluorescently labeled secondary agent.

25 The concept for reliably making that pneumatic interface involves a compliance component as part of the cartridge. In its preferred form, it is in the form of silicone rubber layer as part of the cartridge that mates with a robust, rigid port component contained in the instrument. The rigid component contains a number of vias through which the pneumatic actuation is provided in the form of pressurized air or vacuum.

30 One of the important features is the novel arrangement by which both a compliant component and a rigid component are provided, that are brought together under force to form an airtight seal. The beneficial relationship is that the compliant

component is located on the disposable element and not on the non-disposable side of the reader. The benefit is that the rigid component has a much longer life than a compliant component, as the rigid component would not undergo deformation over time, whereas a compliant component such as silicone rubber and other forms of rubber or even plastics would undergo inelastic deformation which eventually would lead to failure mode in the form of a pneumatic leak.

In the preferred implementation, the rigid component located on the operating instrument is metal, either aluminum or steel, and the compliant component is PDMS or silicone rubber carried by the assay cartridge.

The rubber is exposed and advantageously is provided as an extension of one of the layers within the cartridge. It is provided on the bottom surface of the cartridge, while the wells and reservoirs of the cartridge are provided on the top surface. The thickness of the silicone rubber is approximately 100 microns. In the preferred implementation it spans the entire surface area of the cartridge which could be 120 millimeters by 85 millimeters, and its durometer is about 30 shore A. A clamping system is provided to place pressure to bring the two together to form a seal that is maintained at numerous pneumatic vias. In the example, there are seven vias positioned in close proximity. For example the spacing between vias is approximately 2 millimeters and the via diameter is approximately one millimeter.

One of the important features of the pneumatic manifold interface is that the rigid component on the instrument is constructed to have a small cross-sectional area of contact. In this manner, only a small force is required to create significant physical pressure upon the compliant material. The pressure locally compresses the compliant material, thereby providing assurance of a leak-tight seal. The small area is achieved by providing a pneumatic manifold interface with very small dimensions in both the length and the width. In the example, the entire width of the interface seat is approximately 3 or 4 millimeters wide by 10 millimeters long, that includes all seven of the vias. When the cartridge is in operating position on the operating/reading instrument, its end with pneumatic interface vias rests on the rigid pneumatic interface part of the instrument, the other end of the cartridge being supported by a fixed seat which holds the cartridge in location. X, Y direction constraint is provided by a set of four corner retaining stands,

these being tapered to enable easy insertion of the cartridge into the thus-formed receptacle pocket.

Pressure is applied to the cartridge only in one location to obtain stability in the Z coordinate. The force is applied downward through the cartridge in one
5 embodiment by a roller connected to a leaf spring, the roller arranged to contact the top surface the cartridge and provide a downward force which compresses the compliant material on the cartridge against the pneumatic manifold. In another implementation, as shown here, a simple releasable clamp applies the pressure.

There are alternate techniques to achieve the clamping force as will be
10 understood by a skilled person, including spring-loaded mechanisms, roller mechanisms, and motorized rack and pinion. A pneumatic solenoid or an electrical solenoid can similarly apply force to maintain the connecting pressure.

Cost effectiveness and simplicity are usually the objective when designing the clamping mechanism. A motorized roller is a convenience from a user point of view,
15 but a swing bar as shown is simple, effective, and avoids potential failure modes.

The other significant feature on the movable stage is the pneumatic interface manifold. The pneumatic control lines, the seven different pneumatic control lines in this implementation, are controlled by solenoid valves that are carried on the X, Y stage and connected via flexible hose to a pneumatic manifold in pneumatic
20 communication with a vacuum pump and a pressure pump.

An important advantage of putting the pneumatic valves on the stage is that only two flexible pneumatic lines and a flexible electrical control cable are required to move during the motion process of the stage, so that the pneumatic channels connecting the solenoid valves to the cartridge are hard fixed channels as part of the
25 pneumatic interface. One of the advantages is reliability of using fixed machined channels in a robust metal or plastic component compared with flexible tube connectors that over time tend to fail. Another beneficial feature of having the pneumatic interface as a fixed machined component mounted on the stage is its contribution to the desired speed of the assay. This relates to the ability to minimize the dead volumes of the
30 pneumatic passages. This is important in the operation of the cartridge because the valves and the pistons change states as a result of switching from either a vacuum state to

a pressurized state. The speed at which that occurs is directly proportional to the dead volumes in those channels. For example, switching from pressure to vacuum requires the vacuum to be completely drawn on whatever volume is contained downstream of the solenoid valves. Using the features just described, all of the downstream channels from the solenoid valves are extremely small, and the distance between the solenoid valves and the chip is maintained in a very short distance. Yet another advantage of having the pneumatic interface as a fixed machined component mounted on the stage having a low dead volume is in the ability to use low flow rate, and therefore inexpensive vacuum and pressure pumps. Since the volume of the pneumatic lines downstream of the solenoid valves and the rate of states changes determine the average flow rate, it is desirable to keep the dead volume low so as to allow the use of smaller, low flow rate pumps.

Speed is important because a large number of actuations must occur throughout the assay protocol. There can be tens of thousands of actuations and even tens of milliseconds or hundreds of milliseconds difference can add up to a substantial loss of time.

There are two different active components on the cartridge, valves and pistons. The purpose of the valves is to determine which reagents flow and to which channels they flow. The pistons are the primary components for motivating the fluid. They provide the positive and negative displacement to the reagents located on the cartridge, and so are the primary elements for motivating fluid.

The features contained on the stage are the pneumatic interface with the solenoid valves and cartridge and the clamping device. That is all that is on the stage that moves. The cartridge on the movable stage is exposed to a fixed heater plate underneath, supported only 4 or 5 millimeters below the surface of the cartridge and exposed face-to-face for radiant heat transfer. The bottom surface of the cartridge where the active capture elements are contained in the microfluidic channels must be maintained at a constant temperature between 35 and 37 degrees C. The heater plate extends in the dimension that is actually slightly larger than the surface area of the cartridge to cover its range of travel. A uniform temperature profile is thus maintained over the surface of the cartridge. The biggest advantage of that is that the temperature of the cartridge is easily maintained without having to control the temperature of the entire reader enclosure. Thus

there is no concern about heating the electronics and other sensitive components within the enclosure. Temperature control and stability is only provided at the critical surface.

Further, it is easier to control the temperature much more precisely in this fashion, using radiant heat, than it is using a convective process. It also enables temperature
5 stabilization to be achieved much quicker than by a convective process. This is important for antibody kinetics. Antibody binding is temperature dependent so accurately controlling that temperature is an improved way of controlling the rate of the kinetics.

This is particularly important because many of the assays desired to be run are “single point assays”. The assay is run for a fixed amount of time and the resulting
10 fluorescence signal is proportional to the concentration of the analyte in the sample. Any other parameters that affect the fluorescence needs to be controlled precisely so that the variable is the concentration of the analyte. Temperature is a significant parameter that affects the binding kinetics between the antigens and the capture antibodies. And so that needs to be controlled so that potential source of variation it is taken out of the equation.
15 Also, a convenient feature for a user to be able to just turn the instrument on and within a minute or two provide their cartridge and execute an assay run.

One of the features of the instrument is to excite a fluorescence signal using a laser and then capture that fluorescence with an objective lens while the stage is translated. A well-known epifluorescence configuration is employed in which the
20 excitation signal, provided by a laser or laser diode, is sent through an objective lens and then the returning fluorescence is captured by the same objective lens and sent to an imaging CCD camera. The heater plate which held fixed directly underneath the cartridge is provided with a hole that allows both the excitation and the emission signal to propagate through to the fixed optical system.

All objective lenses have a so-called “working distance.” Key features of an objective lens include numerical aperture and magnification, which will determine the ability of the objective to capture the fluorescence intensity in a very efficient manner and image that back to the CCD camera. The working distance for typical 10x objectives is somewhere between 5 and 12 millimeters. It is important to maintain a distance of
30 somewhere between 5 and 12 millimeters between the objective and the bottom surface of the cartridge.

This critical distance is achieved despite the intervening presence of the heater plate. This is achieved by the heater plate being a thin aluminum plate, about one eighth of an inch thick with very thin heater strips, e.g., 1/32" thick, adhesively attached to the aluminum heater plate.

5 The total distance between the objective and bottom of the cartridge is approximately 12 millimeters, the thickness of the plate is a small fraction of that, it is 4-6, and the plate itself has a hole that allows the light to transmit through. The hole is sized such that the objective is brought up into the hole itself, fitting partially into the plate.

10 Sequence of Operation

 The sequence begins with placing the cartridge onto the cartridge receptacle pocket, then sealing that cartridge using a clamping mechanism, then after
15 warm-up, actuating the pneumatic valves which forces the reagents including the buffers and the samples and the detection antibodies to flow in a very specific sequence for a specific period of time allowing incubation to occur. The incubation results in the binding of the unknown antigen in the sample to the capture moieties contained in the cartridge. The various reagents flow in a given sequence with intermediate wash steps followed
20 finally by a fluorescence scanning process

 In some cases, the stage remains stationery throughout the entire incubation process. In other cases, described herein, the stage is moved during incubation to enable visual monitoring by use of tracers in the fluid. The final reagent
25 process is to flow detection dye or fluorescent dye through the channels which then binds to the immobilized secondary antibody. During this process, if not previously done using tracer dyes, it is necessary to move the stage because it is during this process that the fluorescence dye found in the channels is used as an identification beacon for identifying the location of the channels. The optical system is actually used in coordination with motion control system to identify the location of the channels by exciting with the laser
30 the fluorescence in those channels and the locations of those channels are identified as a result of an increased signal where the channels come through.

The bench top unit implementation described here contains no liquids and no liquids flow between cartridge and the bench top unit.

In the following, we describe other novel details of a preferred bench top unit.

5 Referring to the Figures, the bench top unit has just a few key subsystems. The subsystem that holds the cartridge. The cartridge is placed into a little receptacle area and located in that receptacle area is the pneumatic interface boss that has limited end surface area (“lip area”) for contact with the cartridge. It protrudes off of the surface that is the highest surface. One end of the cartridge sits on that boss. The other end of
10 the cartridge sits on a small rail on the other side of this containment area. These are corner guides that make it easier to place the cartridge. A small arm contains on it a little spring loaded containment clamp. The spring loaded clamp bar comes down and rests on the top surface of the cartridge, and pushes the cartridge down on to the pneumatic boss.

On the opposite end is a lock and catch that holds the device in its
15 clamping position. The user pulls down on part of the arm until it clicks and locks into the catch. Because of leverage, the user need not apply great force. A force of 4 or 5 pounds is effective to push the cartridge down against the pneumatic boss sufficient to guarantee a sound pneumatic interface seal.

Also on the subassembly are, not shown here, there are a number of
20 pneumatic channels that lead back to the manifold with valves located on it, carried by the X, Y stage. These are the ports for vacuum and pressure. Each of the actuation ports is controlled by a solenoid valve in the valve bank. It can switch each one of these ports to either vacuum or pressure.

In the epi-fluorescent optical system is a laser diode, red laser diode, a
25 collimator lens, a cylindrical lens, and three filters. An excitation filter ensures any of the excitation light is within a certain wavelength band. There is a dichroic beam splitter which has a high reflectivity for the red of excitation 640 nanometers, but very low reflectivity for the deeper red that comes back as a result of the fluorescence, around 680-690 nanometers. The reflects 640, but 680 transmits. The 680 coming through hits
30 another filter, the emission filter. This allows only a small band – it blocks all red, and it allows a small band. Following this is a focusing lens onto a camera, called a tube lens.

A cylindrical lens in the infinity space between the collimator and the injector provides a stigmatic beam at the target. This produces a laser beam at target of very long elliptical profile. The beam is approximately 500 microns long by about 8 microns thick. It is like a line. The instrument scans that line down the channels to illuminate the whole width of the channel.

Scanning an array of fixed micro-flow elements

The novel arrays of elements described above are useful only if effectively read after the fluid assay is performed. The following scanning apparatus, procedures and methods for automatically scanning a microfluidic chip effectively solves the problem with arrays of micro-flow elements, and in particular, micro-length tube elements.

2 Scanner Description

2.1 The Scanner

2.2 The Scan

2.3 Chip Layout

2.4 Find Channels

2.5 Find Elements

2.6 Auto – Focus

2.7 Auto – Exposure

2.8 Fluorescence Scan

2.9 Scan Data Processing

2.9.1 Load Data

2.9.2 Break data into segments

2.9.3 Thresholding

2.9.4 Locate Elements and Background in time history

2.9.5 Aggregate element mean RFU's into results

Introduction - Scanner

The general scanning concepts of invention are given in the block diagrams and flow charts. These are followed by description of a specific, novel implementation. As a precursor to the specific method to be described, the general capabilities of the scanner, a scan, and the general microfluidic chip layout will be described first. These three

sections are then followed by each sub-procedure in sequence that contributes to the overall method for automatically scanning a microfluidic chip.

The Scanner

The scanner utilized in this method is a fixed, inverted epifluorescent microscope
5 equipped with a three axis (x, y, and z) stage for motion of the chip to be read, a CCD camera for bright field imaging and fluorescence detection, a diode-pumped solid-state laser for excitation, and a white LED for bright field illumination. A cylindrical lens is used in the laser optical path prior to any filters to expand the beam size. This allows the excitation of a larger surface area in a single pass and allows for some flexibility when
10 placing the elements in the flow channel during chip manufacturing. All of these scanner components are controllable via a computer as follows: on command x, y, z motion, image acquisition / imaging settings, laser on/off, and LED on/off. The method described herein uses various sequences and combinations of the scanner control / acquisition to orchestrate the automatic scan. See Scanning for a general schematic of the scanner.

The Scan

A scan is comprised of a sequence of steps configured with start / end (x, y) positions, z (focus) position, velocity, and a segment number. When the end position of a step in the sequence is not the start position of the next step, the stage will make a full speed move to the x, y, z position of the start of the next step in the sequence, and no data
20 is collected during this rapid move. While executing one of the steps (moving from start to end in x, y at a fixed z) data is collected versus time. The data that is collected includes time, the step segment number (assigned while configuring a scan step), the present x and y positions, the camera settings (gain, exposure etc.), and information extracted from the images in the video stream from the camera. The information
25 extracted from the camera video is based on a region of interest (ROI). An ROI is defined as a rectangle somewhere in the image. The pixels within the ROI are processed to extract information from the image. For example the mean, median, standard deviation, max, min, etc. of the pixels inside the ROI from a given image are computed and included in the data collected during a step move. At the conclusion of a scan, the
30 data collected throughout the sequence of steps that comprise the scan is written to a file (see sample in) that may then be processed to extract desired information.

The ability to flag various steps in a scan with a unique segment number that is subsequently written into the scan data file (“seeded into the file”) greatly facilitates processing of scan data files and significantly improves the robustness of the various signal processing algorithms. This capability is uniquely made use of a number of times
5 throughout the auto-scan method.

All of the sub-procedures described in the subsequent sections are based on the scan just described above, with the exception of the Auto-Focus sub-procedure.

Chip Layout

A priori knowledge of the chip features / layout is necessary to facilitate
10 automatic scanning. A chip layout is depicted in Scanning. In this Figure, the chip itself is a 25 x 75 mm standard microscope glass slide. Within the chip is the scan zone that contains the elements of interest. The ‘scan zone - zoom’ provides more detail. In particular, Scanning shows that there are a number of fluidic channels (1 thru n left to right in the Figure) and there are a number of elements per flow channel (1 thru n top to
15 bottom in the Figure). It is the elements that are fluorescing are to be scanned in detail. The vertical lines in the middle of the flow channels depict the scan moves that are executed for the purposes of locating the channels, and the horizontal lines above and below the elements depict the scan moves that are executed for the purposes of locating the elements and subsequently performing the fluorescence measurements.

Find Flow Channels

The first step in the automatic scan is to determine where the channels are located relative to the stage x, y positions, and to also determine the skew of the chip in the event that the channels are not exactly parallel with the vertical axis. Predefined x, y positions based on a previously complete homing of the scanner stage are sufficient for
25 guaranteeing that the scan executed will, in fact, pass over all the channel edges, given that the chip is mechanically referenced to the stage. The issue is then to determine precisely where the channel edges are relative to the x, y stage positions. To this end, a ‘find channels’ scan is executed following the horizontal lines in the middle of the channels shown in Scanning . More specifically, the find channels scan is broken into
30 distinct steps such that there is a step across each individual channel at the ‘top’ and the ‘bottom’ of the scan zone that is tagged with a unique segment number in order to

facilitate subsequent data processing. Further, the scan is done in bright field (i.e. laser off, LED on), the ROI used is very narrow in width and extends the full height of the image (as show in Scanning), and the z position is intentionally ‘defocused’ from a nominal focused z position of zero, as established by homing the stage.

5 Alternatively, the ‘Find Channels’ routine can be done during the ‘detect’ flow phase of the chip assay. In this mode, the channels are filled with fluorescent dye. The scan to find the channels is then done as described above but the scanner is in fluorescence mode (i.e. laser on, LED off). This has the advantage that the signal to noise ratio is very high.

10 An example of the data collected during a ‘find channels’ scan is given in Scanning and a zoom in to a single channel scan is given in Scanning. In this example the ‘find channels’ scan has eight steps, one for each channel crossing above and below the elements.

A scan data file from a find channels scan is processed on a per scan segment basis. Consequently, the data processing operates on a set of data as depicted. The data processing proceeds as shown in Scanning. In the Figure, the data processing will produce a scan configuration that can be used for the find micro-length tube elements procedure, or it will throw an error that will halt the auto-scan procedure. The information collected during this procedure is useful for:

- 20 • Defining the Find Elements Scan.
- Defining the Fluorescence Scan.
- Collecting Chip Quality Control data about:
 - Variations in channel width
 - Variations in channel to chip reference edge

25 **Find Micro-length tube Elements**

The primary goal of find elements is to locate the first micro-length tube element in each flow channel. This information is then subsequently used for executing the auto-focus and auto-exposure procedures. The find channels procedure must be a precursor to this procedure in that the scan utilized by find elements is built via knowledge of the channel positions. The find elements scan is broken into a segment per channel and follows the horizontal lines as depicted in Scanning. This scan is done in bright field (i.e.

laser off, LED on), and the ROI used is wider than a channel in width and has a height greater than the length of a micro-length tube element. This ROI is shown in Scanning. Further, the scan z position is intentionally ‘defocused’ from a nominal focused z position of zero, as established by homing the stage.

5 An example of the data collected during a ‘find elements’ scan is given in Scanning, and the processing sequence of the ‘find elements’ scan data is given as a flowchart in Scanning. The results of processing this scan data are the x, y position of each element in each channel. The information collected during this procedure is useful for: The x, y positions to execute Auto-Focus.

- 10
 - The x, y positions to execute Auto-Exposure.
 - The x, y positions for processing a Fluorescence Scan.
 - Validating that the predetermined number of elements are in fact present on the chip.
 - Collecting Chip Quality Control data about element placement.

15 **Auto – Focus**

This procedure takes as input the x, y positions of the first micro-length tube element in each channel as determined by the ‘find elements’ procedure. For each of these positions the procedure moves to the given x, y position and conducts a sweep of z from a negative position thru zero to a positive position. While the z sweep is taking
20 place the full images from the camera video stream are run thru a Sobel edge detect filter and then the resulting image standard deviation is computed. The end result is a set of data as shown in Scanning. For each segment (i.e. at the x, y position of an element), the resulting z position versus standard deviation plot is then used to find the z position at the maximum value of the standard deviation (See Scanning for details). The z positions, at
25 the maximum standard deviation, from each segment are the ‘in focus’ z positions for each channel on the chip. The information collected during this procedure is useful for: Setting the focus for the Fluorescence Scan,

- Gauging the degree to which the chip / and or stage is not flat with respect to the optical system.

Auto – Exposure

The purpose of this procedure is to select the appropriate camera exposure setting in order to efficiently utilize the range of the camera given the fluorescence level of the micro-length tube elements. Too short of an exposure will lead to dark images, poor
5 signal to noise, and underutilizes the camera range. Too long of an exposure will lead to saturated images that cannot be used for collecting a fluorescence measurement. The degree to which a micro-length tube element fluoresces is dependent on the concentration of the targeted capture agent, e.g. antibody, in the sample under investigation and therefore will not necessarily be known in advance. As a result, the best exposure setting
10 must be determined in-situ, for each fluid channel in the chip.

This procedure takes as input:

- The x, y positions of the first micro-length tube element in each channel as determined by the ‘find elements’ procedure.
- Maximum pixel value range
- 15 • Exposure range and start value
- Scan Half Length
- Data Extract Half Length

From this input, a scan, as depicted in Scanning, is constructed. The auto-expose procedure then follows the sequence given in Scanning. The ROI used for this procedure
20 is the same as that used for the fluorescence scan discussed in the next section. This procedure is done with the LED off and the Laser on. To avoid significant photo-bleaching effects the velocity of this scan is selected to minimize the laser exposure incurred by the element. The end result of this process is the optimal exposure setting per channel to be used in the fluorescence scan discussed next.

Fluorescence Scan

Using the (x, y) positions, z(focus) and camera exposure settings found from the results of the ‘Channel Find’, ‘Element Find’, ‘Auto-Focus’, and ‘Auto-Exposure’ the Fluorescence Scan(FS) can be constructed. As with all measurements from the scanner, an ROI is used to collect pixel intensity values. The ROI for the fluorescence scan is a
30 rectangle oriented with the long side perpendicular to the flow channel and positioned in the image on top of the laser cross section (Scanning & 28. The size of the ROI is

determined by the size of the laser spot, the width of the fluorescent region on the micro-length tube element, the width of the channel containing the element, the number of pixels needed for a measurement and the scan speed. These parameters can be predetermined and optimized empirically and therefore do not change for each FS. The FS starts at a location upstream of the first micro-length tube element in the channel to be scanned. The scanner is put into fluorescence mode (LED: Off, Laser: On) and the camera exposure time and z position (focus) are set. A scan is performed (see section 0). Scanning depicts a snapshot of an element during a FS.

Scan Data Processing

This sections discussion is available as a flowchart shown in Scanning.

Load Data

The data collected from the FS is loaded into memory and the mean ROI value is plotted vs. time (Scanning). This Figure depicts the mean ROI value for all the channels scanned vs. time.

Break data into segments

To process this data, it is broken up into segments where each segment consists of one flow channel's worth of data (). A peak detection algorithm is used to determine the element positions in the channel with respect to the background signal. The micro-length tube element positions found during the 'Element Find' can also be used to locate the elements in the segment.

Thresholding

Since the relative signal intensities for all micro-length tube elements in one channel are approximately equal, k-means clustering can be used to separate the pixels associated with the background from the pixels associated with the micro-length tube element. The outputs of the clustering algorithm are centroids representing the mean background value and the mean element value. The mid-point between these two centroids is used as a threshold. Thresholding must be done on a channel-by-channel basis due to differing background and exposure settings per fluid channel.

Locate Elements and Background in time history

Once a suitable threshold is found, the mean time history gets filtered using a Savitzky-Golay (SG) filter and the peak detect algorithm identifies all threshold crossings

larger than a predetermined width, thereby rejecting of most of the high frequency noise in the data. Using the found threshold crossings the time history is further broken up into an element signal component and a background signal component. The element signal component comes from the section of the channel with the fluorescent micro-length tube element in it. The background component comes from the 'empty' section of the channel adjacent to, but downstream of the fluorescent element (). This allows each micro-length tube element to have its own background-offset correction. The center of each component is found and the data points $\pm 25\%$ of the element width are then extracted to create an average value for each component (See the highlighted points in Scanning). Only points about the center of the element and background are used in order to eliminate element edge effects. Since the signal rides on a background offset, the average of the background points is subtracted from the average of the element points and the result is normalized for camera exposure and finally stored as that element's mean RFU (Relative Fluorescence Unit). This is performed for each micro-length tube element in each channel on the chip.

Aggregate element mean RFU's into results

Statistics are done on all micro-length tube elements' mean RFUs for each channel and outliers are removed by finding the lowest % CV among all combinations of element means. A minimum number of micro-length tube elements must be retained for statistical purposes. These result values can now be applied to a standard dose curve to determine capture agent, e.g. antibody, concentration.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A method of flowing a fluid with a tracer in a microfluidic channel of an assay device and detecting the tracer for determining the channel location or condition of the channel.

2. The method of claim 1 of conducting an assay in which an assay fluid having a desired property must be present within a microfluidic channel at a given phase of the assay protocol, including the step of providing the assay fluid with a detectable tracer that is benign (e.g. inert) to the respective phase of the assay, and, during conduct of that phase of the assay, under required assay conditions, monitoring a selected region of the microfluidic channel with a detection system to detect the tracer, and comparing the detected results with a standard of acceptable results.

3. The method of claim 1 conducted to determine the precise location of a portion of a microfluidic channel relative to a detection system, including the step of providing a fluid with a detectable tracer, and performing a detection operation that locates the microfluidic channel or a portion of it by a detection system that detects the tracer.

4. The method of claim 3 conducted during a step of an assay in which the tracer is inert with respect to an assay fluid in which it is carried.

5. The method of claim 4 wherein a detectable property of the tracer is used to verify the assay phase.

6. The method of claim 4 wherein different values of the detectable property of the tracer is used to confirm the phase of an assay, e.g. different and unique concentrations of the a tracer are used for each phase of an assay.

7. The method of claim 6 wherein the property of the tracer is fluorescence intensity.

8. The method of claim 6 wherein the property of the tracer is optical density.

9. The methods of any of the foregoing claims, in which the detection steps are performed by an epi-fluorescent system employing a light to excite fluorescence within a

microfluidic channel, and to translate the beam with relation to the channel over a set of channels or along the channel for detecting position, monitoring, or assay-reading purposes.

10. The method of claim 9 wherein the light source is a laser.

5 11. The method of claim 10 wherein the laser beam has an aspect ratio of at least 2:1.

12. The method of any of the foregoing claims in which relative movements along a channel are used to index between monitoring locations, and to progressively read assay results, e.g. from one or a set of immobilized detection elements, such as
10 micro-length tubes (or glass nano-reaction vessels).

13. The method of any of the foregoing claims in which relative movements along a channel are used to determine the locations of immobilized detection elements, such as micro-length tubes (or glass nano-reaction vessels).

14. The method of any of the foregoing claims in which the microfluidic flow
15 channel has a flow axis, along which a series of discrete, axially-spaced apart, transparent hollow flow elements are secured in fixed position, each hollow flow element having at least one axially-extending flow passage through its interior, the elements having interior and exterior surfaces extending in parallel in the direction of the channel axis, and end surfaces extending transversely to the axis, the surfaces of the elements exposed to liquid
20 in the channel, and assay capture agent fixed to the interior surface of the elements for capture of an analyte in liquid flowing through the interior of the hollow flow elements, the device constructed to enable light to be transmitted into and out of the elements transversely to the flow axis for excitation and reading of fluorescence from captured analyte.

25 15. A device for performing the method of any of the foregoing claims.

16. The assay method or device of any of the foregoing claims associated with a positive-displacement pump arranged to introduce a segment of liquid sample, and cause

the sample to move back and forth with respect to a hollow flow element to produce capture of analyte only in the interior surface of the element, and to repeat this action for successive segments of liquid sample.

5 17. The assay method or device of any of the foregoing claims in which capture agent on the interior surface of a hollow element in the channel is configured to define a code.

18. The assay method of claim 17 in which the code is a bar code.

10 19. The assay method of claim 14 or a device for performing the method in which the active capture agent is an antibody.

15 20. The assay method of claim 14 or a device for performing the method in which the active capture agent is an antigen.

21. The assay method of claim 14 or a device for performing the method in which the active capture agent is an oligomer.

20 22. A micro-length tube element for use in an assay device comprising a hollow body with a through passage for liquid flow, wherein active capture agent for a given analyte resides on at least a portion of the interior surface of the hollow body for interaction with fluid sample, the capture agent on the interior surface of the element being configured to define a code.

25 23. A micro-length tube element for use in an assay method or device comprising a hollow body with a through-passage for liquid flow, wherein active capture agent for a given analyte resides only on a portion of the interior surface of the hollow body for interaction with fluid sample; the exterior surfaces of the micro-tube element being free
30 of active capture agent, and un-reactive with the analyte in the sample and in which the capture agent on the interior surface of the element is configured to define a code.

24. The micro length tube element of claim 22 or 23 in which the code is a bar code.

5 25. The micro length tube element of any of the foregoing claims 22-24 in which the active capture agent is an antibody.

26. The micro length tube element of any of the foregoing claims 22-24 in which the active capture agent is an antigen.

10 27. The micro length tube element of any of the foregoing claims 22-24 in which the active capture agent is an oligomer.

28. Reading code on a discrete hollow flow element, according to the following steps:

15 (A) provide hollow flow element with code pattern written in capture agent on inside surface of the element; preferably provide as a micro-length tube element; preferably the code represents the identity and/or concentration of capture agent on the element;

20 (B) Pick and place the hollow element into the flow channel;

 (C) Conduct analyte capture step by sample flow through channel, attaching analyte molecules to capture agent in the code pattern on inside surface of hollow element;

25 (D) Attach fluorophore tag to captured analyte molecules by flow through the channel; complete the fluid assay;

 (E) Stimulate and detect pattern of fluorescent emission from tagged analyte through the wall of the hollow element;

 (F) Conduct pattern analysis, identify pattern of light emission from captured analyte and match the read pattern to stored code table;

30 (G) Use the coded information associated with the specific code pattern of the hollow element; present e.g. by print-out or associate the data with stored or transmitted

assay data;

(H) And, or, use the detected information in data form in computer logic as part of an algorithm determining analyte concentration.

5 29. The method of claim 28 in which step A is provided according to the steps of

(A) Provide micro-length tube element uniformly coated with capture agent on inside tubular surface with no active capture agent on outside cylindrical surface;

(B) With laser beam, deactivate or remove the capture agent according to the blank spaces of a pre-determined code pattern, preferably a bar-code pattern;

10 (C) Place thus-coded micro-length tube element in flow channel constructed to enable sample flow both through the interior and past the exterior of the element, capturing analyte.

15 30. Reading code on a discrete hollow flow element, and quantifying assay data, according to the following steps:

(A) Provide hollow carrier element with code pattern written in capture agent on inside surface of the element; preferably provide as a micro-tube element; preferably the code represents the identity and/or concentration of capture agent on the element;

(B) Pick and place hollow element into flow channel;

20 (C) Conduct analyte capture step by sample flow through channel, attaching analyte molecules to capture agent in the code pattern on inside surface of hollow element;

(D) Attach fluorophore tag to captured analyte molecules by flow through the channel; complete the fluid assay;

25 (E) Stimulate and detect pattern of fluorescent emission from tagged analyte;

(F) Substantially simultaneously with step E, read intensity of emission from discrete hollow carrier element (preferably, a micro-length tube element), quantify the magnitude of detected emission; determine the concentration of analyte in the sample as a function of this quantification;

30 (G) Conduct pattern analysis, identify pattern of light emission from captured analyte and match the read pattern to stored code table;

(H) Use the coded information associated with the specific code pattern of the hollow element; present e.g. by print-out or associate the data with stored or transmitted assay data;

(I) And, or, use the detected information in data form in computer logic as part of an algorithm determining analyte concentration.

31. The method of claim 1 in which the tracer comprises a substance not employed in the conduct of the assay.

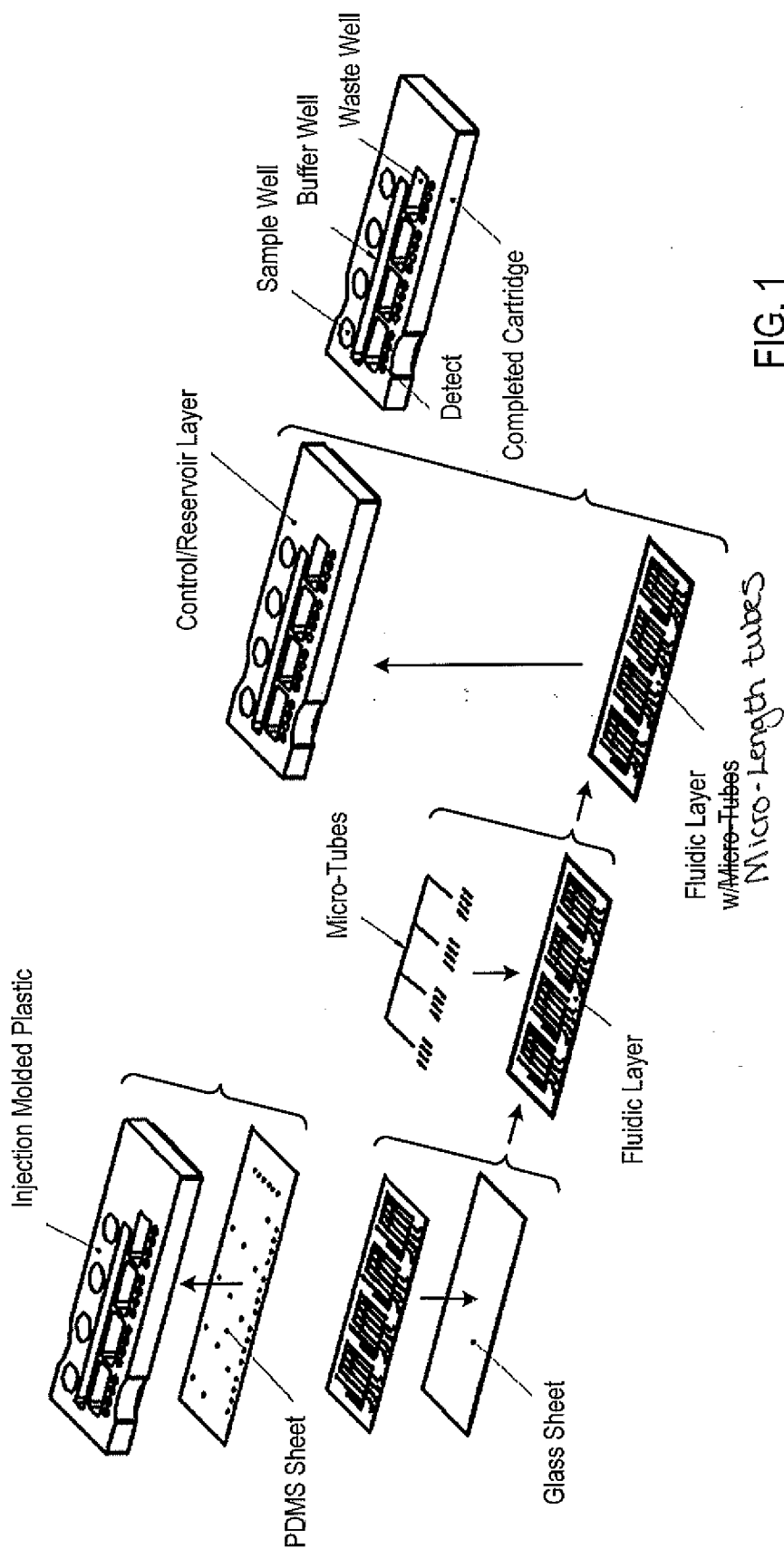
32. The method of claim 1 in which the tracer is a substance employed in the assay.

33. The method of claim 32 in which the tracer is a fluorescent tag employed to be captured at the site of analyte to enable reading of the assay.

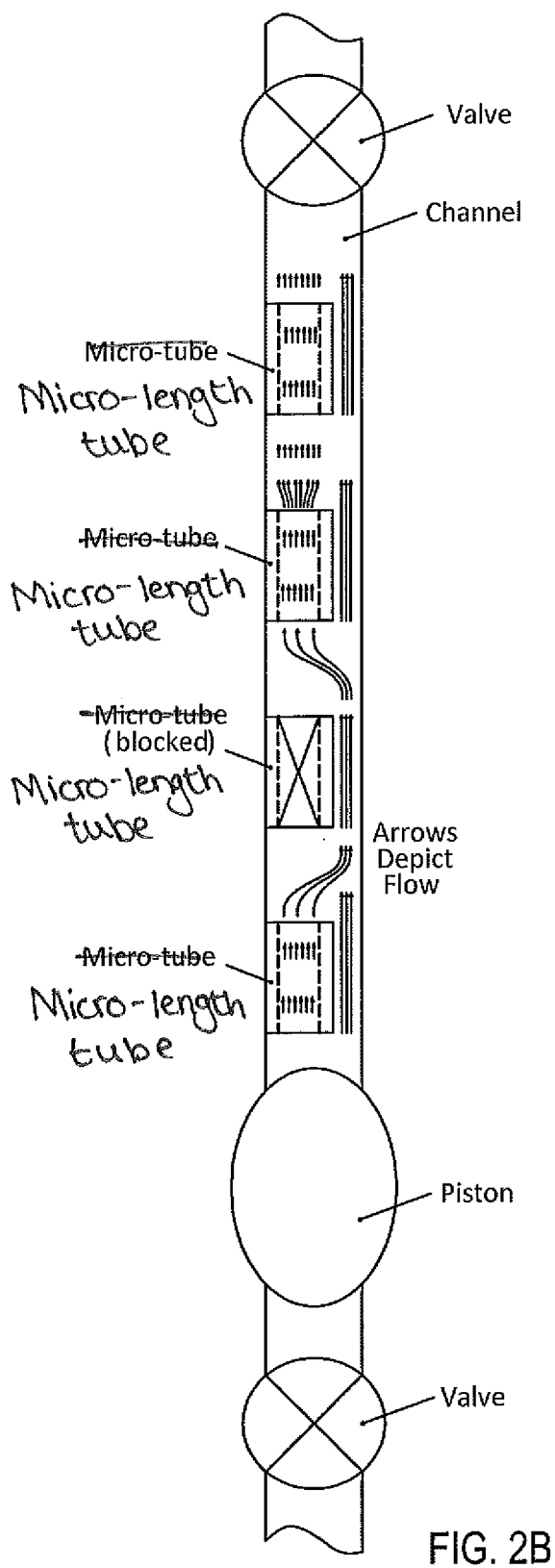
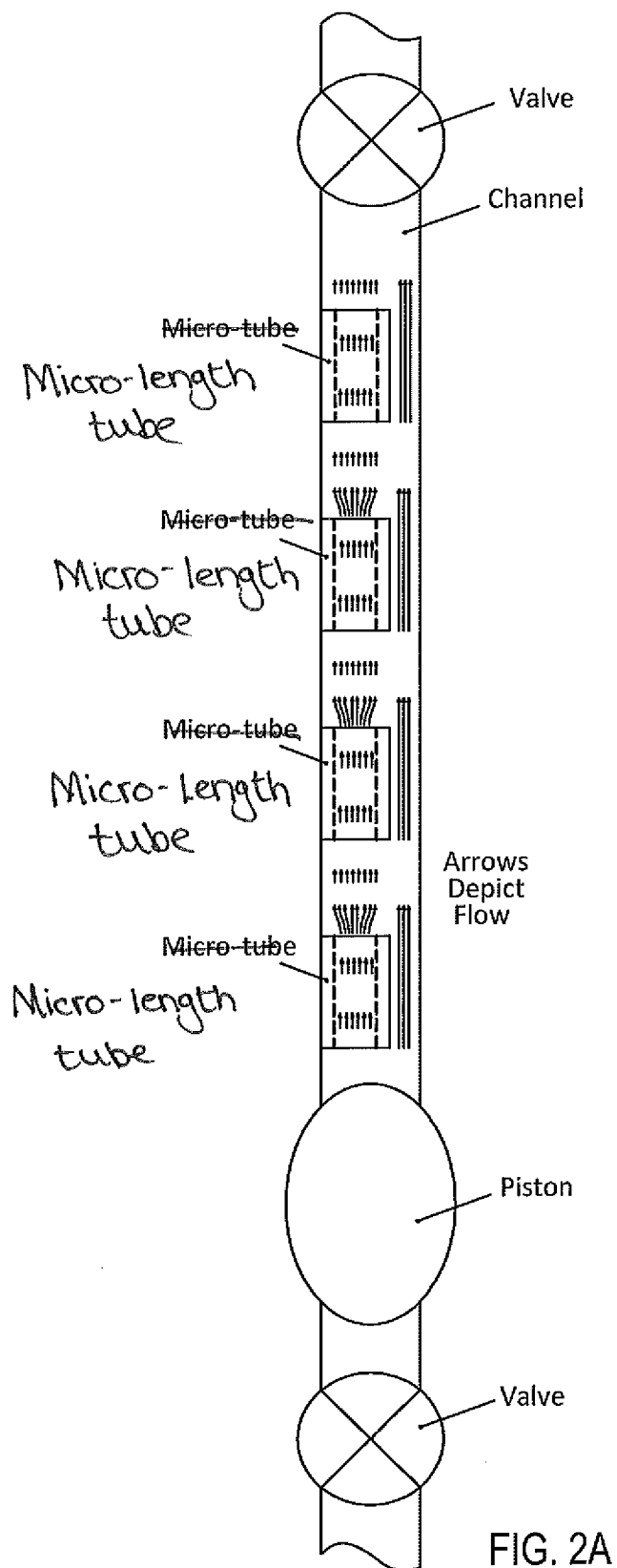
34. The method of claim 33 in which the fluorescent tag is capable of fluorescent emission under stimulation, the method including exciting the fluorescence.

35. The method of claim 34 in which the fluorescent tag is capable of fluorescent emission by light stimulation, and the step includes reading the fluorescence by an epi-fluorescence detection system.

36. Apparatus for performing the method of any of the foregoing claims.



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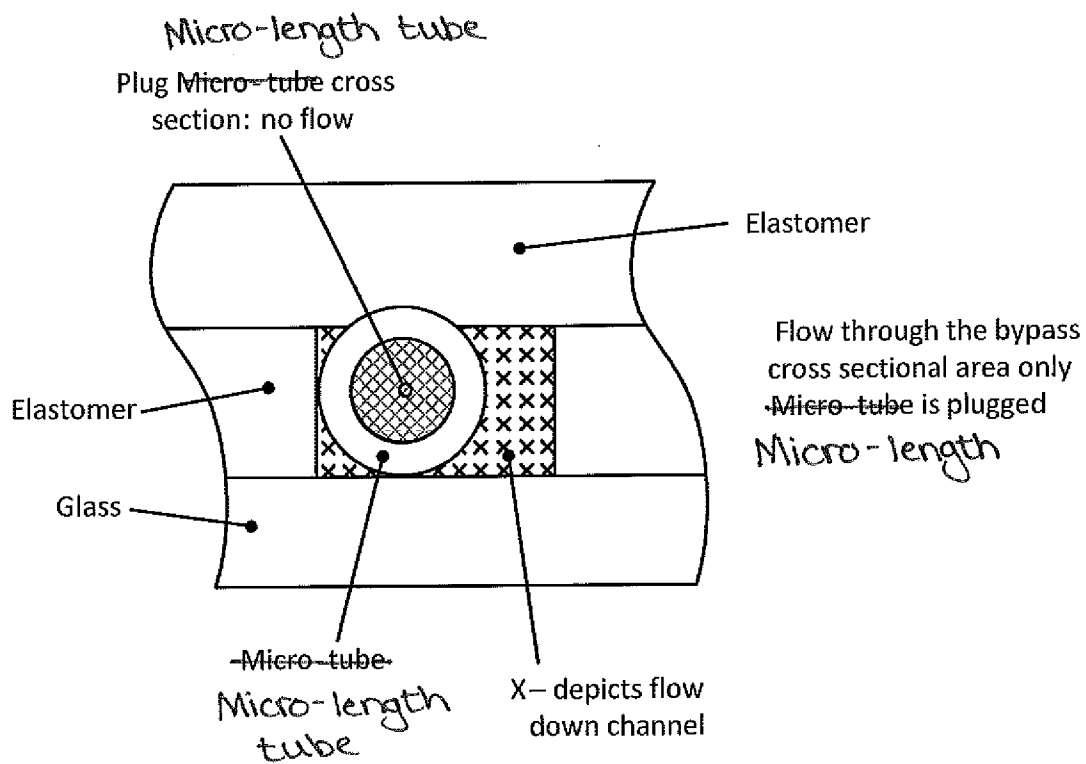


FIG. 2C

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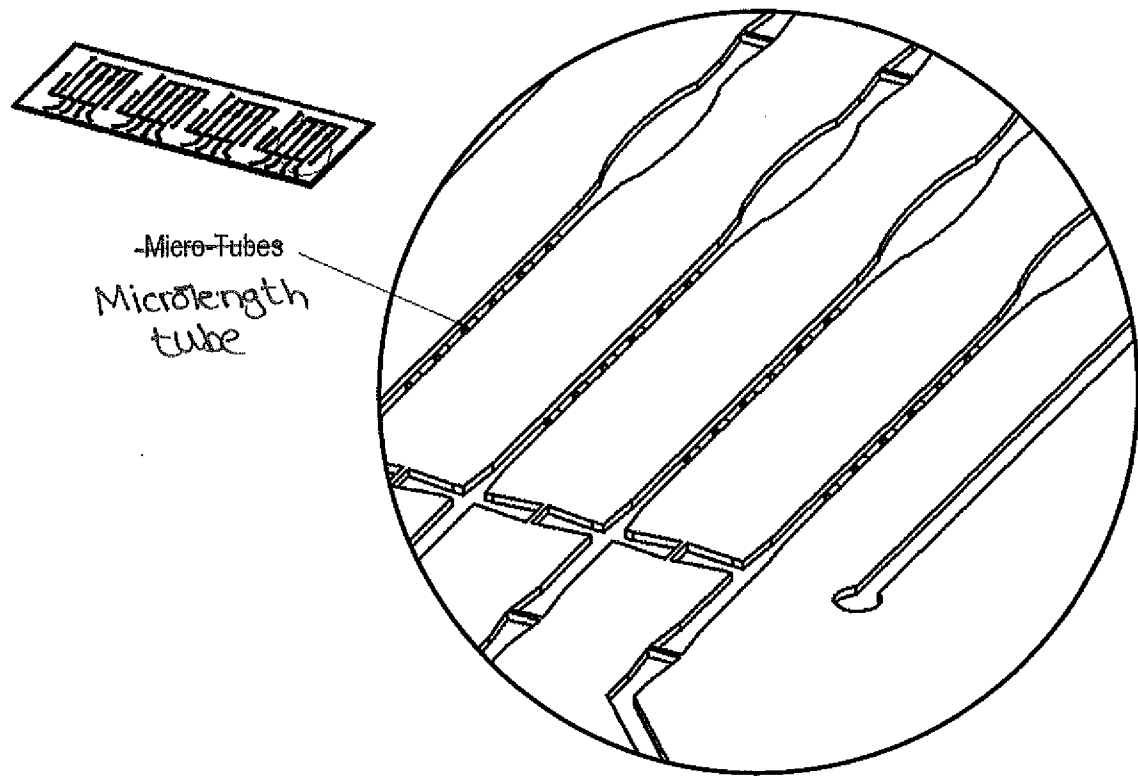


FIG. 3

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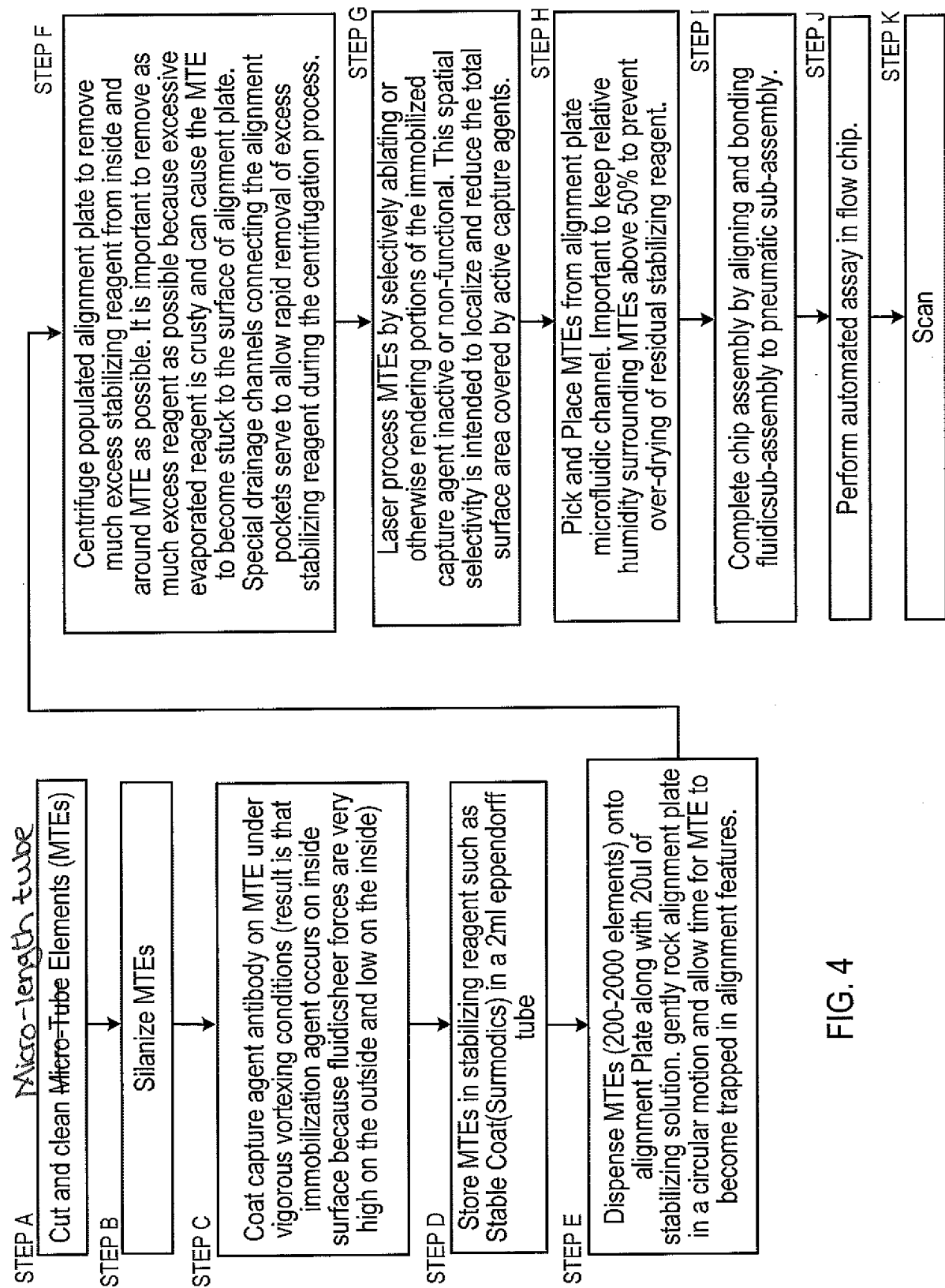
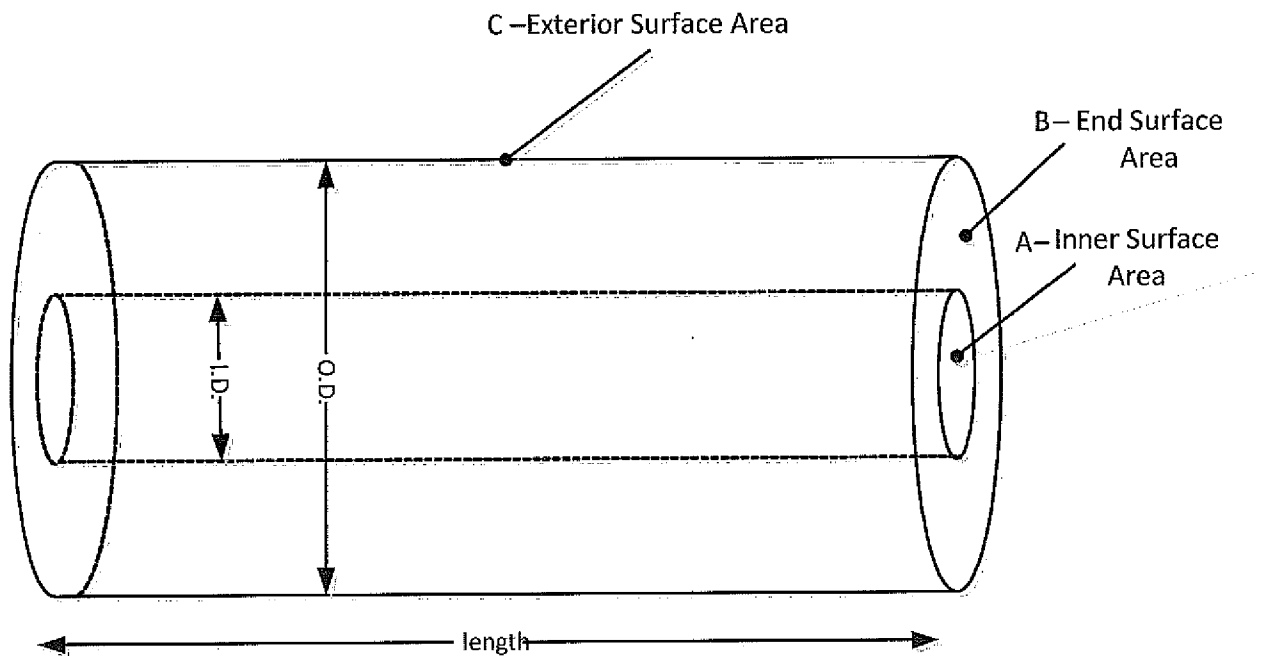


FIG. 4

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Total Surface Area, $T = A + 2 * B + C$

% Reduction of Surface Area without exterior coating = $1 - (A + 2 * B) / T$

% Reduction of Surface Area without exterior and ends coating = $1 - A / T$

% Reduction of Surface Area without exterior and ends coating = $1 - (A * 0.5) / T$

Example: O.D. = $125\mu\text{m}$, I.D. = $75\mu\text{m}$, Length = $250\mu\text{m}$

Without Exterior : 57% Reduction of surface area

Without Exterior & Ends: 66% Reduction of surface area

Without Exterior & Ends & with 50% code: 83% reduction of surface area

Fig. 6

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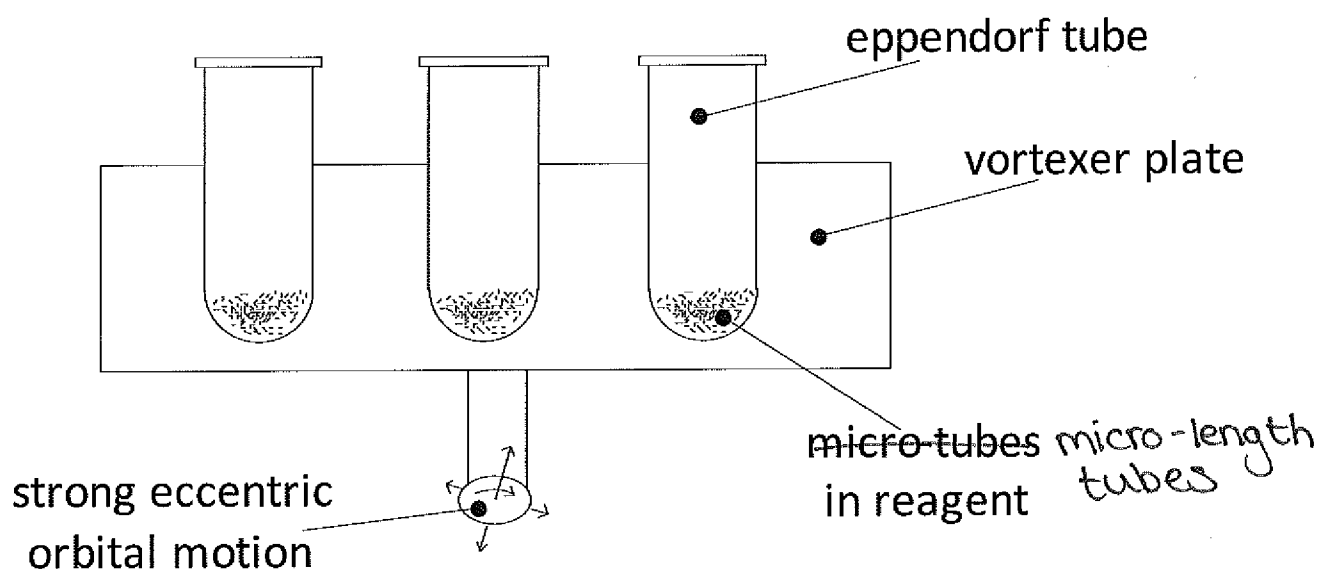


Fig. 7

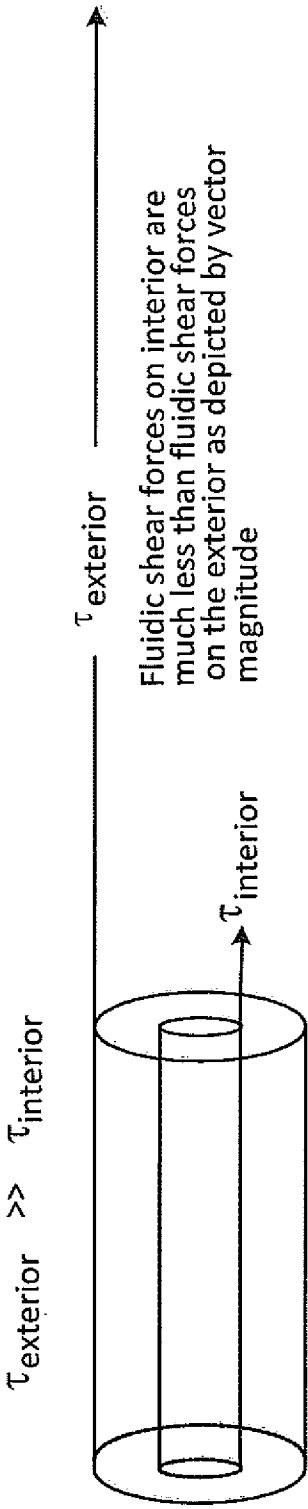
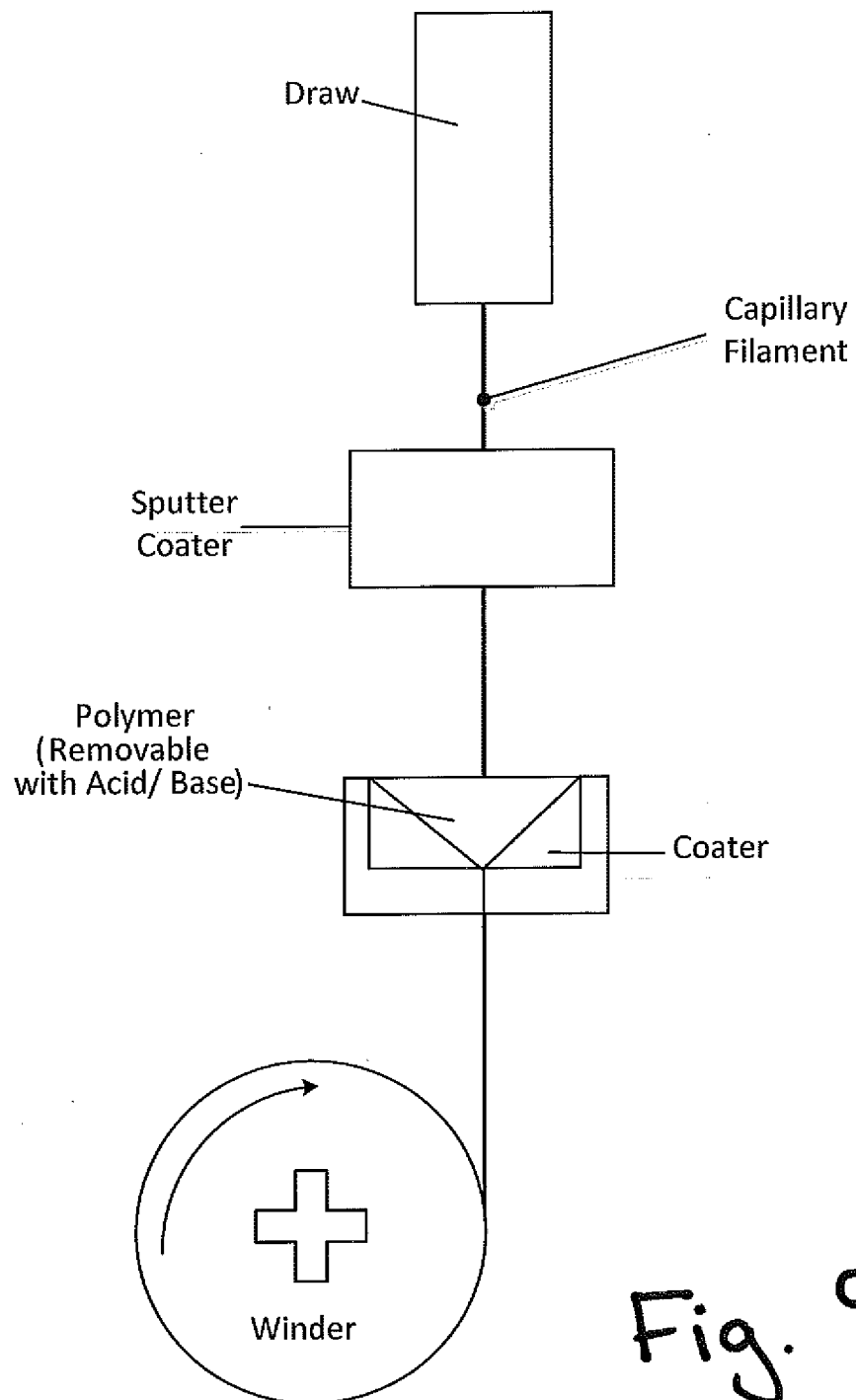


Fig. 8

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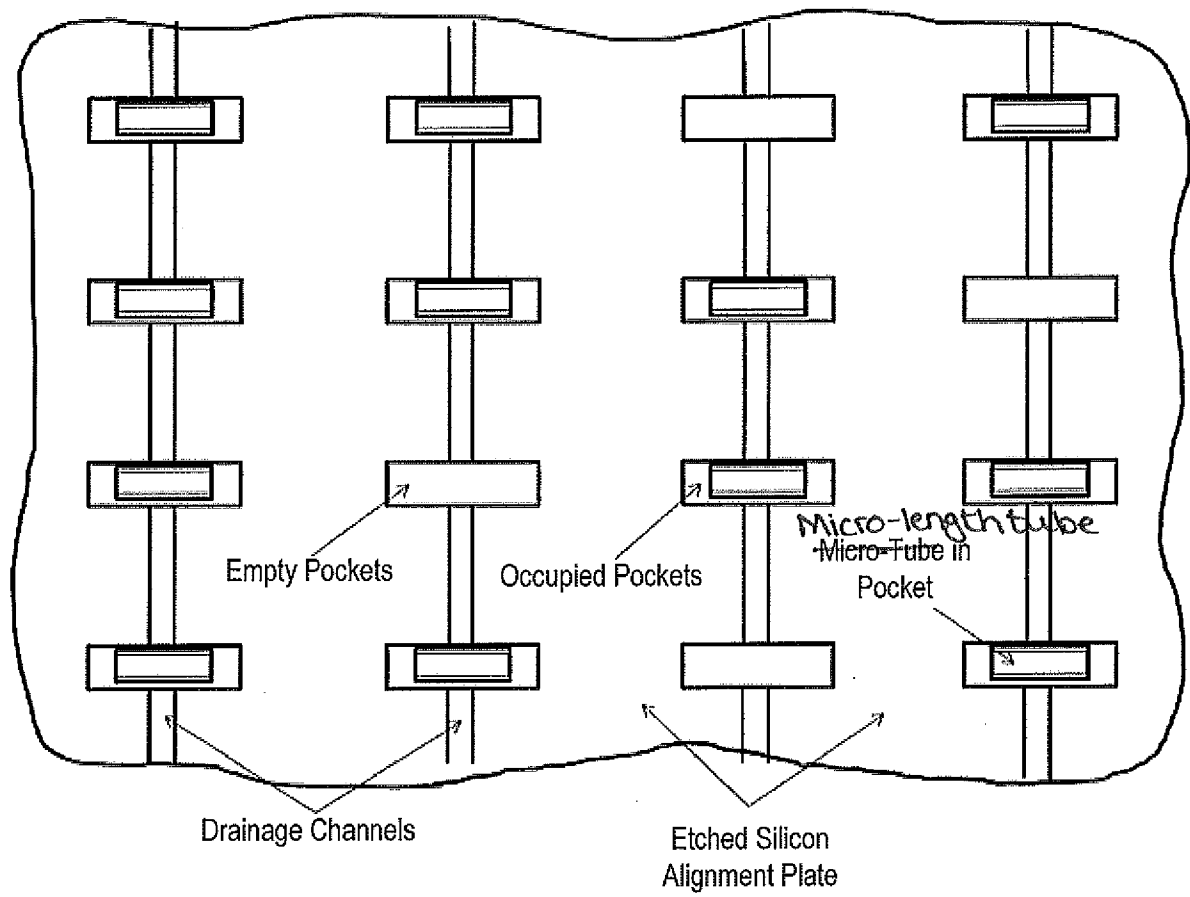


Fig. 10

Pick and place

Fig.

10 A

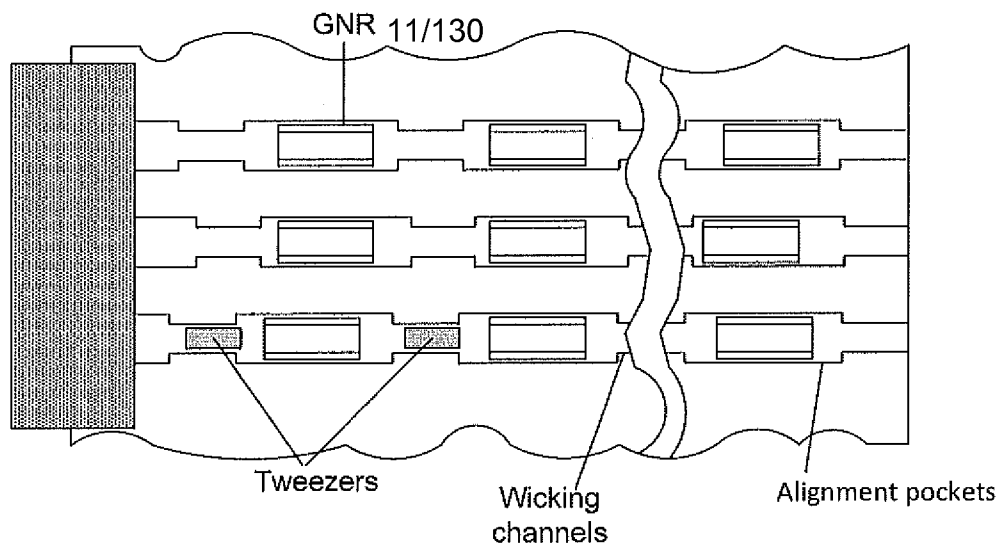


Fig.

10 B

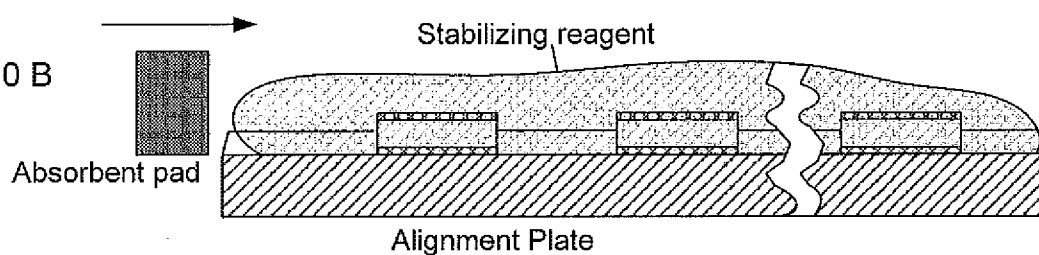


Fig.

10 C

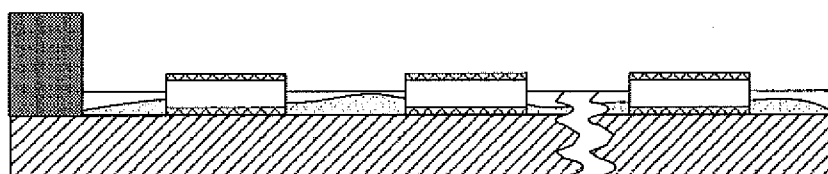
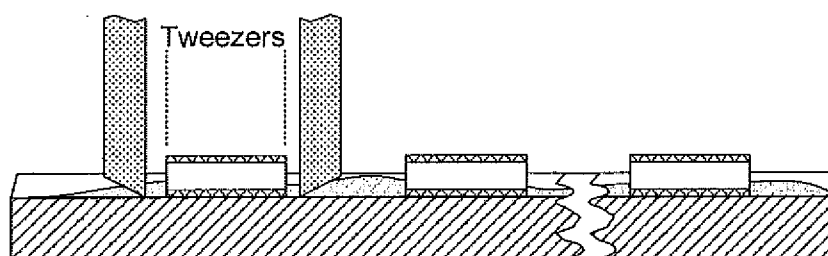


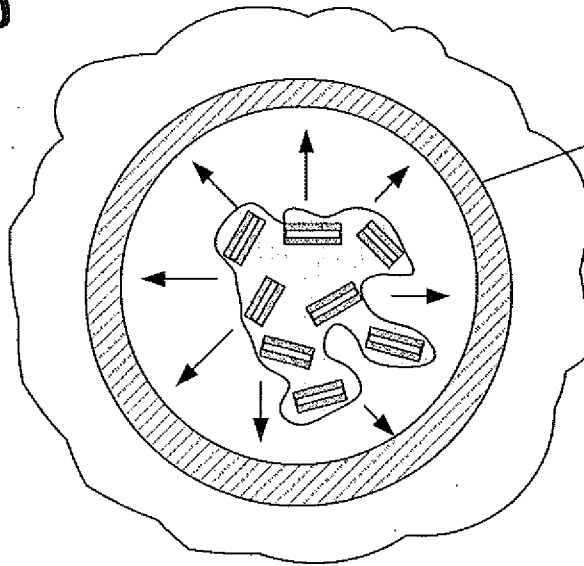
Fig.

10 C'

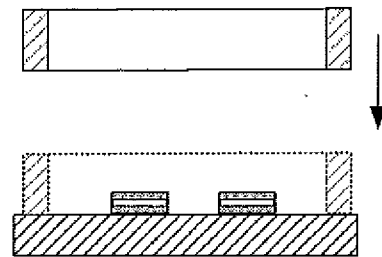
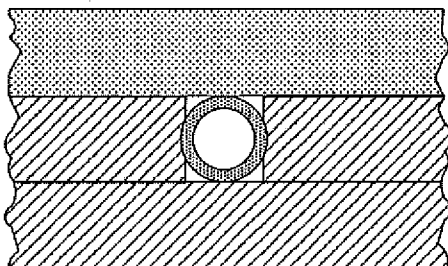
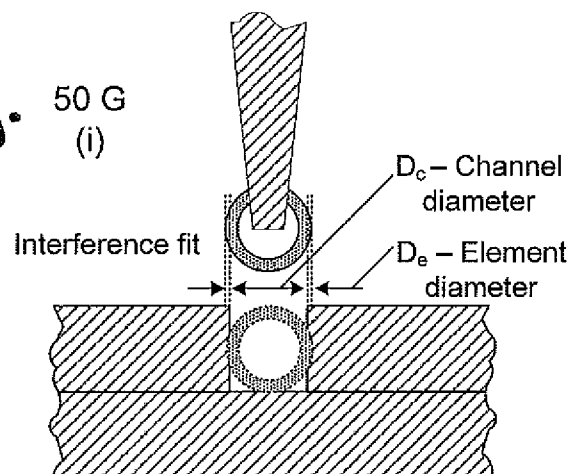


Pick and place

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Fig. 10 D

Absorbent pad

Fig. 10 E**Fig.** 50 H**Fig.** 50 G (i)

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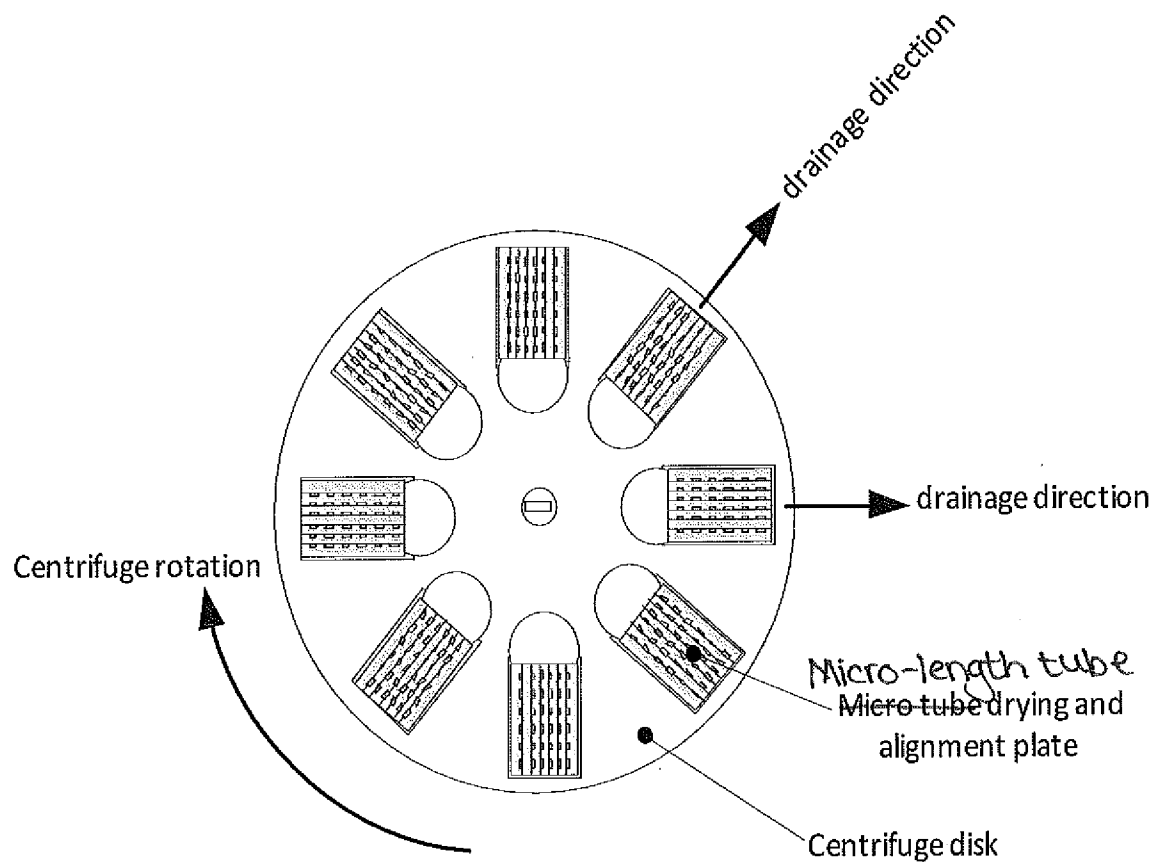


Fig. 11

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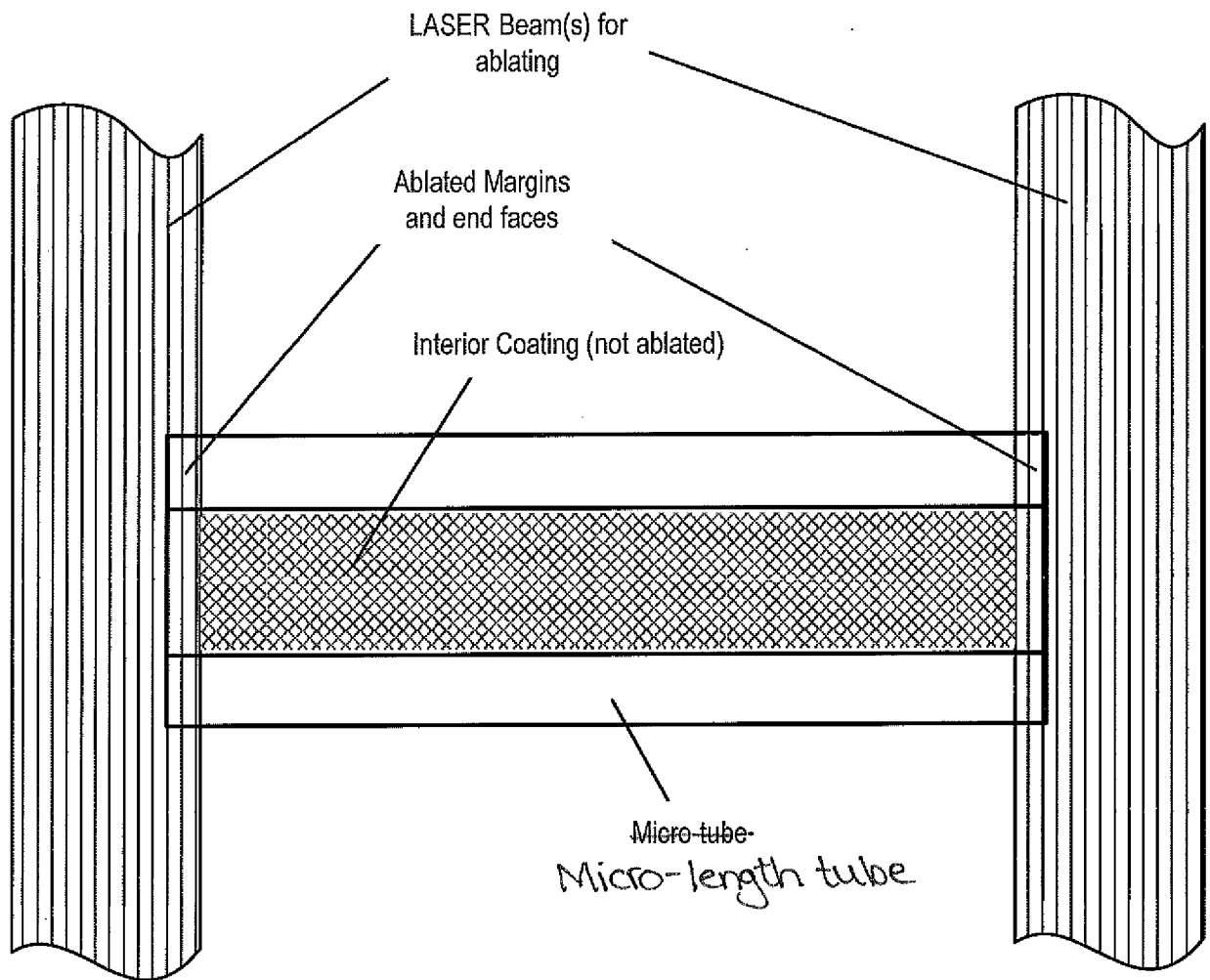


Fig. 12

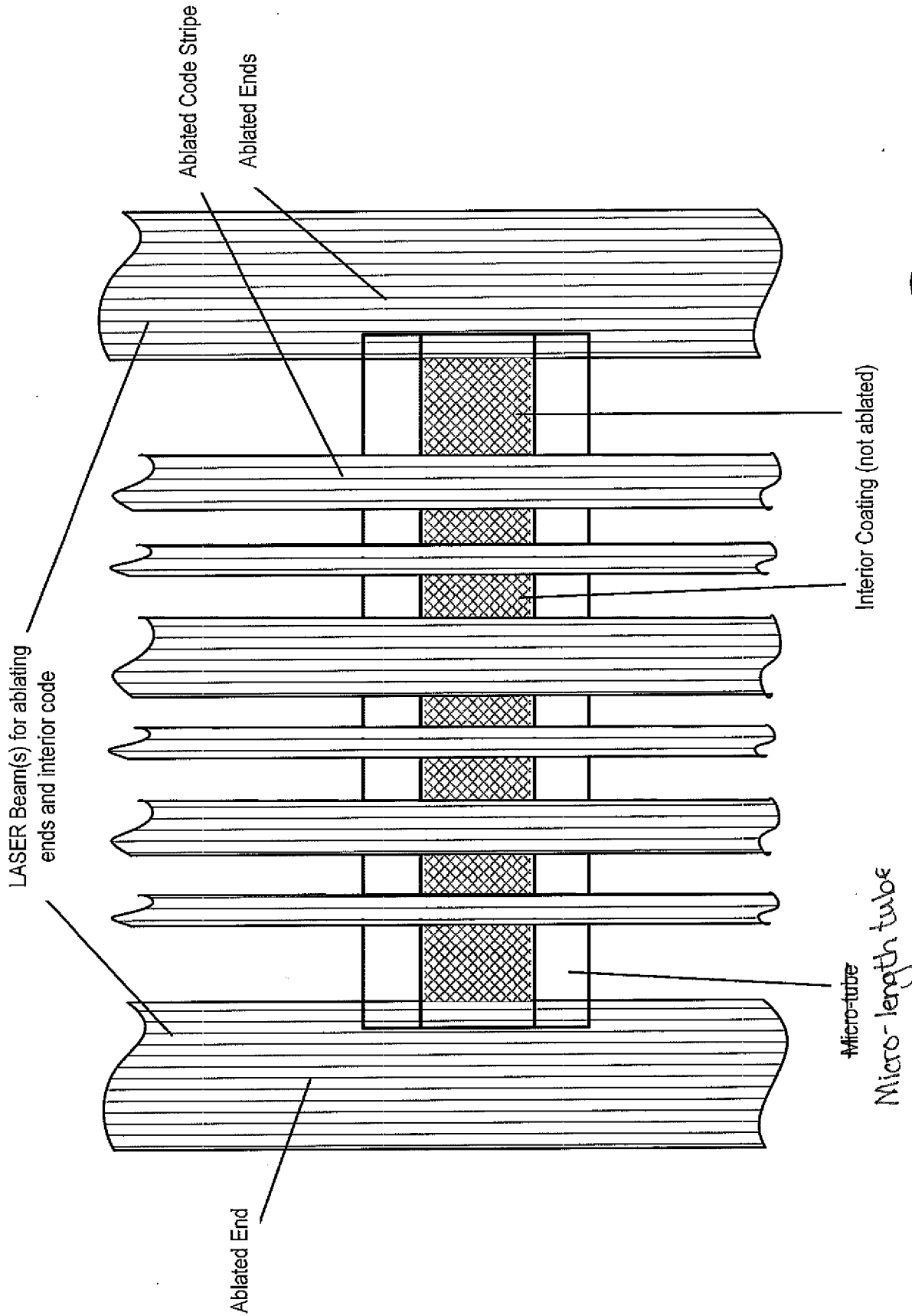


Fig. 13

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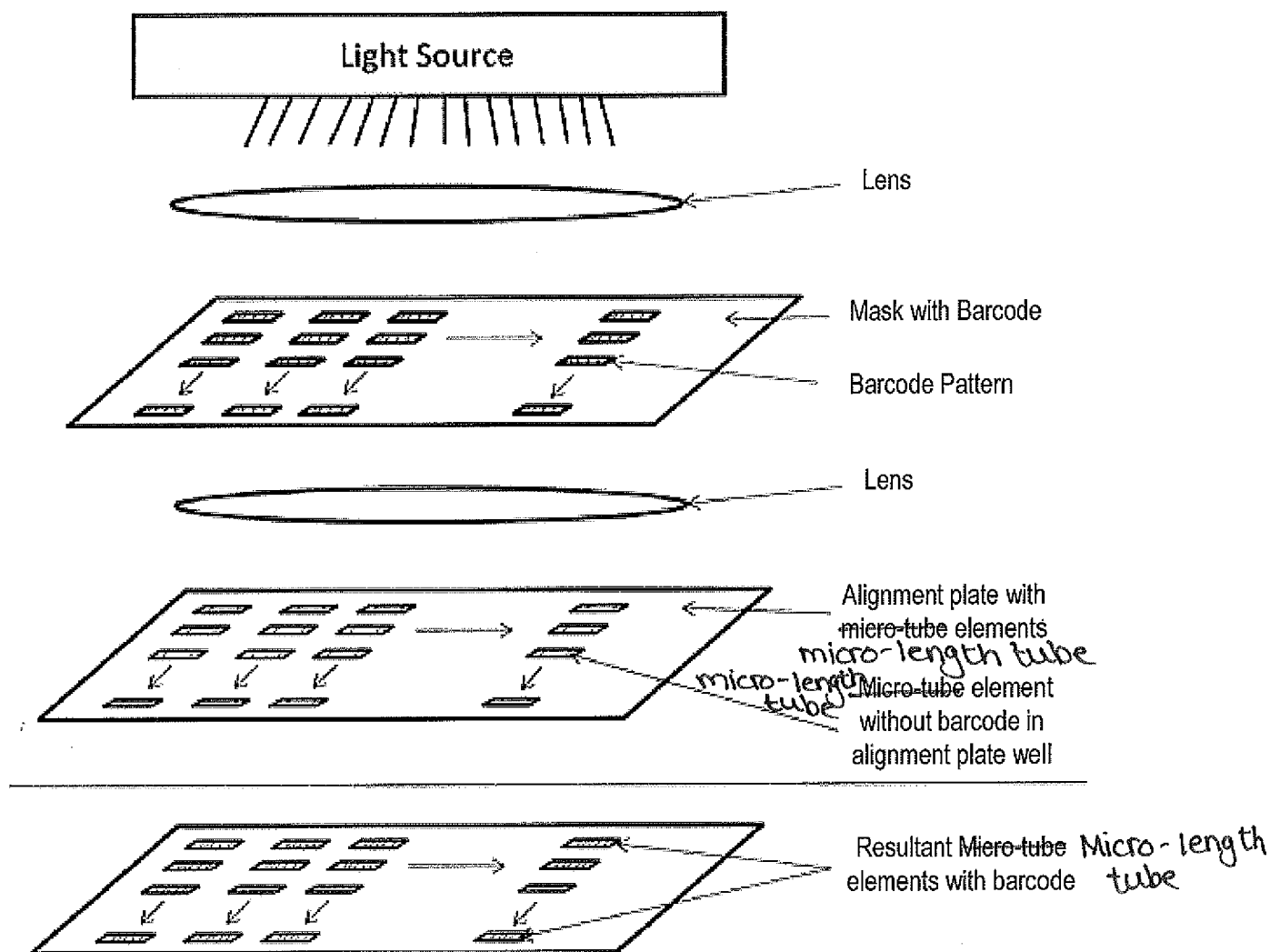


Fig. 14

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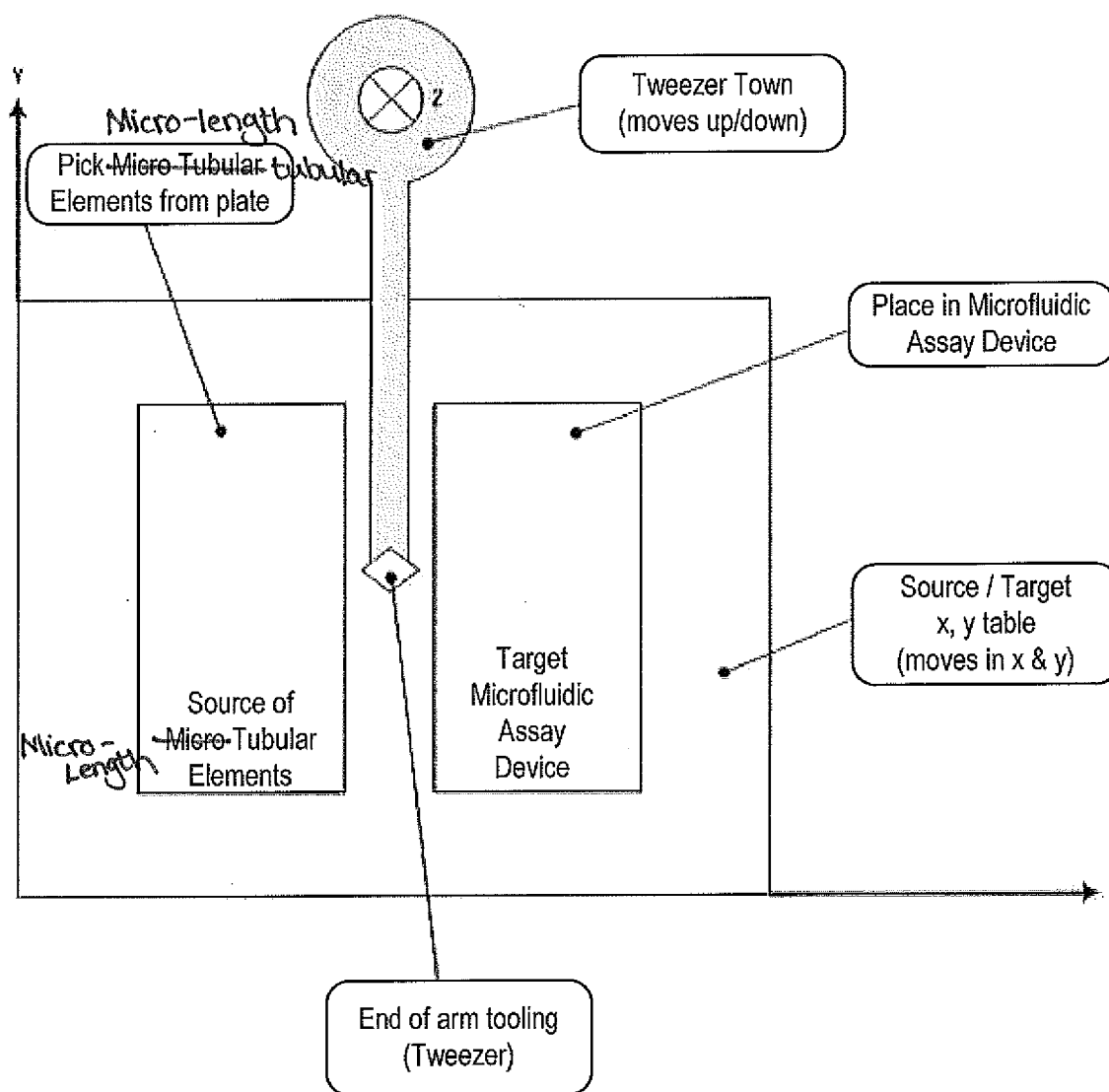
Pick & Place – Top View

Fig. 15

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Pick & Place – Front View

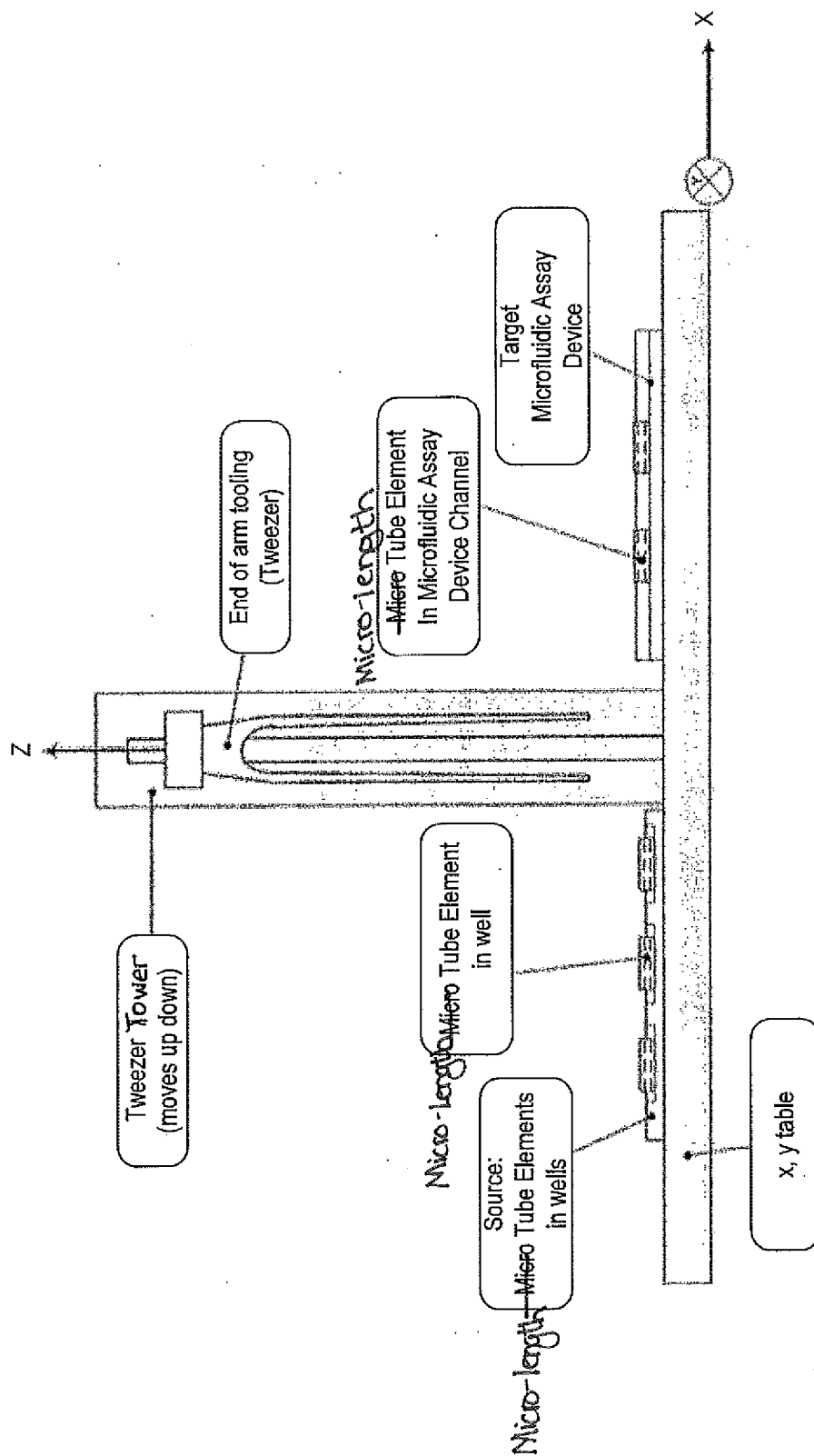


Fig. 16

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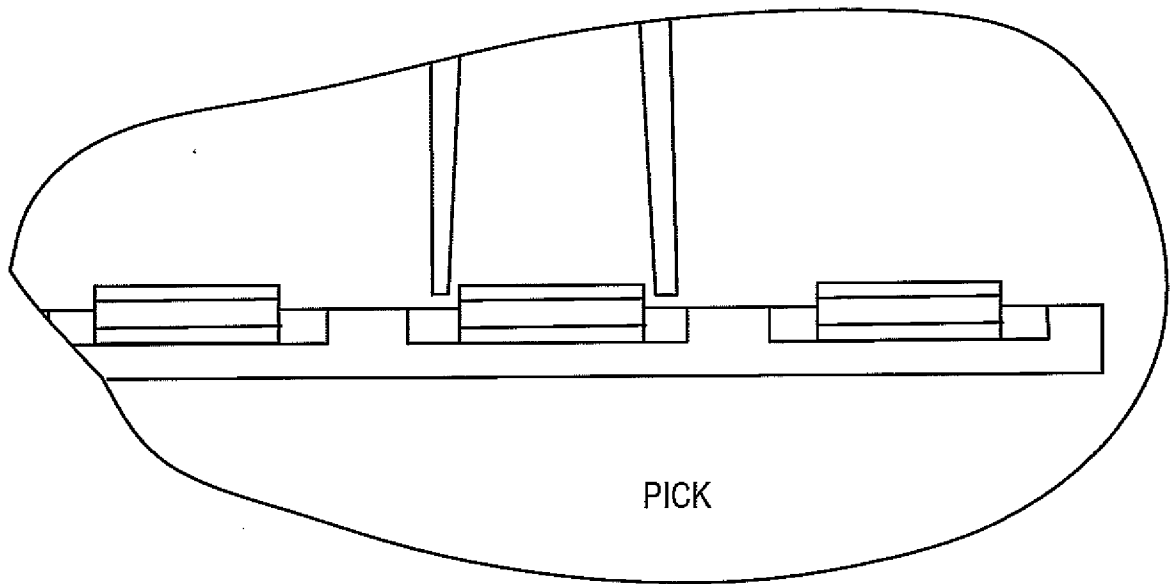


Fig. 17

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PLACE with Tweezer

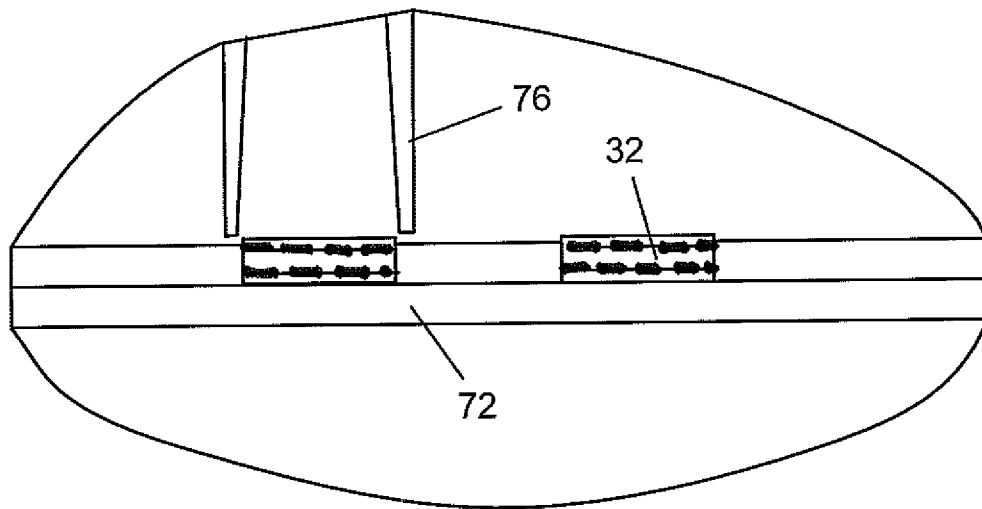
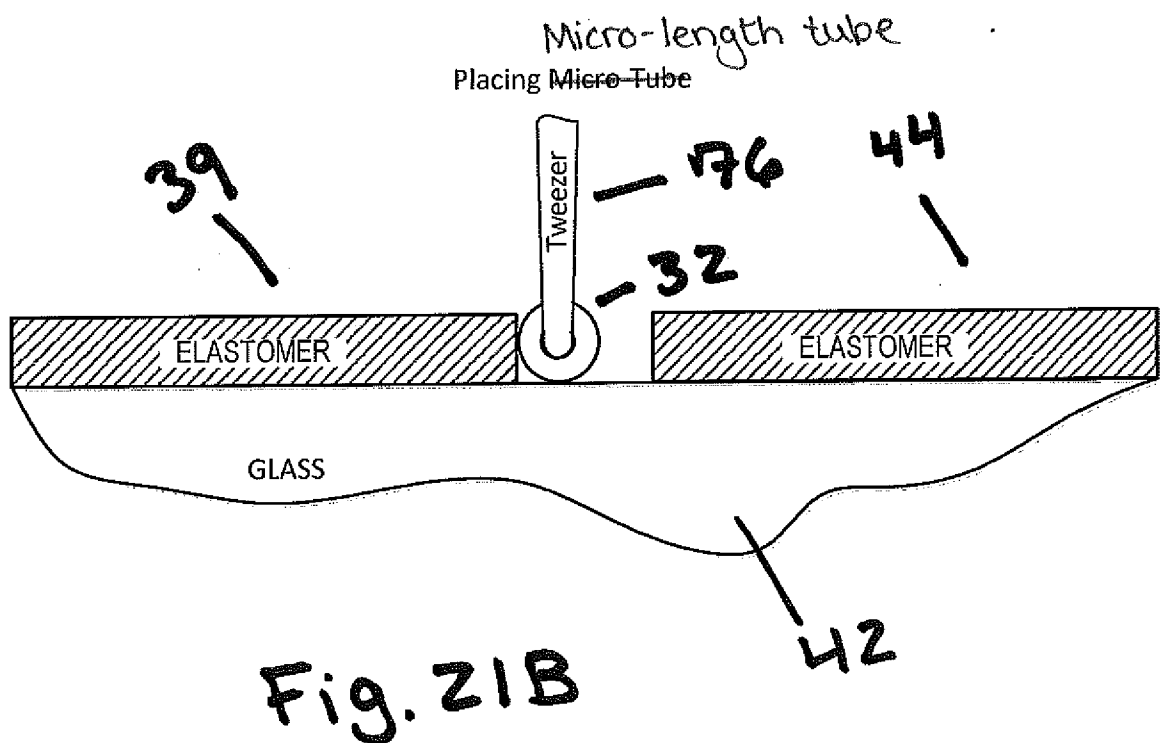
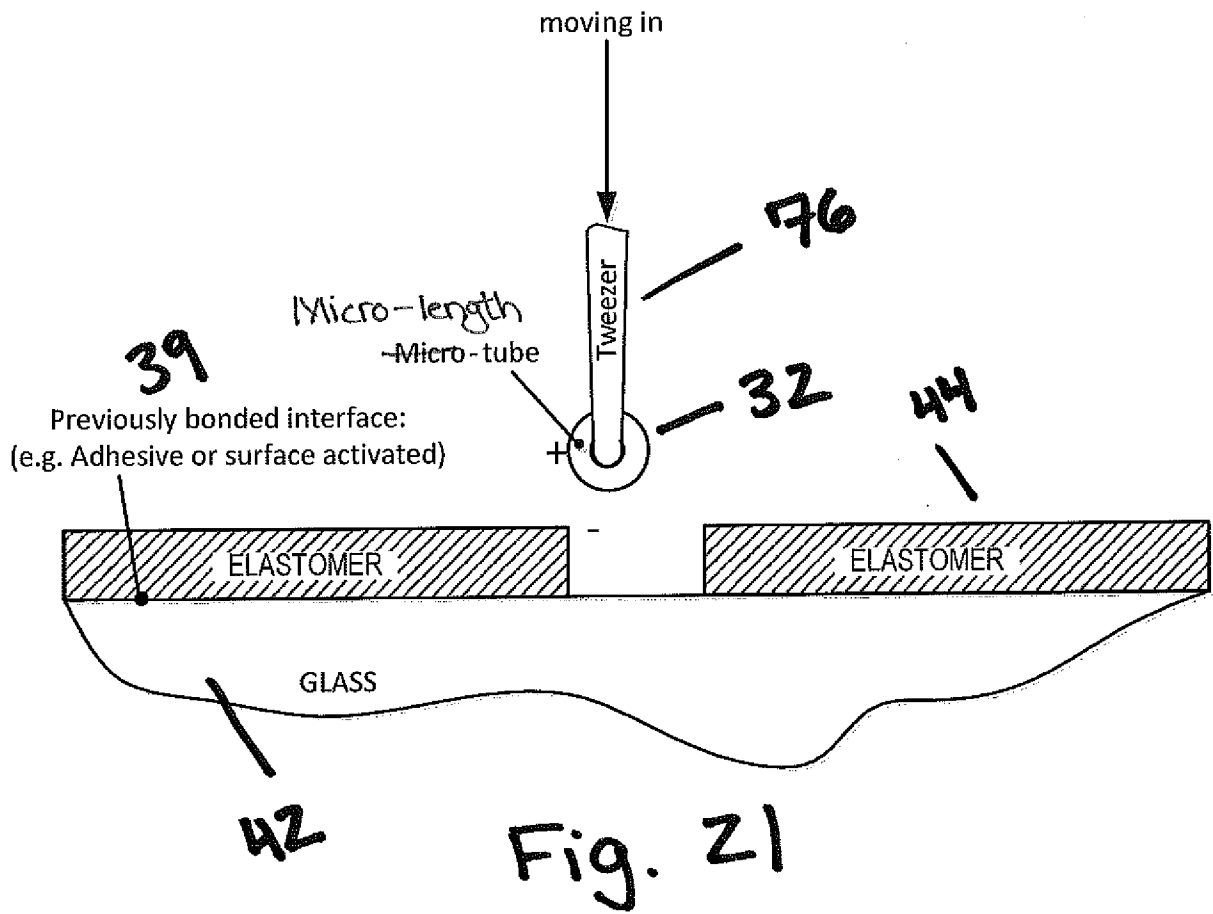
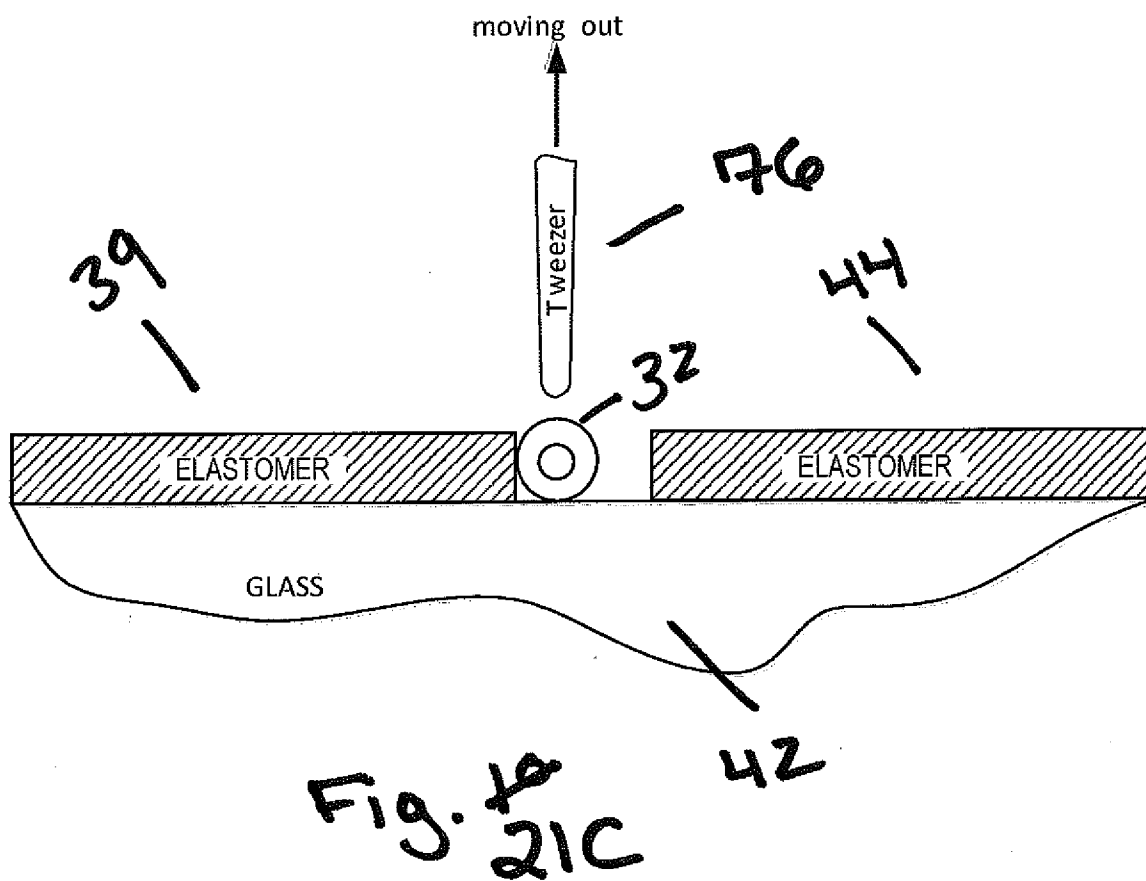


Fig. 19

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Surface
activated
for bonding

Placing channel top

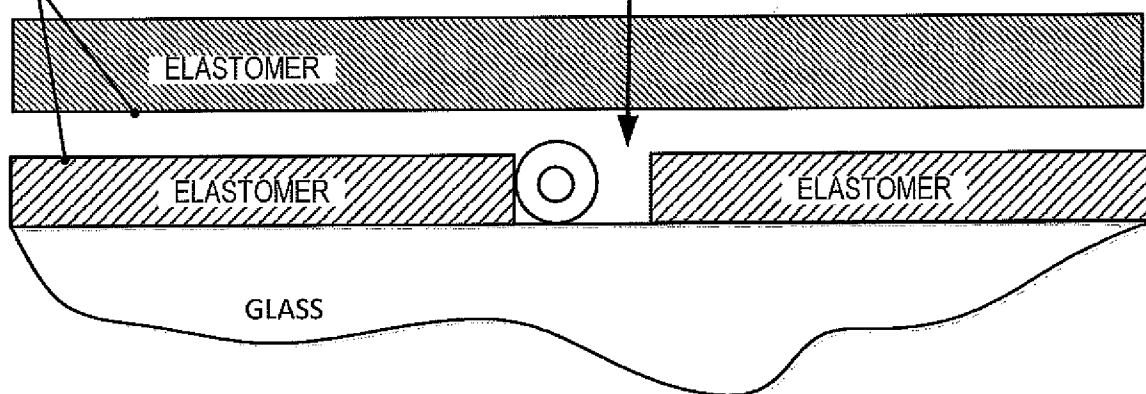


Fig. 21 D

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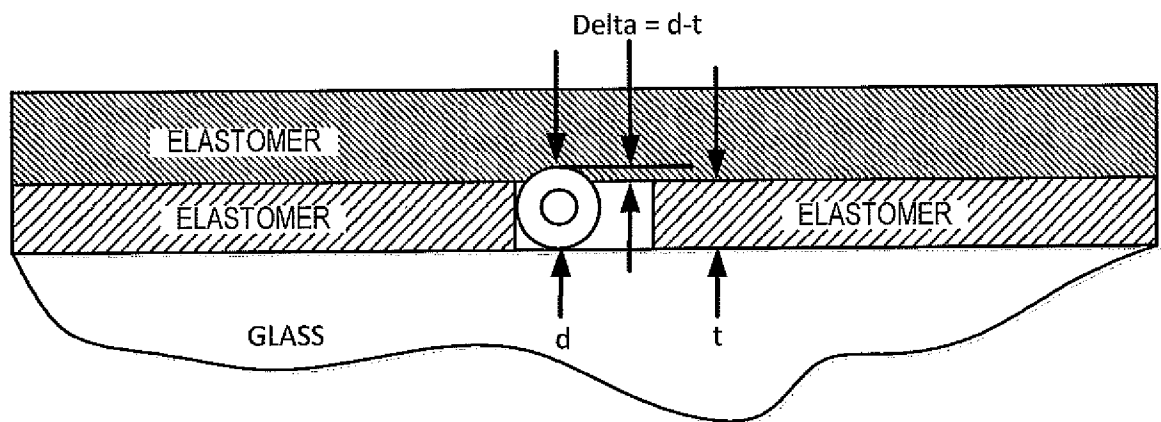
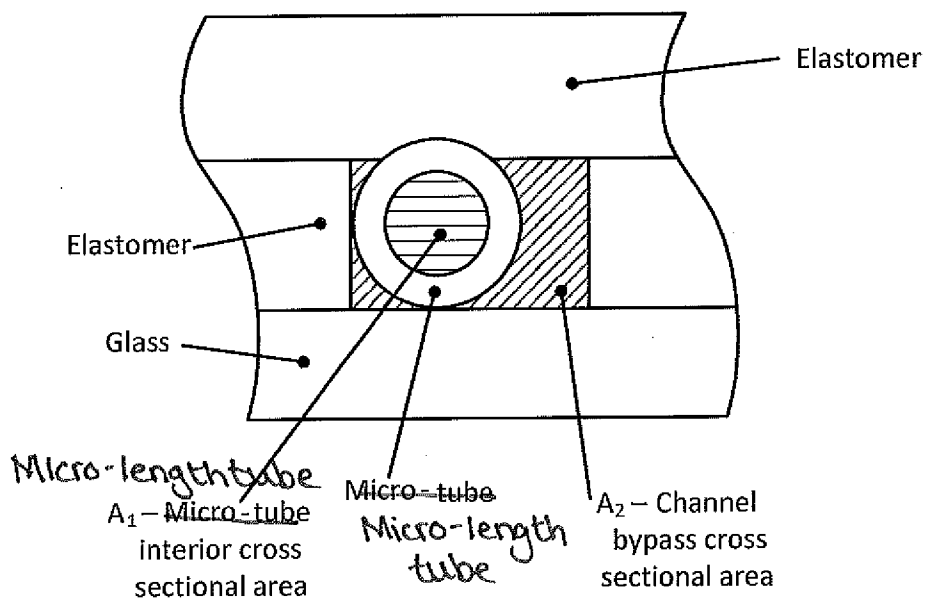


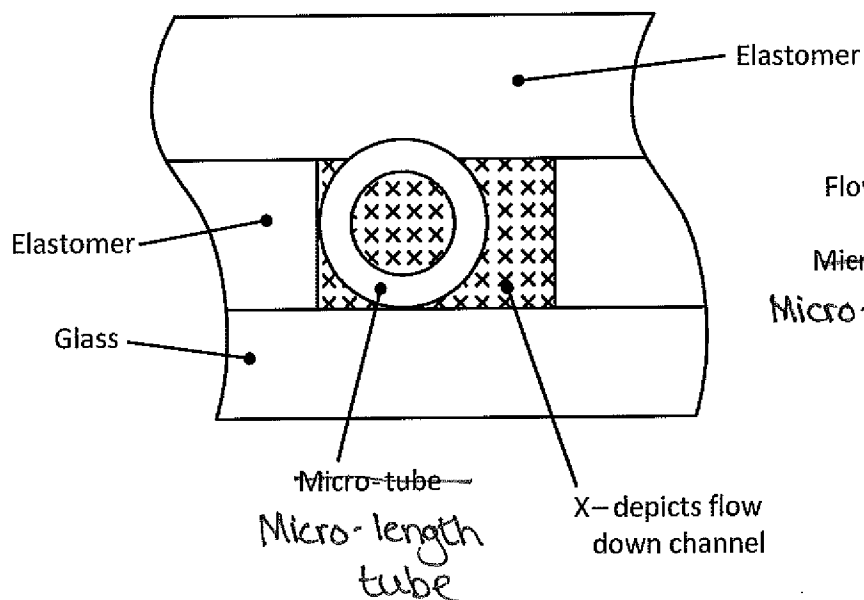
Fig. 21 E

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Total Cross sectional area for flow
 $A_T = A_1 + A_2$

Fig. 22A



Flow through the total cross sectional area
 Micro-tube NOT plugged
 Micro-length tube

Fig. 22B

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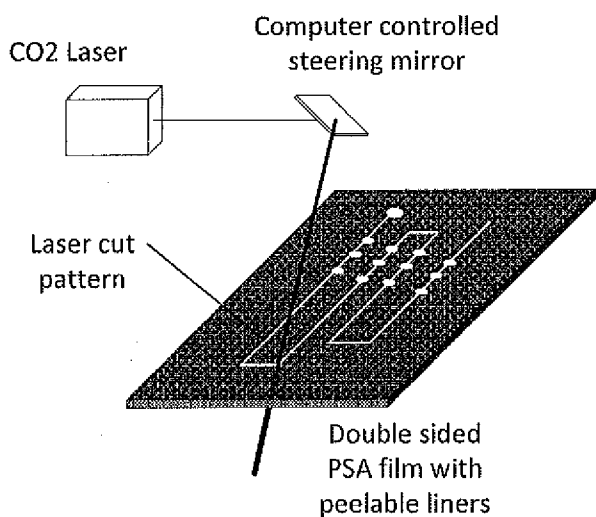
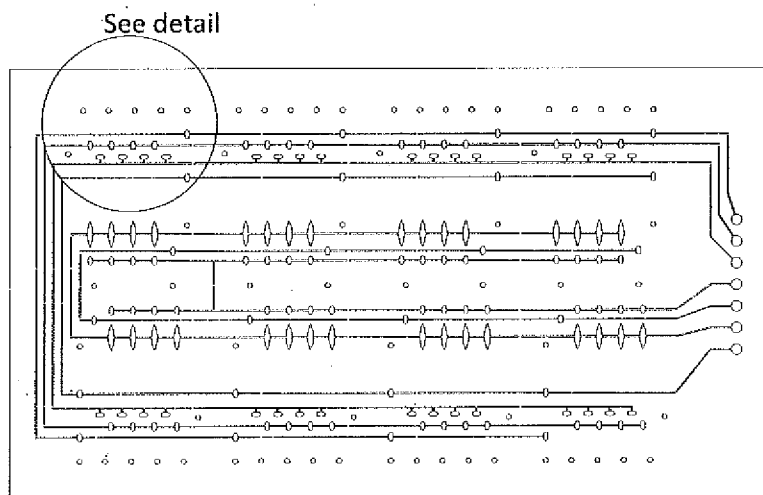


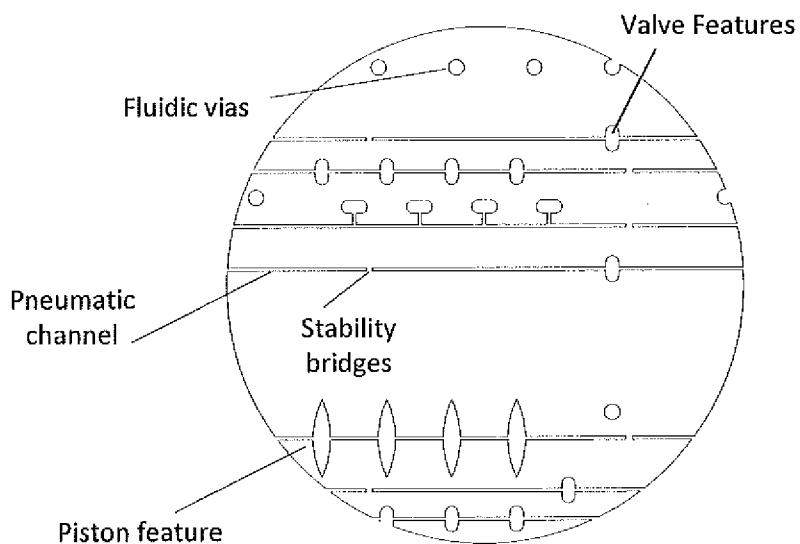
Fig. 23A



Pneumatic control channels and features for integrated valves and pumps

Fig. 23B

Fig. 23C



DETAIL A
SCALE 8 : 1

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Fig.
23D

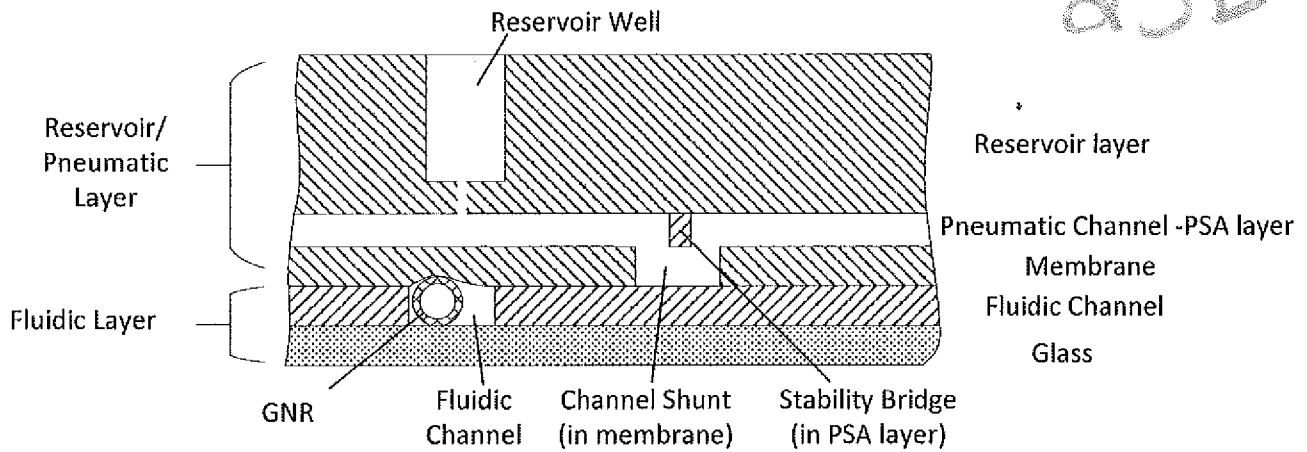


Fig.
23E

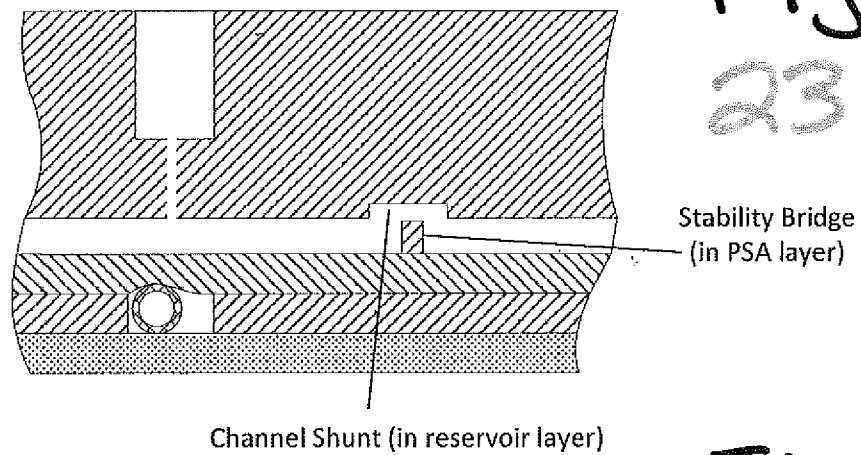
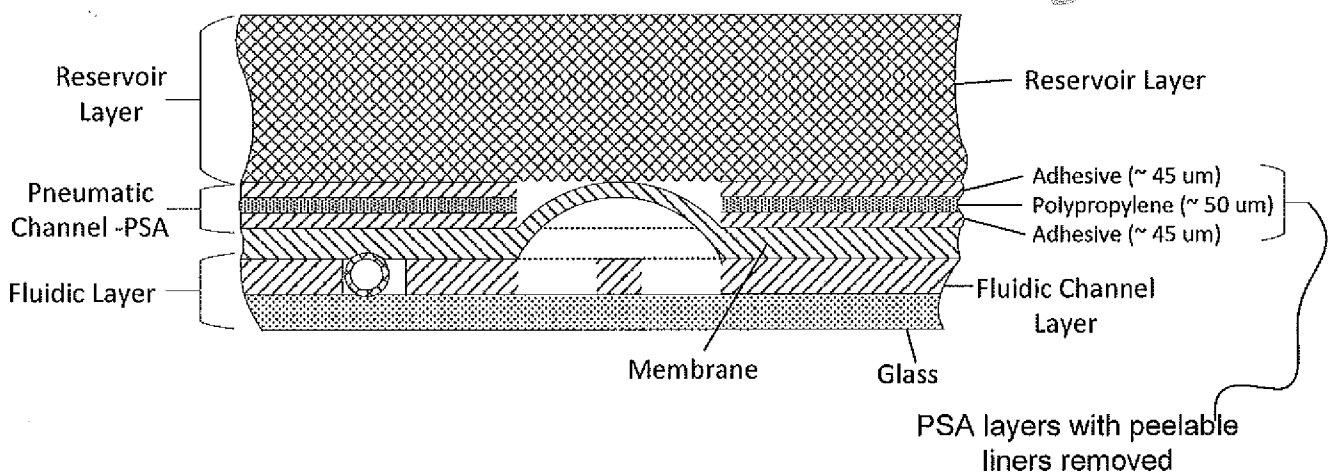


Fig.
23F



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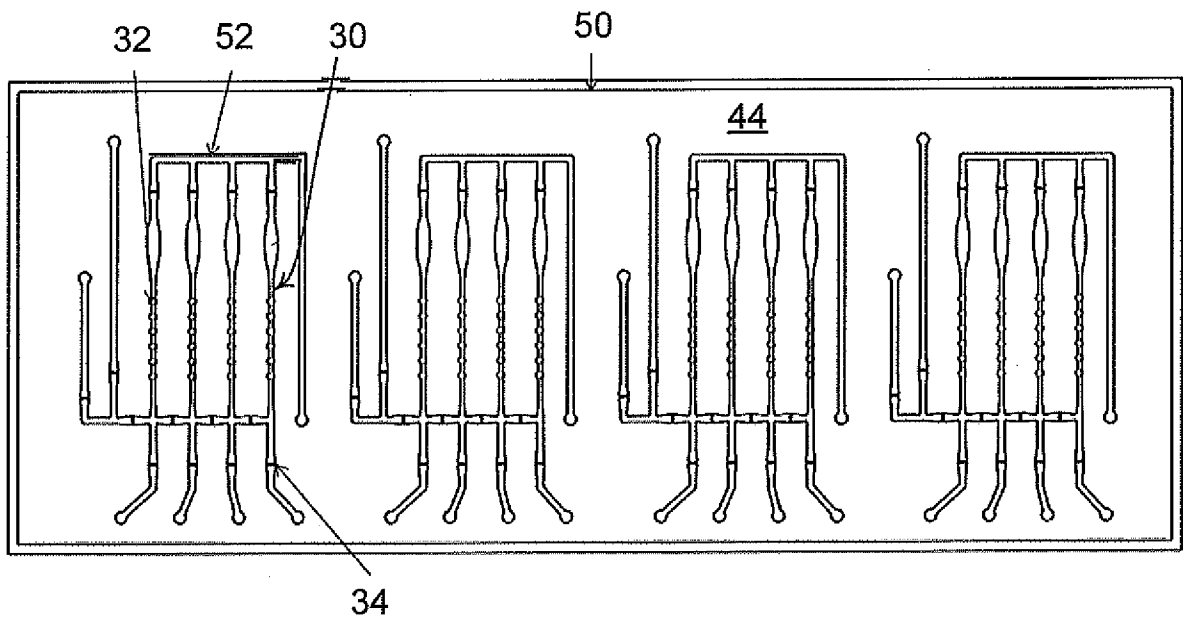


Fig. 24

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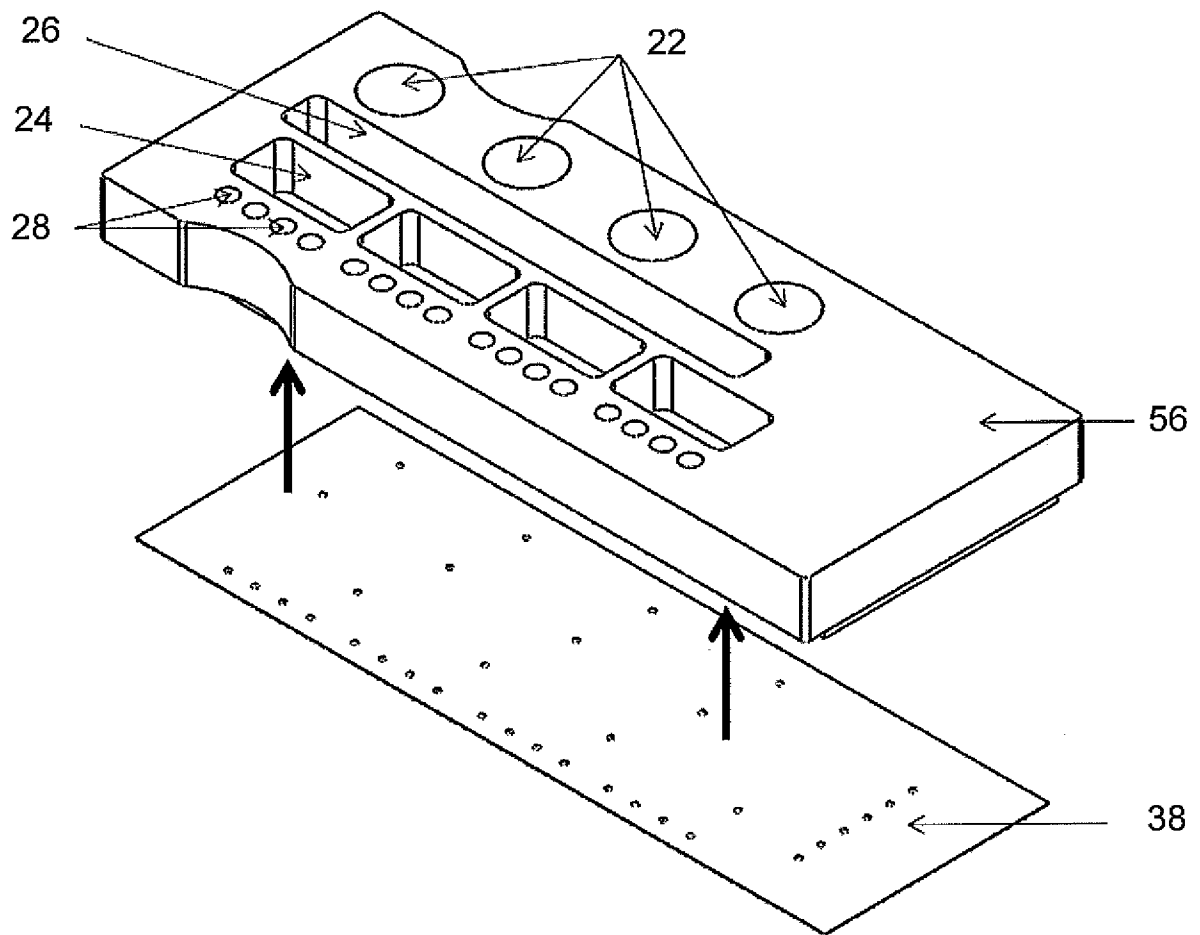


Fig. 25

29/130

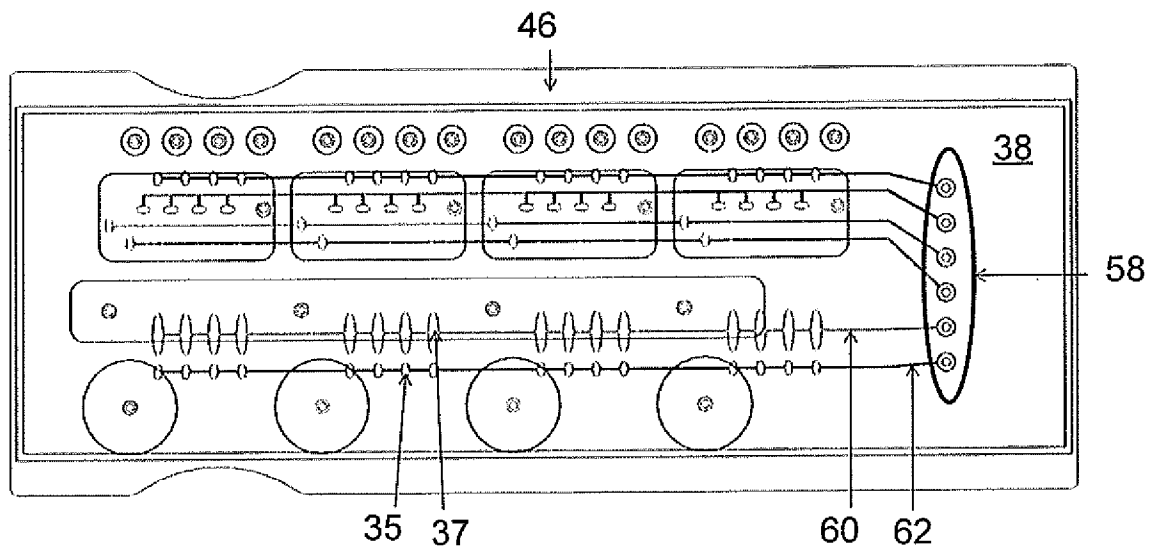


Fig. 26

30/130

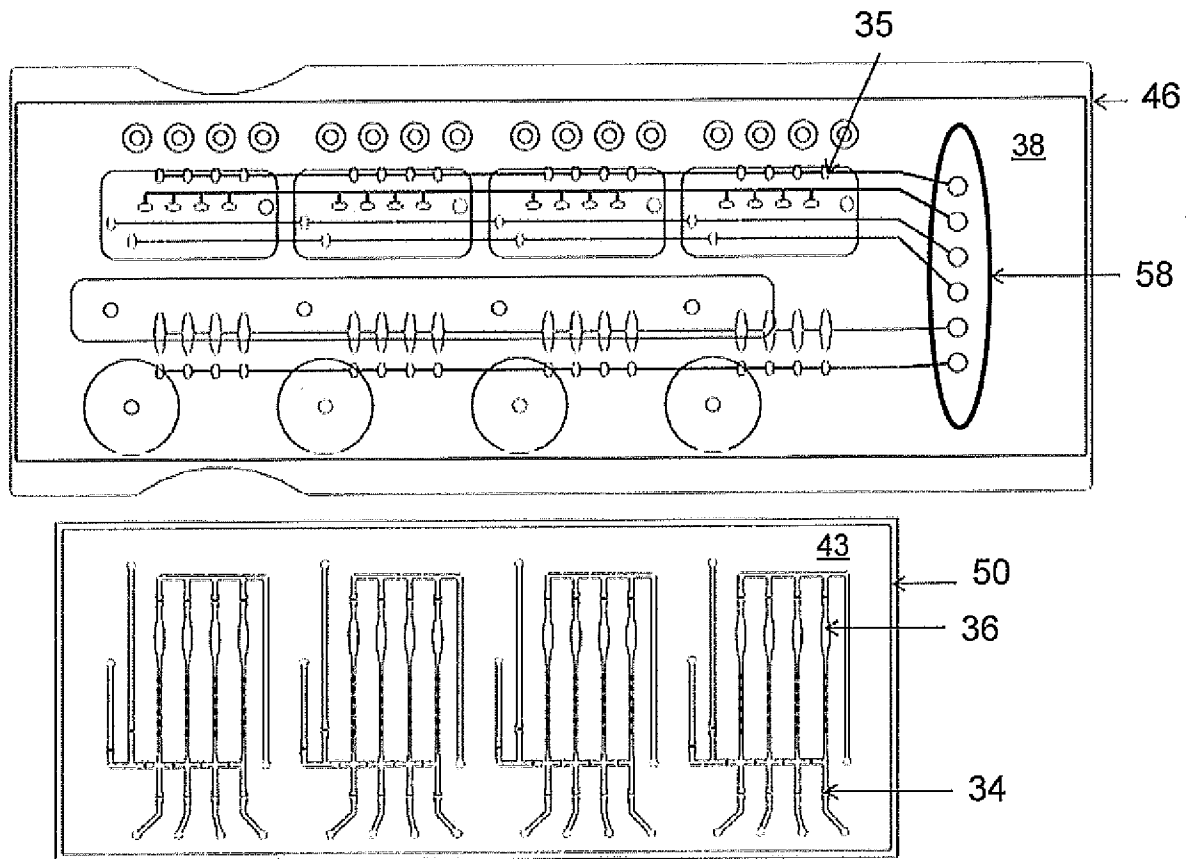


Fig. 27

31/130

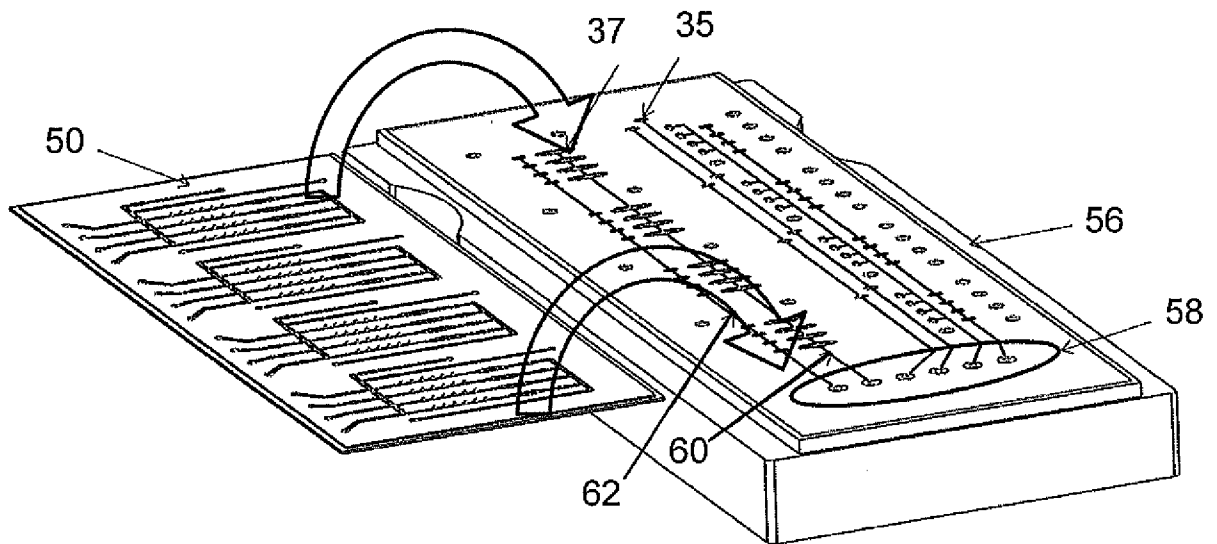


Fig. 28

32/130

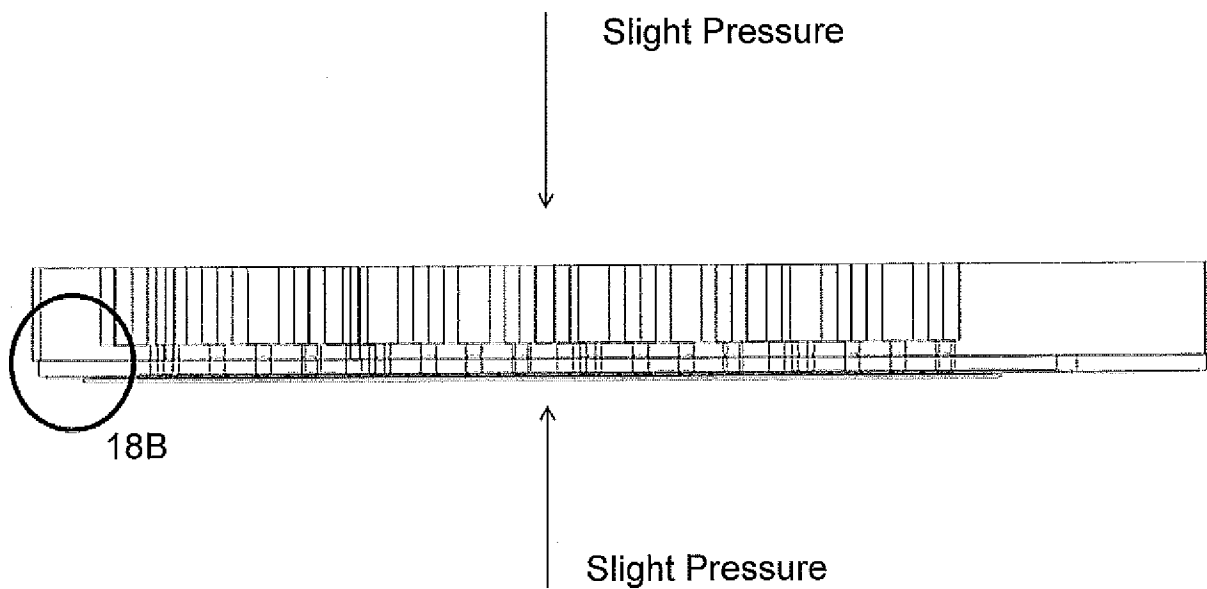


Fig. 28A

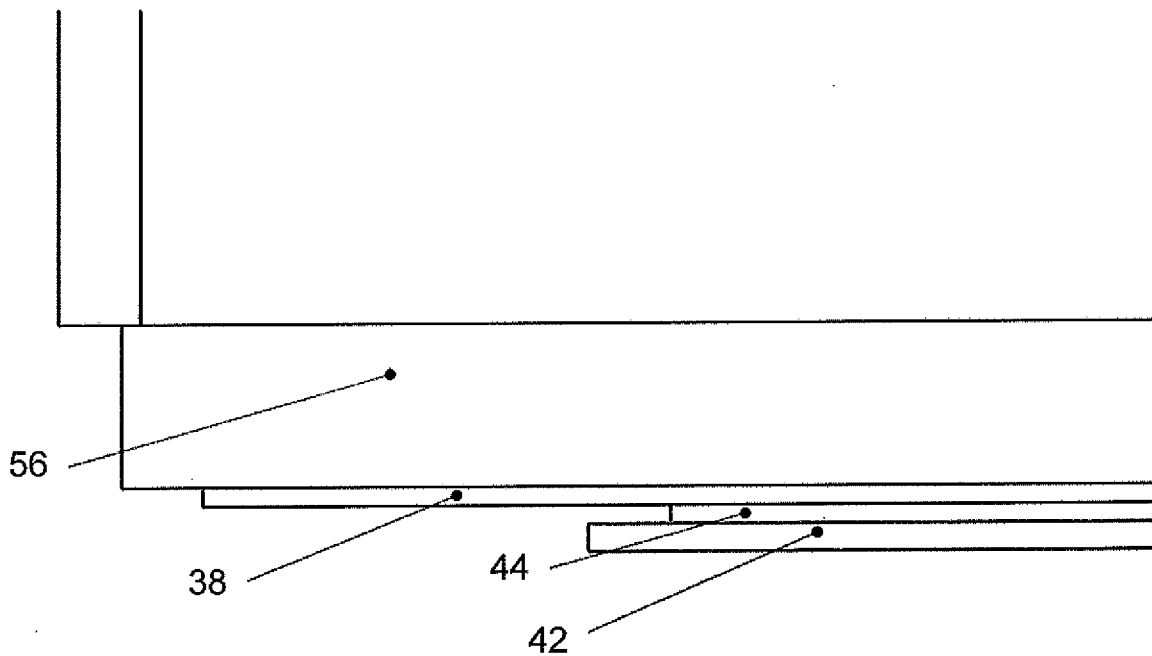


Fig. 28B

33/130

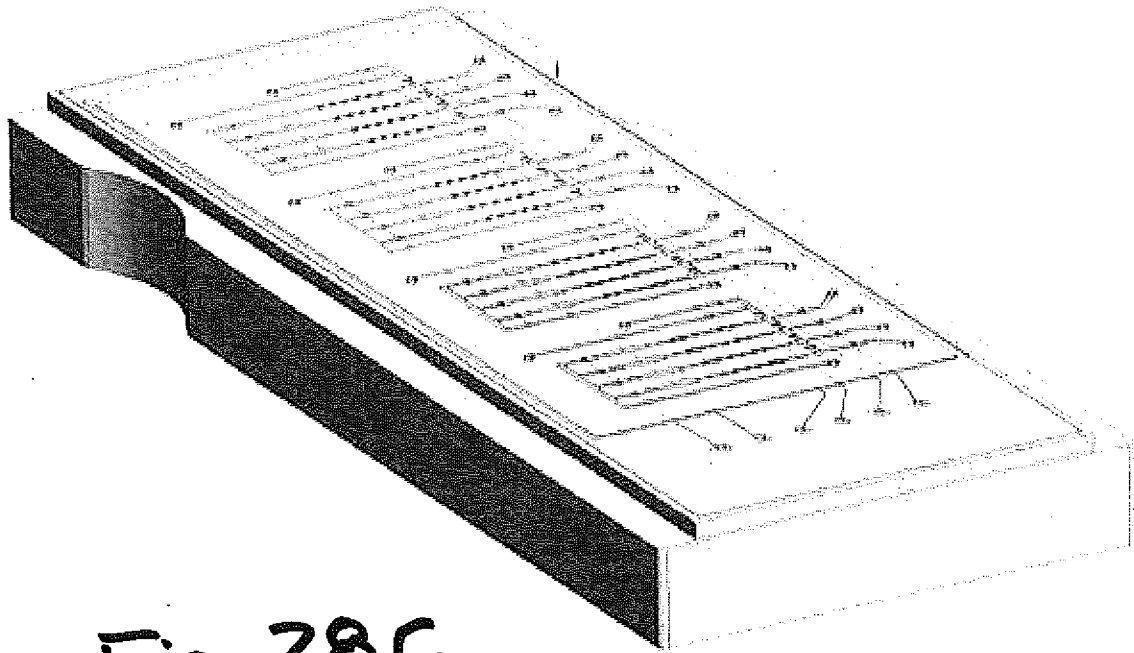


Fig. 28C

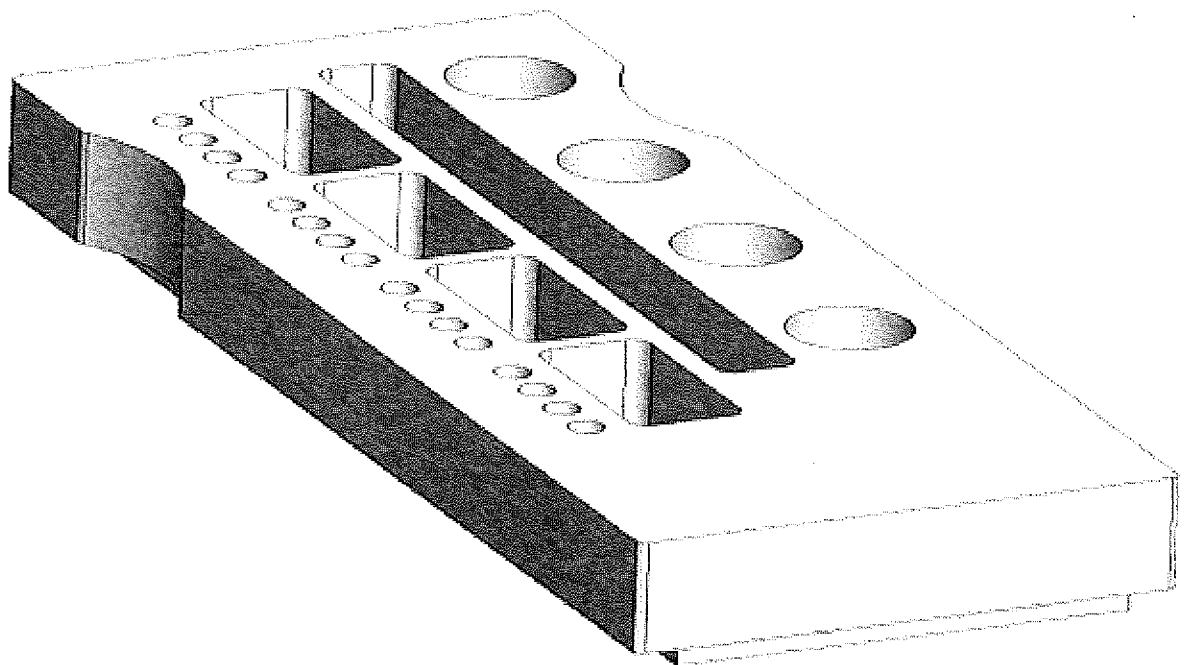


Fig. 28D

34/130

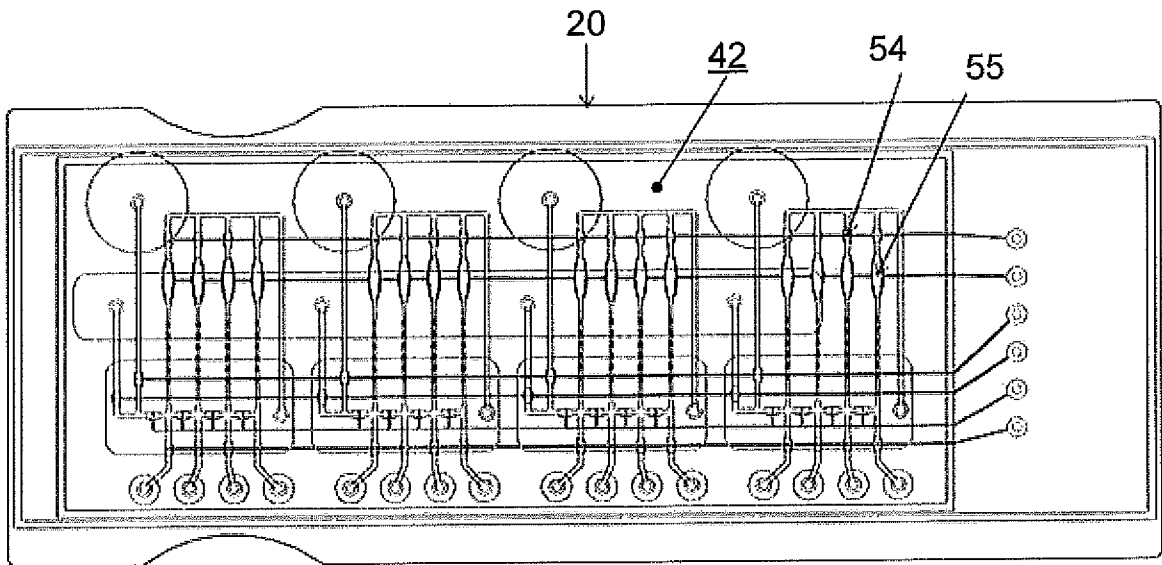


Fig. 29

35/130

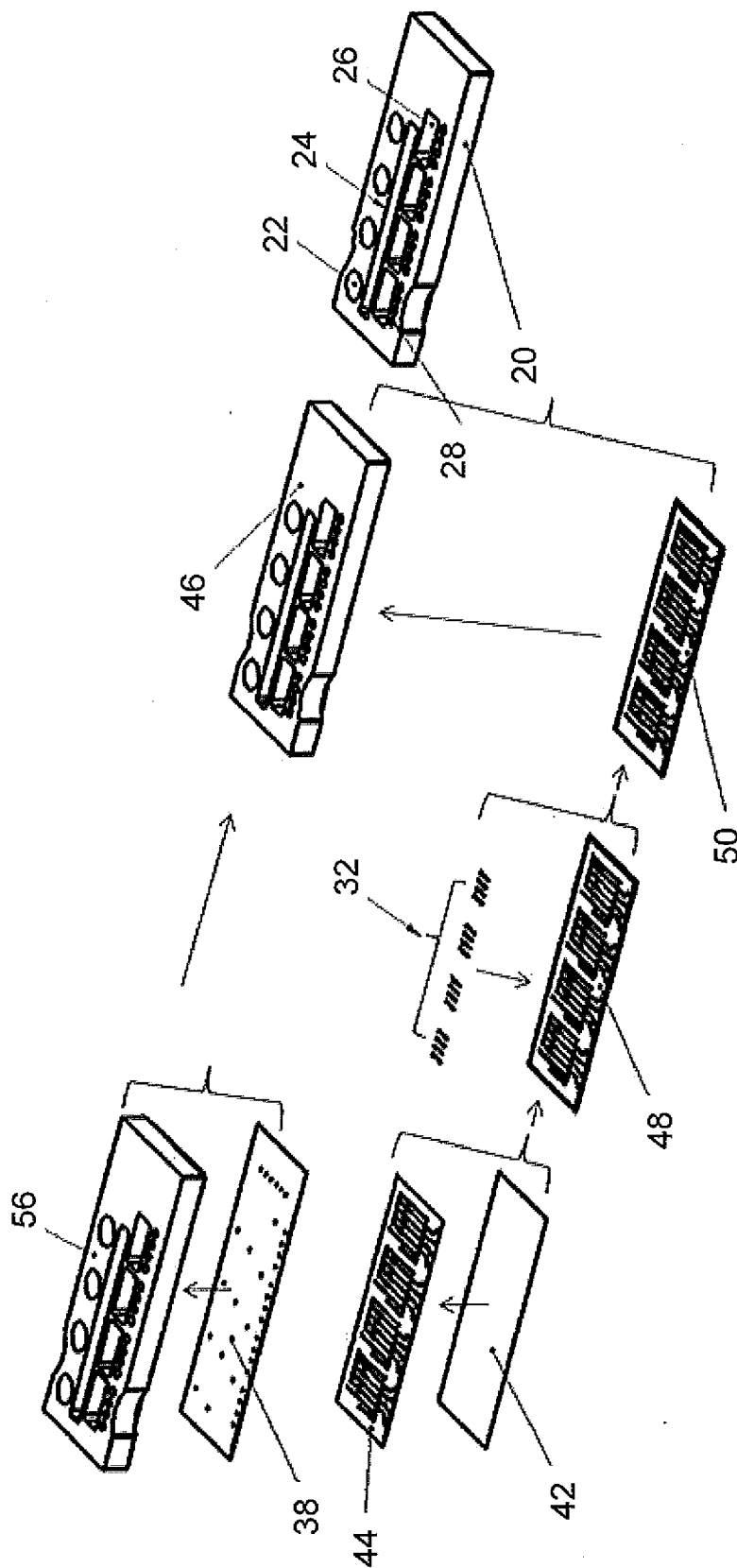


Fig. 30

36/130

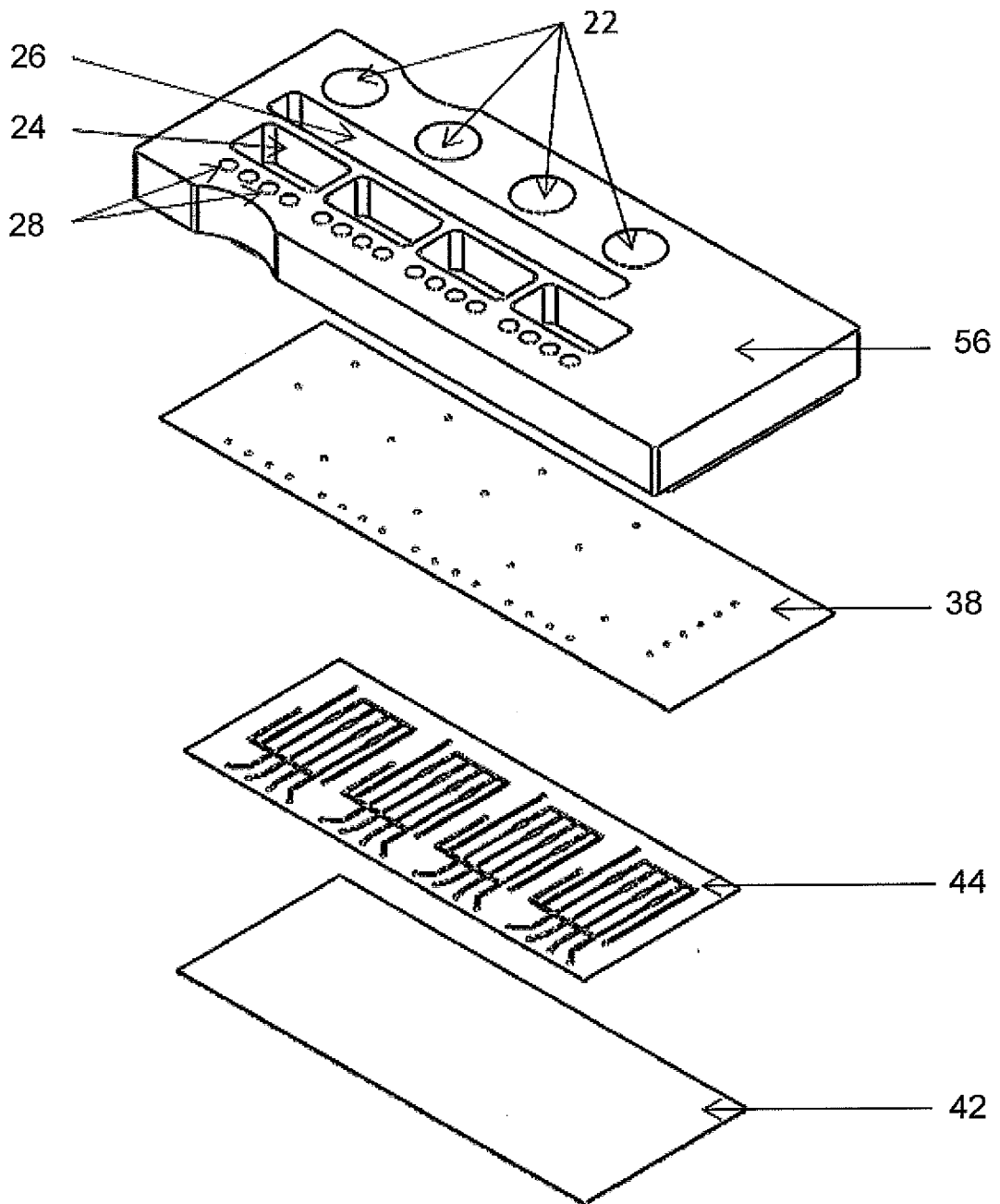


Fig. 30A

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Fig. 31A

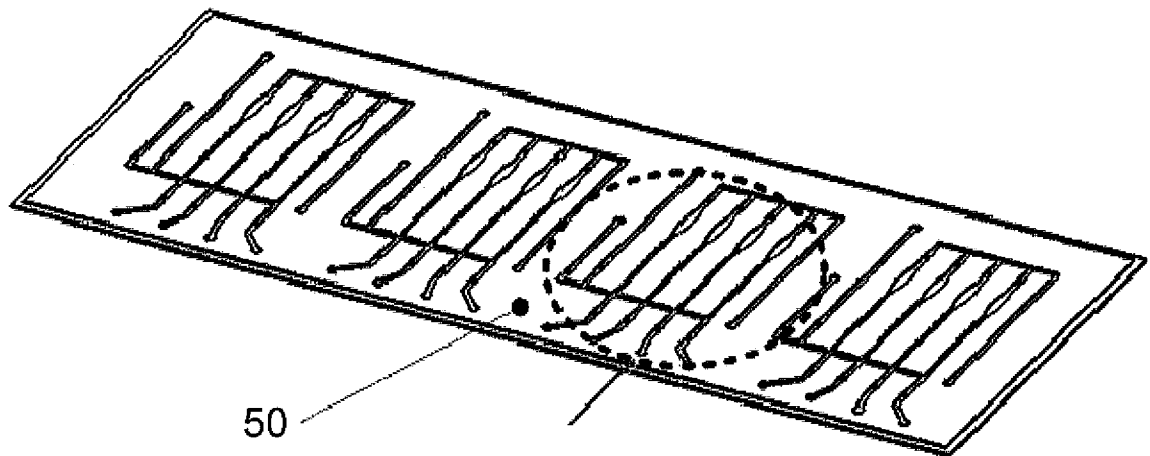


Fig. 31B

38/130

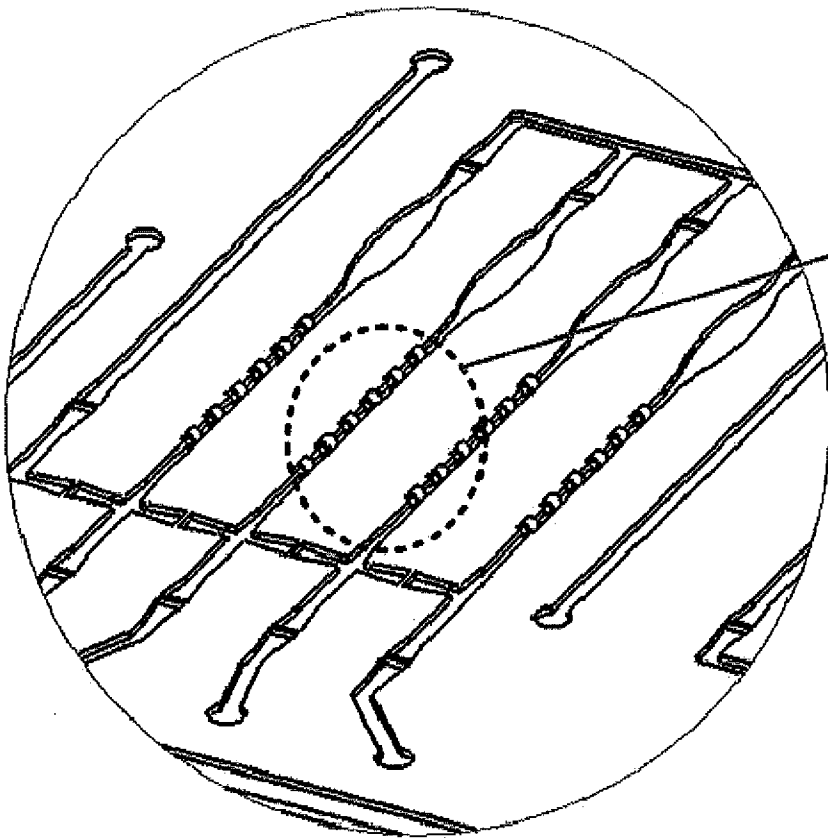


Fig. 31C

Fig. 31B

39/130

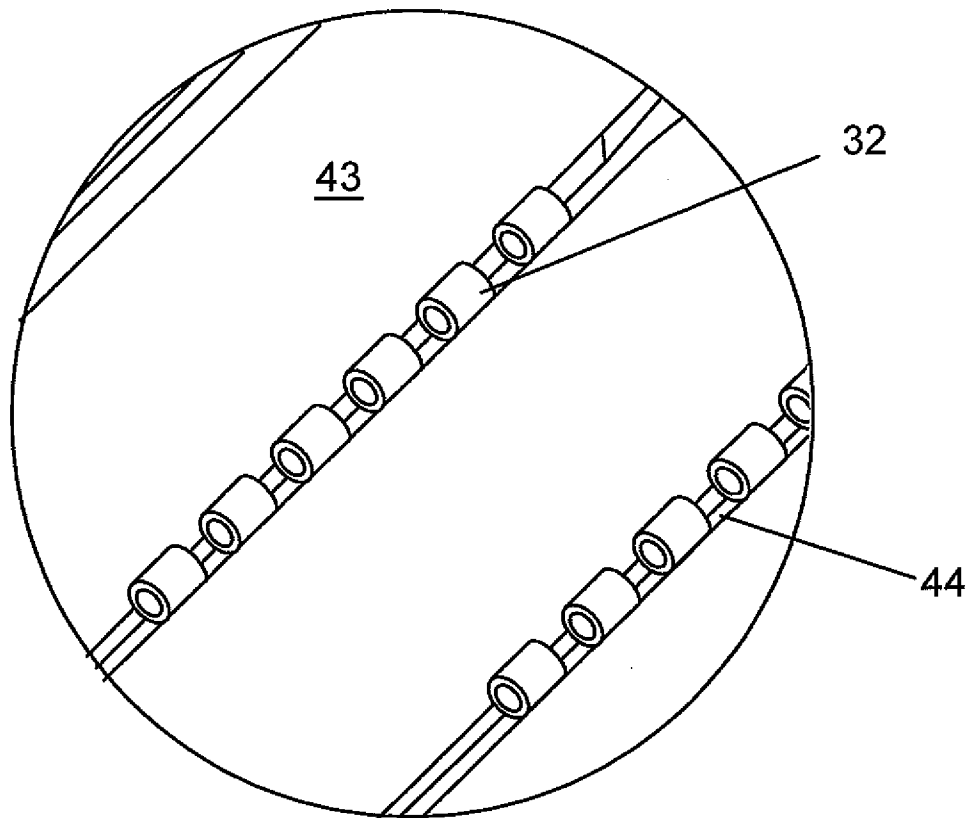
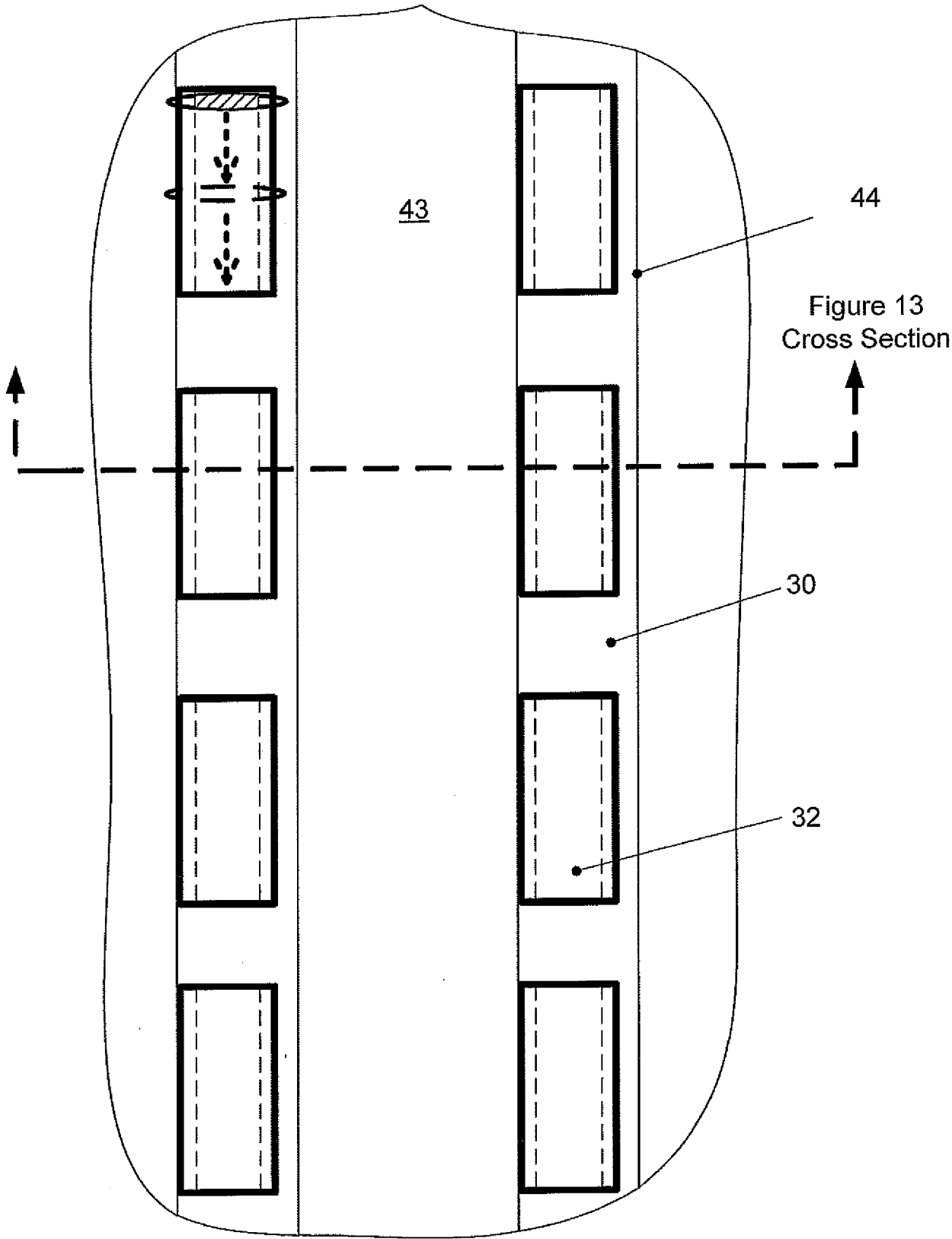


Fig. 31C

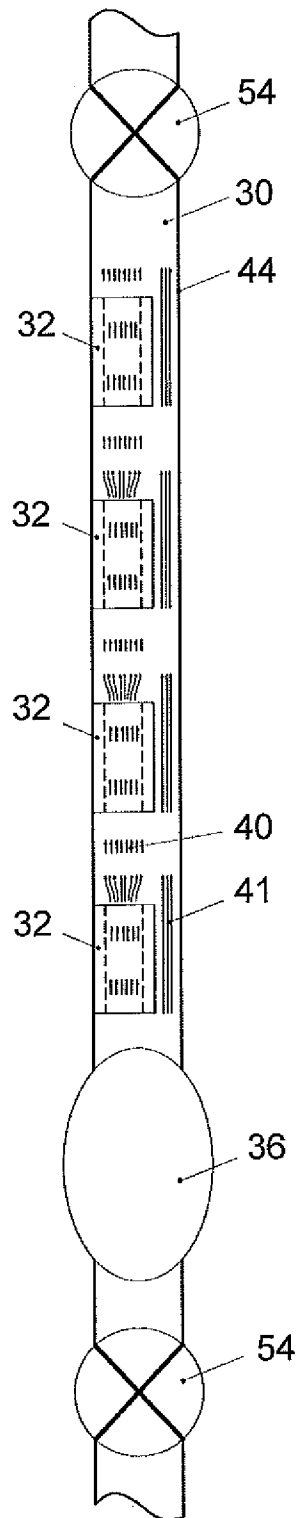
40/130

Fig. 32



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Fig. 33



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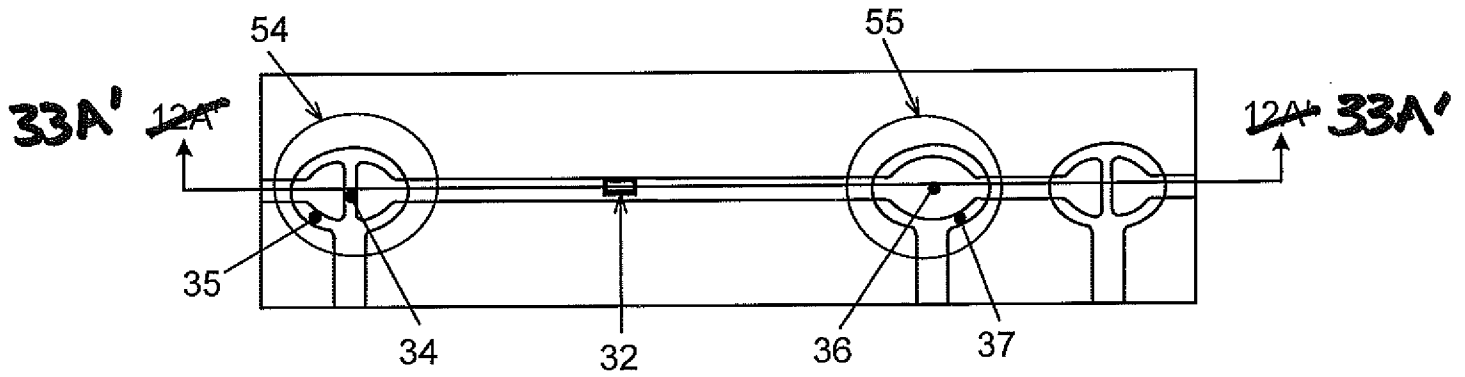


Fig. 33A

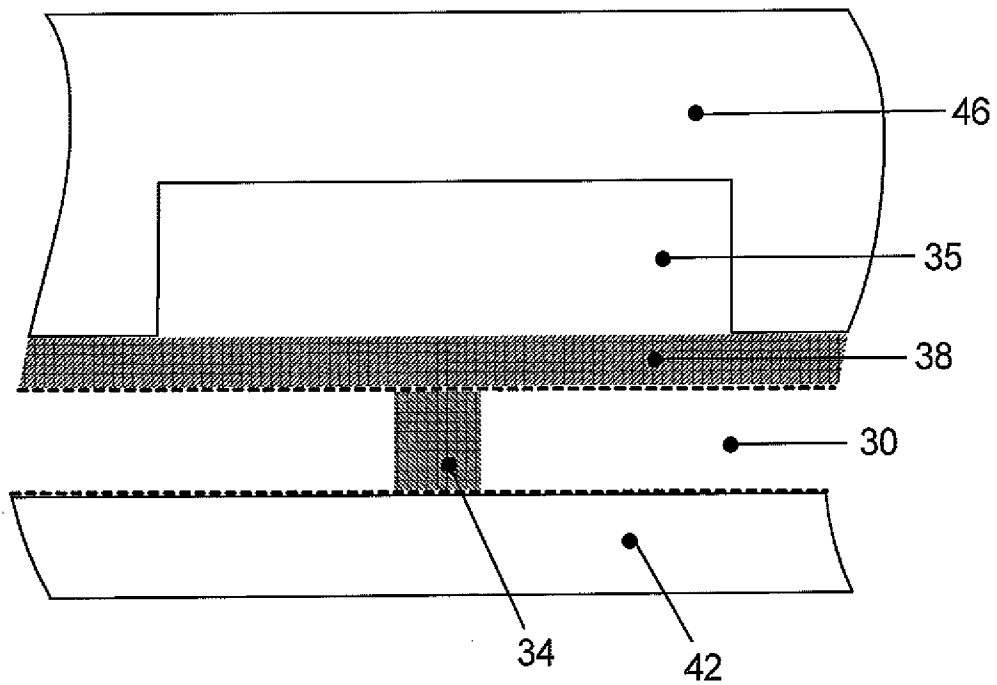
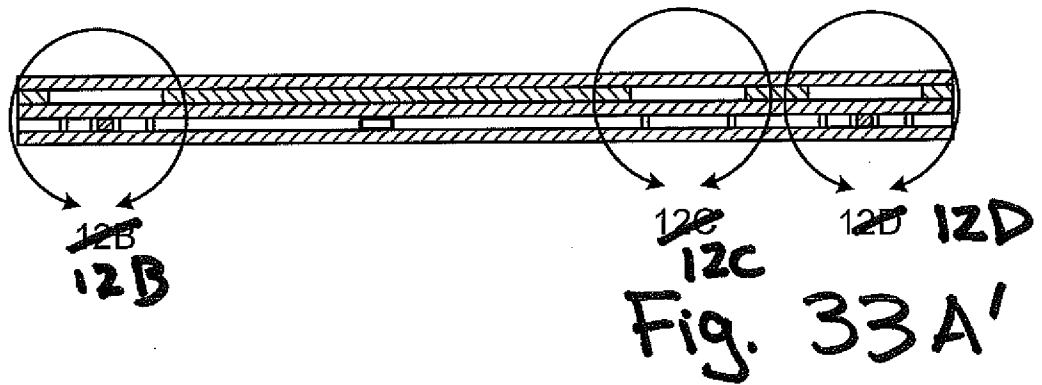


Fig. 33B

43/130

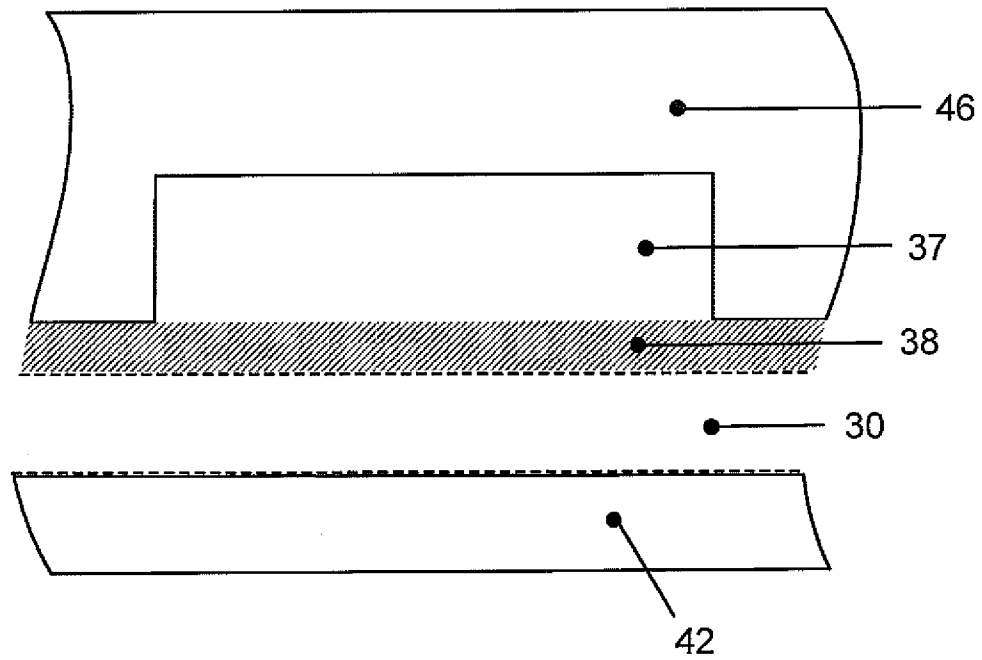


Fig. 33C

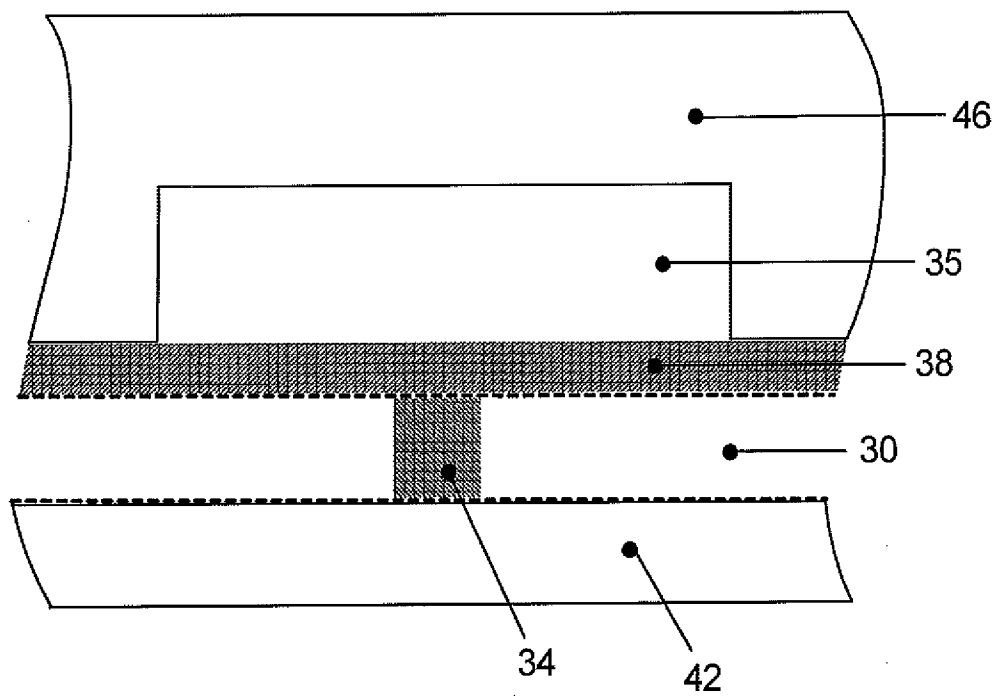


Fig. 33D

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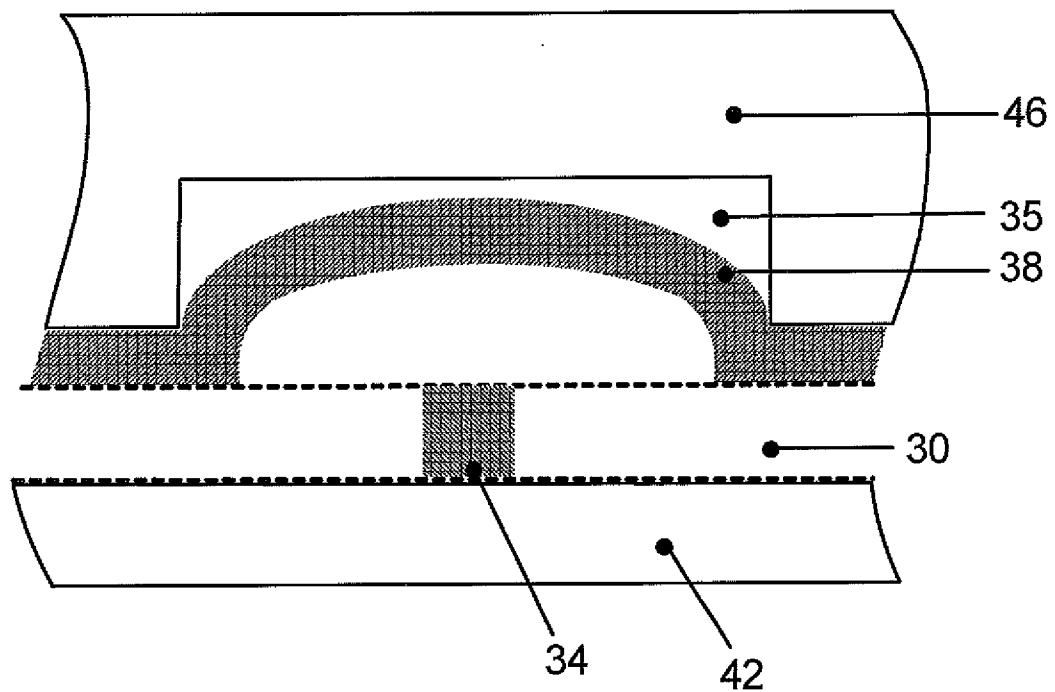


Fig. 33E

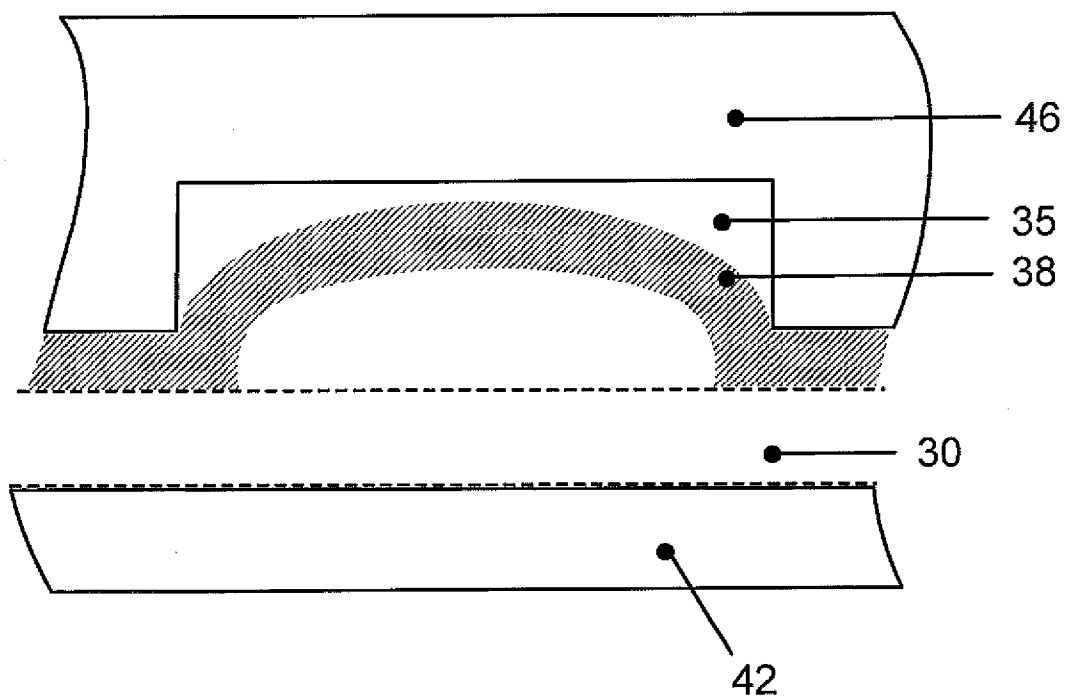


Fig. 33F

45/130

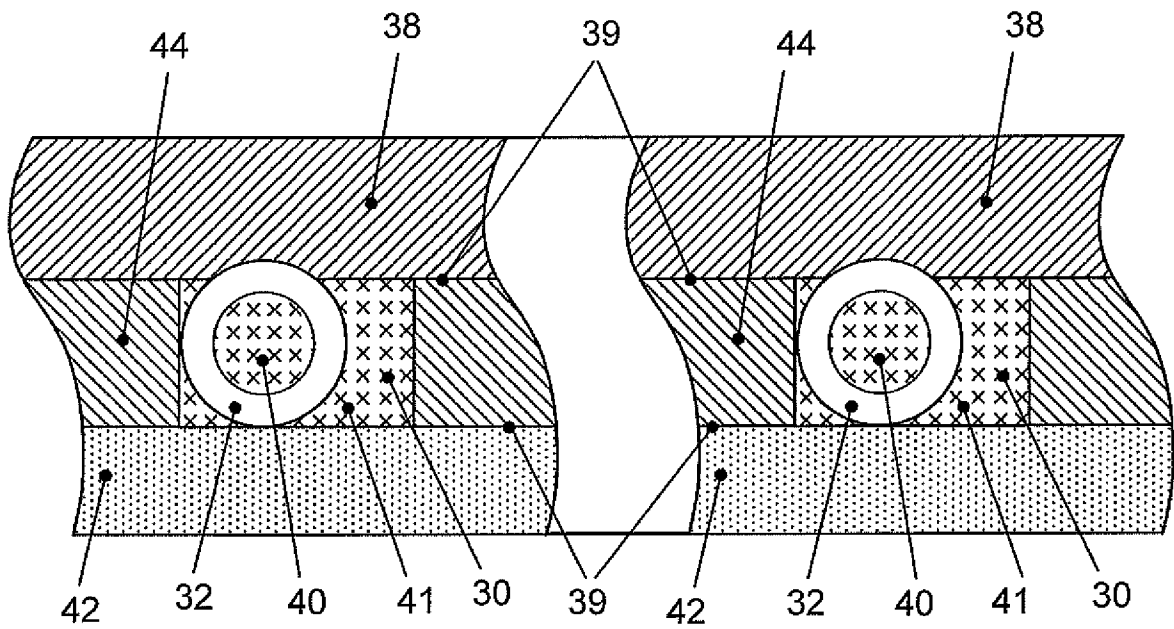


Fig. 34

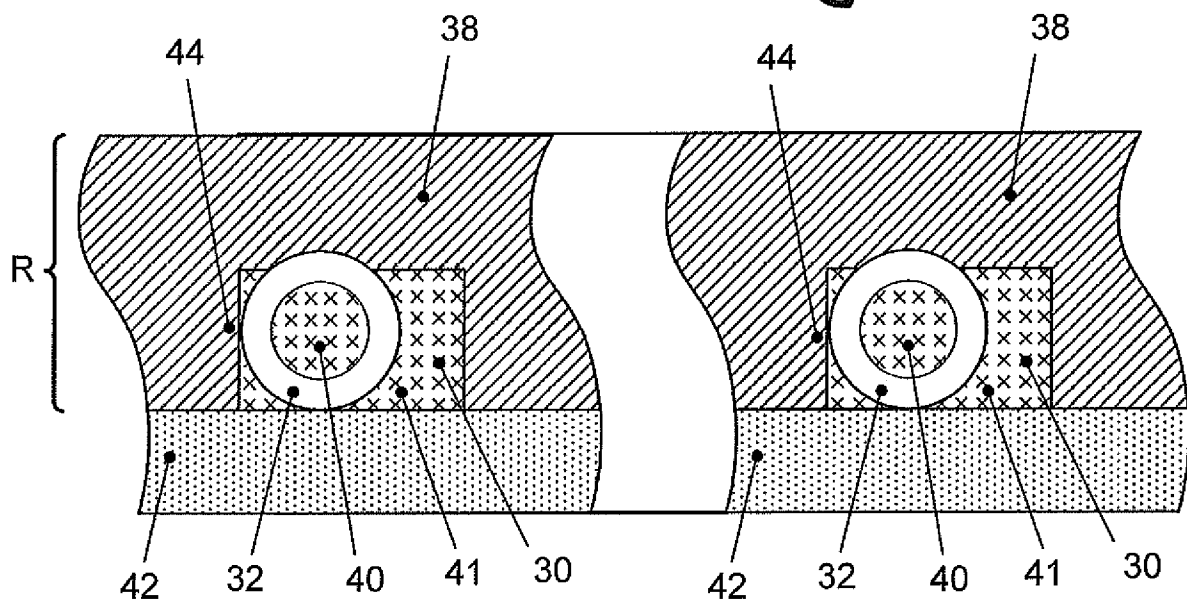


Fig. 34A

46/130

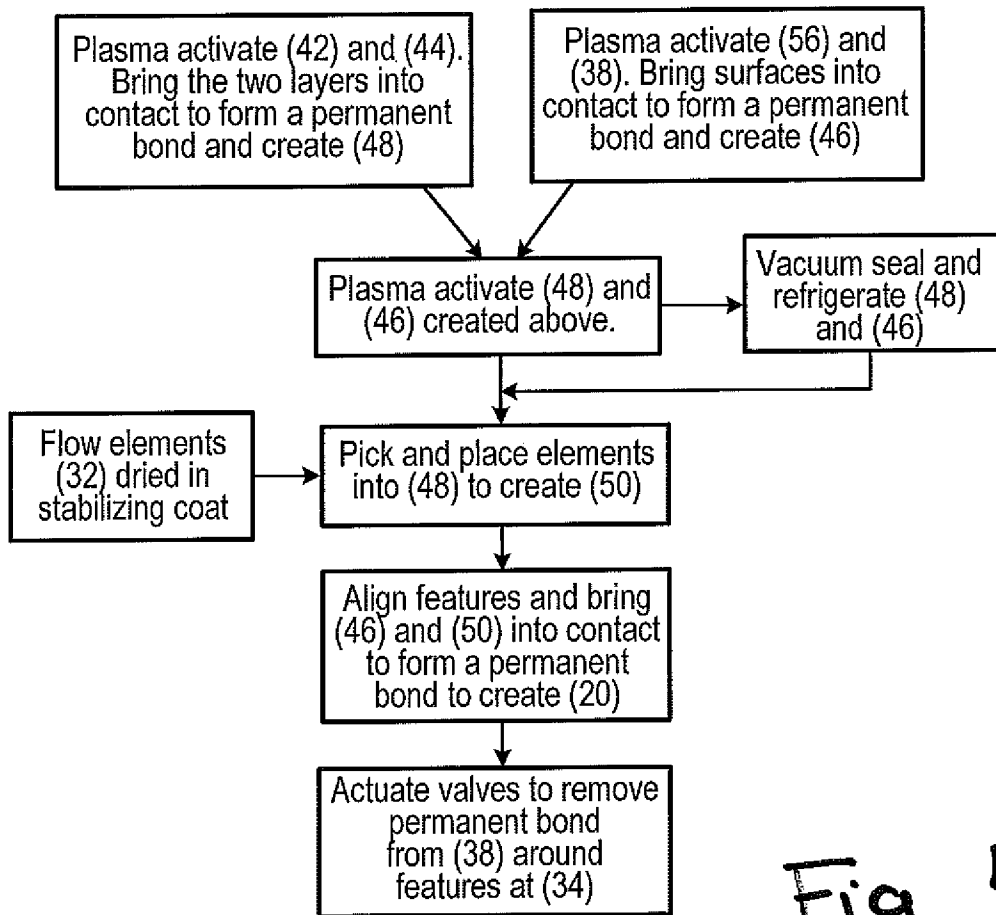


Fig. 41

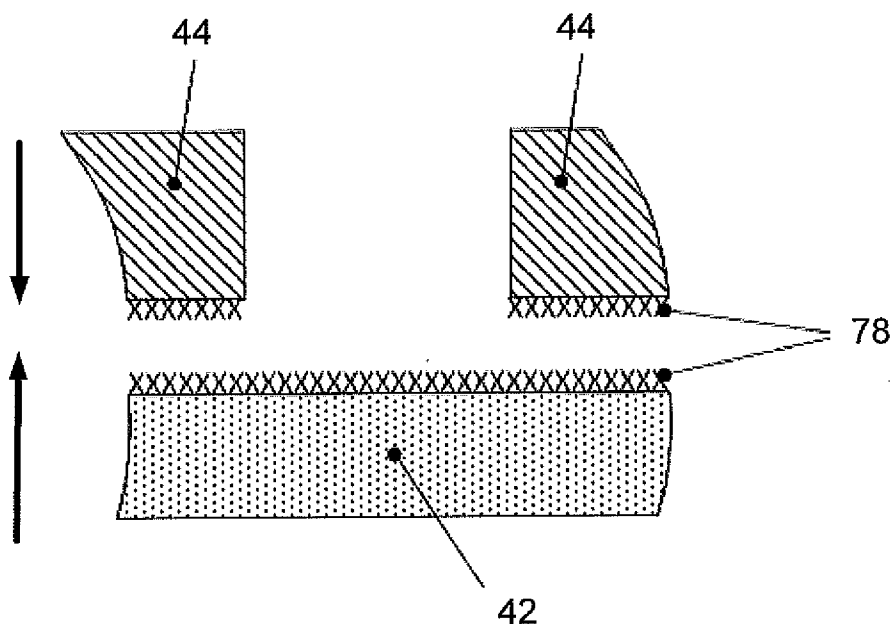


Fig. 41A

47/130

Fig. 41B

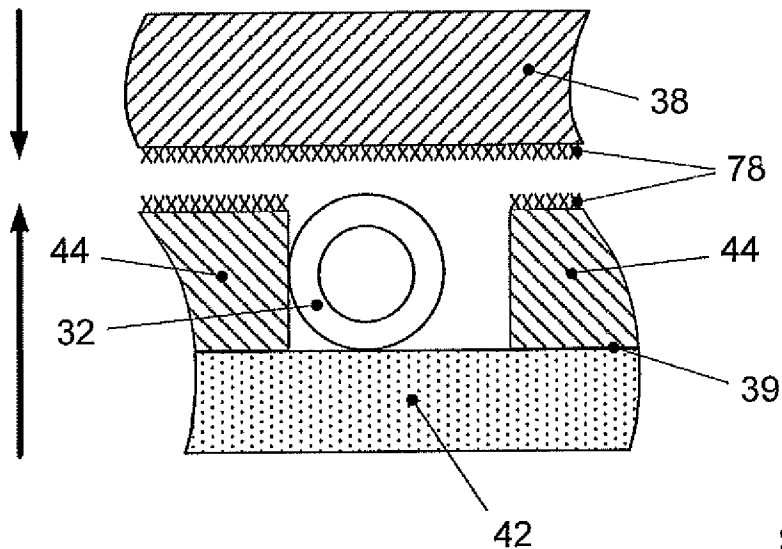


Fig. 41C

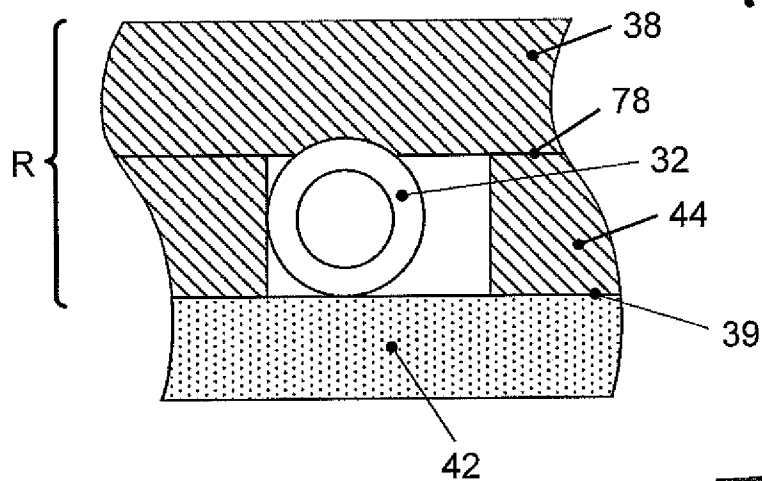
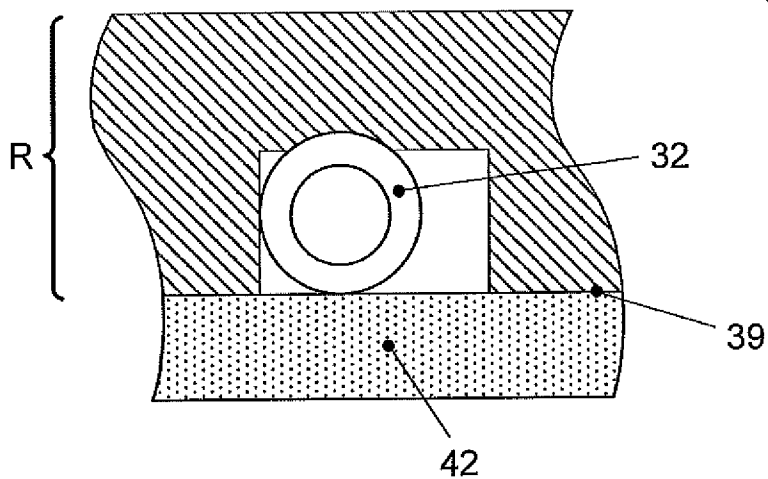
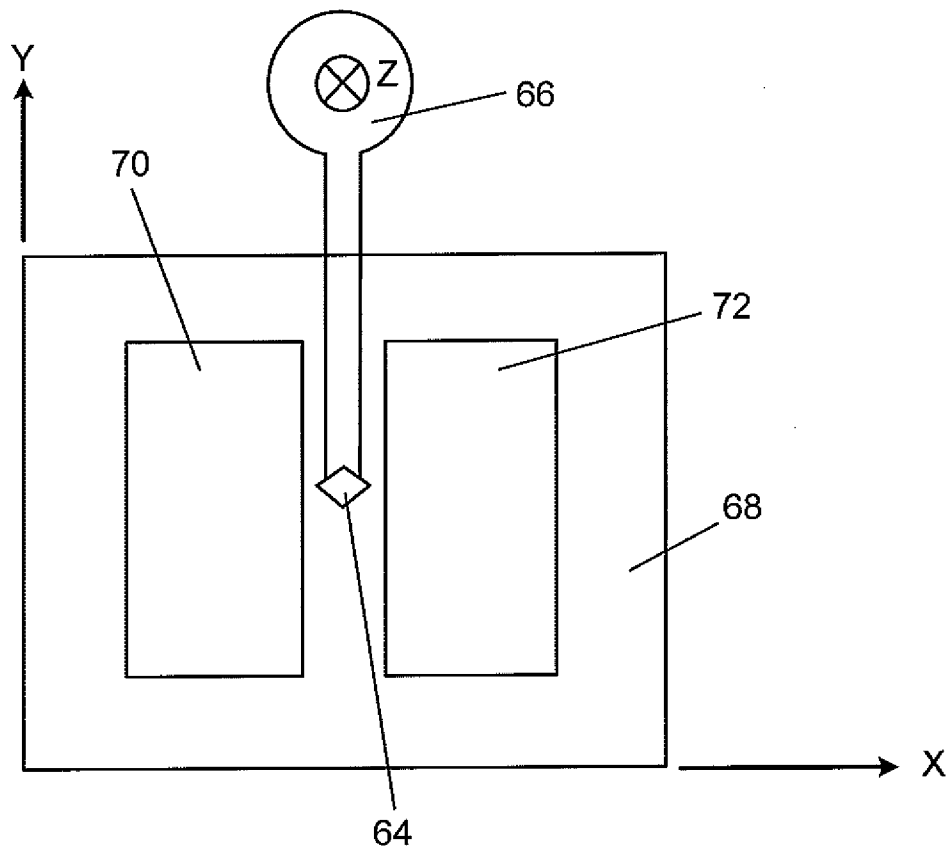


Fig. 41D



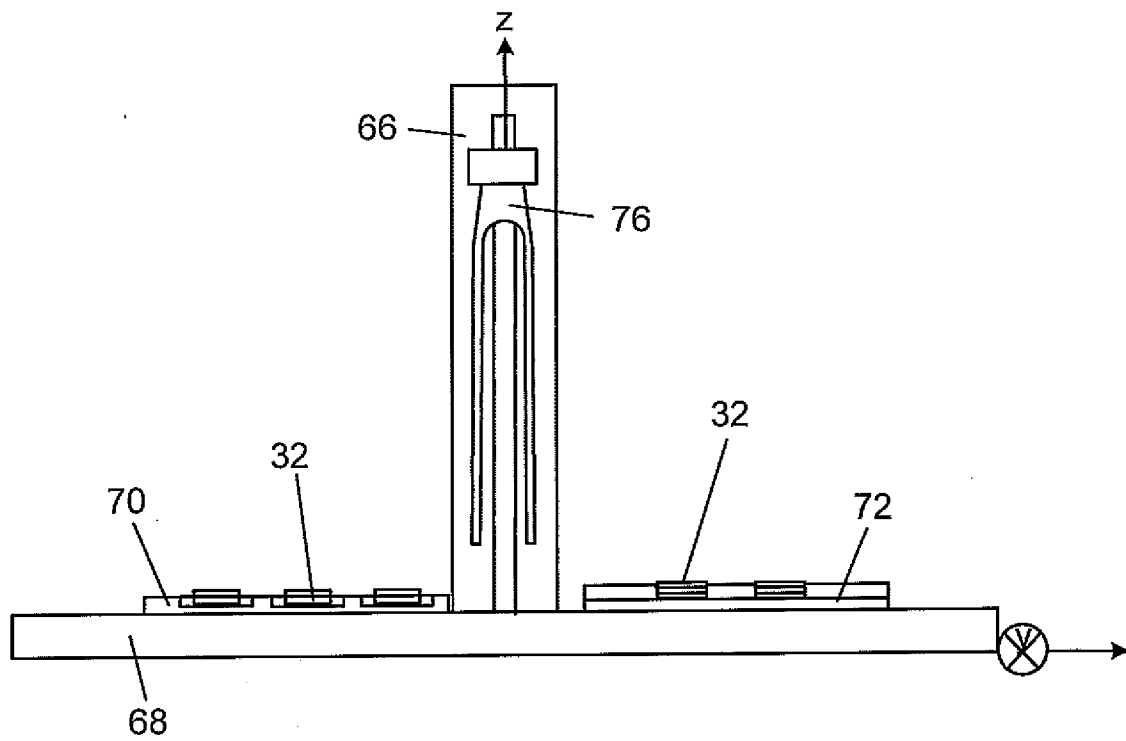
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Fig. 4Z

Pick & Place – Top View

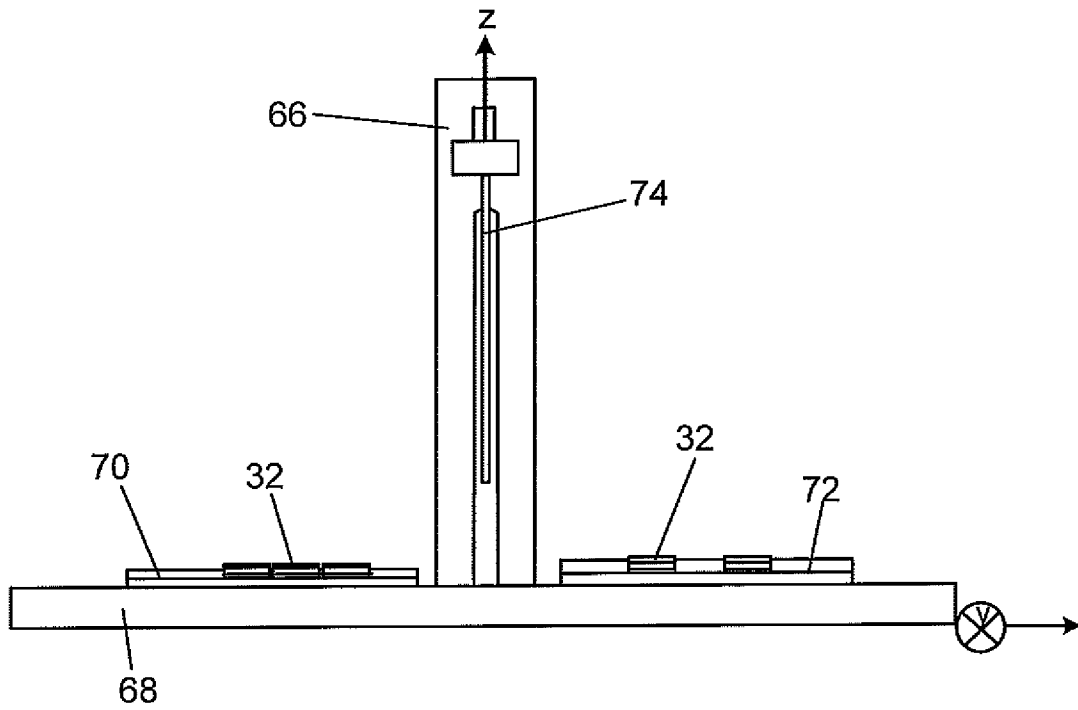
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Fig. 43

Pick & Place – Front View

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Fig. 45

Pick & Place – Front View

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PICK with Vacuum

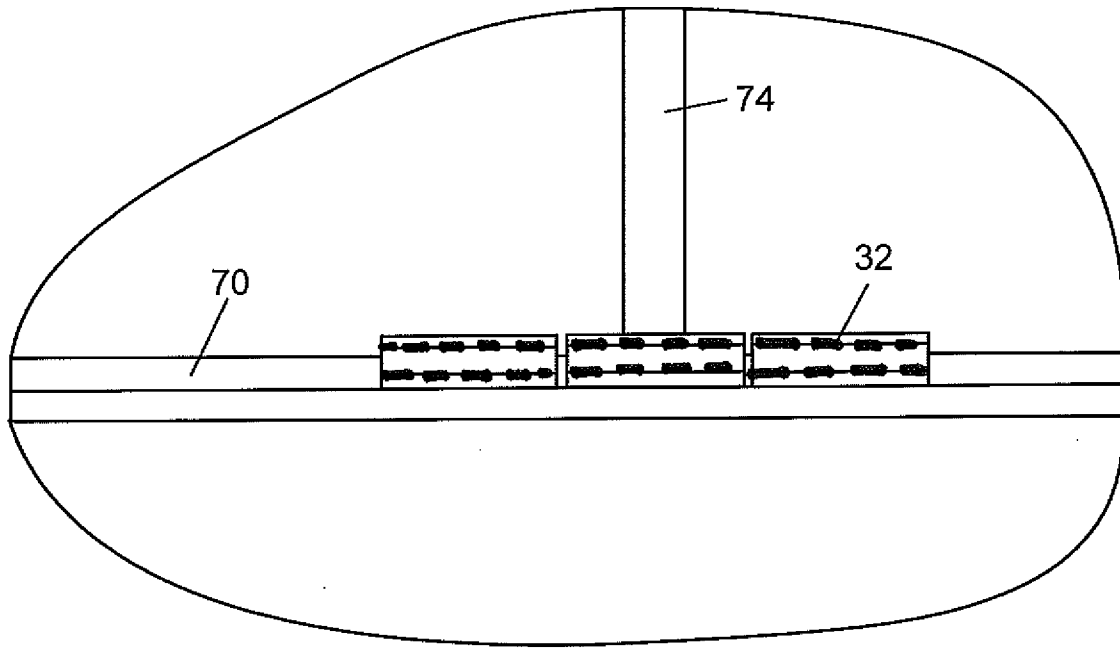


Fig. 46

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PLACE with Vacuum

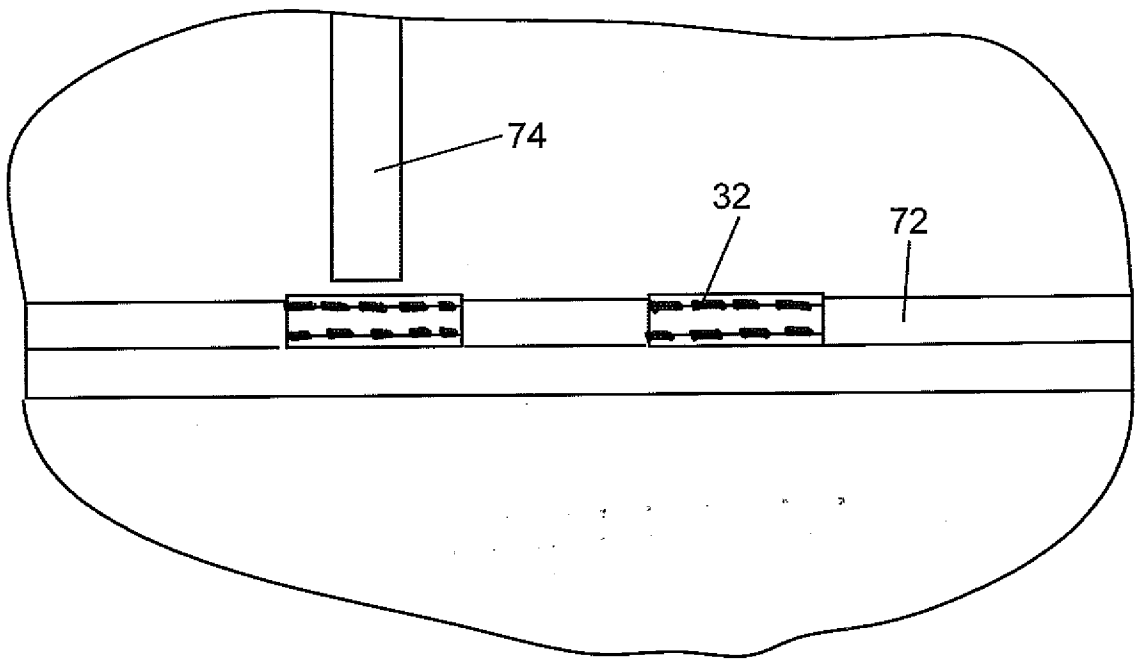


Fig. 47

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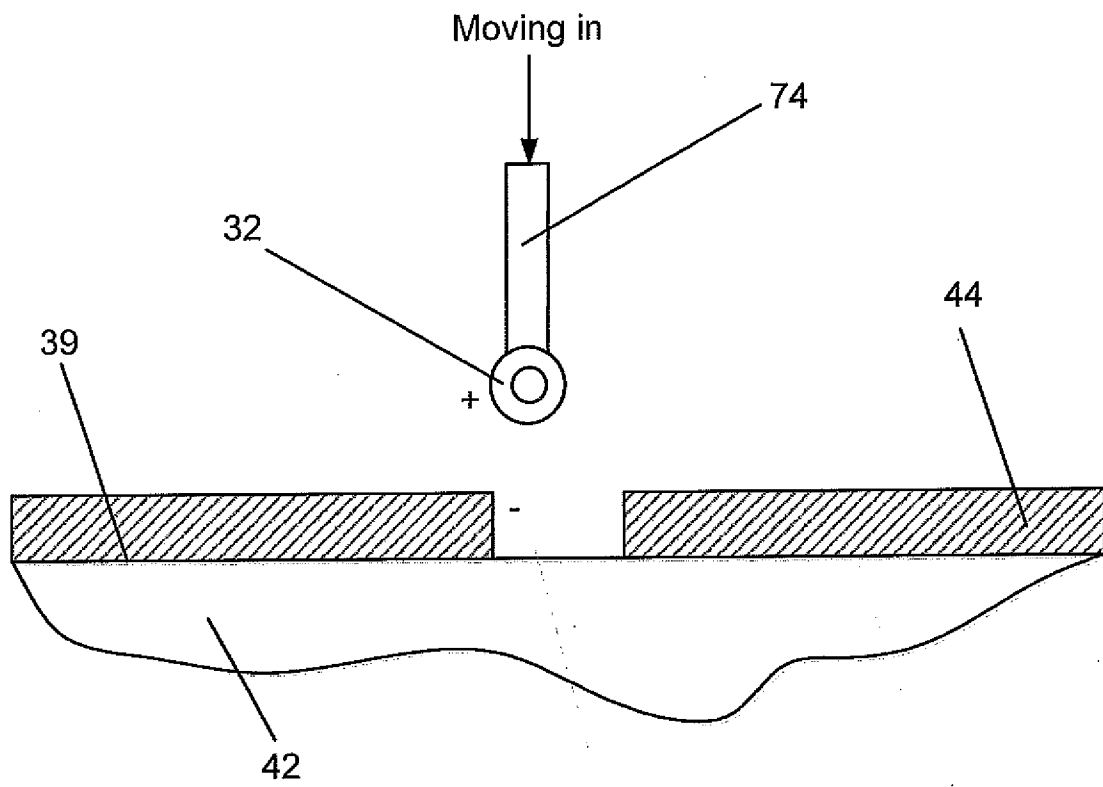


Fig. 48

54/130

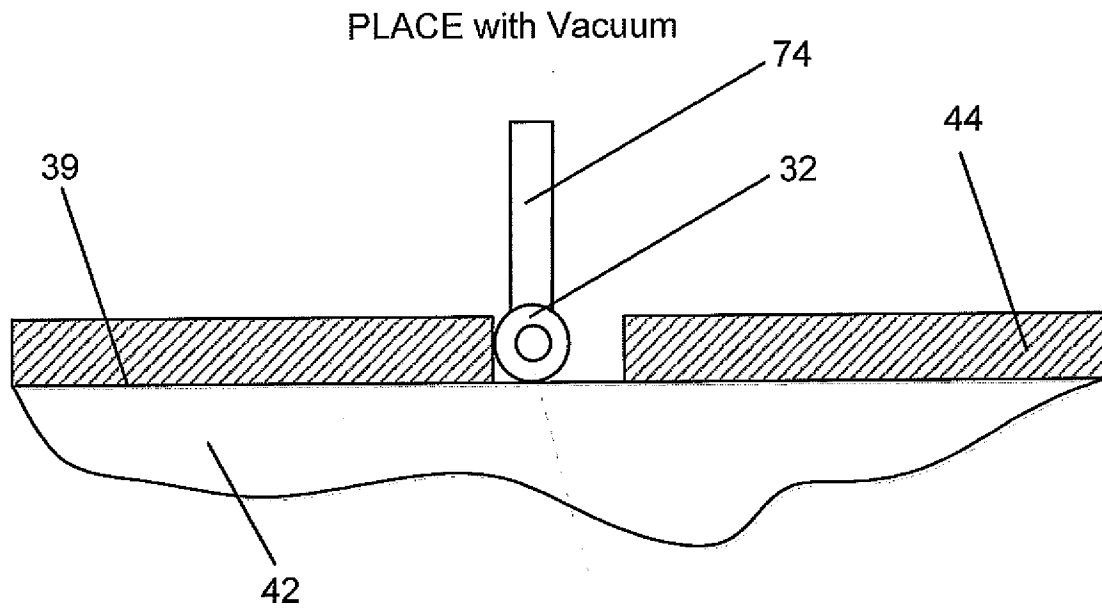


Fig. 49

55/130

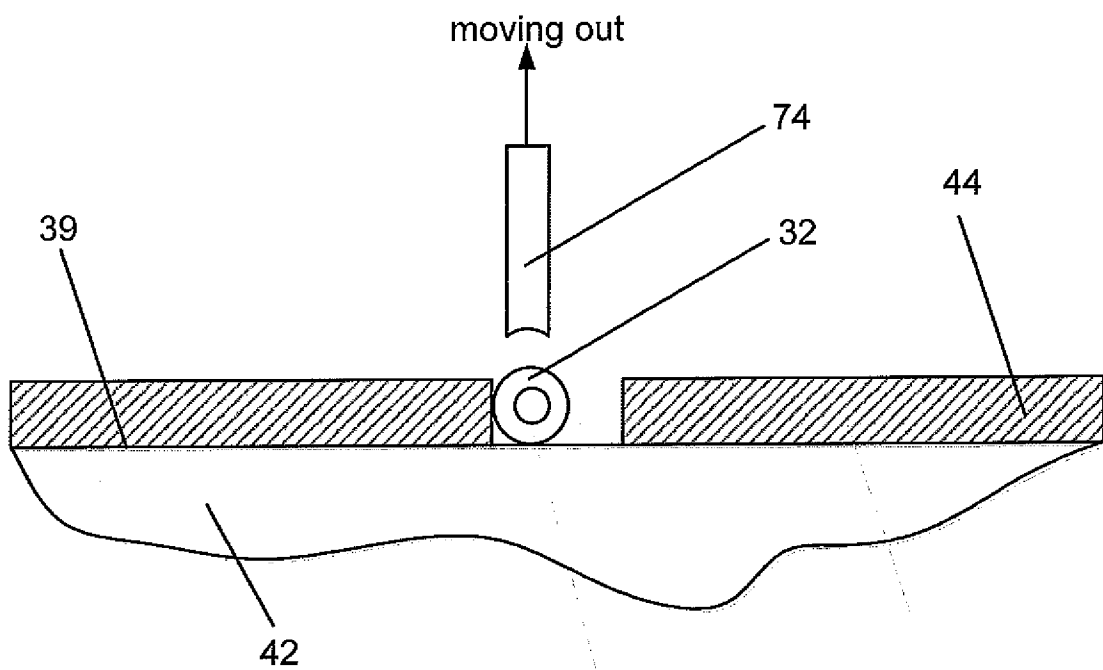


Fig. 49A

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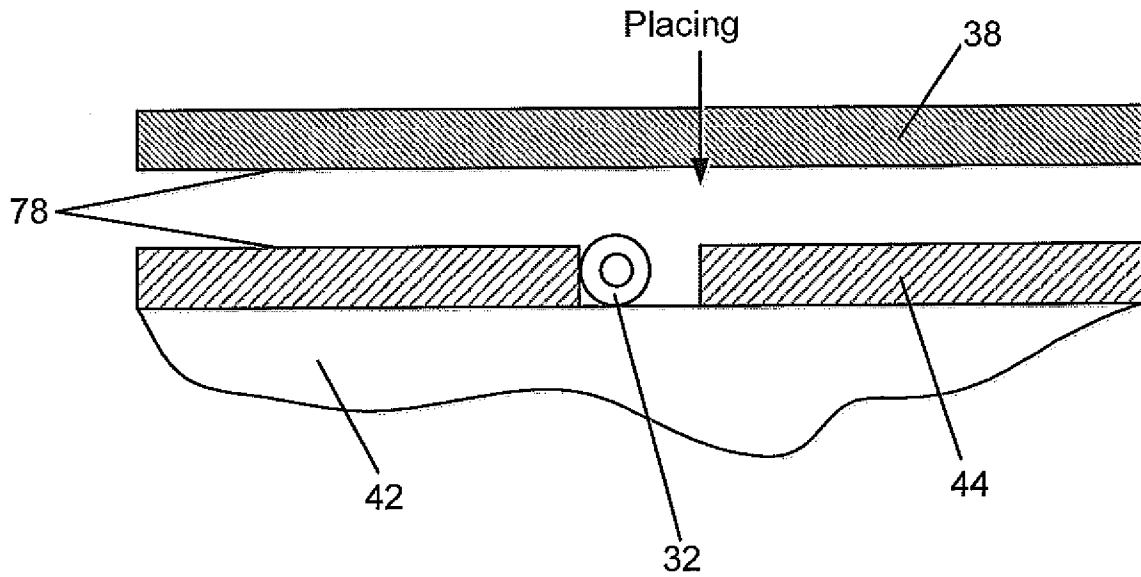


Fig. 49B

57/130

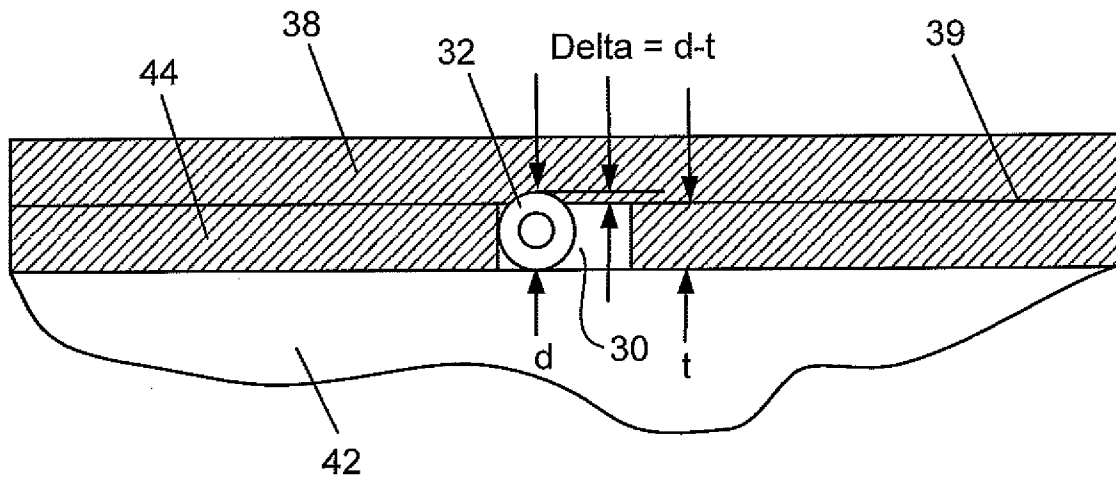
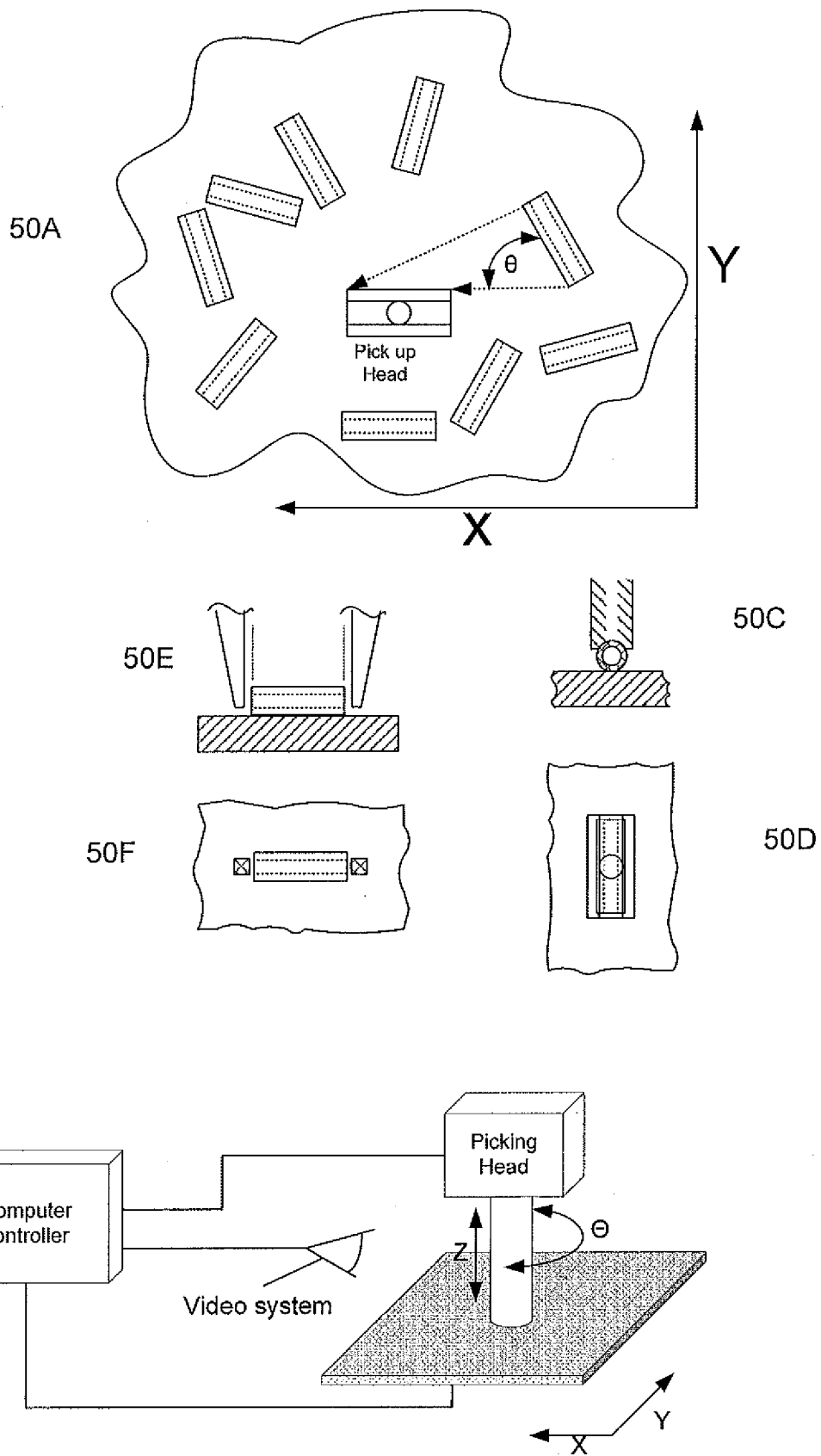


Fig. 50

Pick and place

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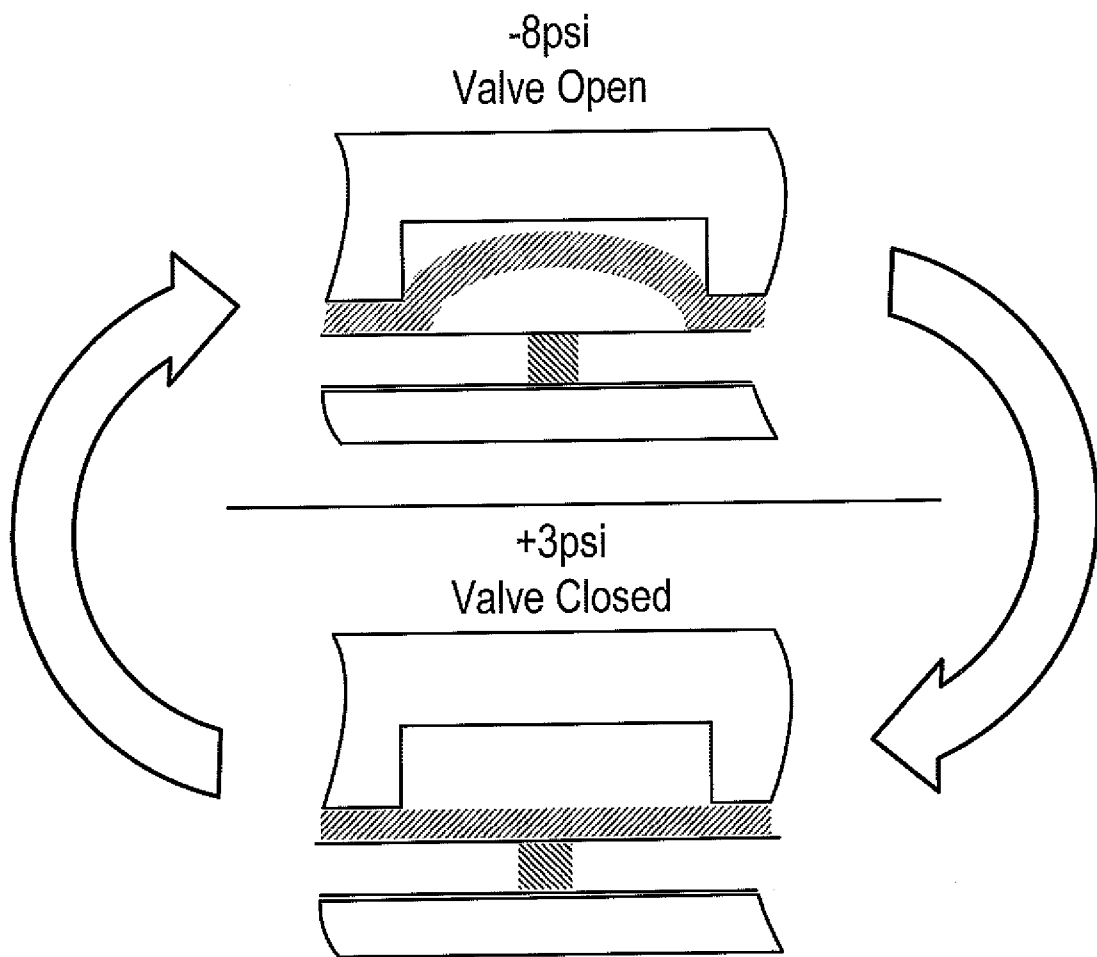
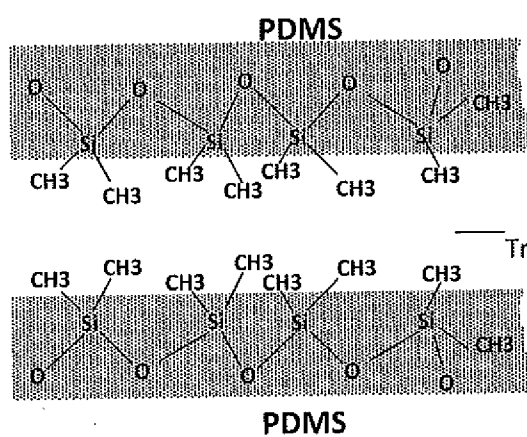


Fig. 51

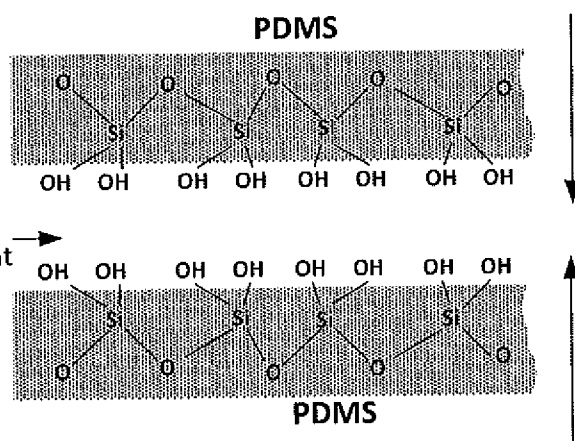
60/130

PDMS Bonding
Chemistry

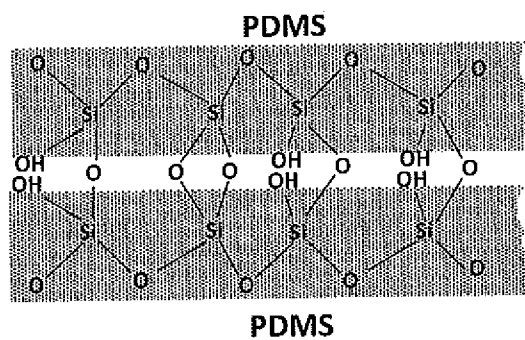
51 A-1



51 A-2

Plasma
Treatment

51 A-3

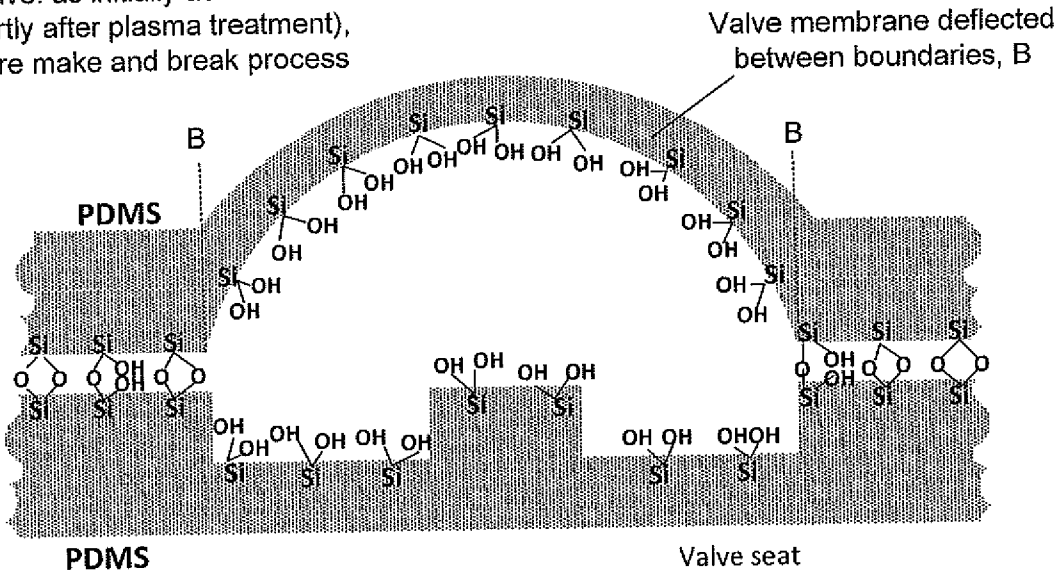


Bonded

51 A - 4A

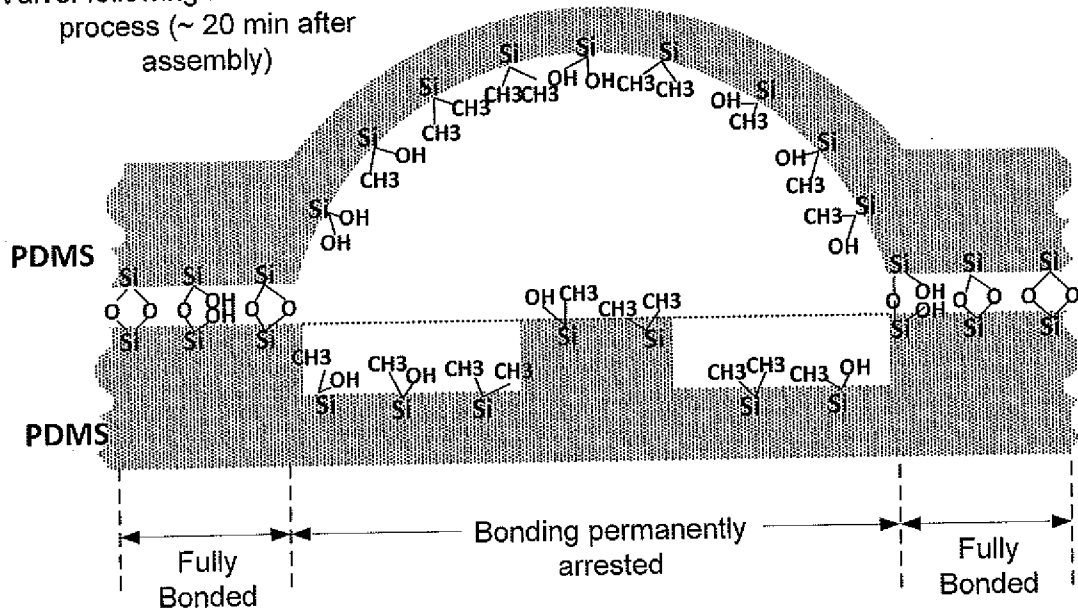
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Valve: as initially assembled
(shortly after plasma treatment),
before make and break process

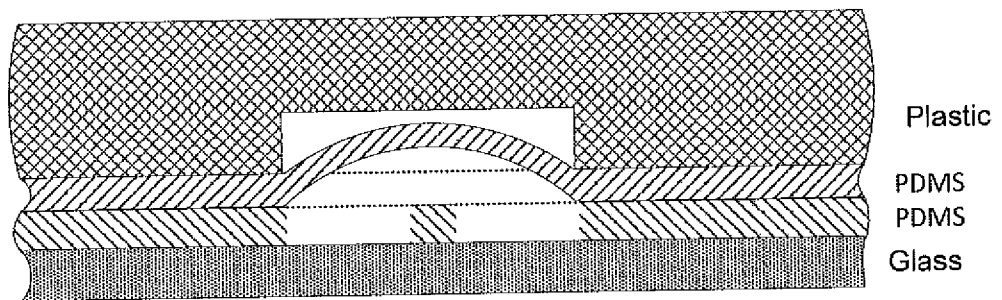


51 A - 4B

Valve: following make and break
process (~ 20 min after
assembly)



51 A - 6



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Make and break
Process Illustration –
used to prepare
multiple valves at once
on a device

Fig 51 C

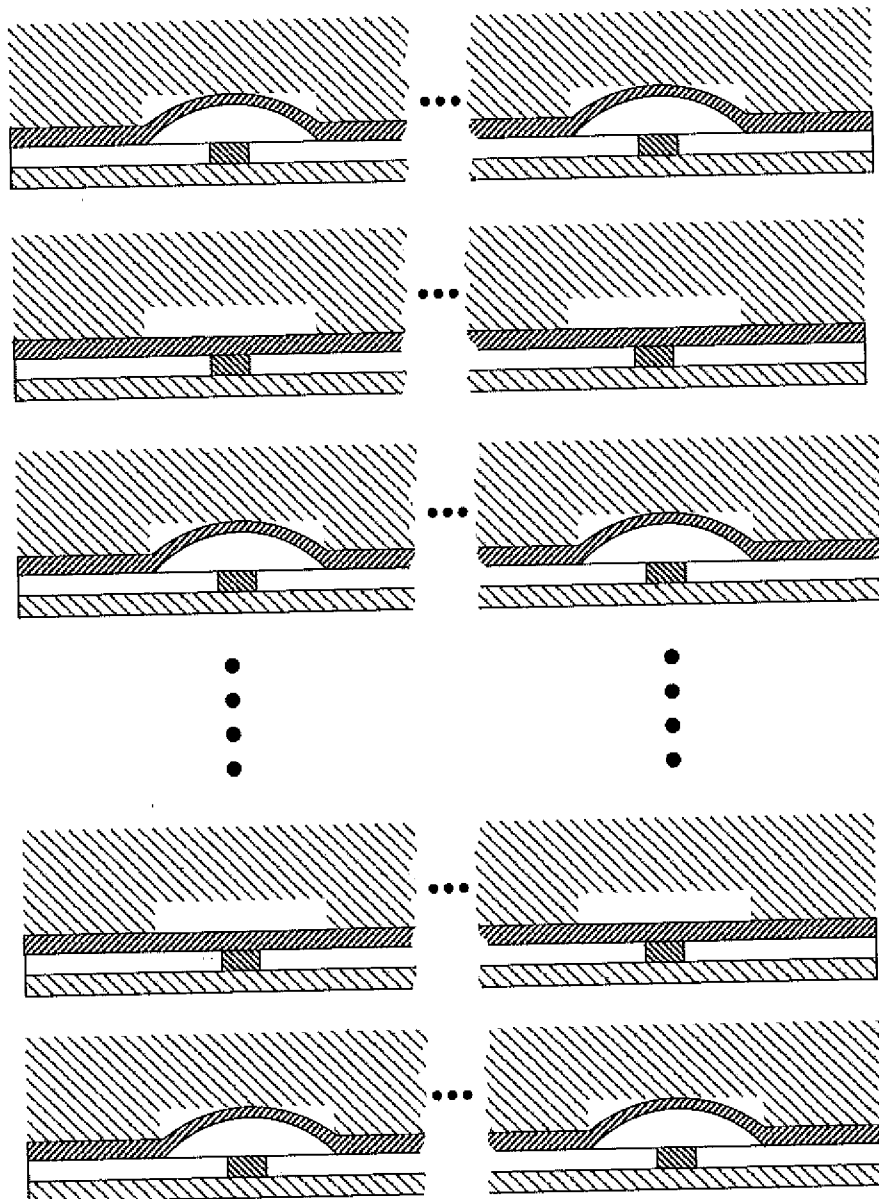
Valve deflected during
initial assembly

1st 'Make' - duration
<1 sec

1st 'Break' - break initial
bonds formed during 1st
'Make'

nth 'Make'

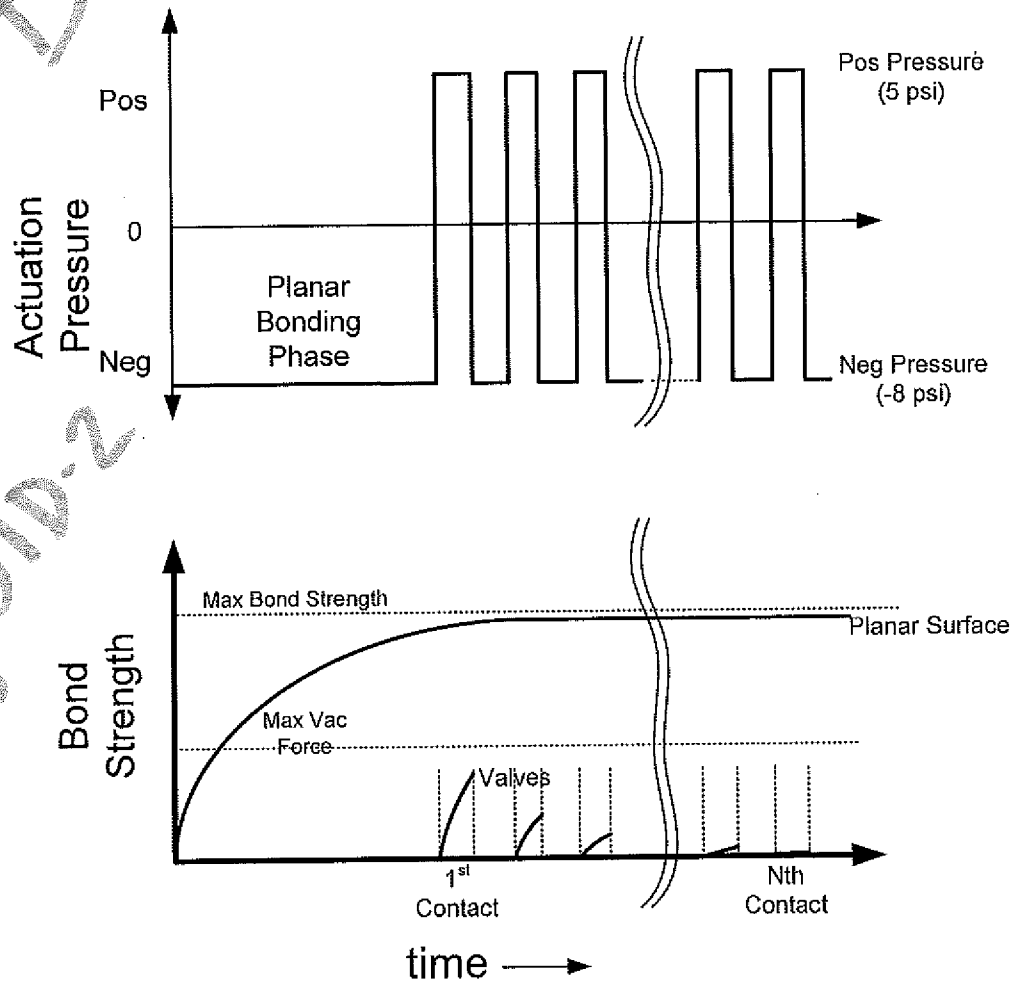
nth 'Break'



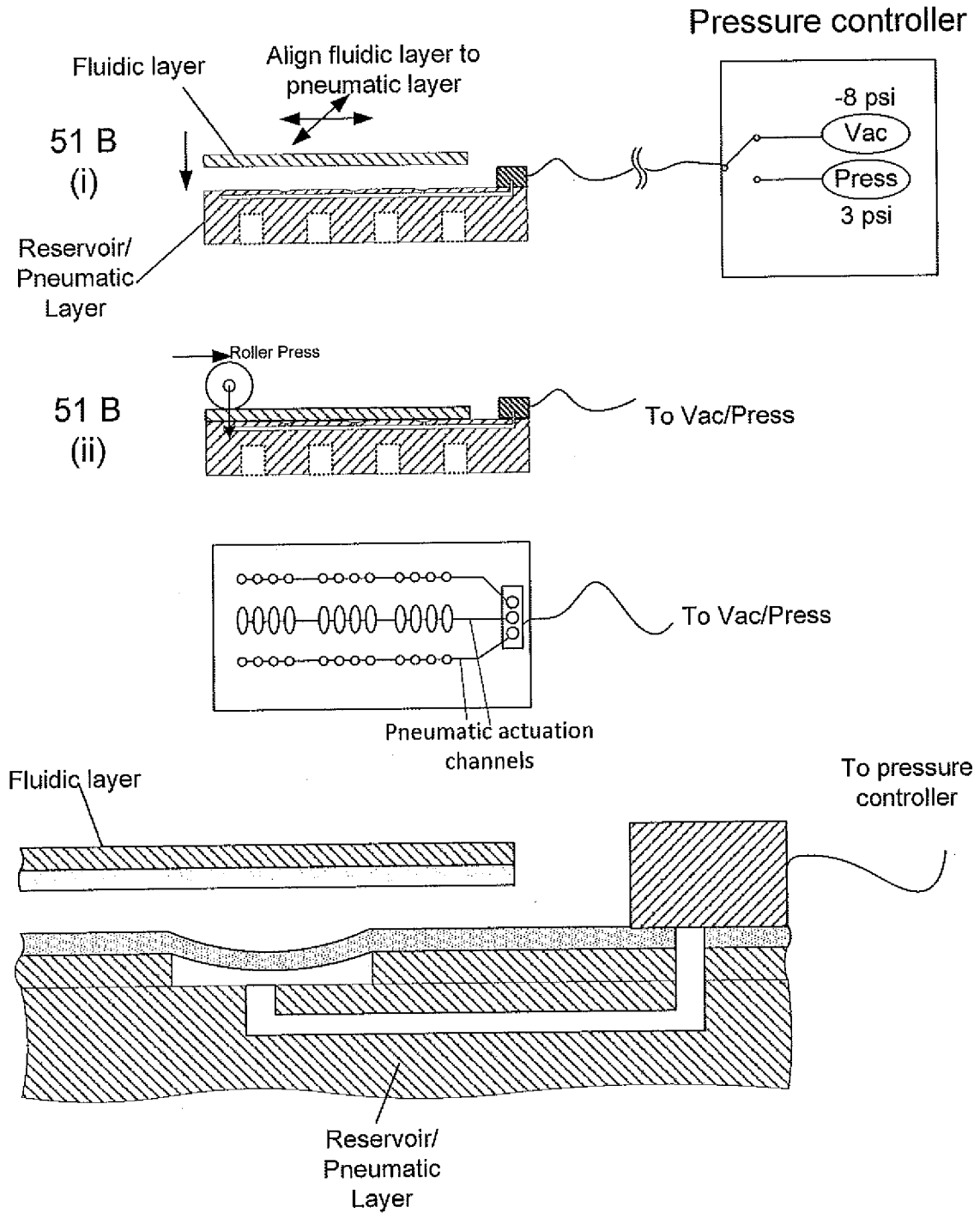
All future bonding on valve
seats permanently arrested

Make and Break

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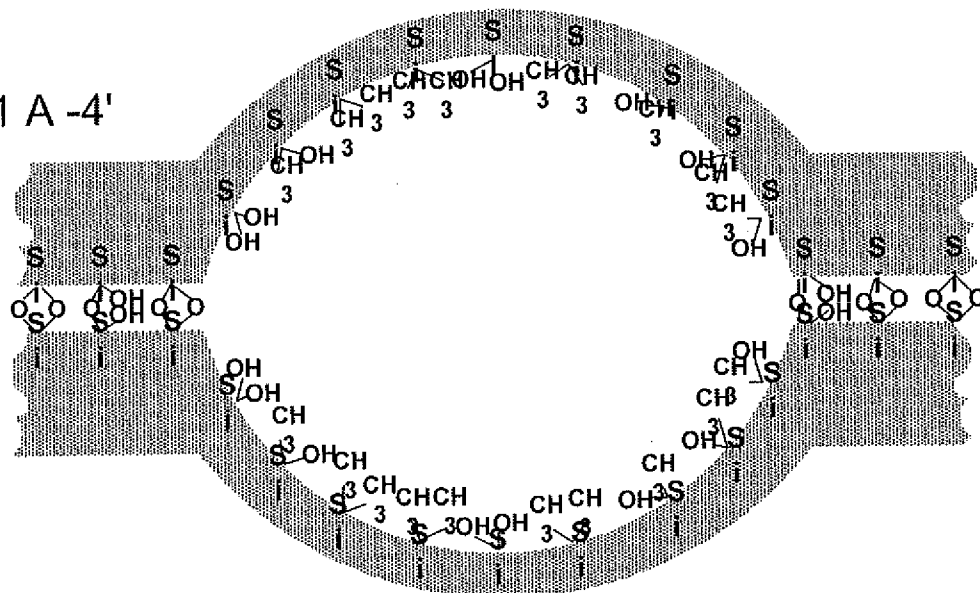
64/130



Make and Break

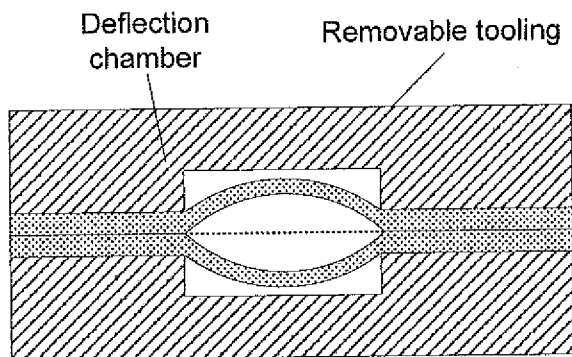
65/130

51 A -4'



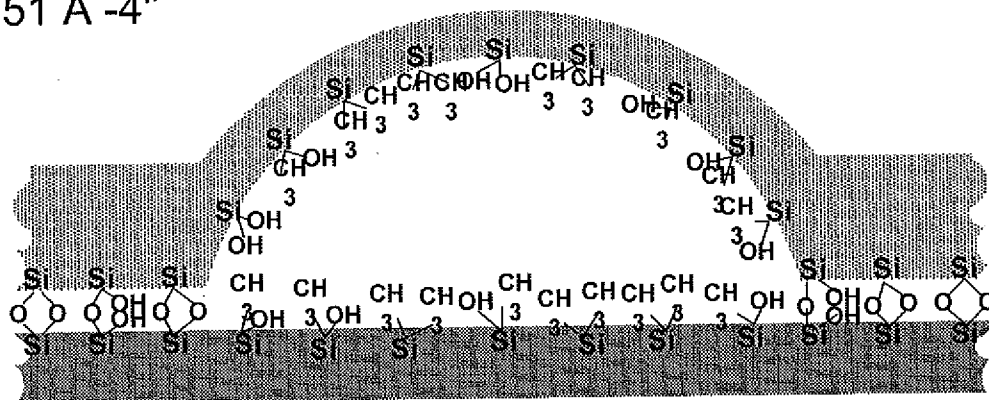
51 A -5

Laminated structure with formed deflectable region

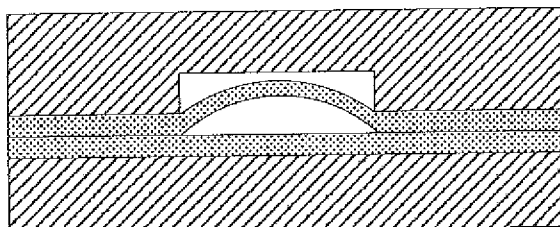


PDMS membrane laminate with non-bonded regions defined by tool

51 A -4''



51 A -7



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Make and Break

Composite
membrane

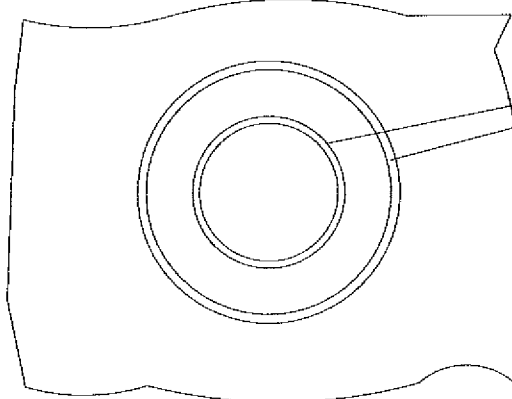
51 A-9

Thin flexible sheet (PET ~ 10 um thick)
PDMS Film (50-200 um)

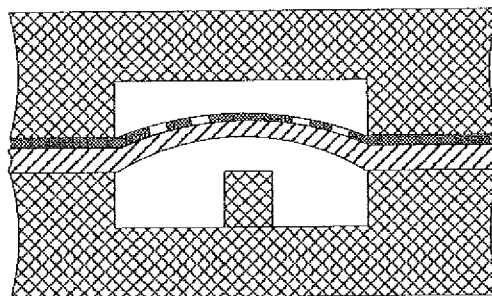
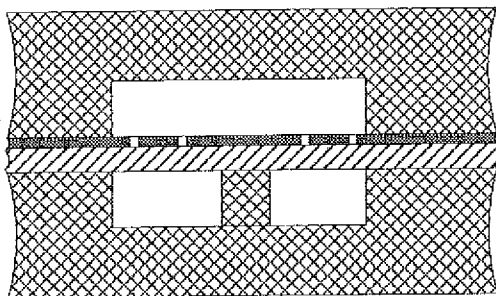
51 A - 10

Thin flexible sheet (PET ~ 10 um thick)
PDMS coating (0.5-5 um)

51 A - 11

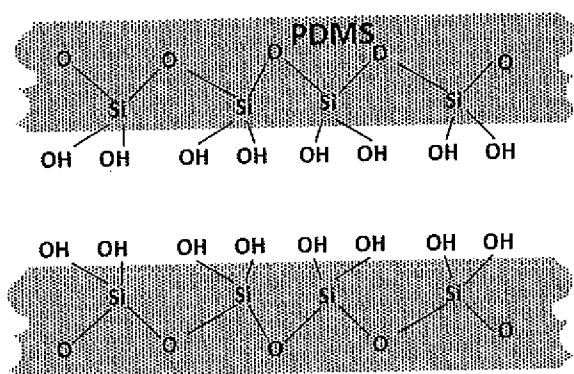
PET/PDMS
LaminationStress relief channels formed in
PET film (~ 25 um wide)

Circular stress relief slots

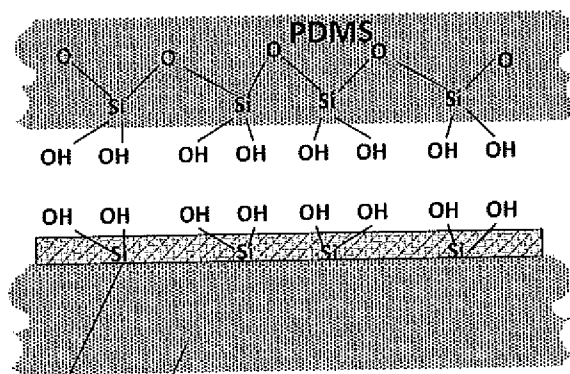
Thin flexible sheet (PET ~ 10 um thick)
PDMS Film (50-200 um)

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Fig. 51A-12



Silicon based rigid materials ex. Silicon, silica or silicates

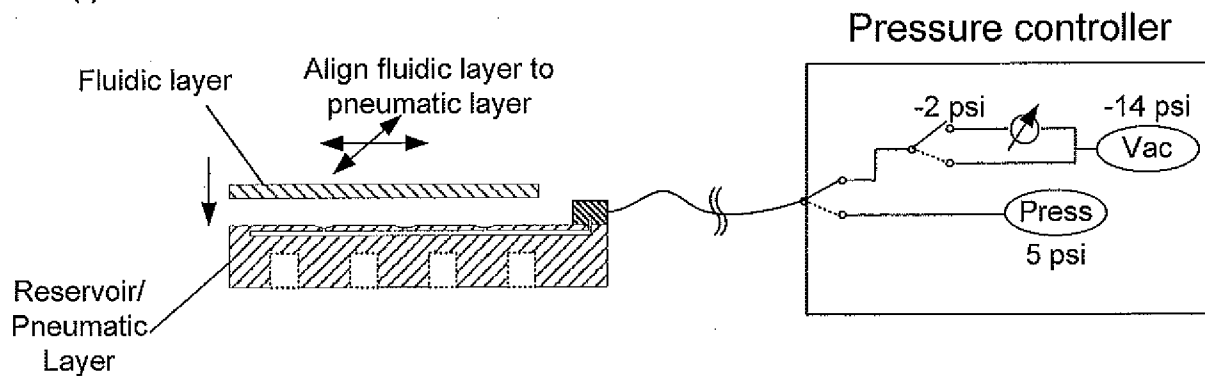


Synthetic resin (coc, cop, pc, polysulfone)

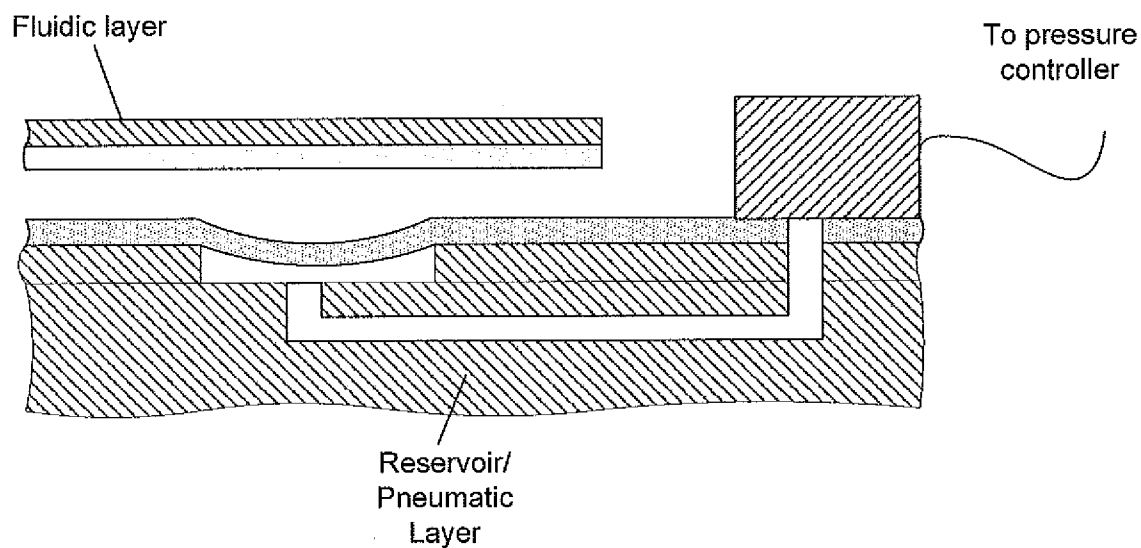
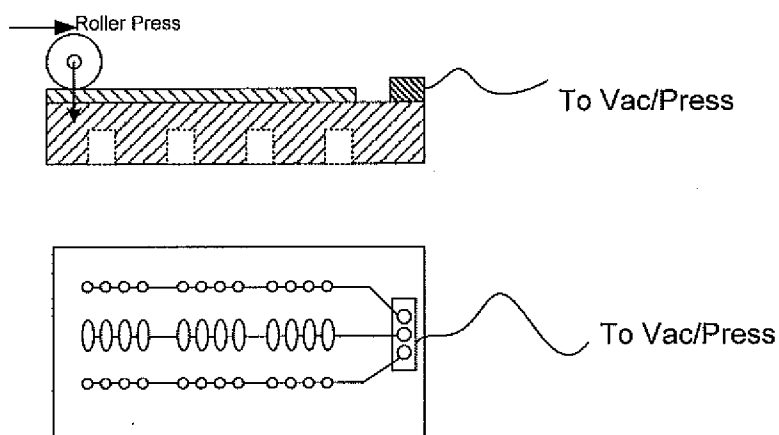
Intermediate bifunctional layer
such as Organofunctional silane
or oxide layer

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51 B (i)

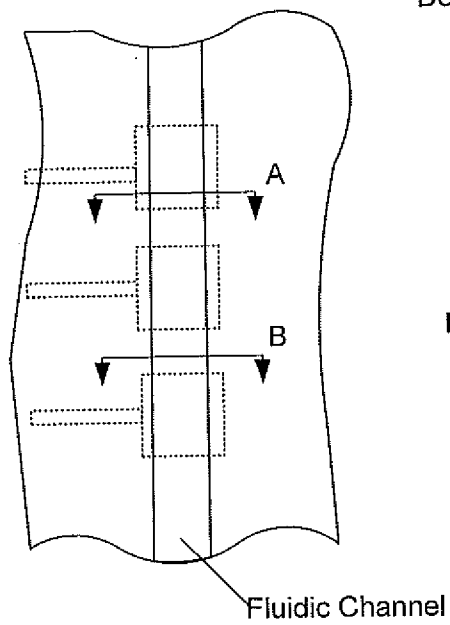


51 B (ii)



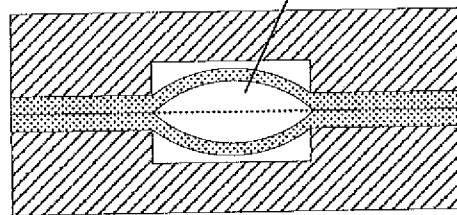
69/130

51 A -16

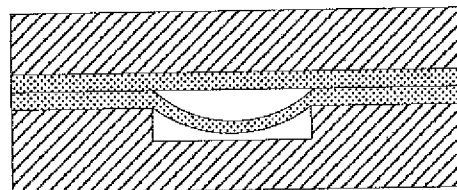


Detail A

Fluidic channel

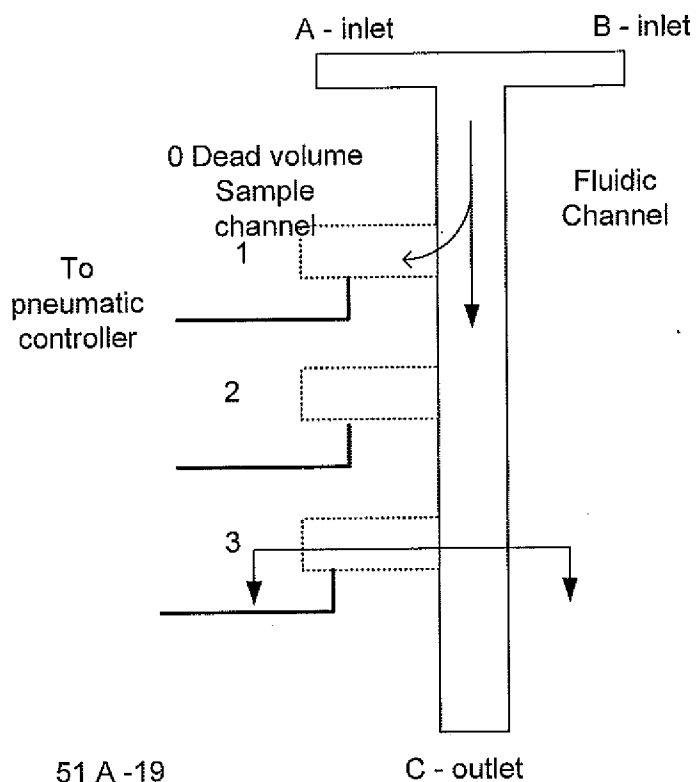


Detail B

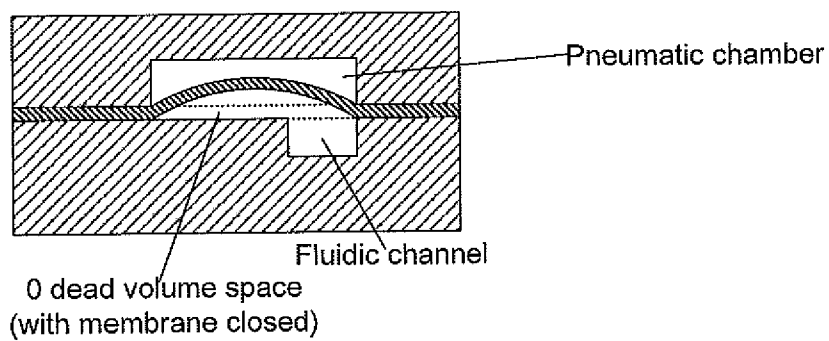


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51 A -18

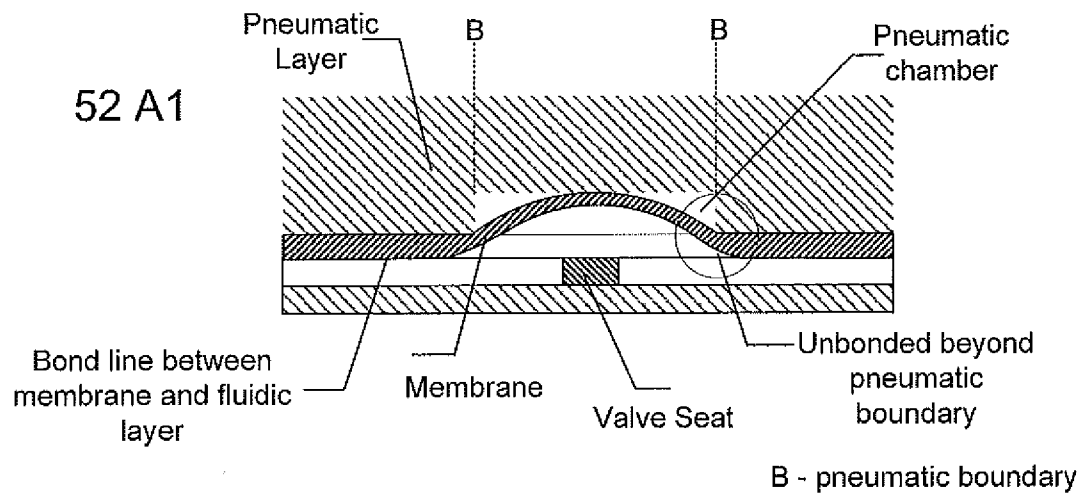


51 A -19

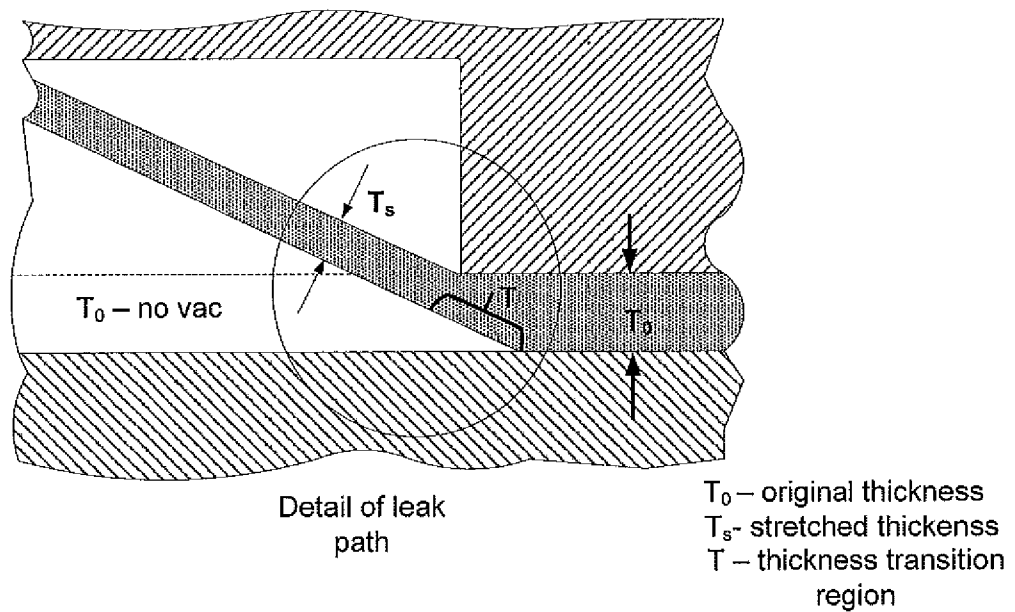


Make and Break

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52 A1m



52 A1 plan

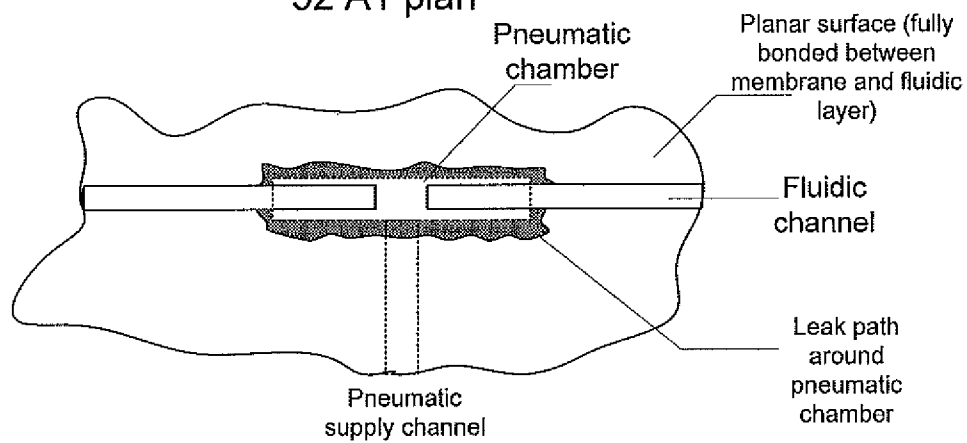
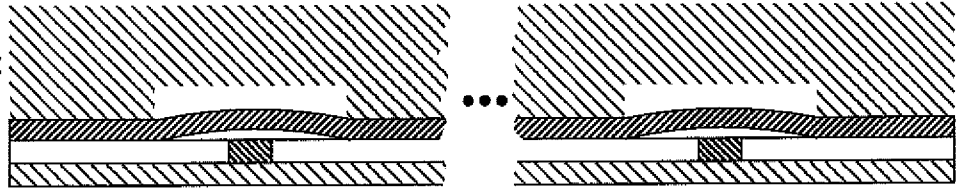
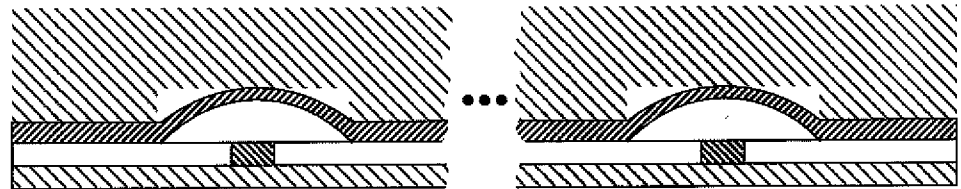


Fig 52 A1N 72/130

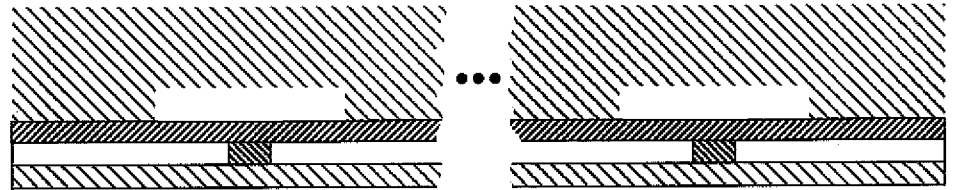
Low vac valve actuation state:
held until planar surface
bonding is completed



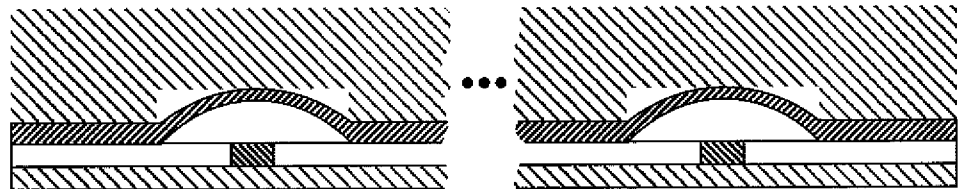
Valve deflected during
initial assembly



1st 'Make' - duration
<1 sec



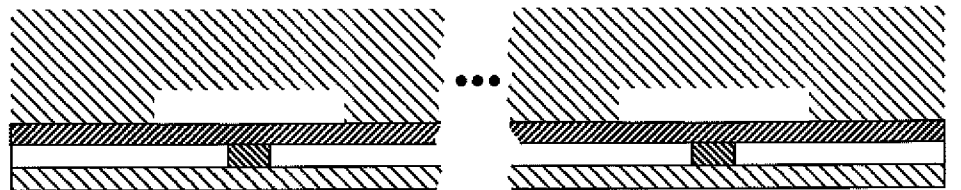
1st 'Break' - break initial
bonds formed during 1st
'Make'



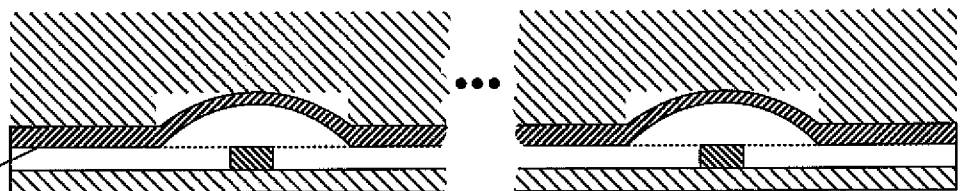
⋮

⋮

nth 'Make'



nth 'Break'

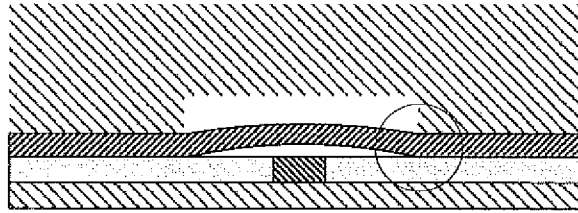


permanent bond

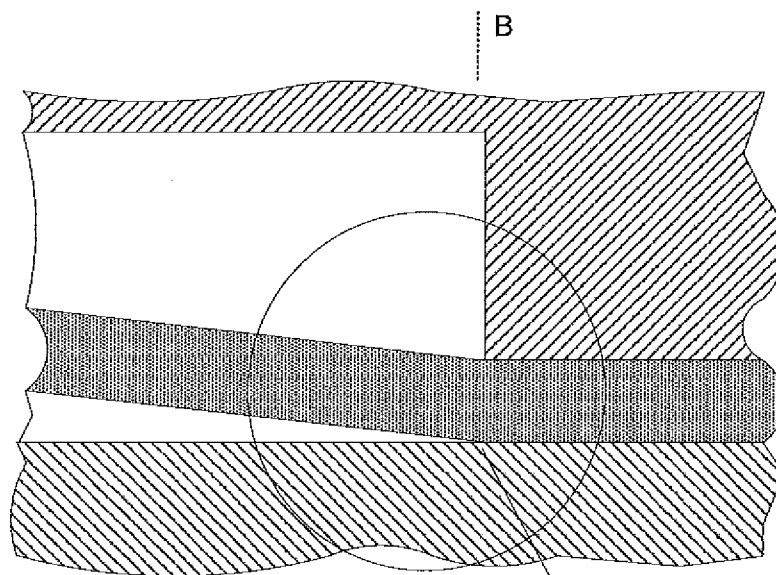
All future bonding
permanently arrested

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Make and Break

52 A2



52 A2m



No leak path exists outside
chamber boundary B

52 A2 plan

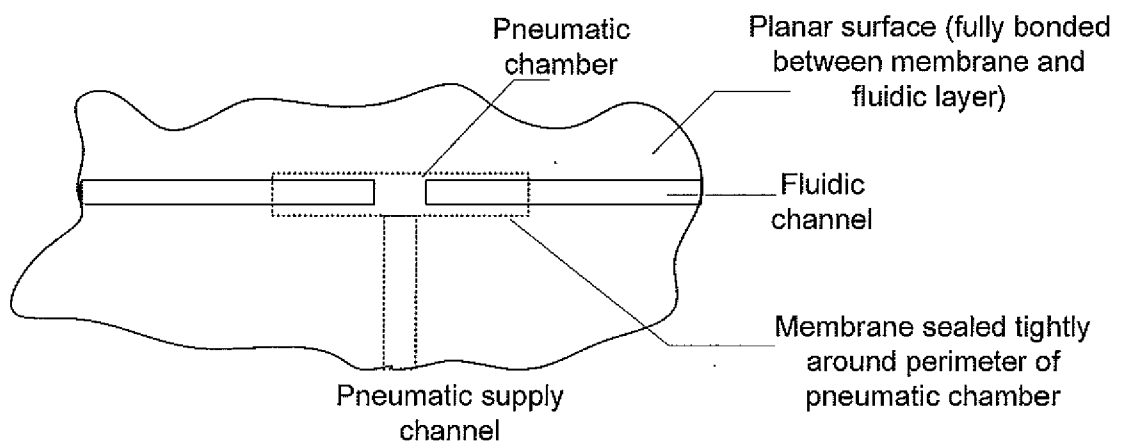
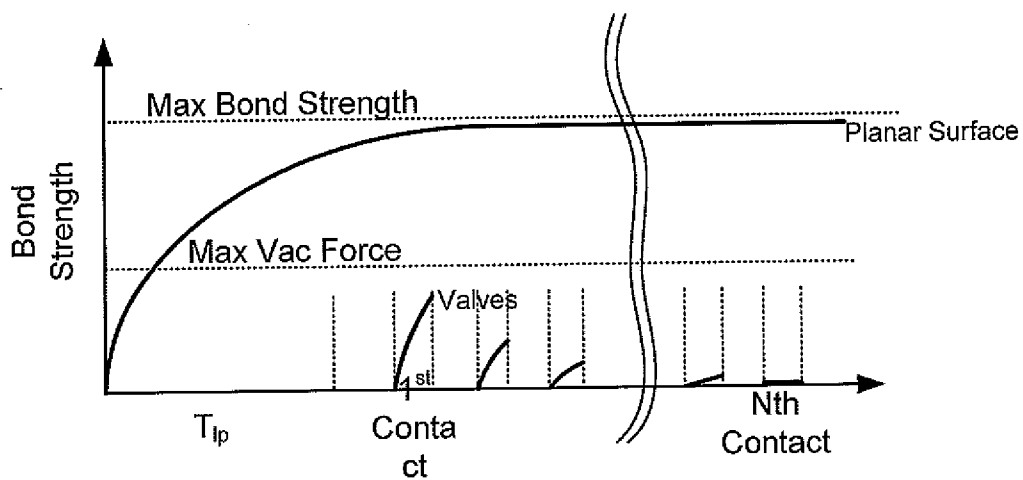
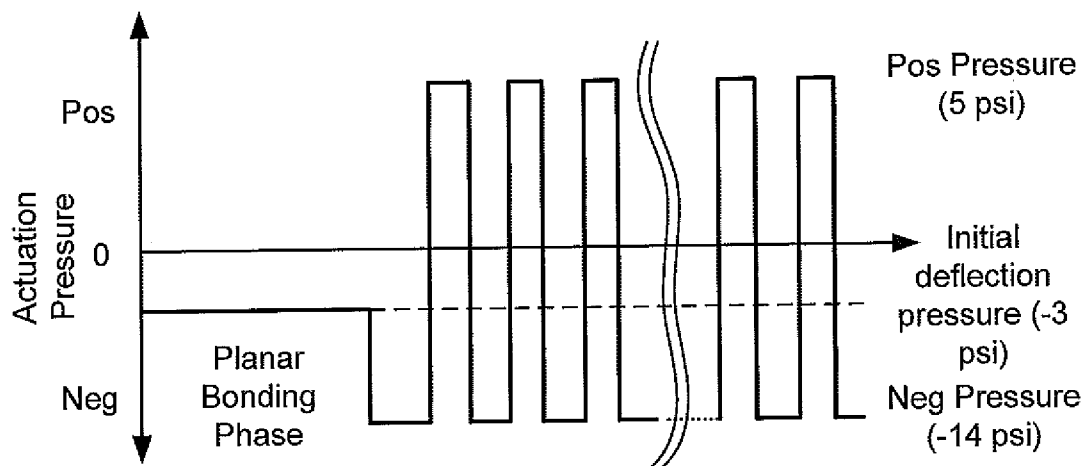


Fig. 50
A2N 74/130



T_{lp} = time of low pressure bonding

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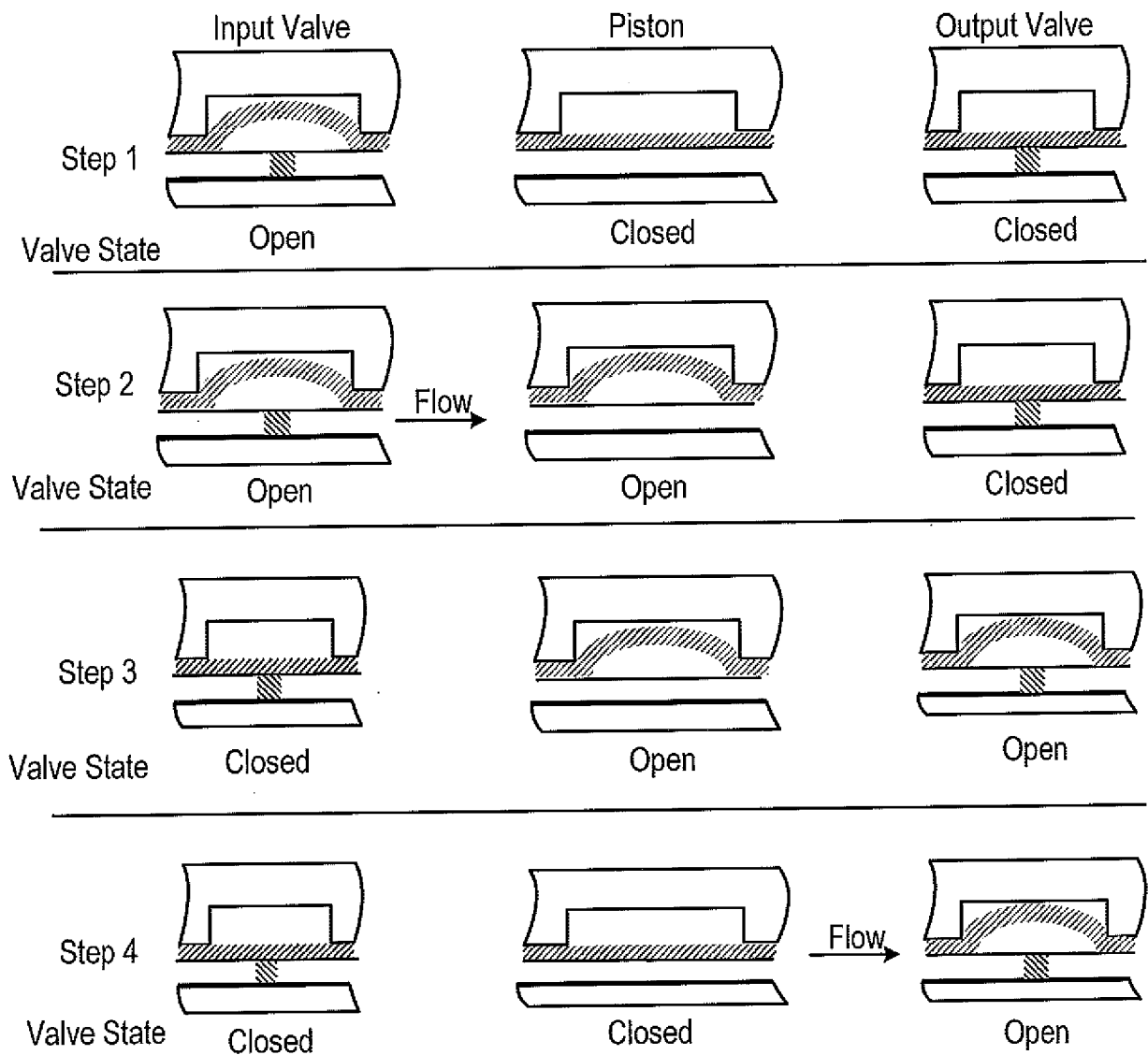
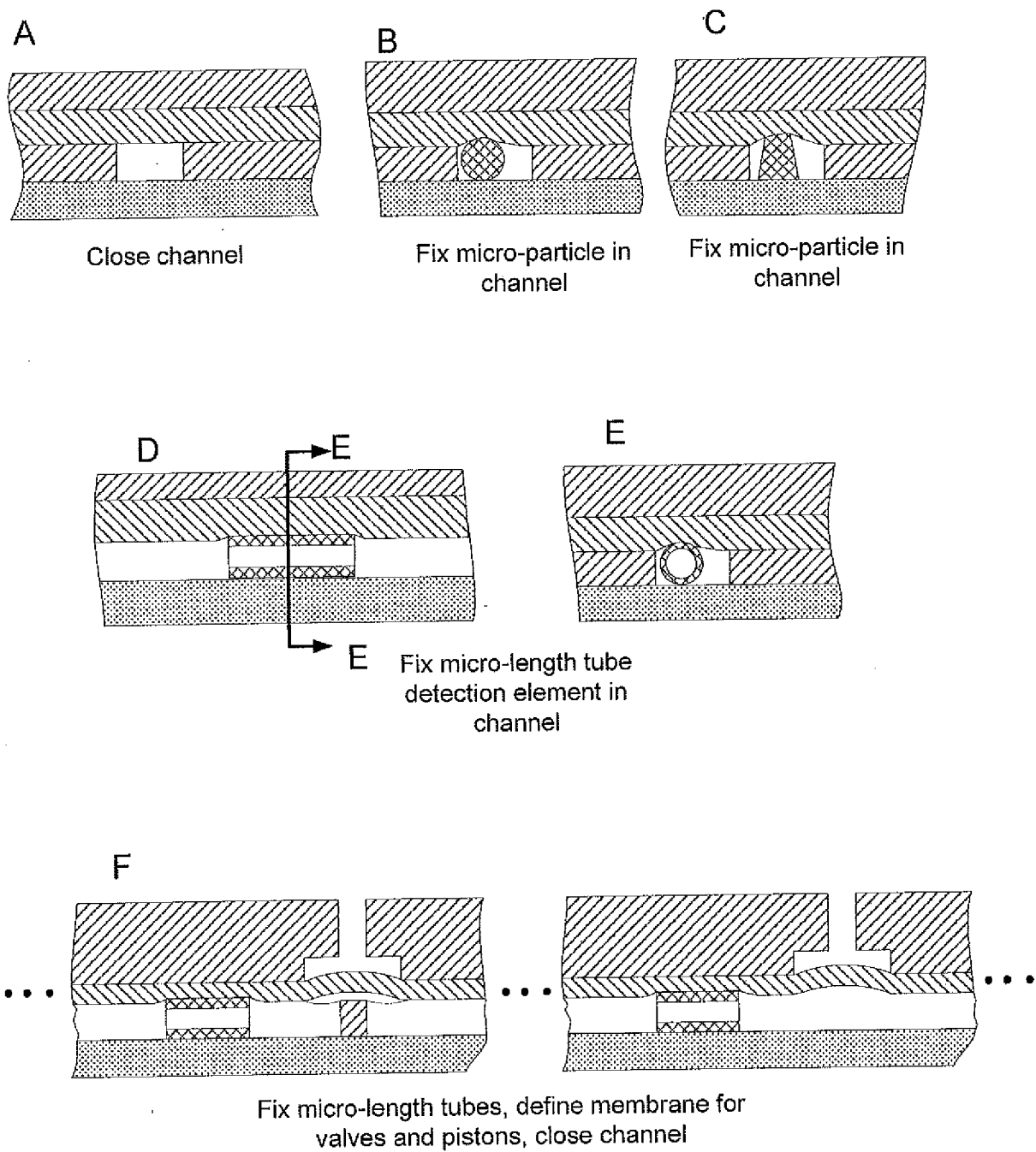


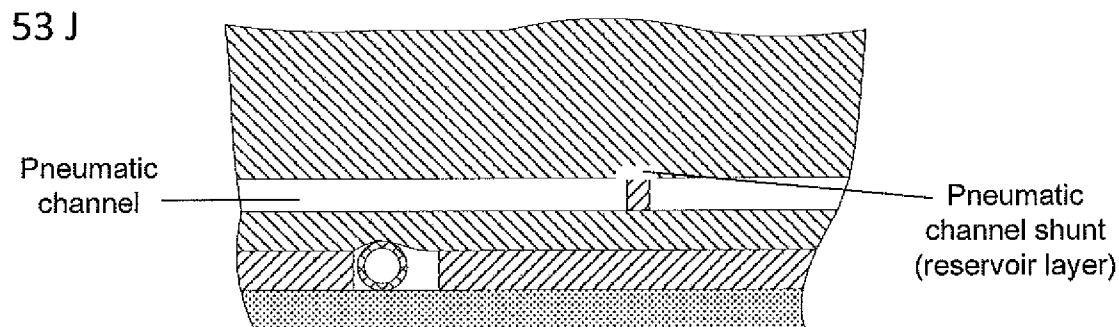
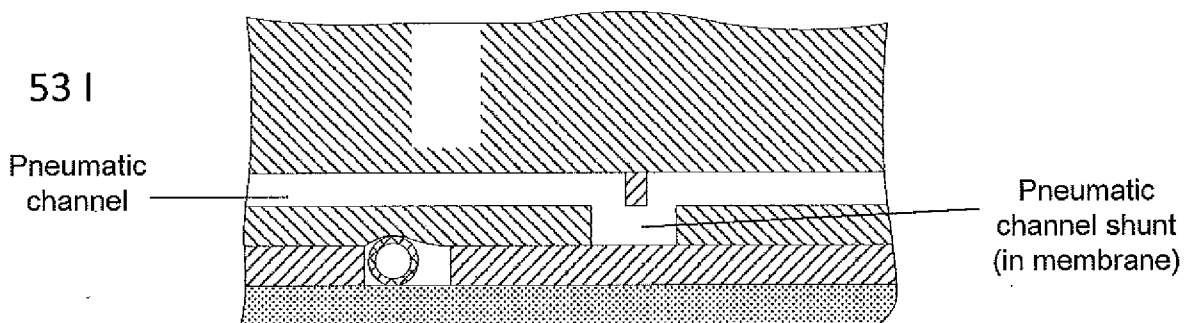
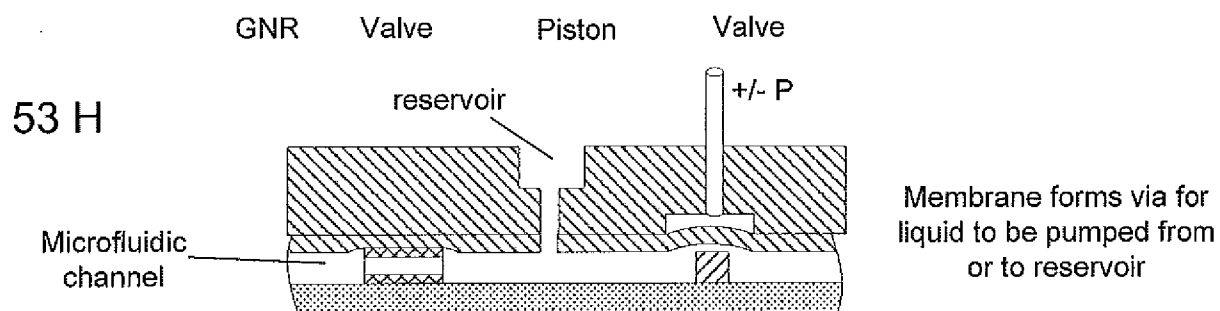
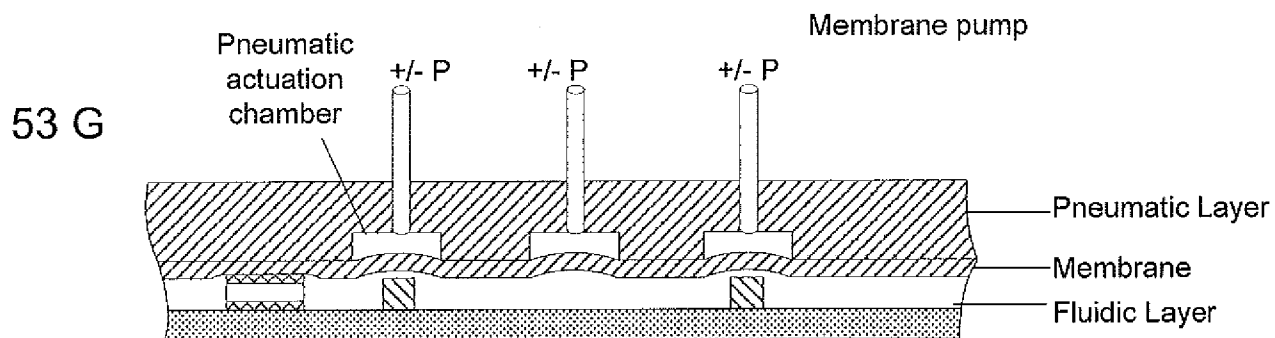
Fig. 53

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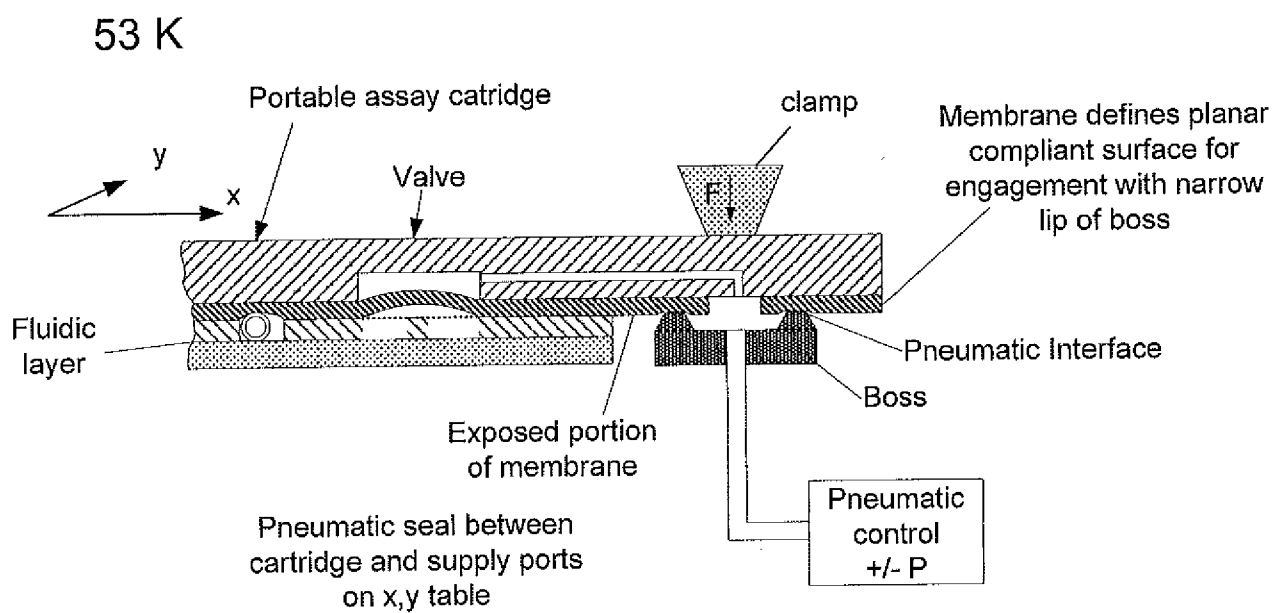
53



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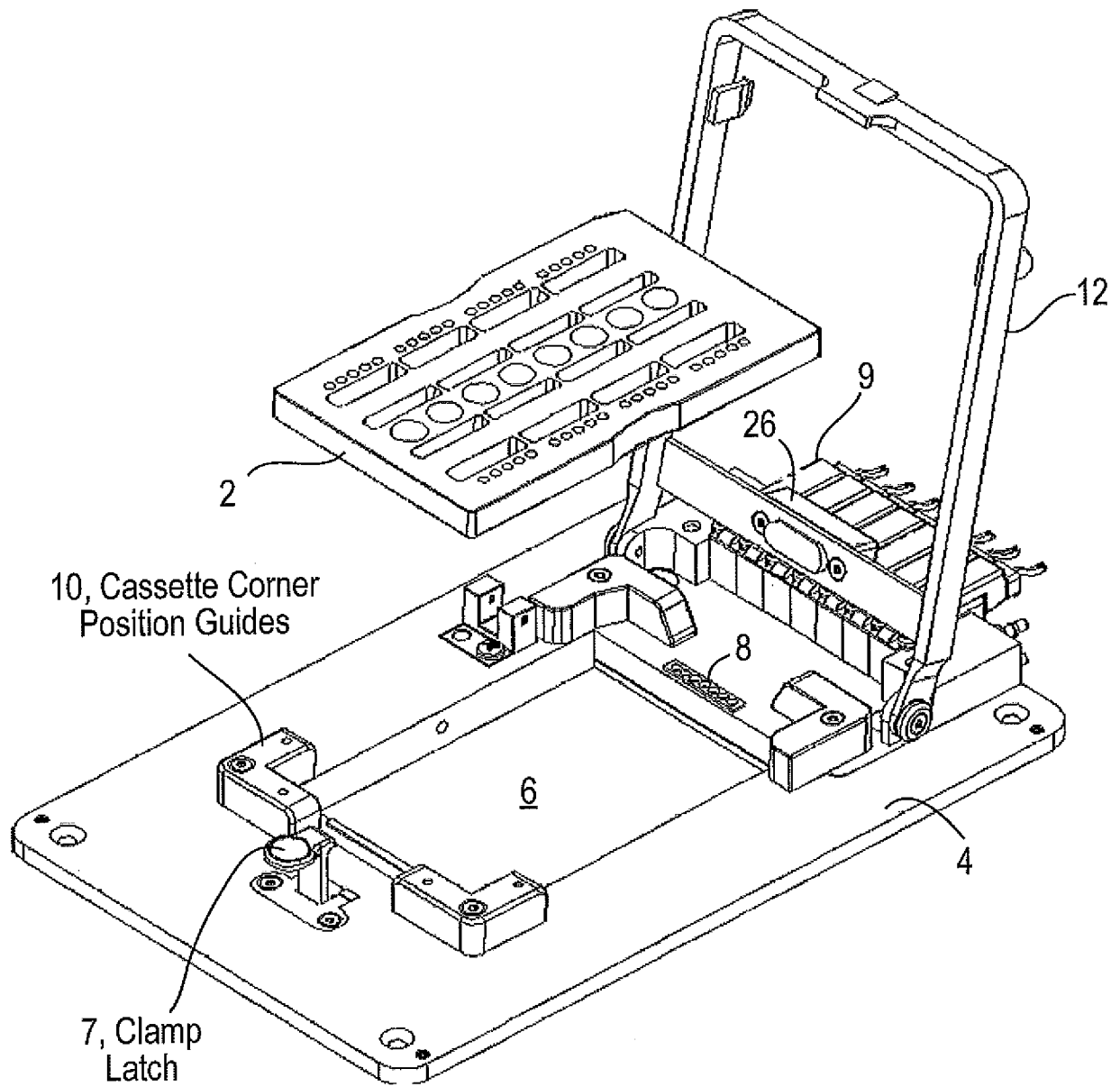


Fig. 54

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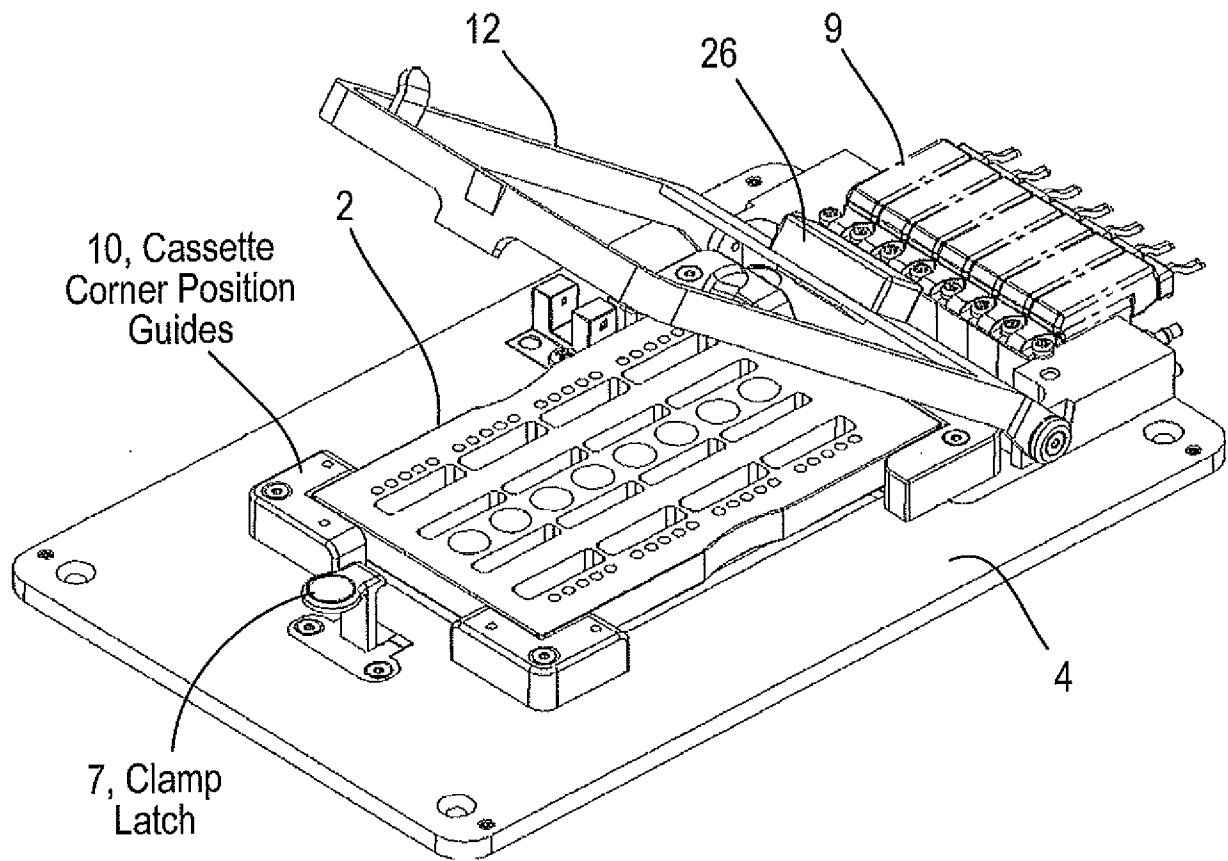


Fig. 54A

Solenoid-actuated three-way valves 9 on moving X,Y stage selectively apply Pneumatic Conditions at Supply Ports defined by raised bosses of Fig. 7A. Conditions supplied are: (1) atmospheric pressure, (2) positive (+) actuating pressure, (3) negative (-) actuating pressure. Only connections to moving X,Y stage assembly are positive and negative pressure lines to manifolds feeding valves 9 and electrical control lines for solenoid coils of the valves

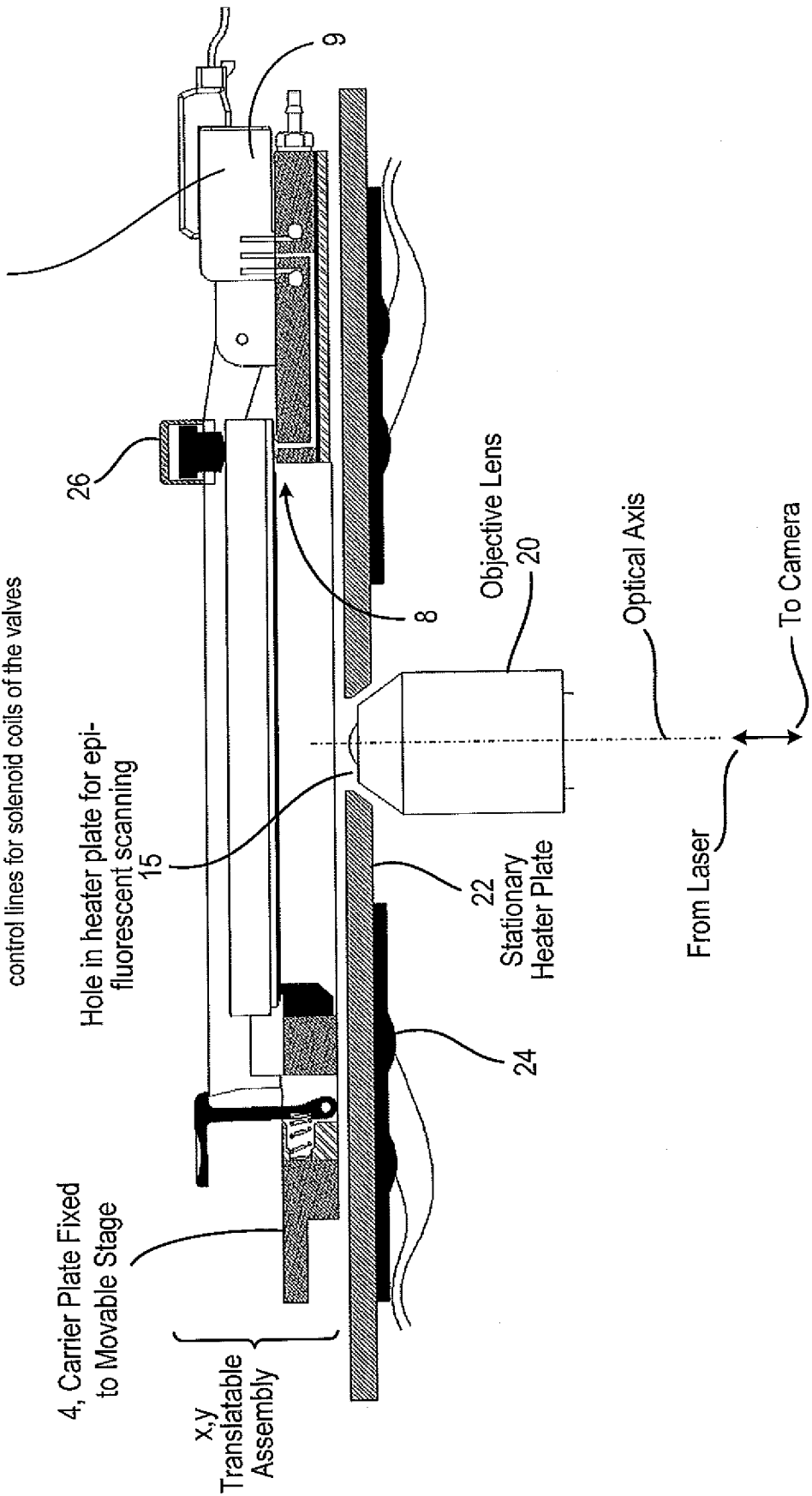


Fig. 55

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Pneumatic Interface Detail

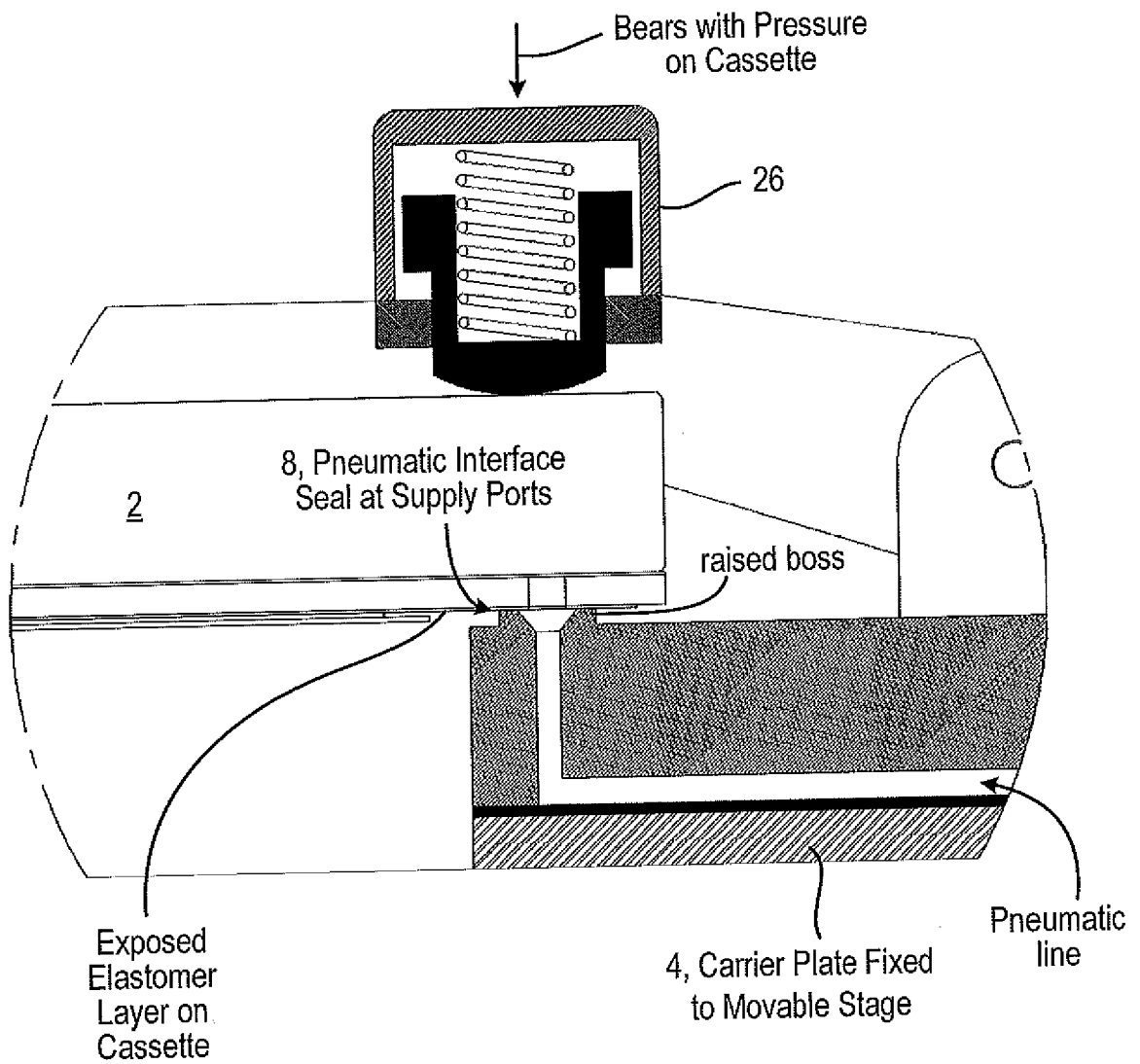


Fig. 55A

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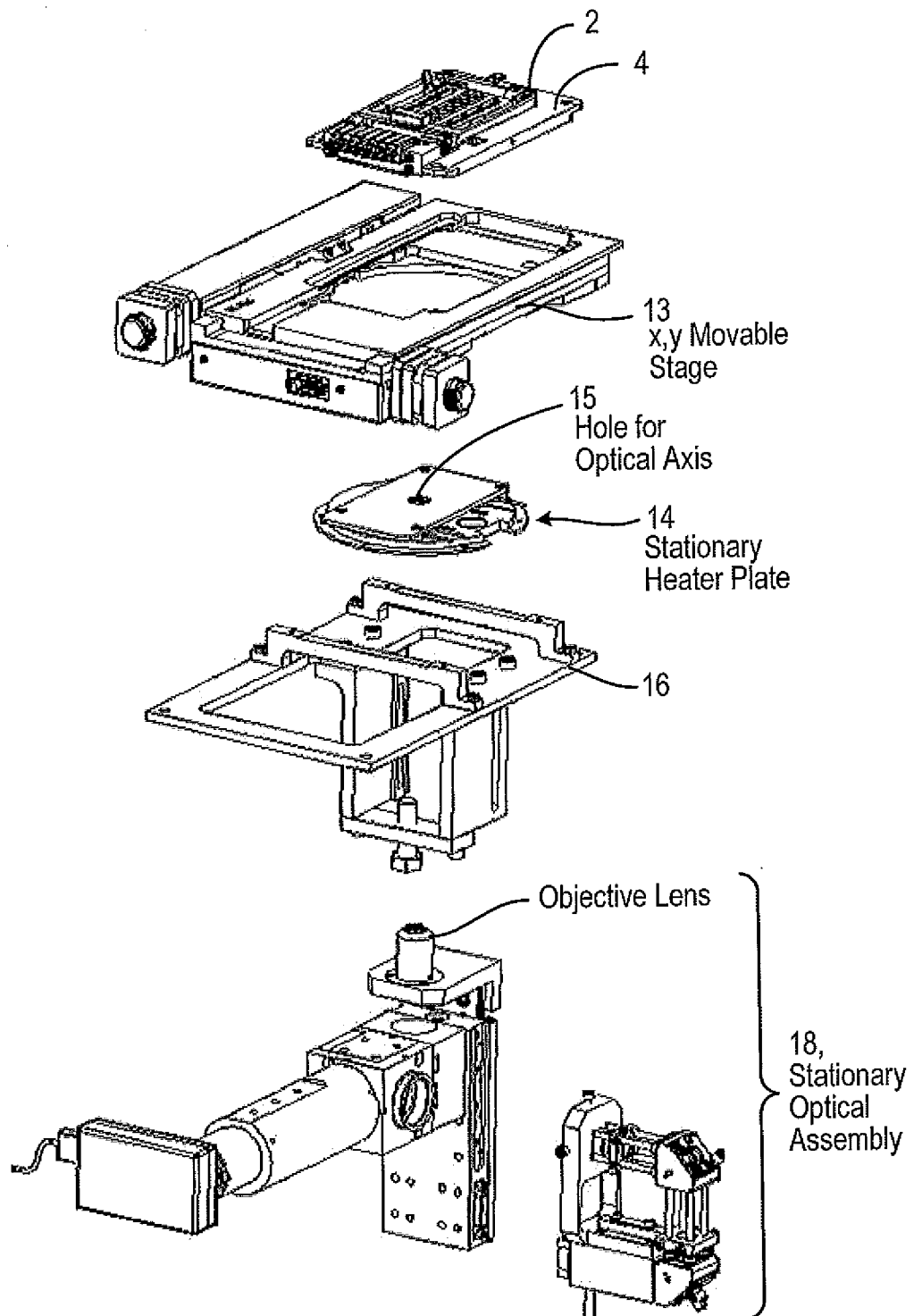


Fig. 56

Fluidic /Pneumatic Architecture

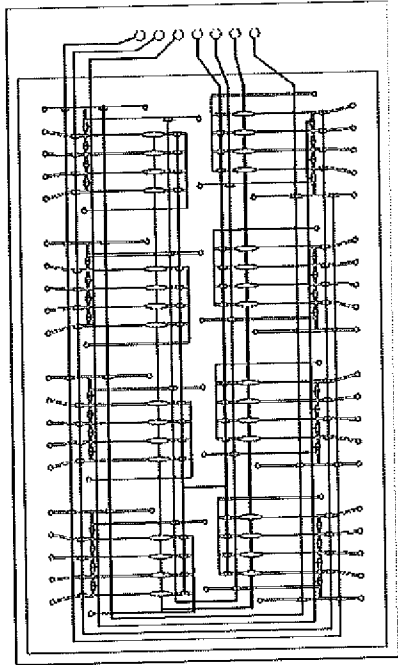
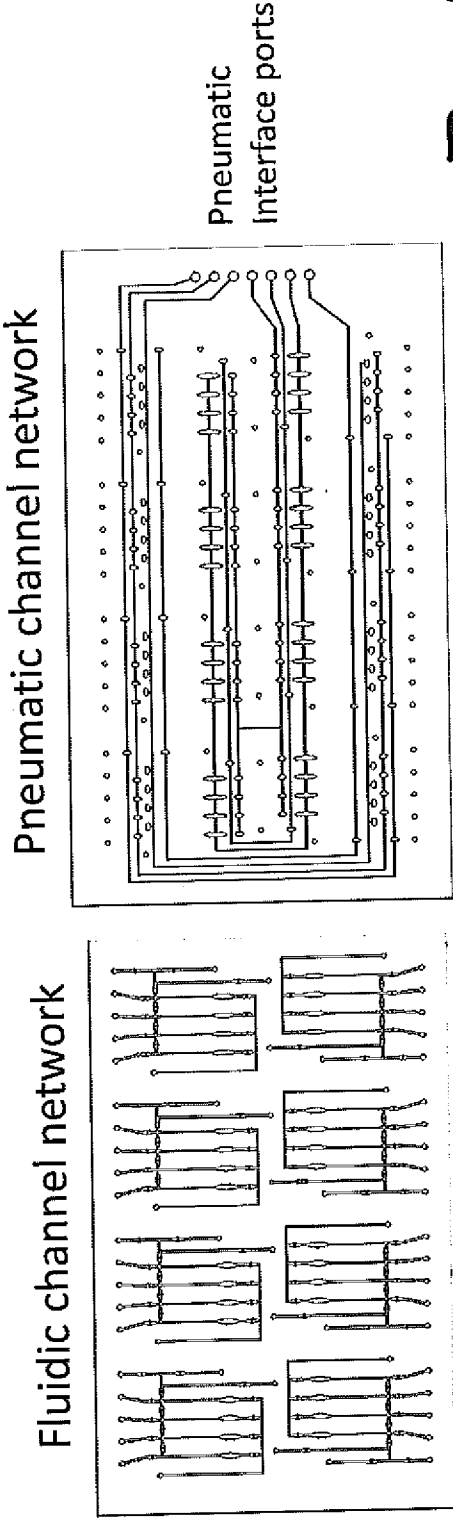


Fig. 57A

Fig. 57B

Fig. 57C

Combined fluidic and pneumatic layers

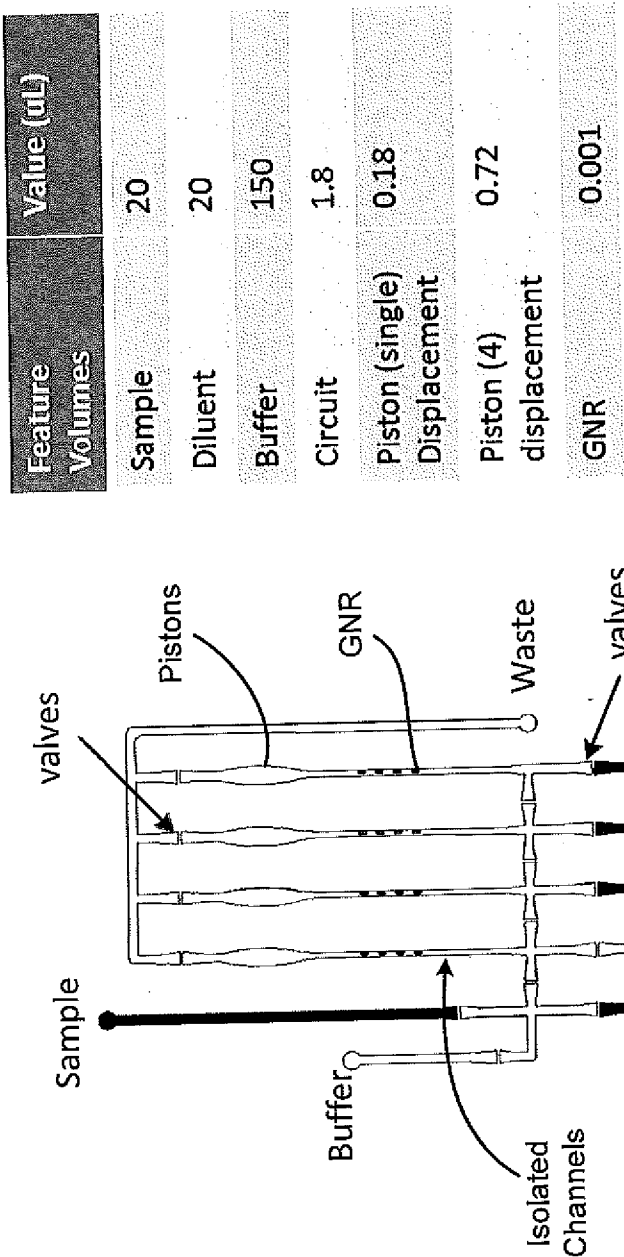
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Fig. 58

Fluidic Architecture and Protocol

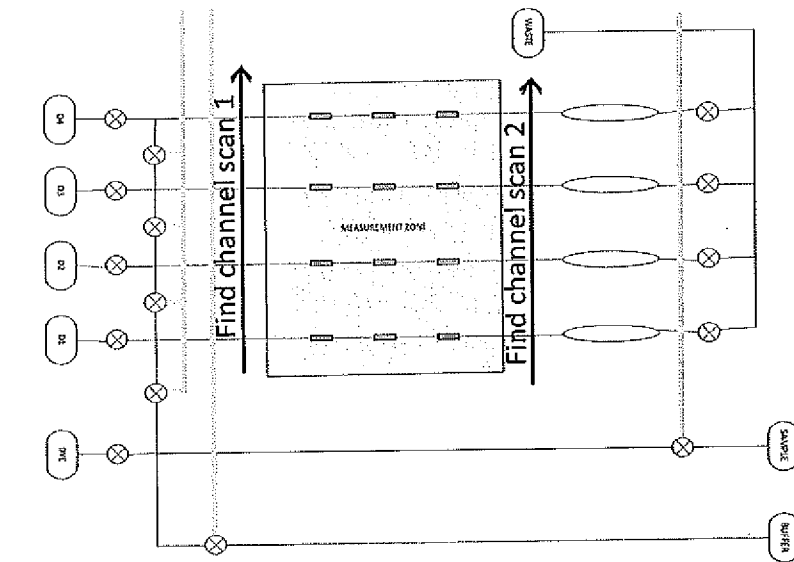
Assay Protocol

- Prime
- Sample
- Wash
- Secondary
- Wash
- Dye
- Wash

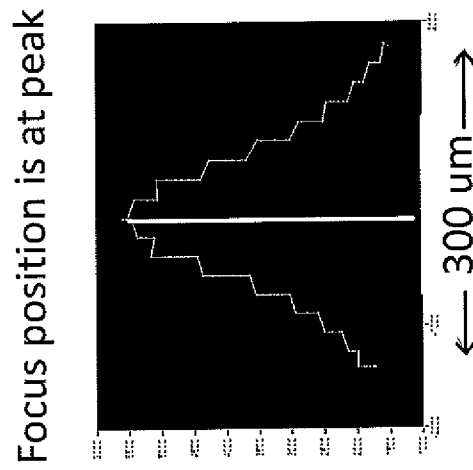
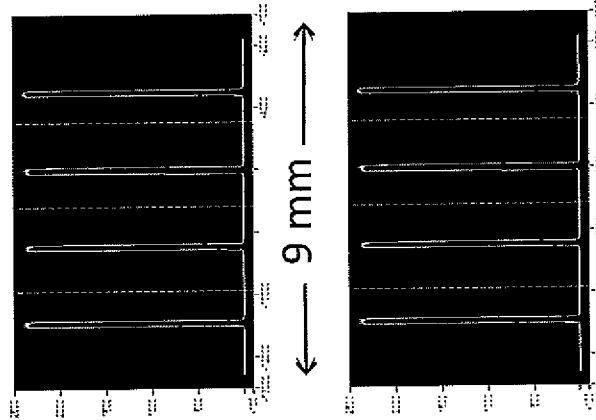


Automatic Scan Setup

- Sample Circuit Layout



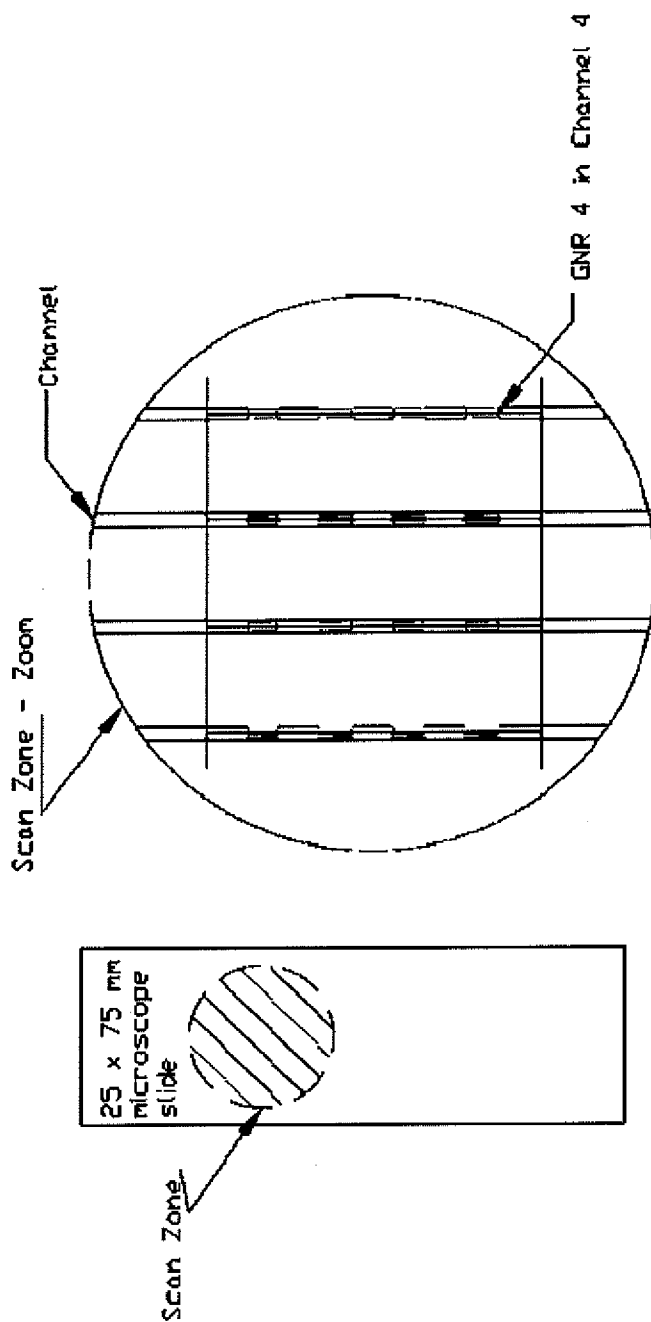
ch1 ch2 ch3 ch4



- Channels with fluorescent dye present produce high contrast signal.
- Channel locations and chip orientation is precisely determined from scan profiles
- Optimal focus is determined by sweeping z

Fig. 6

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Find Channels Scan GNRs
Find GNRs and Fluorescence Scan

Fig. 62

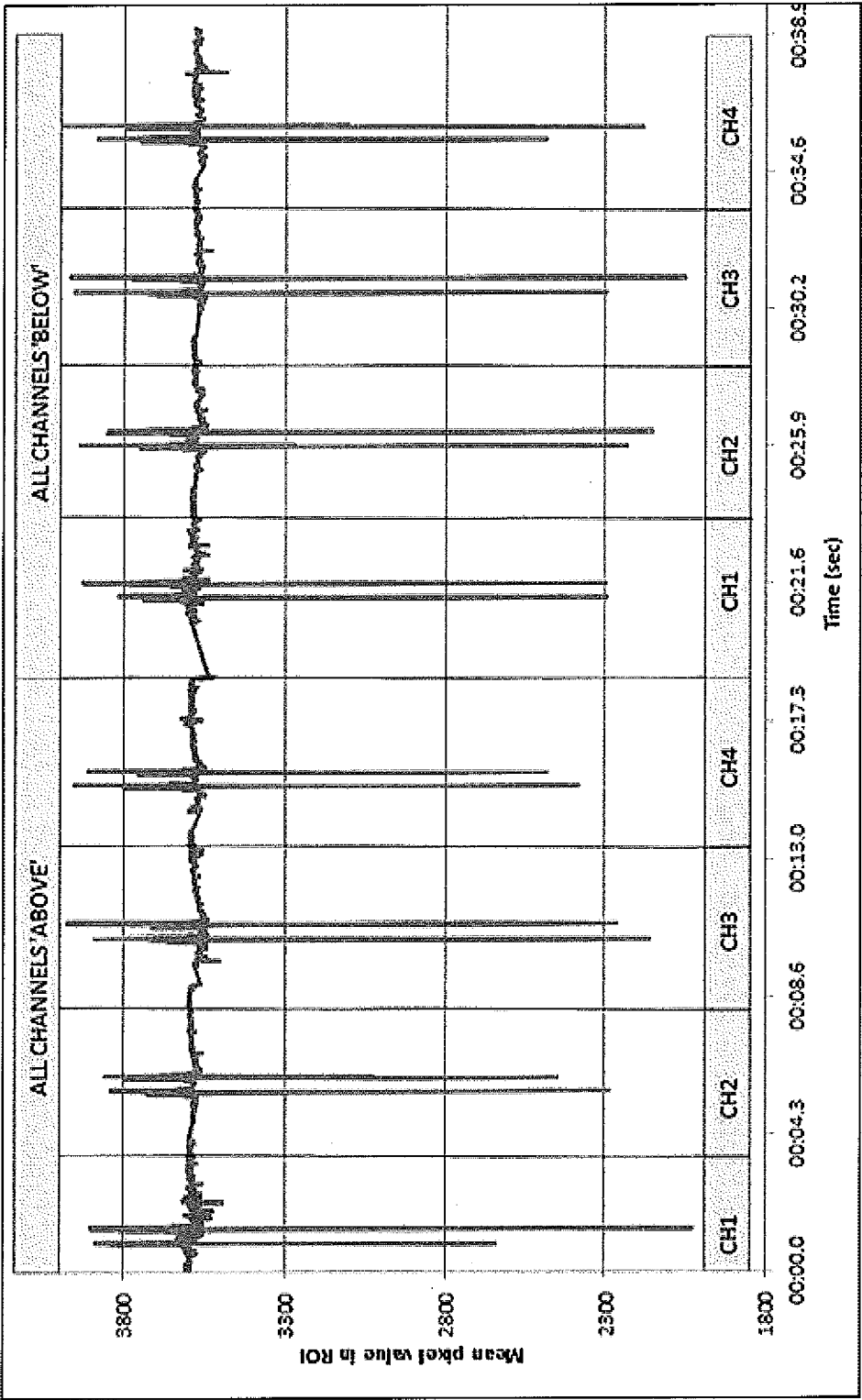


Fig. 63

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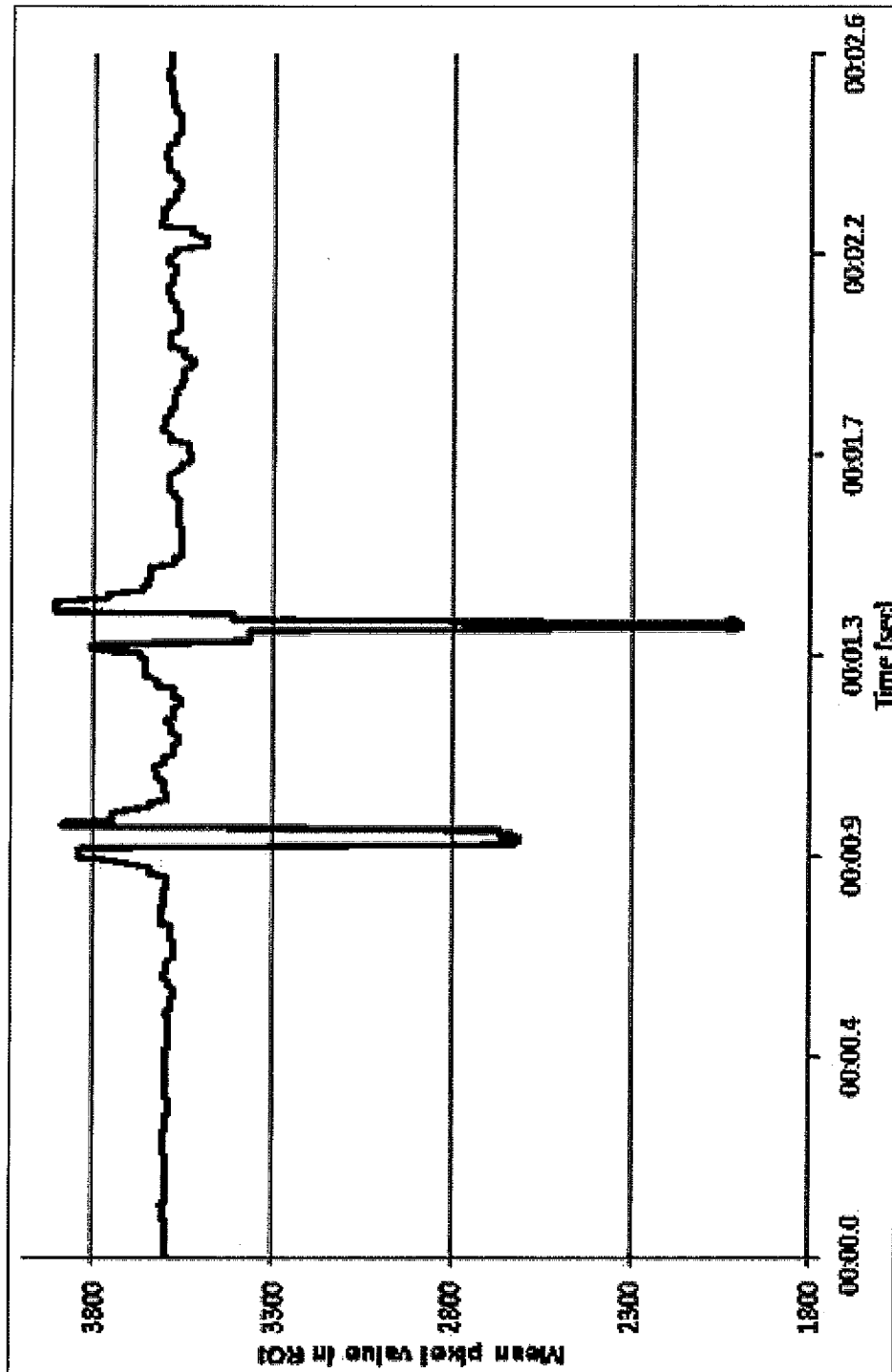
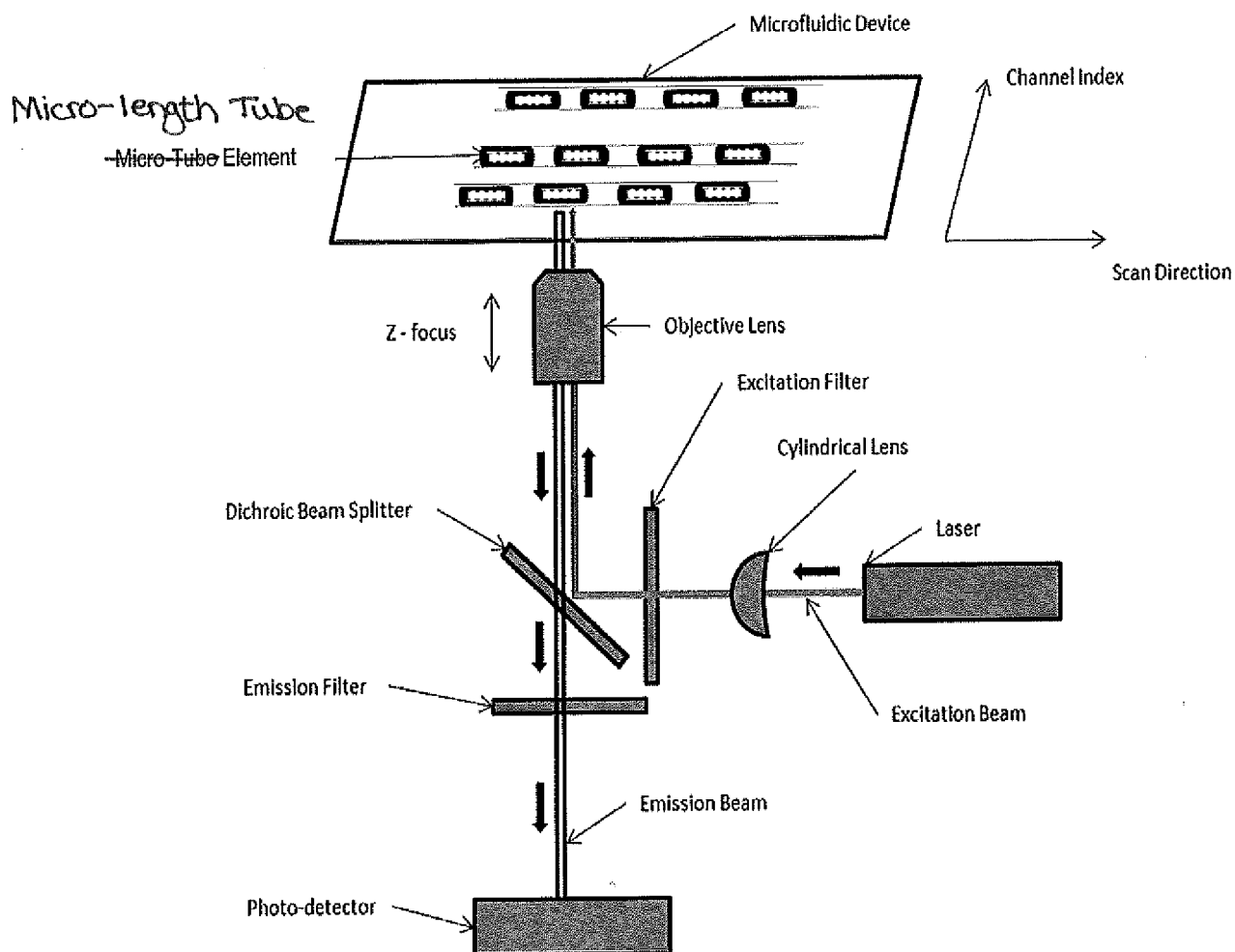


Fig. 64

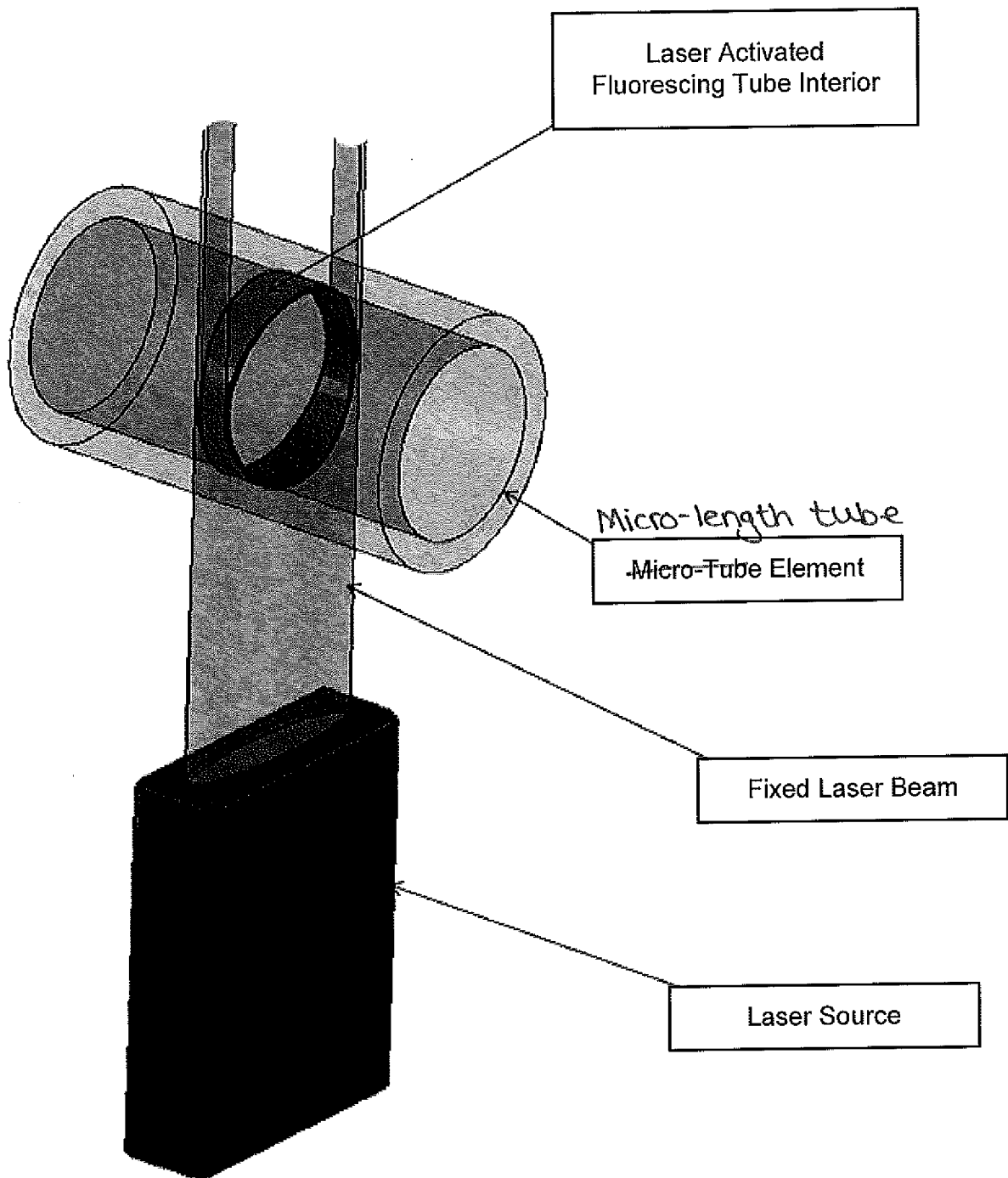
90/130

Fig. 65**Scanning Figure 1 - General Schematic for
Epifluorescent Scanning Microscope**

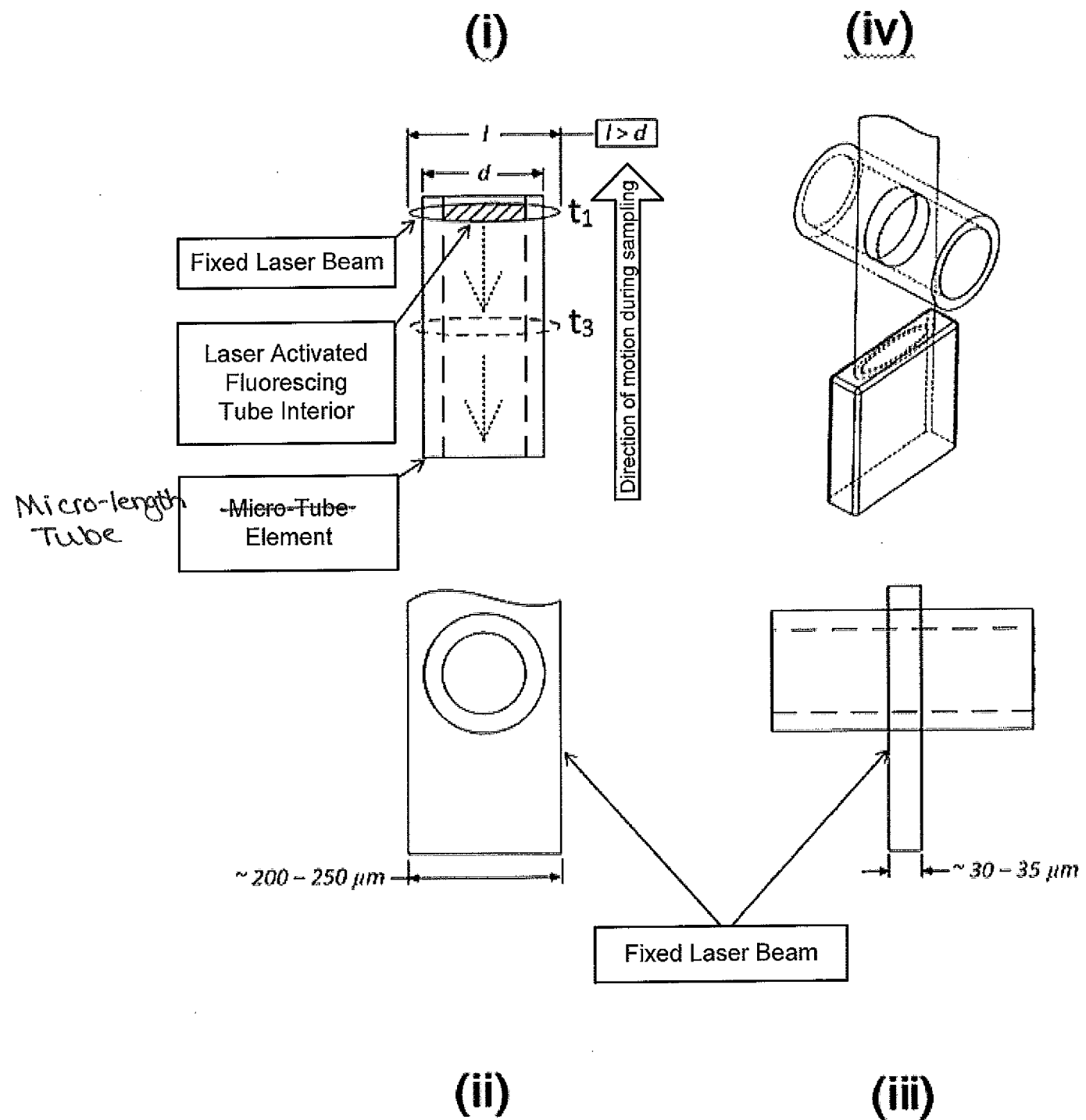
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Fig. 66

Scanning Figure 2 - Laser Beam Shape Isometric View



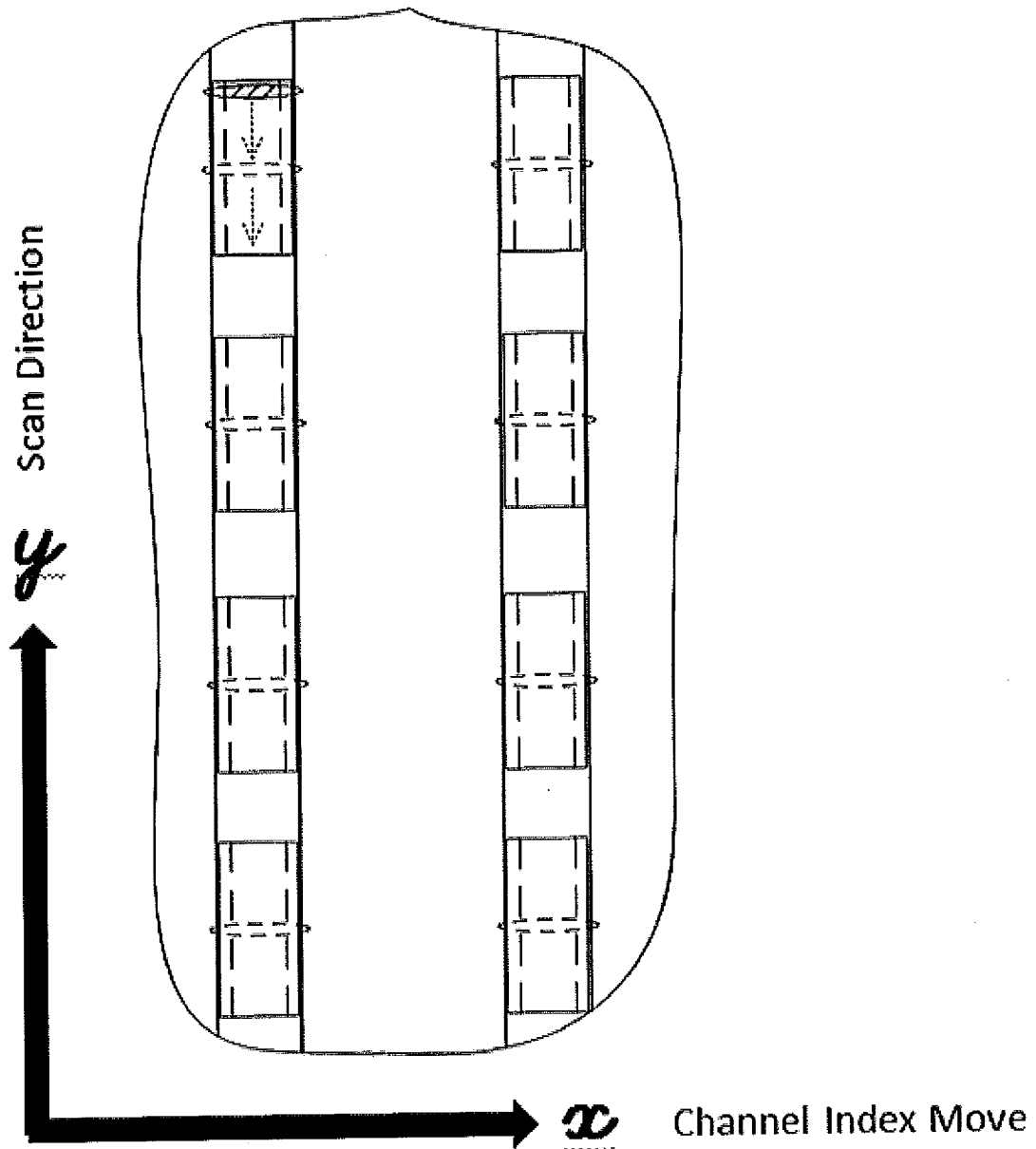
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Fig. 67**Scanning Figure 3 - Laser Beam Shape Layout View**

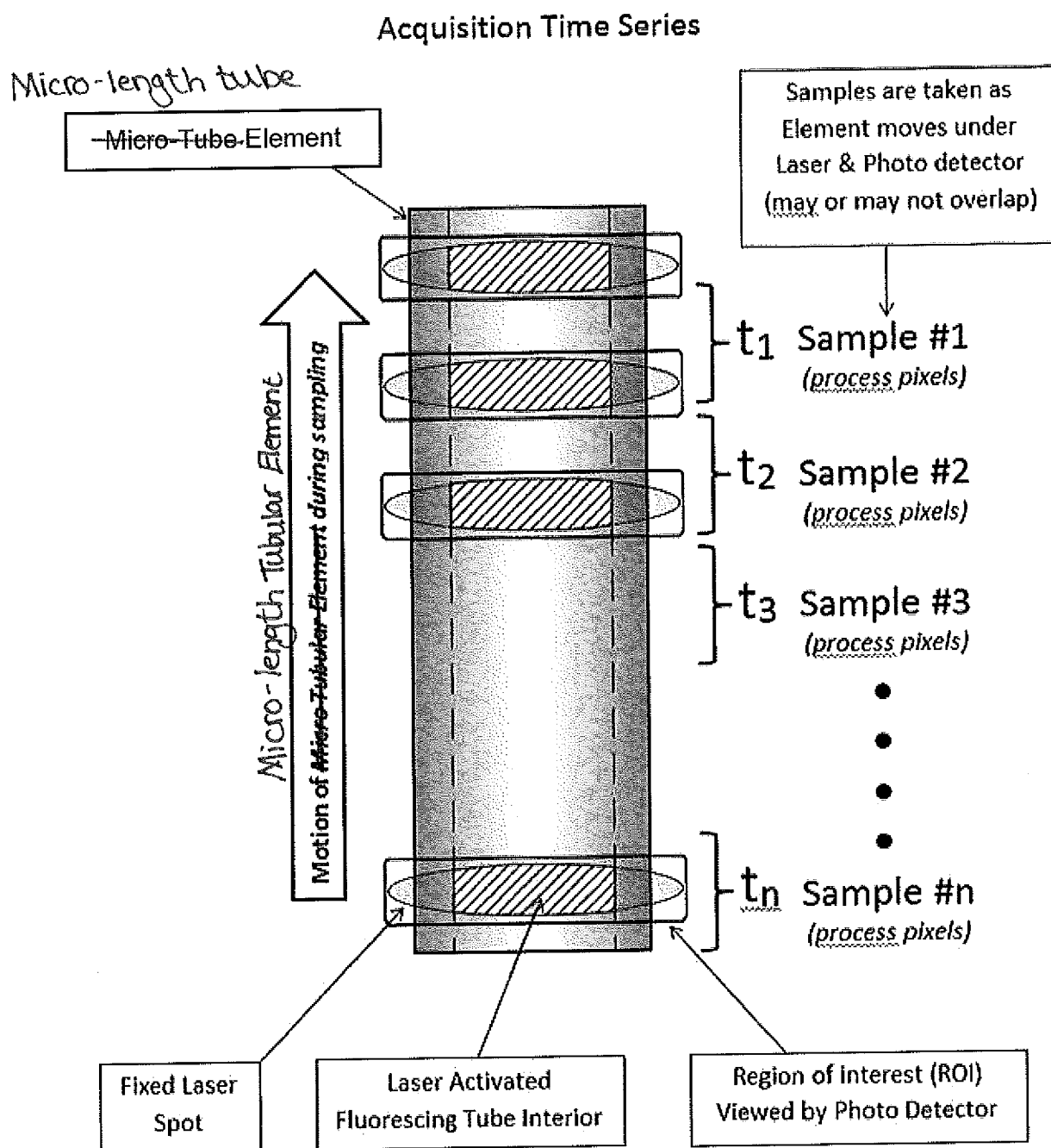
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Fig. 68

Scanning Figure 4 — Micro-tube element scan schematic
Micro-length tube

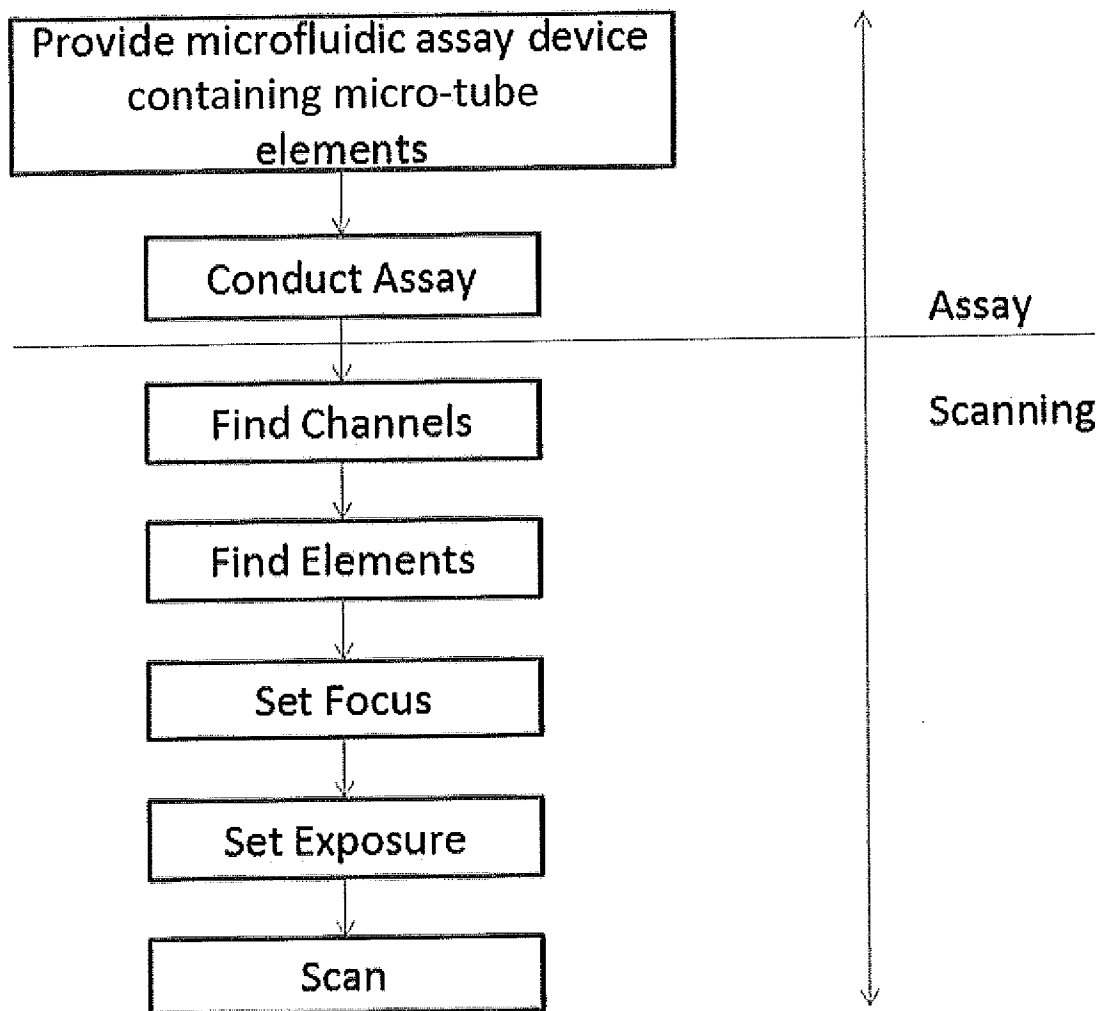


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Fig. 69**Scanning Figure 5 - Acquisition Time Series**

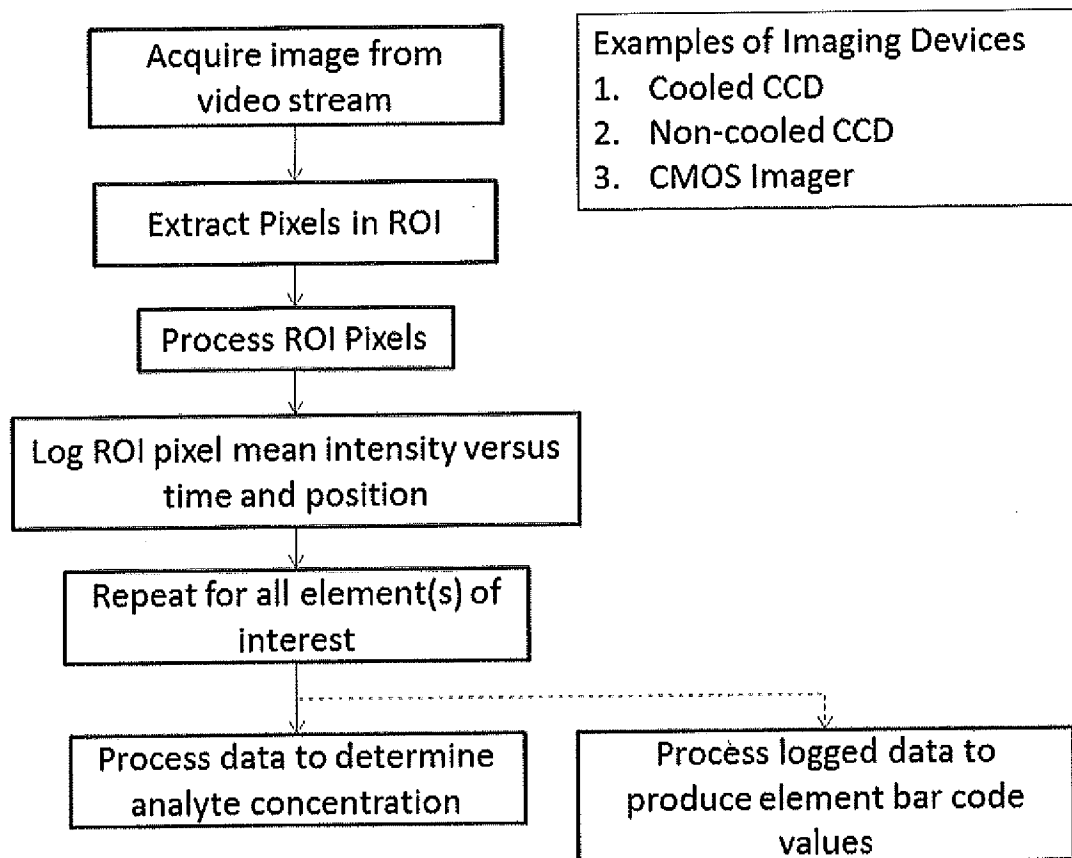
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Fig. 70

Scanning ~~Figure 6~~ - Overall Scan Sequence

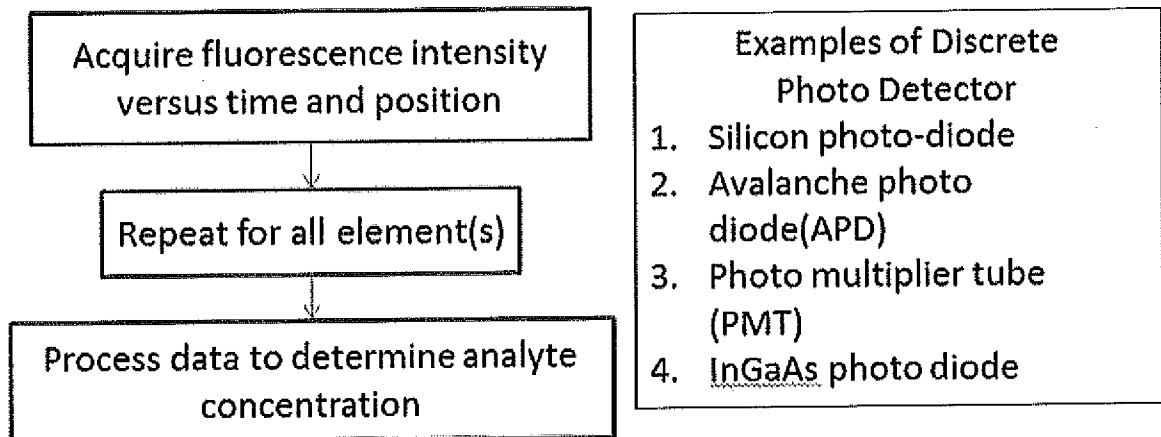
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Fig. 71

~~Scanning Figure 7~~ - Scan Sequence – Imaging

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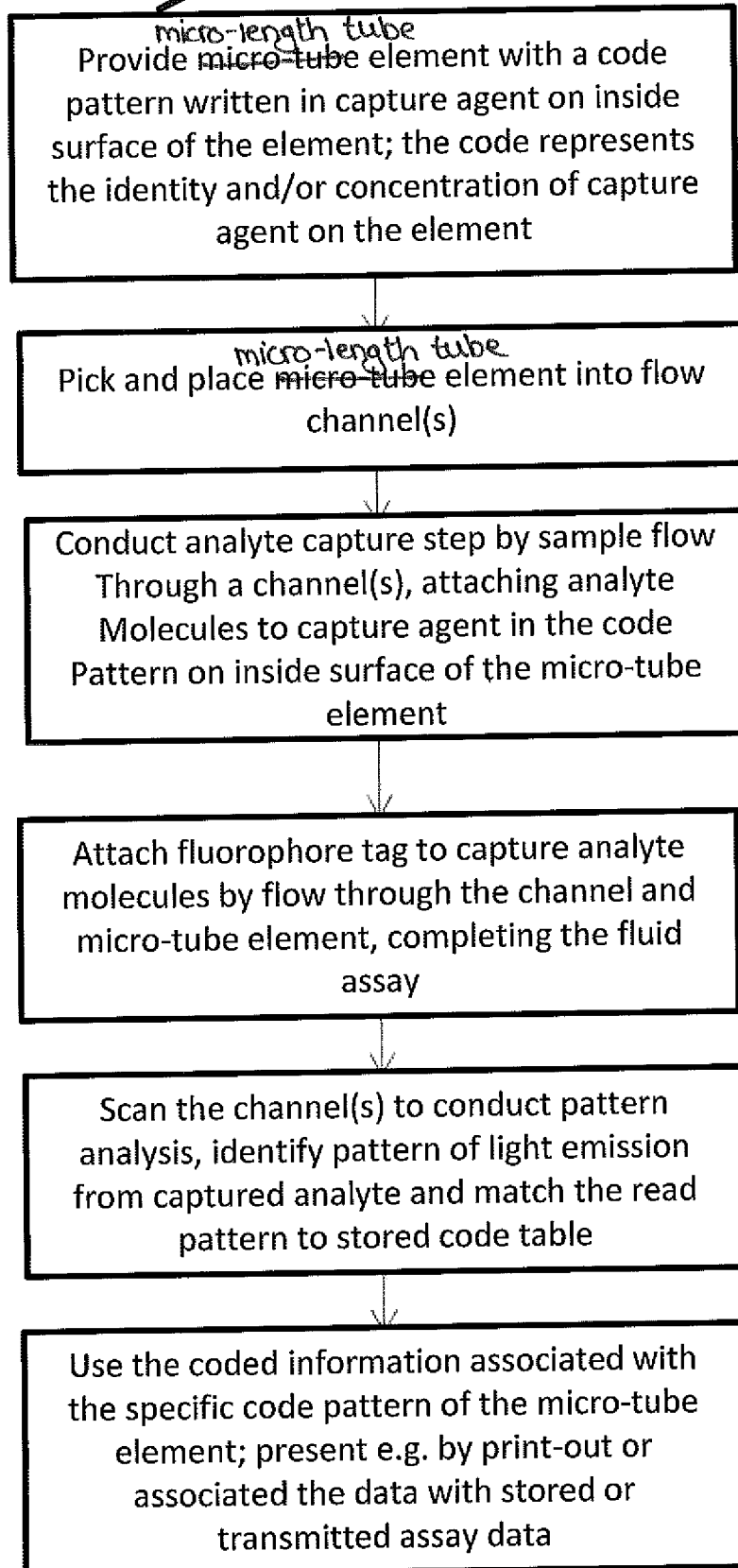
Fig. 72

~~Scanning Figure 8 - Scan Sequence – Discrete photo detector~~

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Fig. 73

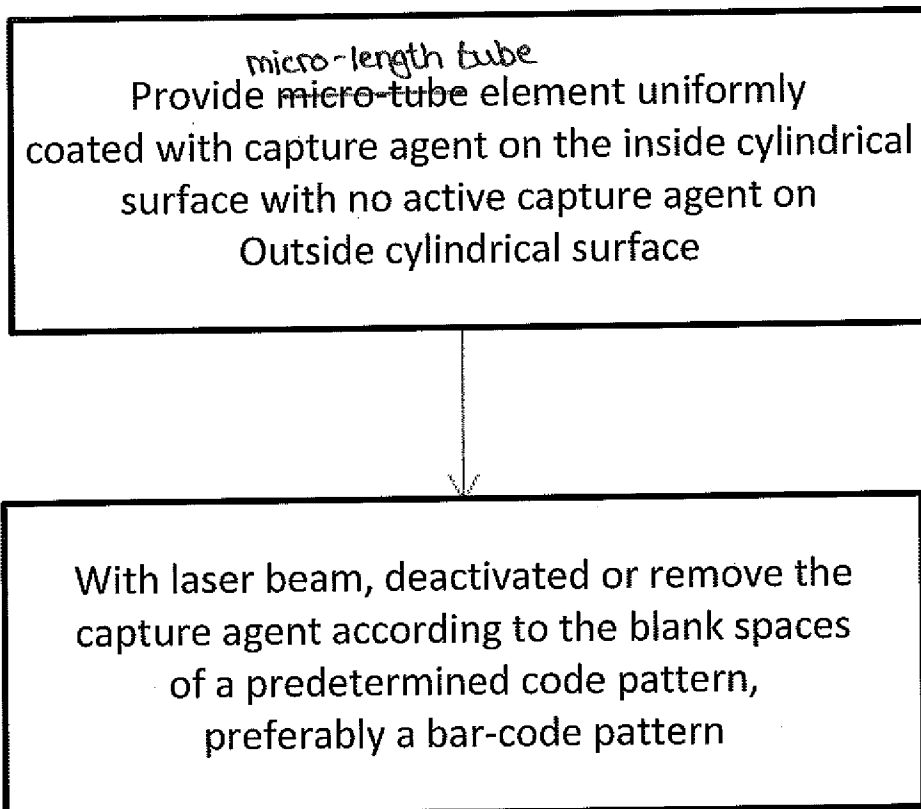
Scanning Figure 9 – Reading Code from Micro-tube flow element



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Fig. 74

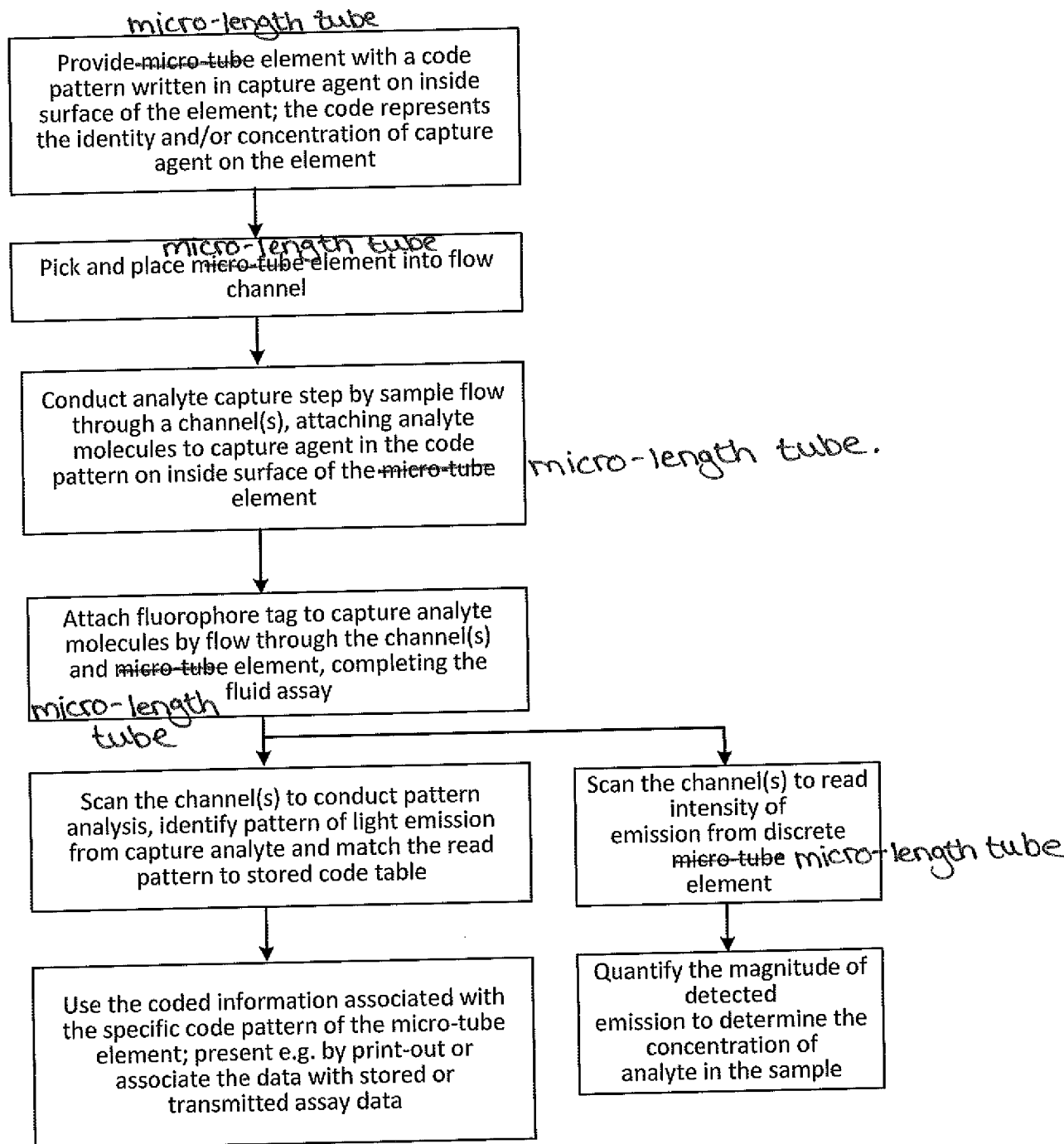
Scanning Figure 10 - Preferred Implementation of previous figures first block



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Fig. 75

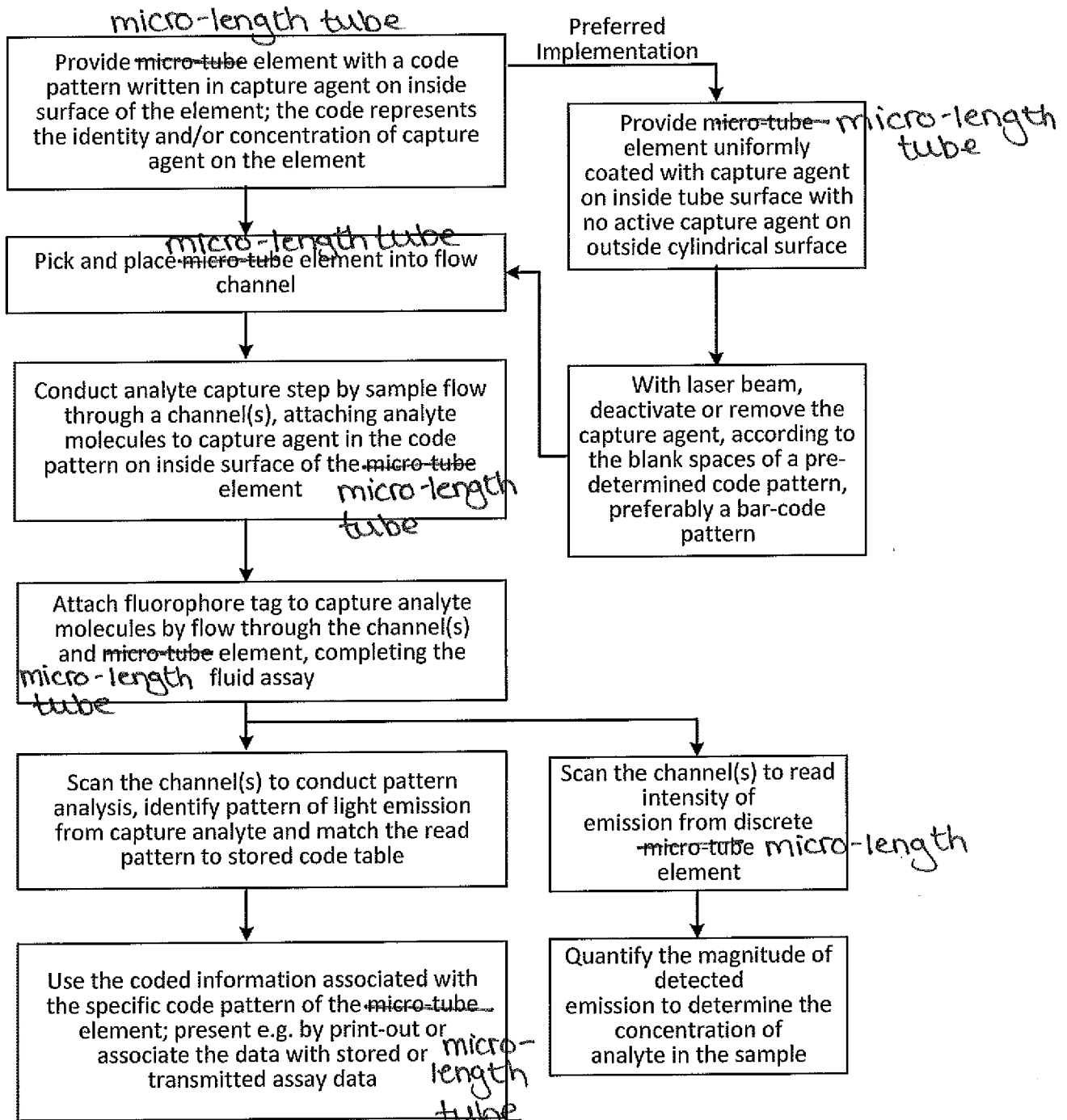
Scanning Figure 11 - Reading code and analyte quantity from ~~micro-tube~~ *micro-length tube*
~~flow element simultaneous~~



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Fig. 76

Scanning Figure 12 Reading Code Consolidated



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Fig. 77

Scanning Figure 13 - Bar Code in ~~Micro-tube~~ Element
Micro-length tube

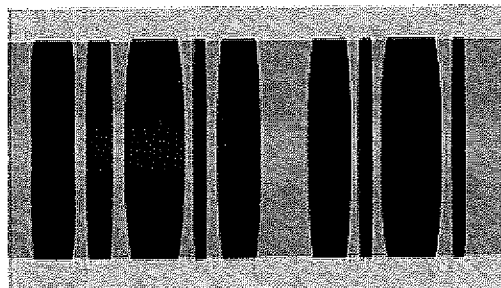
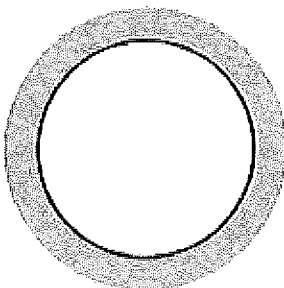
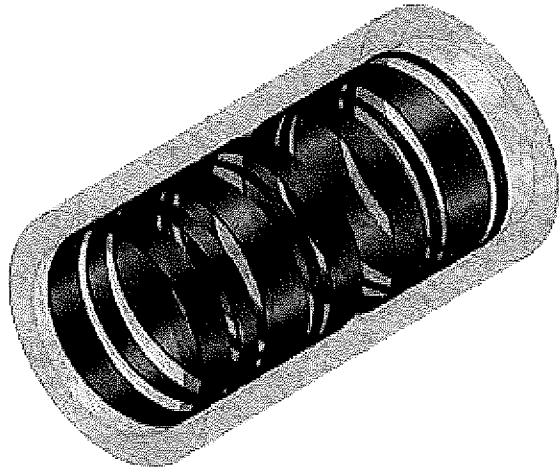
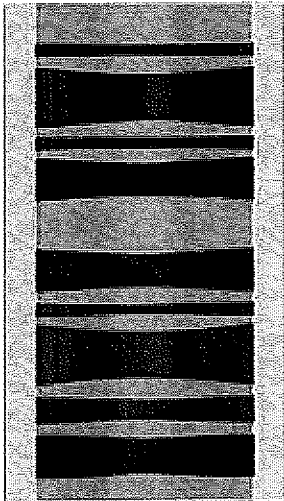


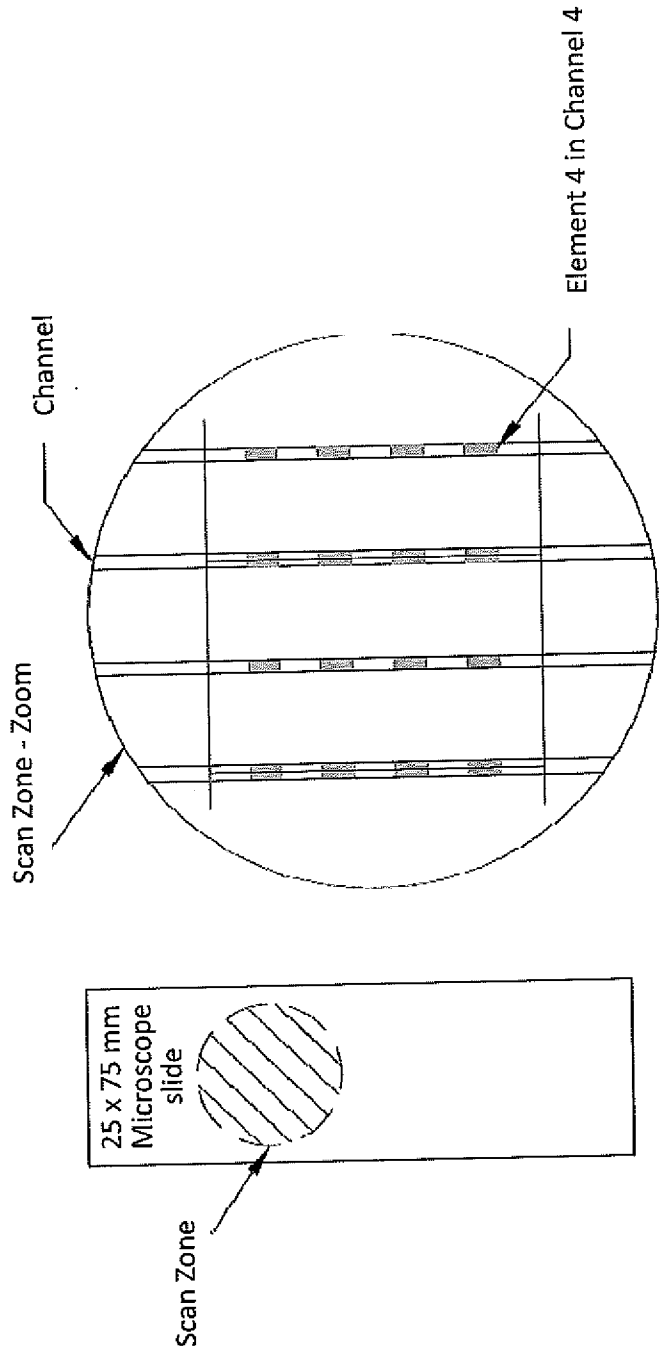
Fig. 78

Scanning Figure 14 - Scan Data File Snippet

Timestamp	Gain	Exposure	xPos	yPos	Center	Median	RMS	Mean	StdDev	Max	Min	Channel	Velocity
25:18.6	1	10	561	-207	11	12	12.59831	12.41033	2.168574	20	6	1	200
25:18.6	1	10	561	-207	11	12	12.59977	12.41679	2.139898	19	6	1	200
25:18.7	1	10	1099	-207	11	12	12.59977	12.41679	2.139898	19	6	1	200
25:18.7	1	10	1099	-207	16	13	12.71469	12.54103	2.094641	21	5	1	200
25:18.7	1	10	1093	-207	14	13	12.74004	12.56383	2.111963	19	5	1	200
25:18.7	1	10	1093	-207	14	13	12.74004	12.56383	2.111963	19	5	1	200
25:18.7	1	10	1086	-207	18	13	12.96235	12.78609	2.130713	20	6	1	200
:	:	:	:	:	:	:	:	:	:	:	:	:	:
:	:	:	:	:	:	:	:	:	:	:	:	:	:
:	:	:	:	:	:	:	:	:	:	:	:	:	:
25:44.9	1	10	-3906	-207	12	11	11.29127	11.1288	1.908924	18	4	2	200
25:44.9	1	10	-3906	-207	12	11	11.29127	11.1288	1.908924	18	4	2	200
25:44.9	1	10	-3906	-207	12	11	11.29127	11.1288	1.908924	18	4	2	200
25:44.9	1	10	-3906	-207	9	11	11.34321	11.19035	1.856259	17	5	2	200
25:44.9	1	10	1100	2585	11	11	11.25899	11.11056	1.822494	18	4	2	200
25:44.9	1	10	1100	2585	11	11	11.25899	11.11056	1.822494	18	4	2	200
25:44.9	1	10	1100	2585	11	11	11.25899	11.11056	1.822494	18	4	2	200
:	:	:	:	:	:	:	:	:	:	:	:	:	:
:	:	:	:	:	:	:	:	:	:	:	:	:	:
:	:	:	:	:	:	:	:	:	:	:	:	:	:
27:02.5	1	10	-3892	8169	13	12	11.94034	11.78989	1.898861	18	4	n	200
27:02.5	1	10	-3892	8169	13	12	11.94034	11.78989	1.898861	18	4	n	200
27:02.6	1	10	-3892	8169	13	12	11.94034	11.78989	1.898861	18	4	n	200
27:02.6	1	10	-3898	8169	13	12	11.78514	11.63564	1.871547	18	5	n	200
27:02.6	1	10	-3898	8169	10	12	11.68101	11.52888	1.879474	18	5	n	200
27:02.6	1	10	-3898	8169	10	12	11.68101	11.52888	1.879474	18	5	n	200
27:02.6	1	10	-3904	8169	12	12	11.76859	11.61512	1.894712	19	5	n	200

Fig. 79

Scanning Figure 15 - Chip Layout

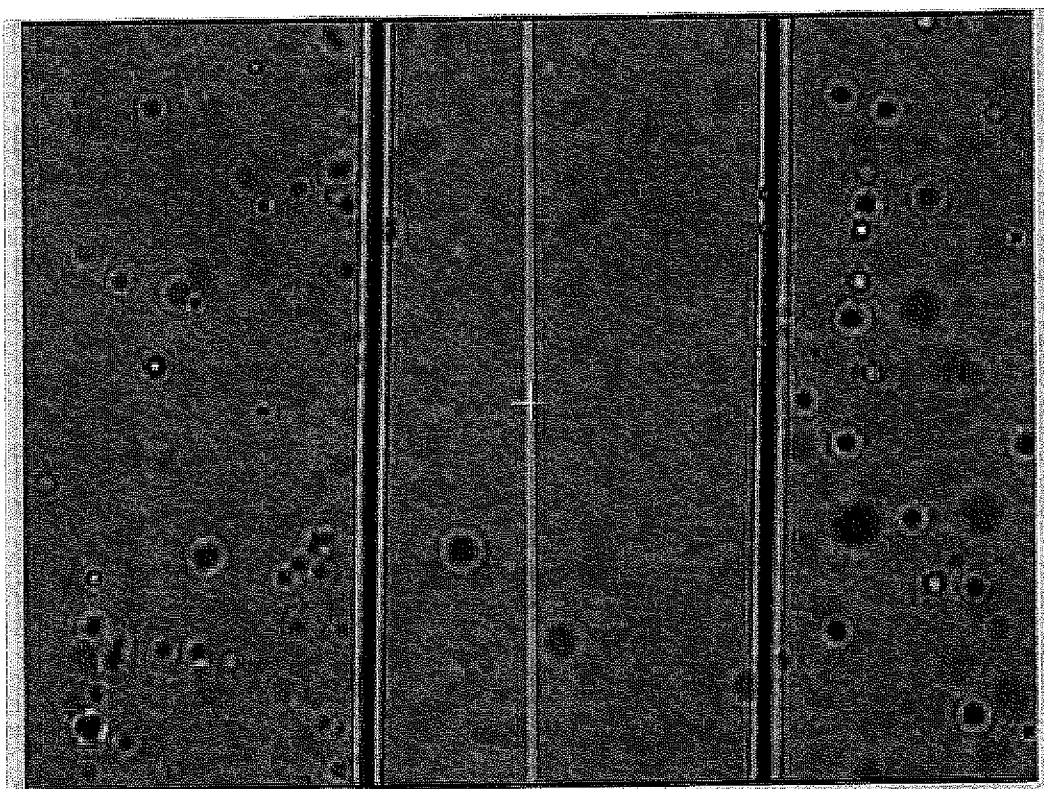


Find Channels Scan Element
Find Elements / Fluorescence Scan

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Fig. 80

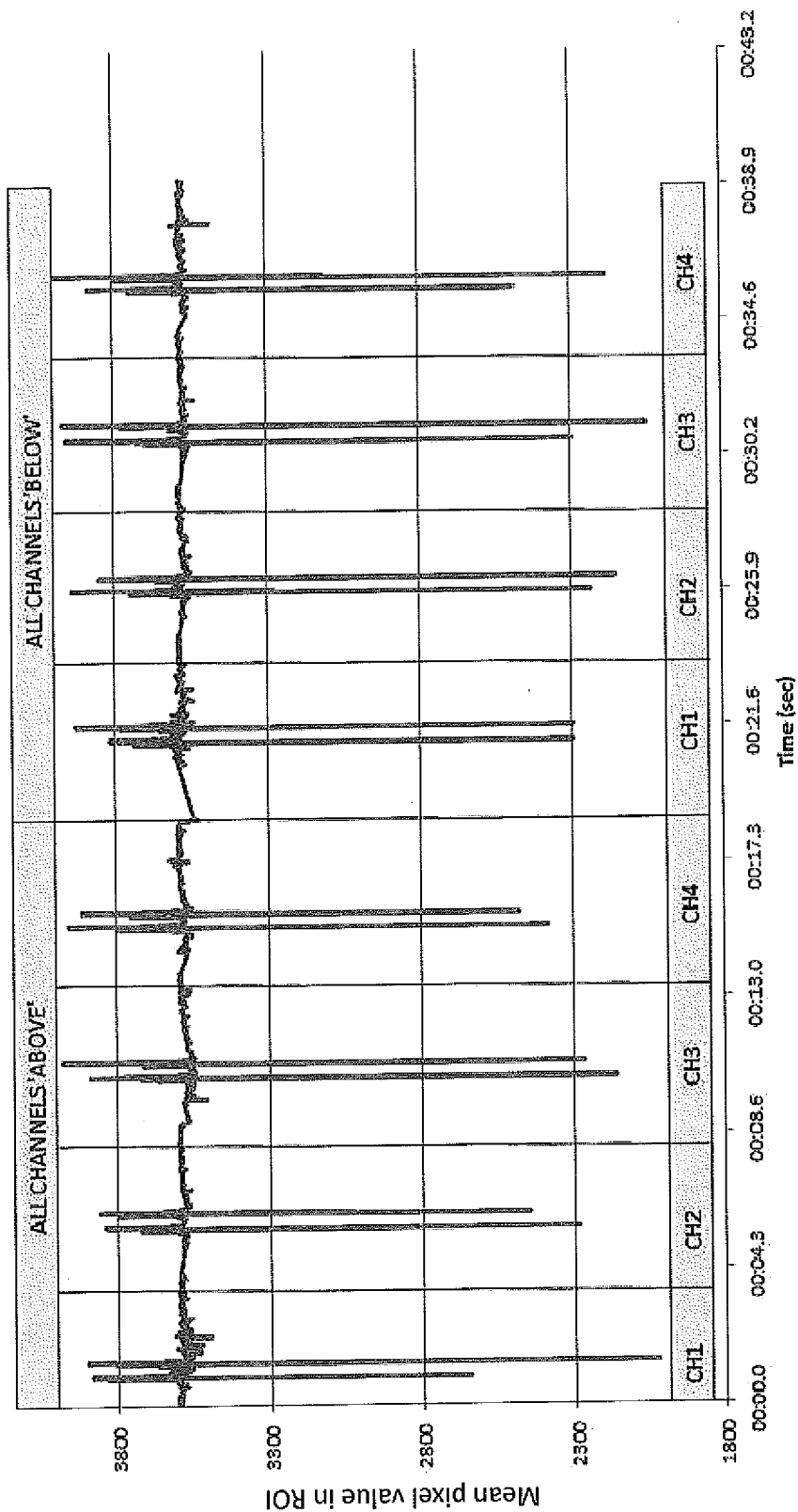
Scanning ~~Figure~~ 16 - Find Channels ROI



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Fig. 81

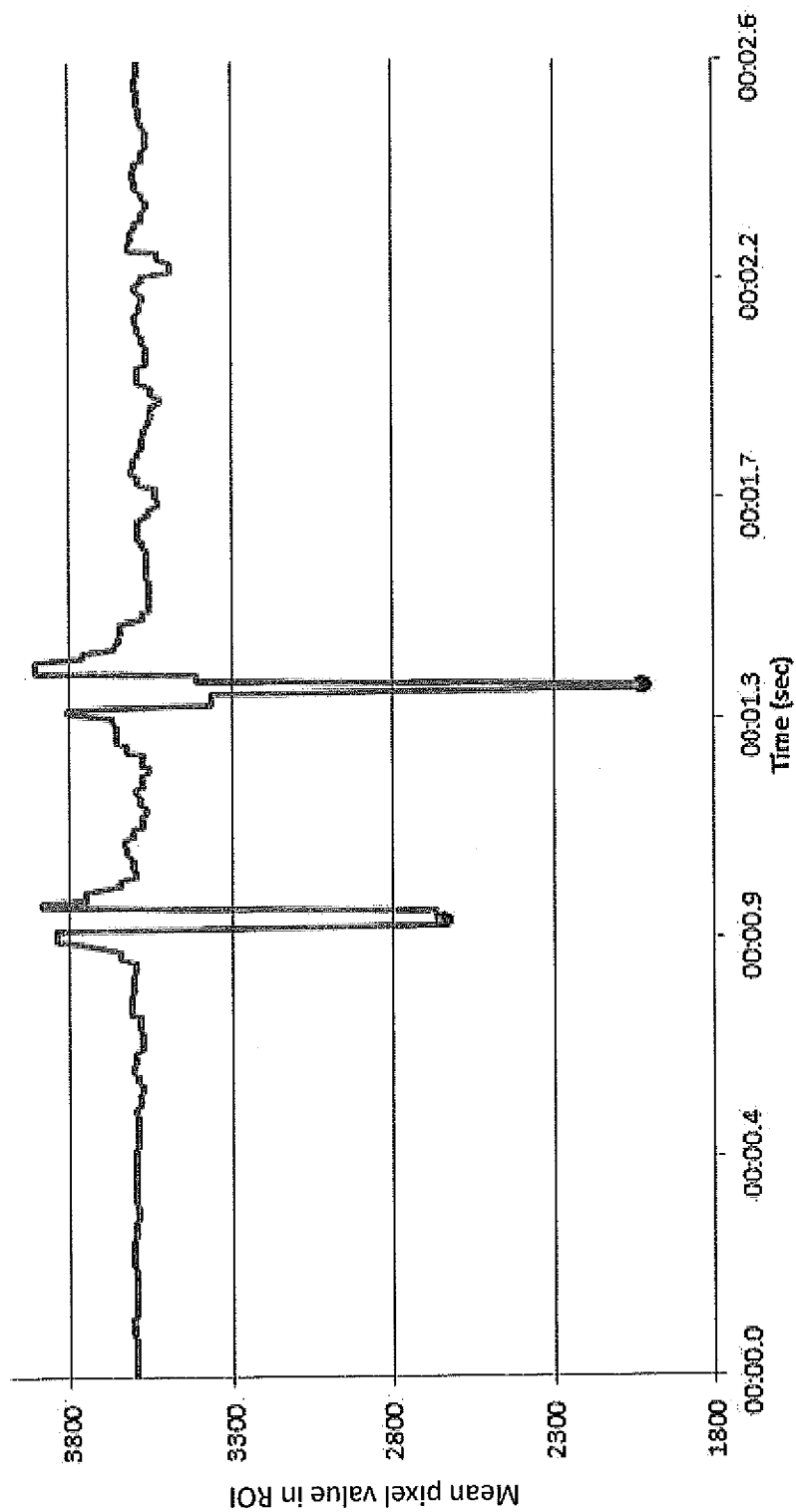
Scanning Figure 17 - Find Channels Scan Plot



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Fig. 82

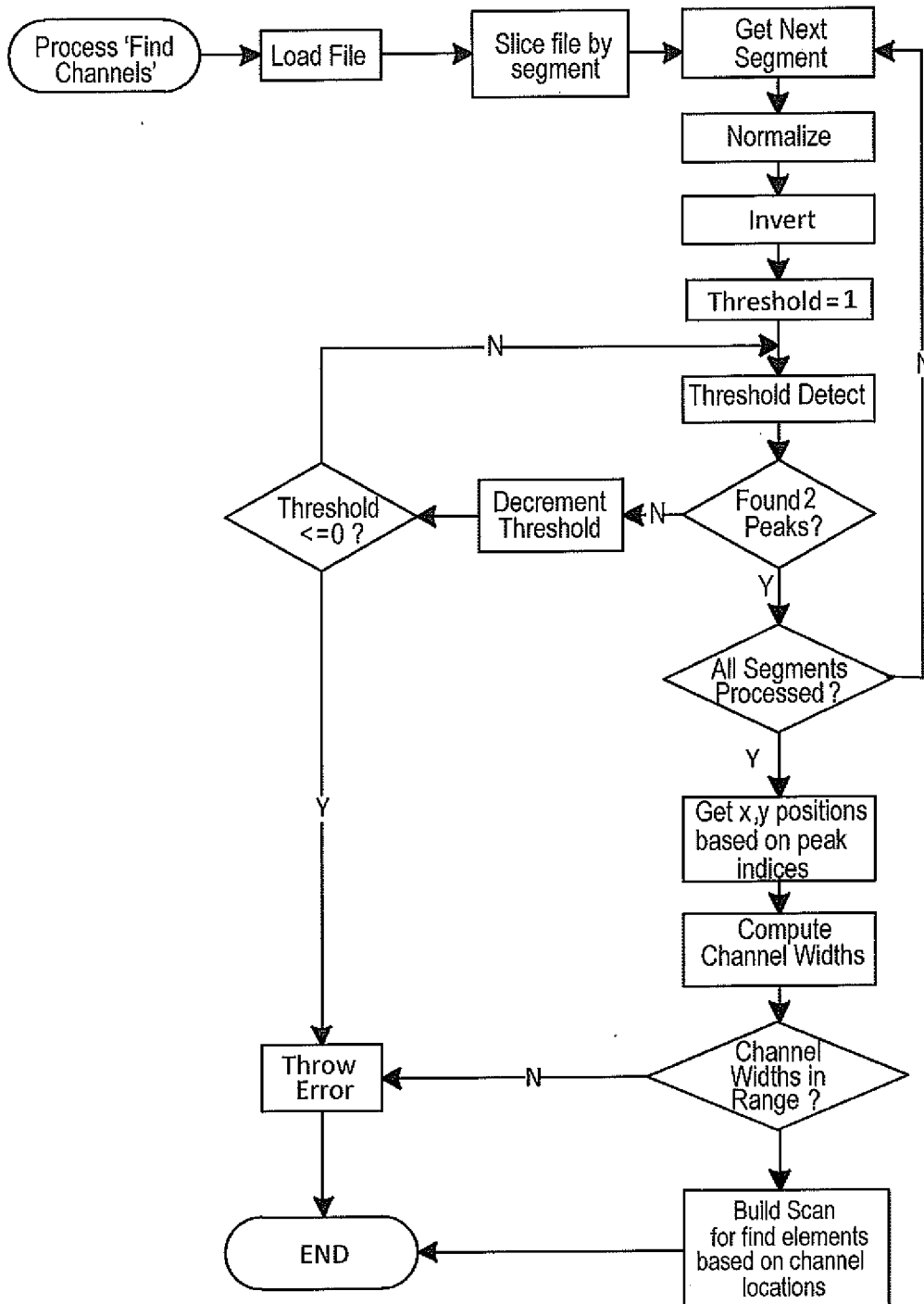
Scanning Figure 18 - Find Channels Data Segment Plot



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Fig. 83

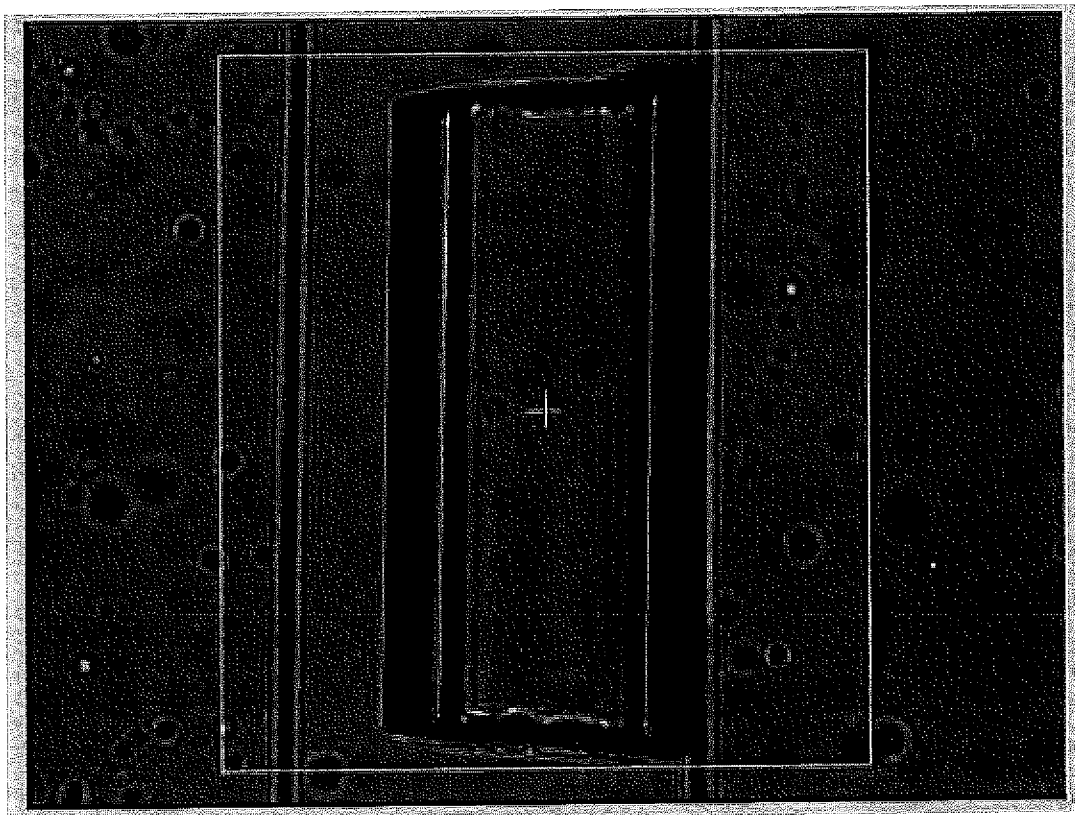
Scanning Figure 18 - Find Channels Processing Flowchart



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Fig. 84

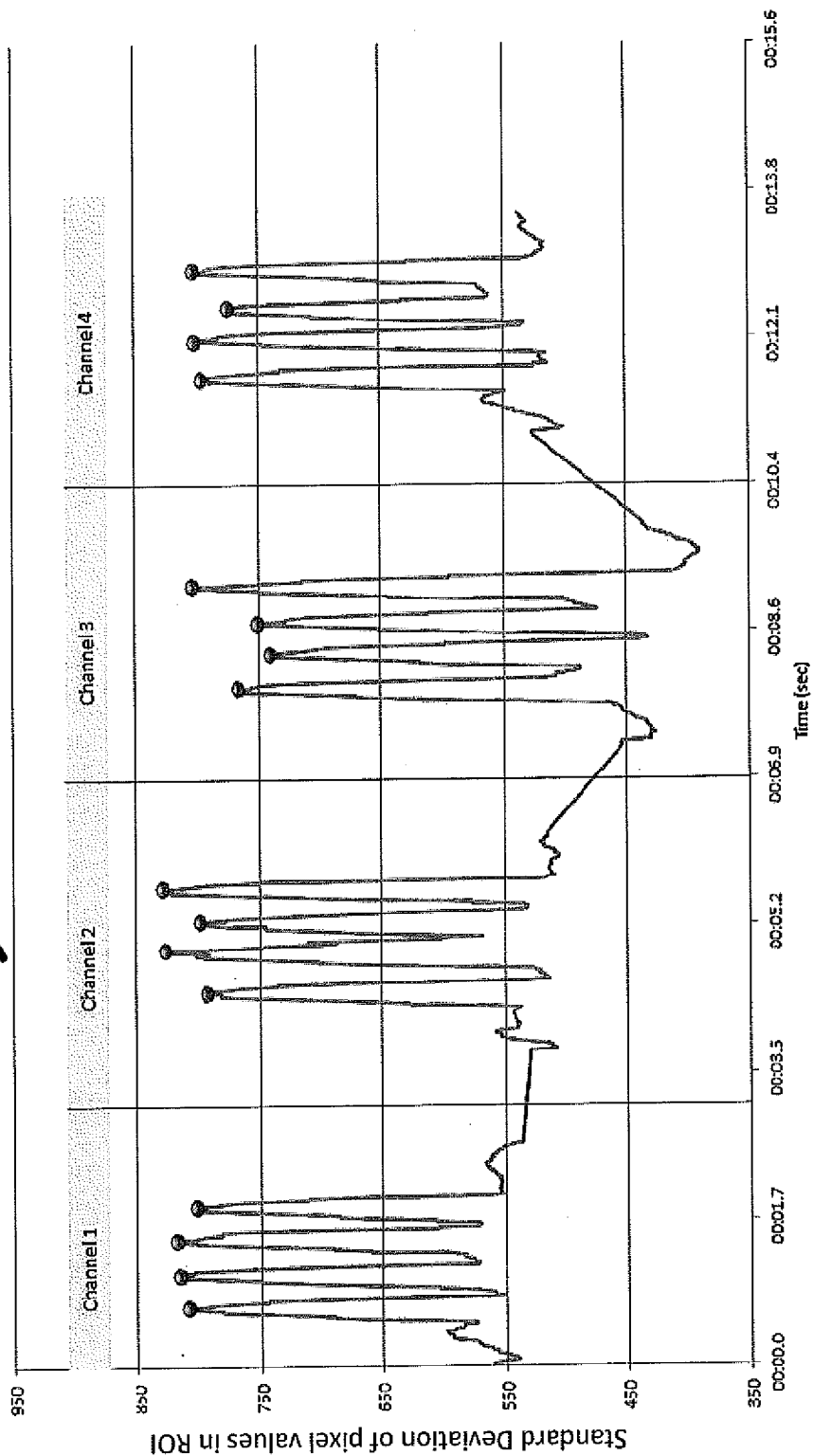
Scanning Figure ~~20~~ - Find Elements ROI



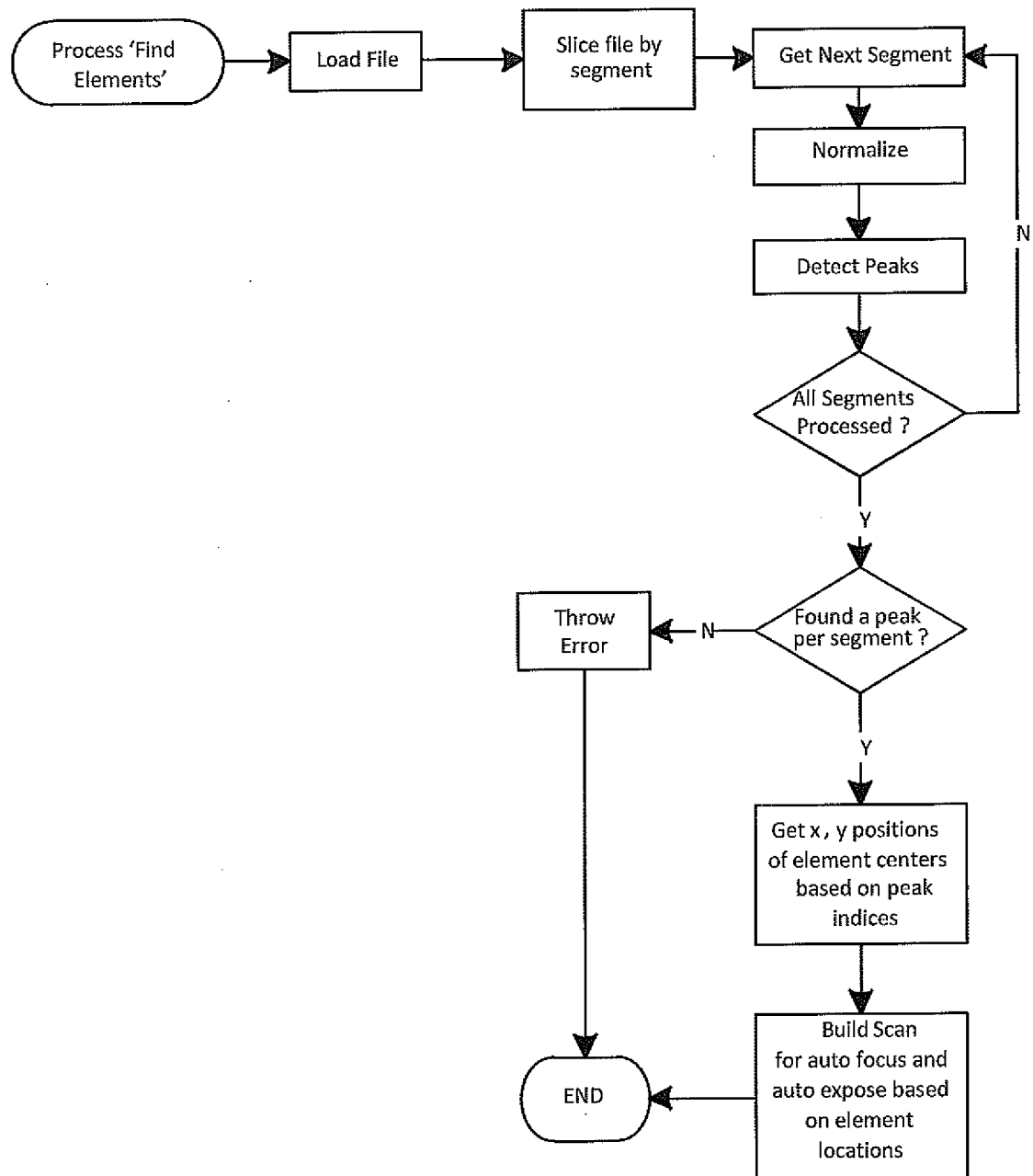
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Fig. 85

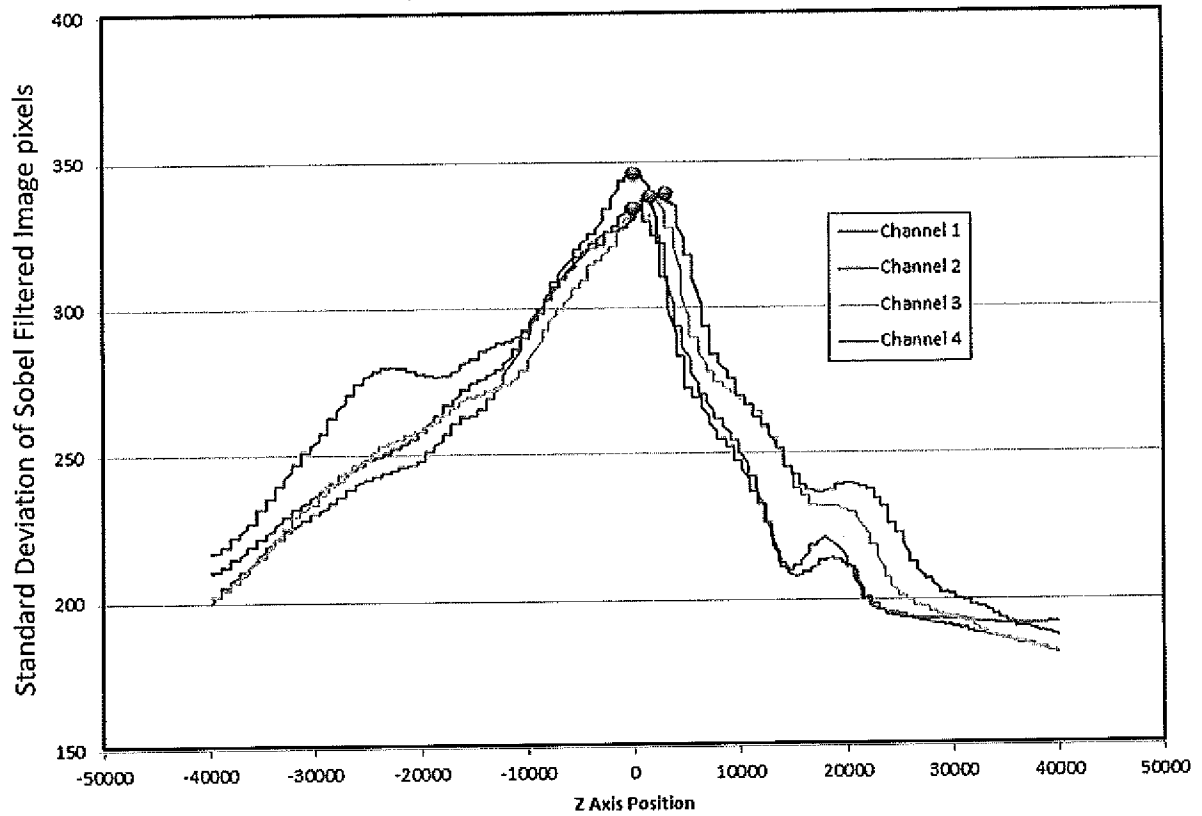
Scanning Figure 21 - Find Elements Scan Plot



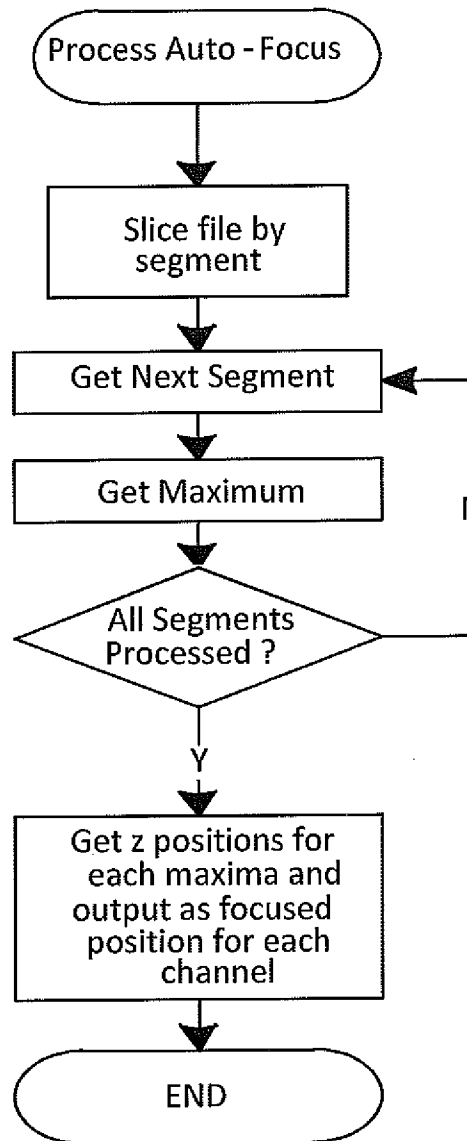
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Fig. 86**Scanning Figure 22 - Find Elements Processing Flowchart**

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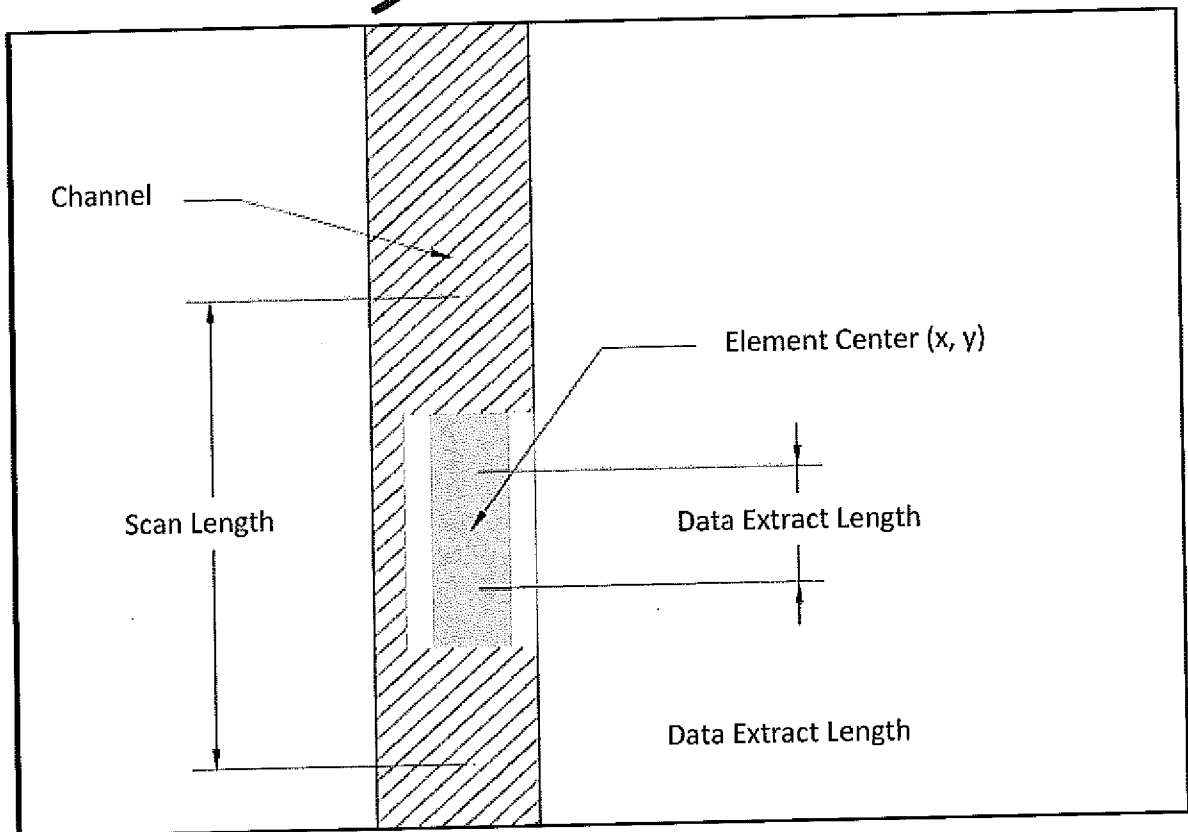
Fig. 87**Scanning Figure 23 - Auto Focus Scan Plot**

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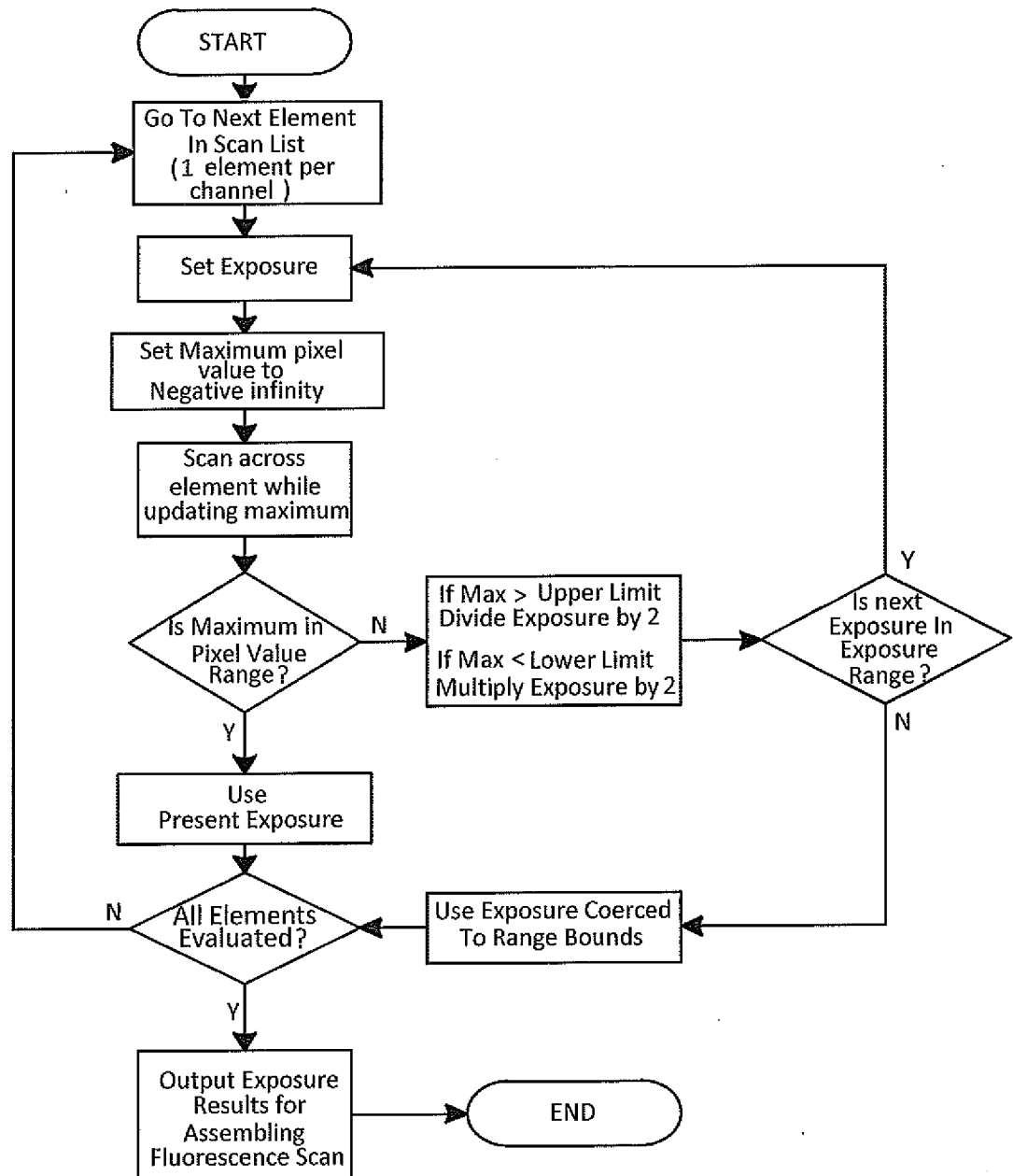
Fig. 88**Scanning Figure 24 - Auto-Focus Processing Flowchart**

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Fig. 89
Scanning Figure 25 - Auto-Expose Schematic



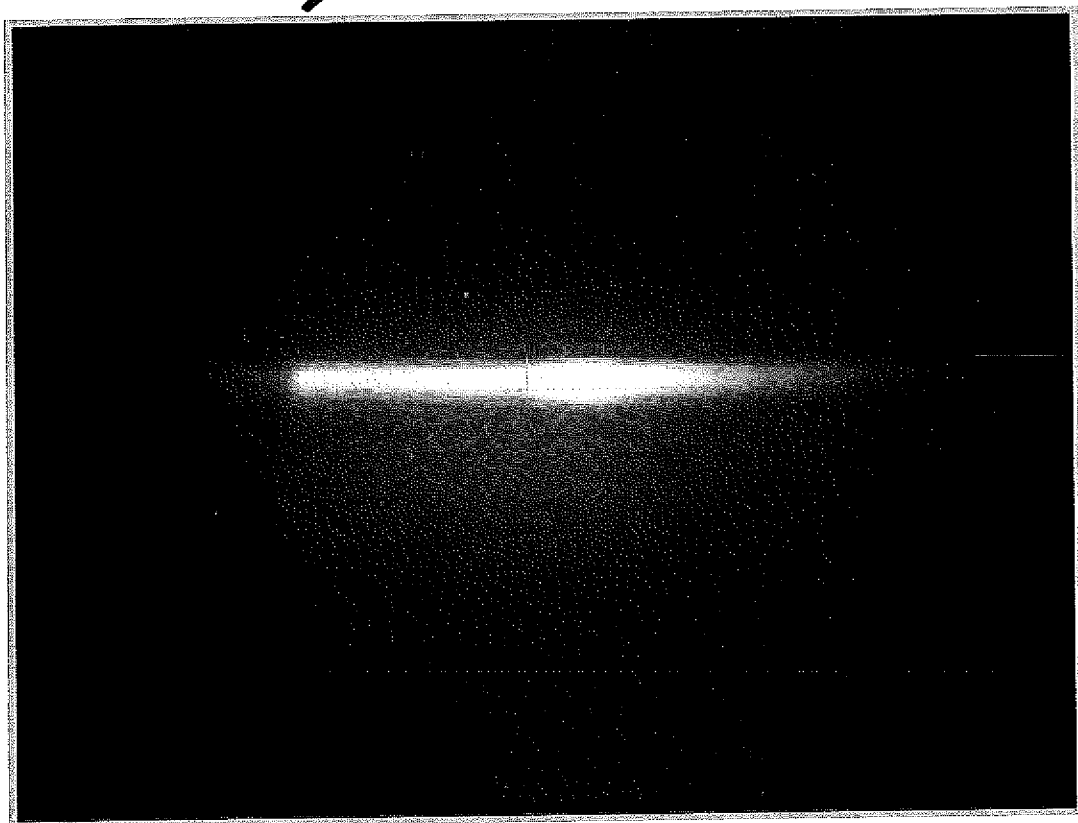
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Fig. 90**Scanning Figure 26 - Auto-Exposure
Procedure Flowchart**

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Fig. 91

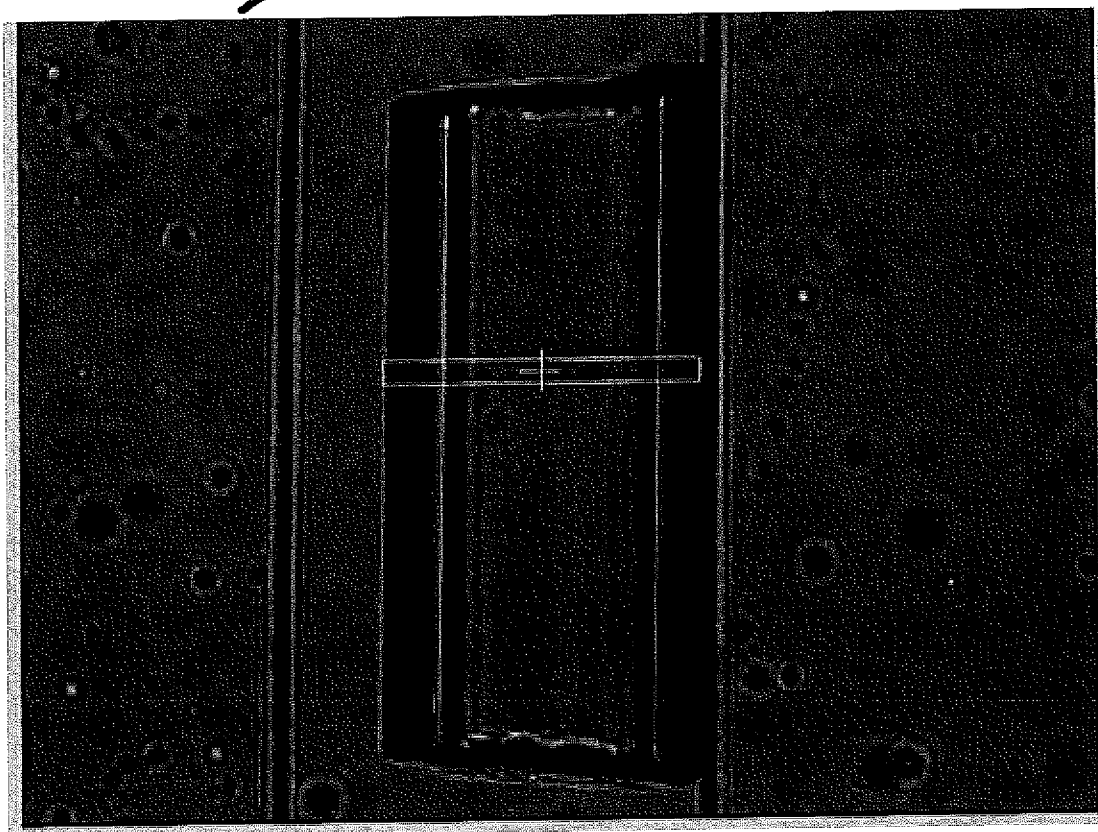
Scanning Figure 27 - Laser/ROI Alignment



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Fig. 92

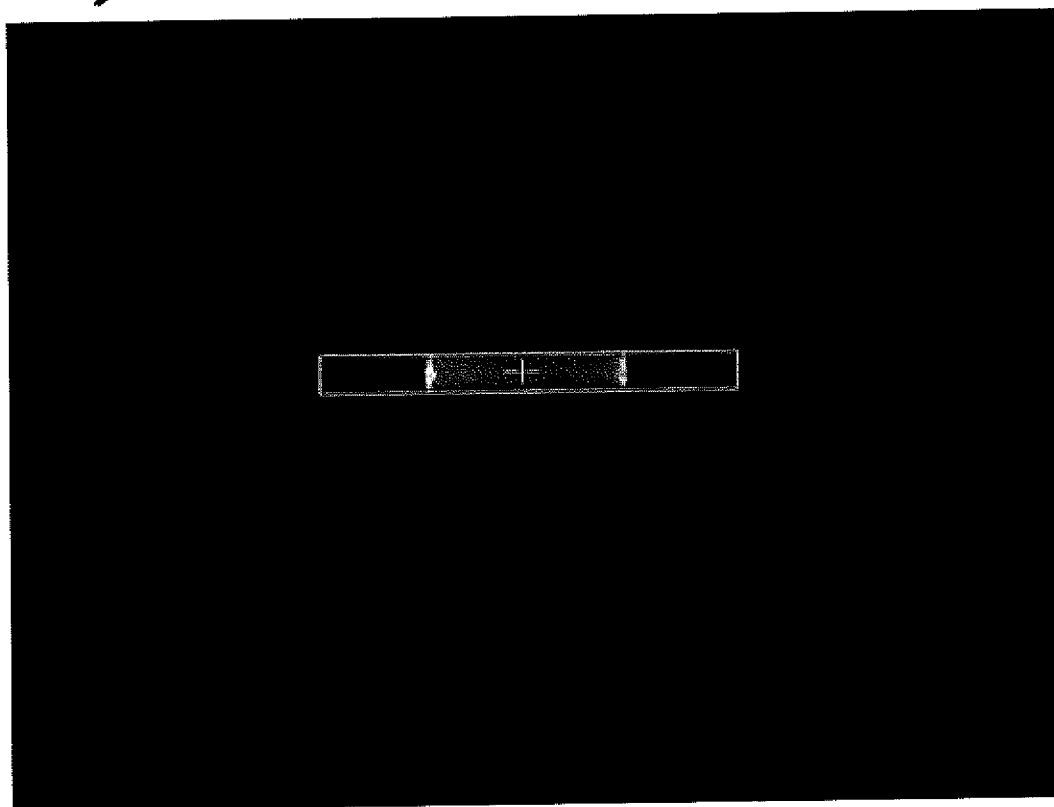
Scanning Figure 28 - Fluorescence Scan ROI, bright field



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Fig. 93

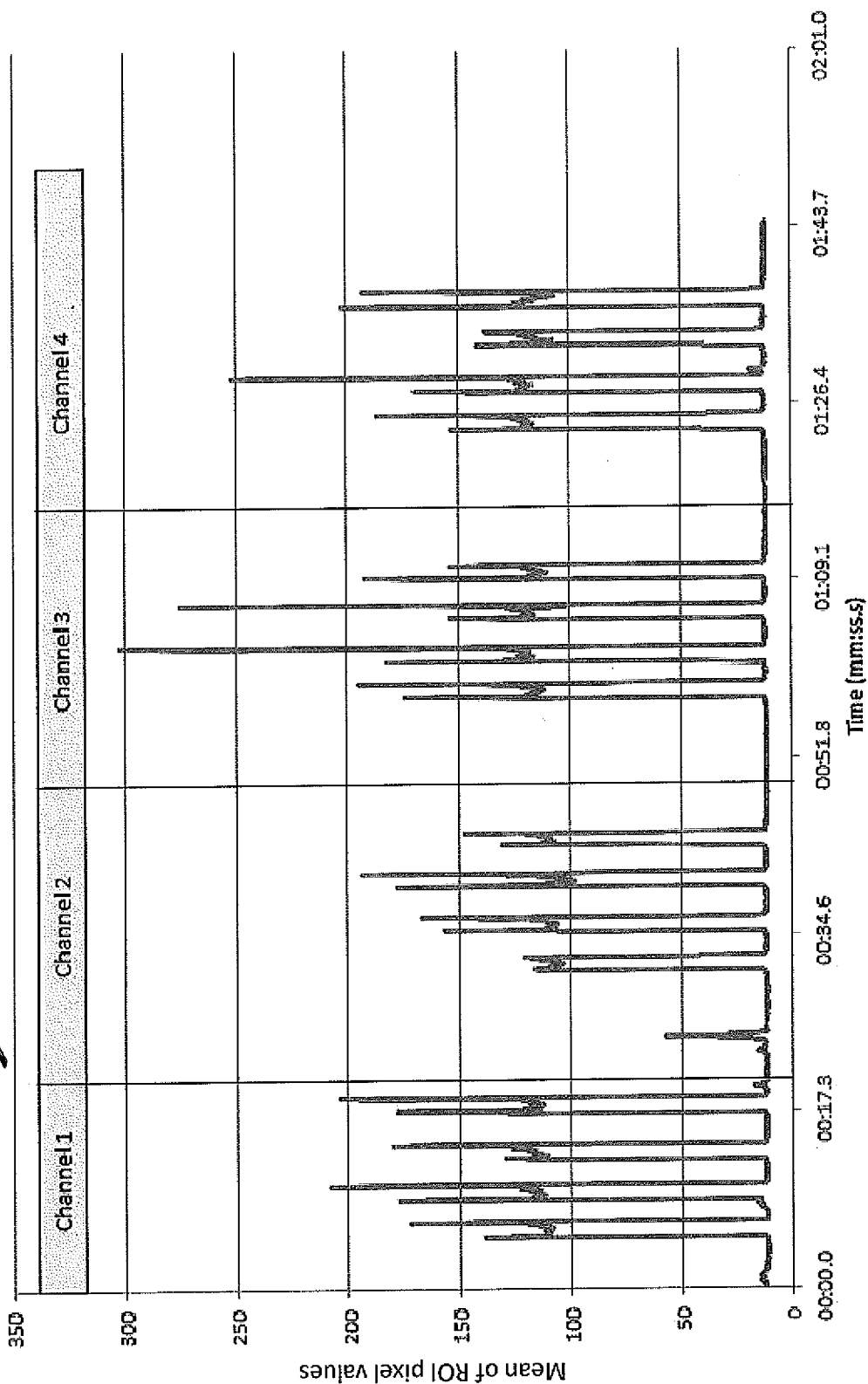
~~Scanning~~ Figure 29 - Fluorescence Scan ROI, laser on, LED off



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Fig. 94

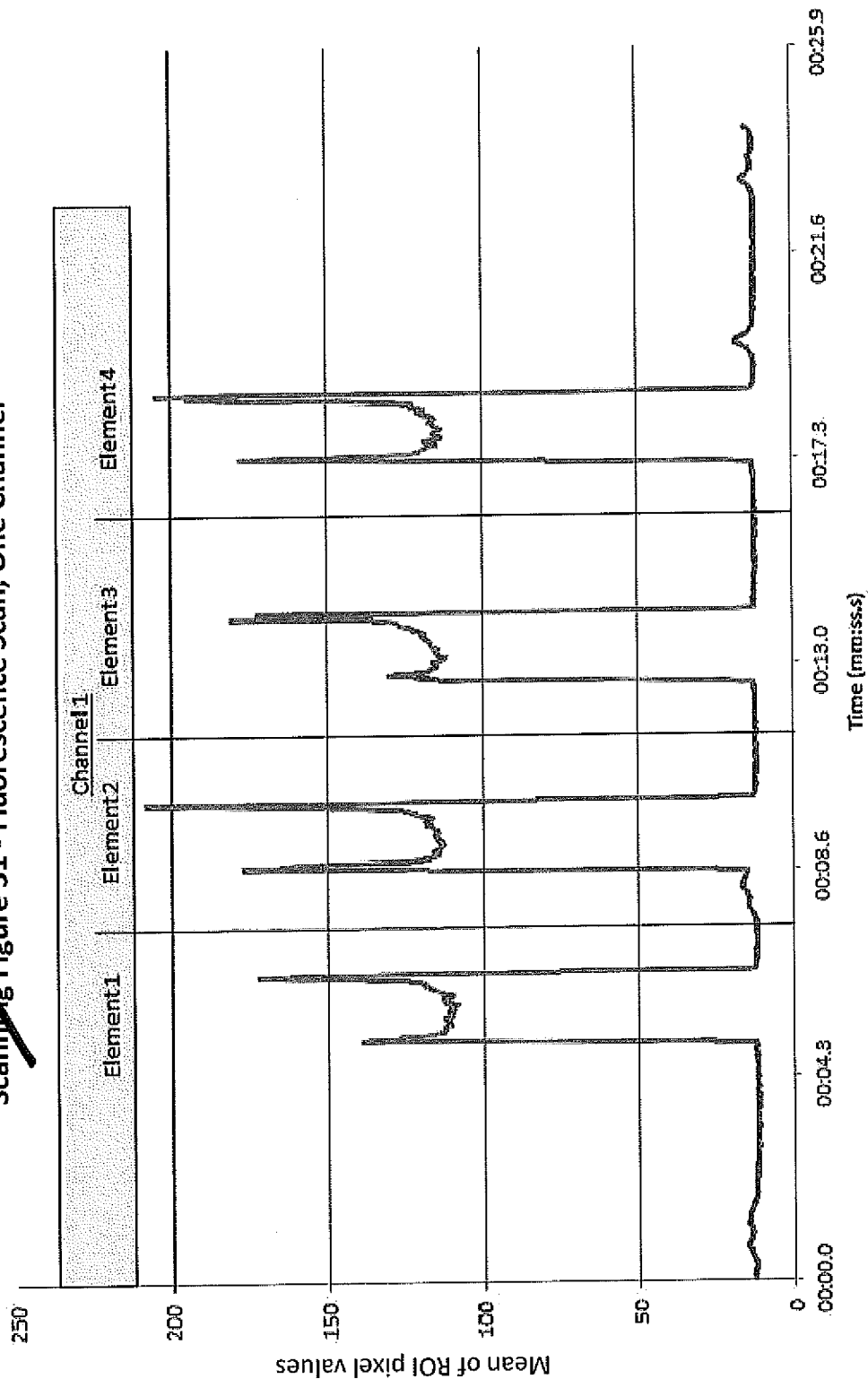
Scanning Figure 30 - Fluorescence Scan Data, Full San



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Fig. 95

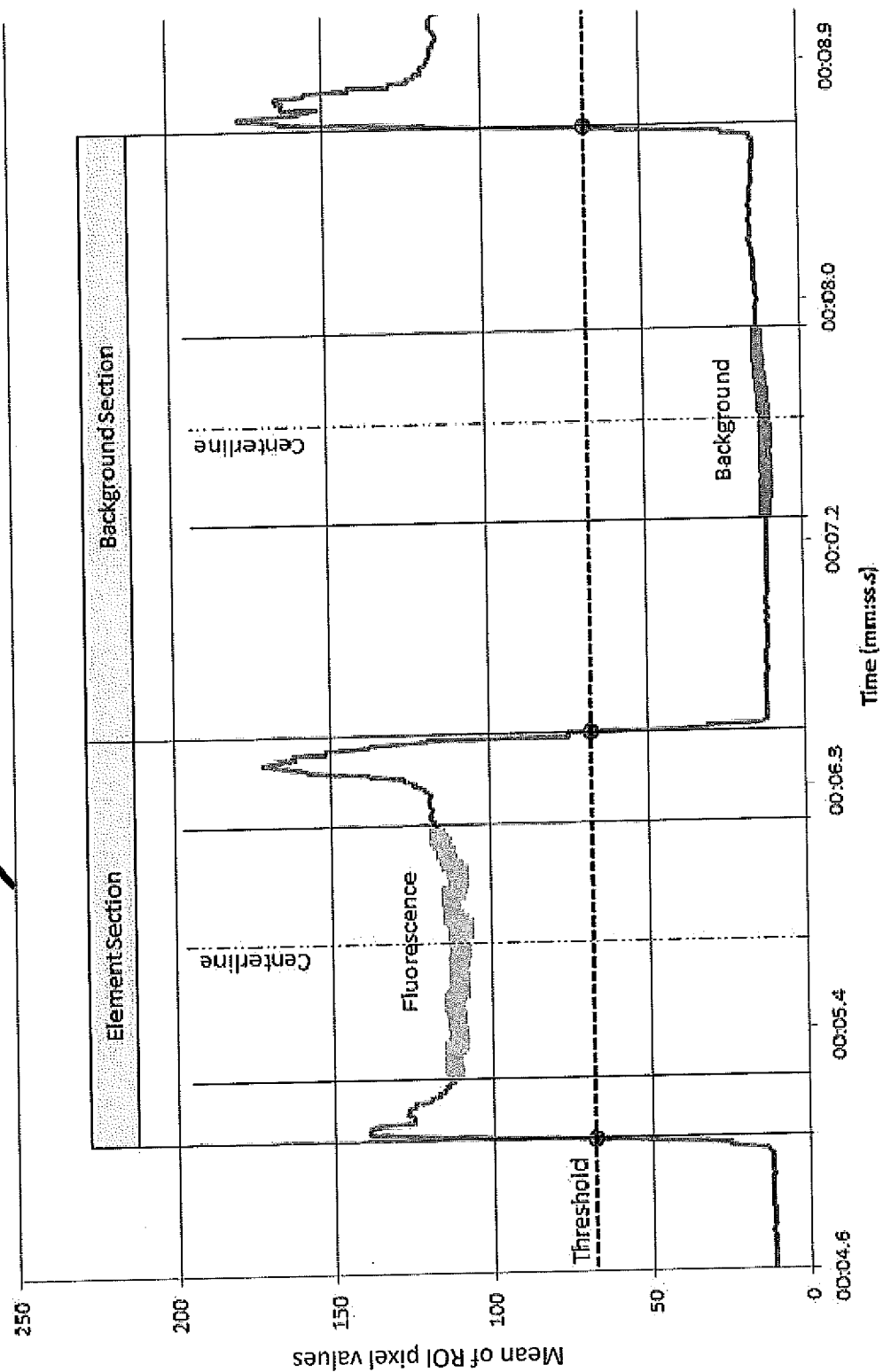
Scanning Figure 31 - Fluorescence Scan, One Channel



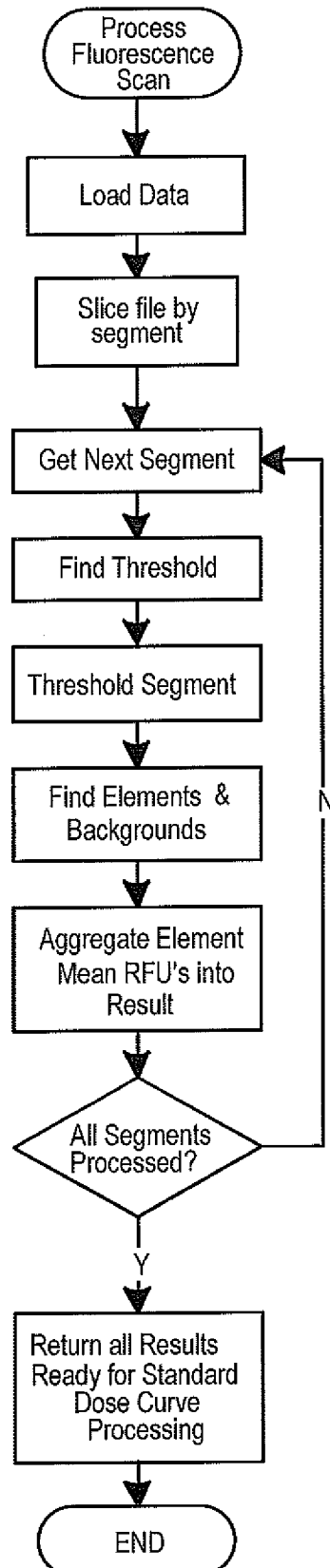
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Fig. 96

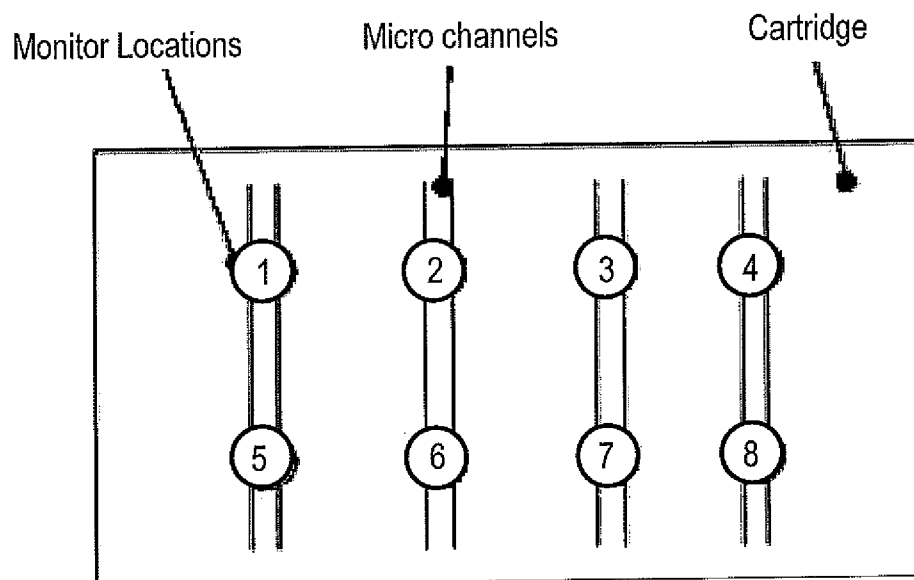
Scanning Figure 32 - Fluorescence Scan, One Element



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Fig. 97**Scanning Figure 33 - Fluorescence scan data processing**

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~~FIG. 1~~

Fig. 98

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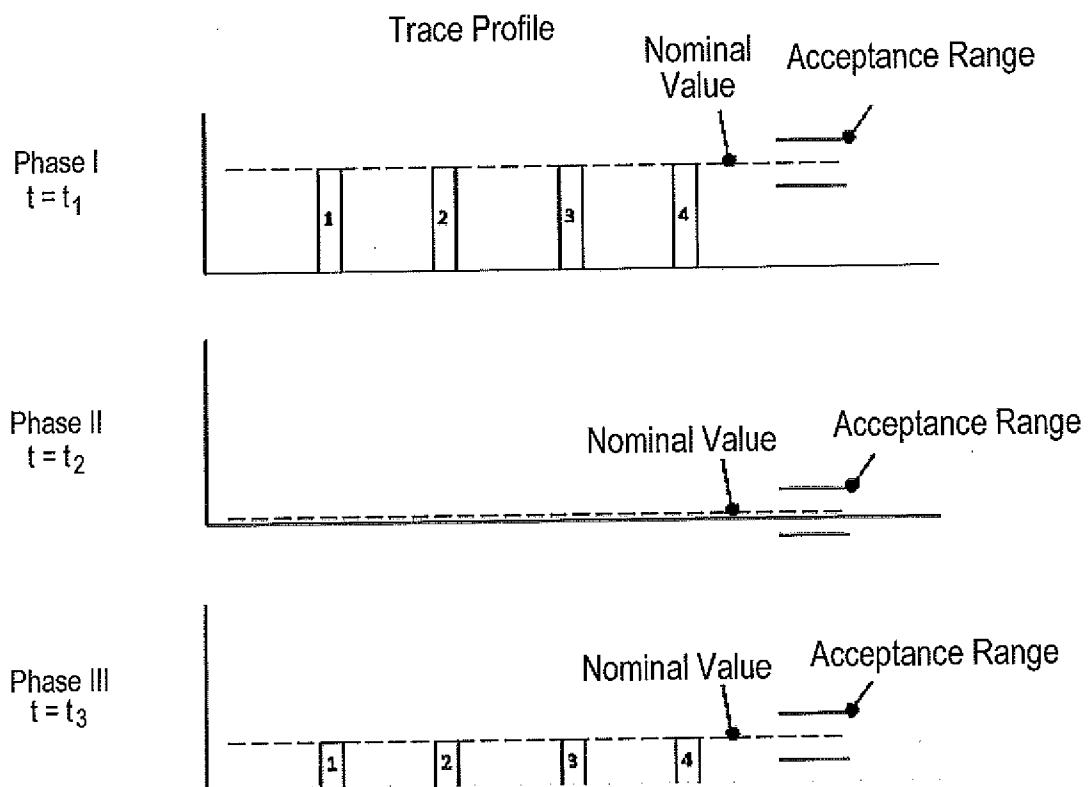
~~FIG. 1A~~

Fig. 98A

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Fig. 99C
~~FIG. 1C~~

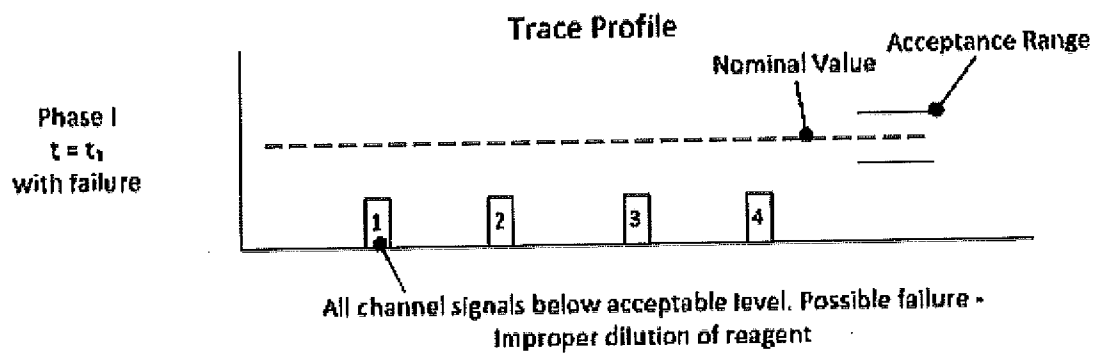
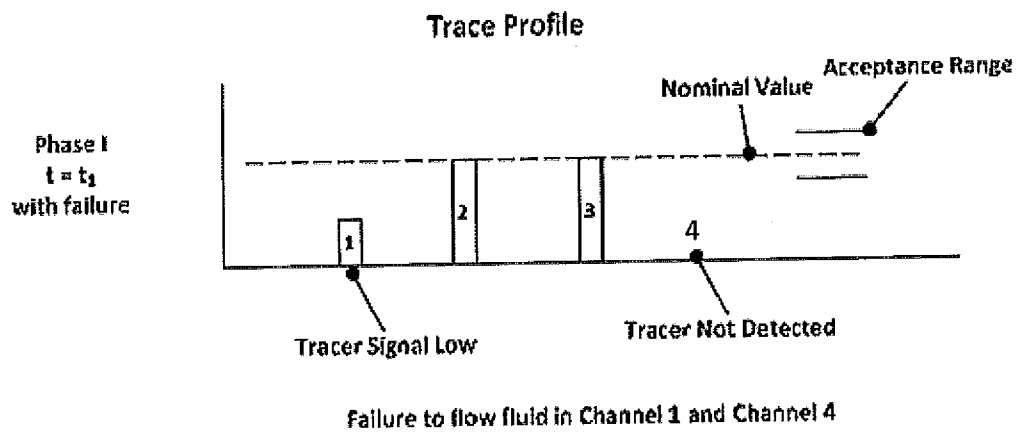


Fig. 98 B
~~FIG. 1B~~



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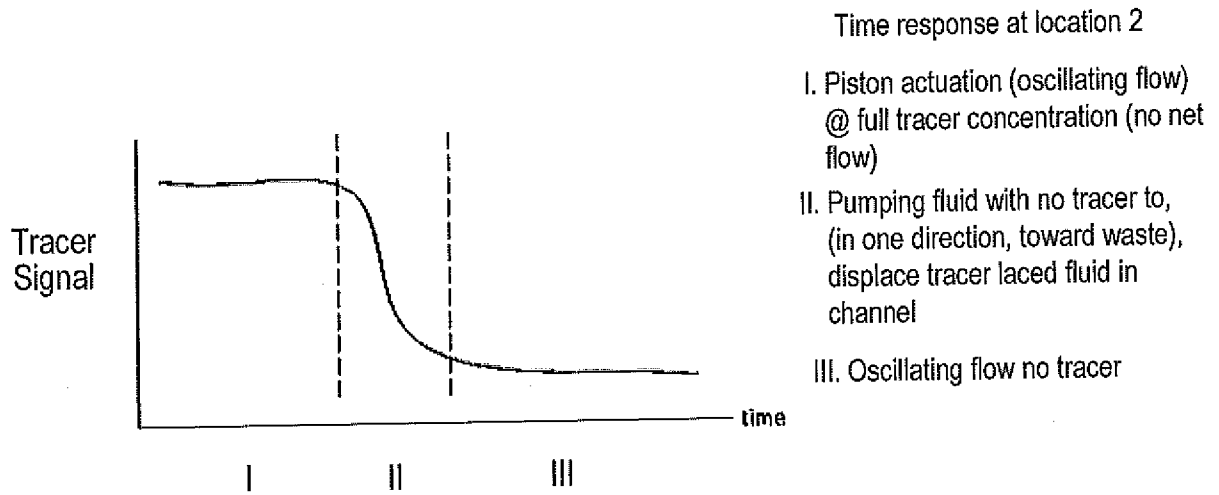
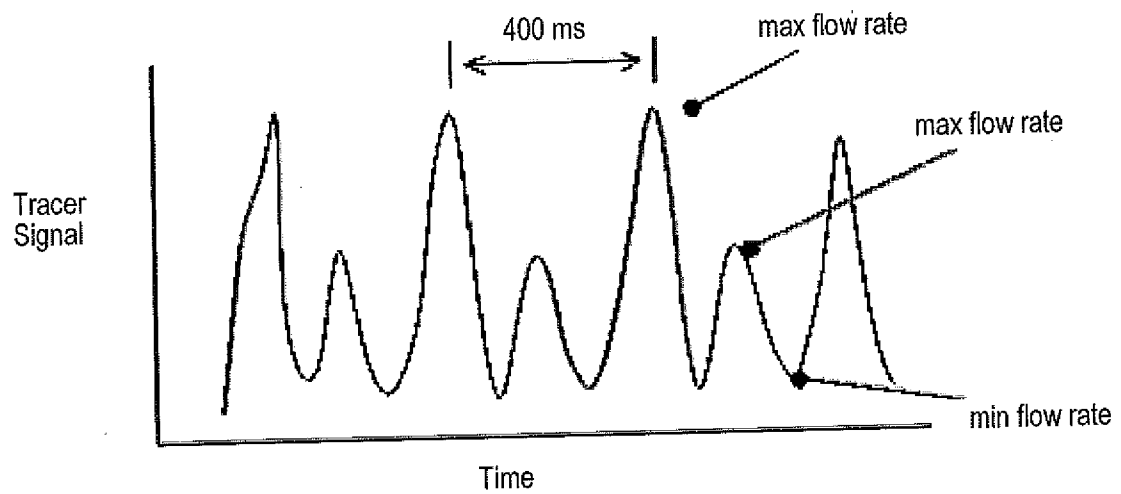
~~FIG. 2~~

Fig. 99

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Fig. 100
~~FIG. 3~~



Oscillator flow monitored at single location
Refer to FIG. 1

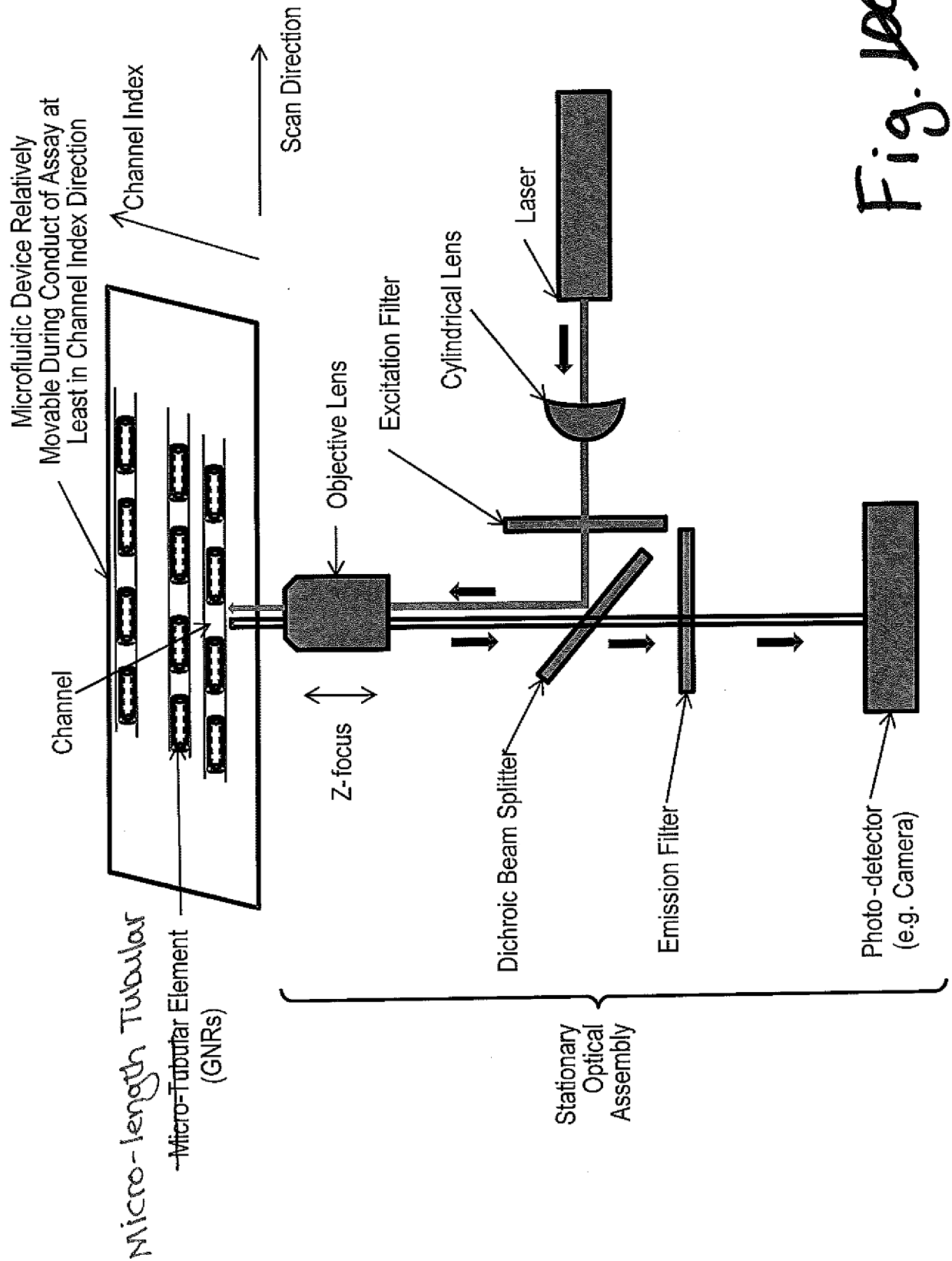


Fig. 100 101

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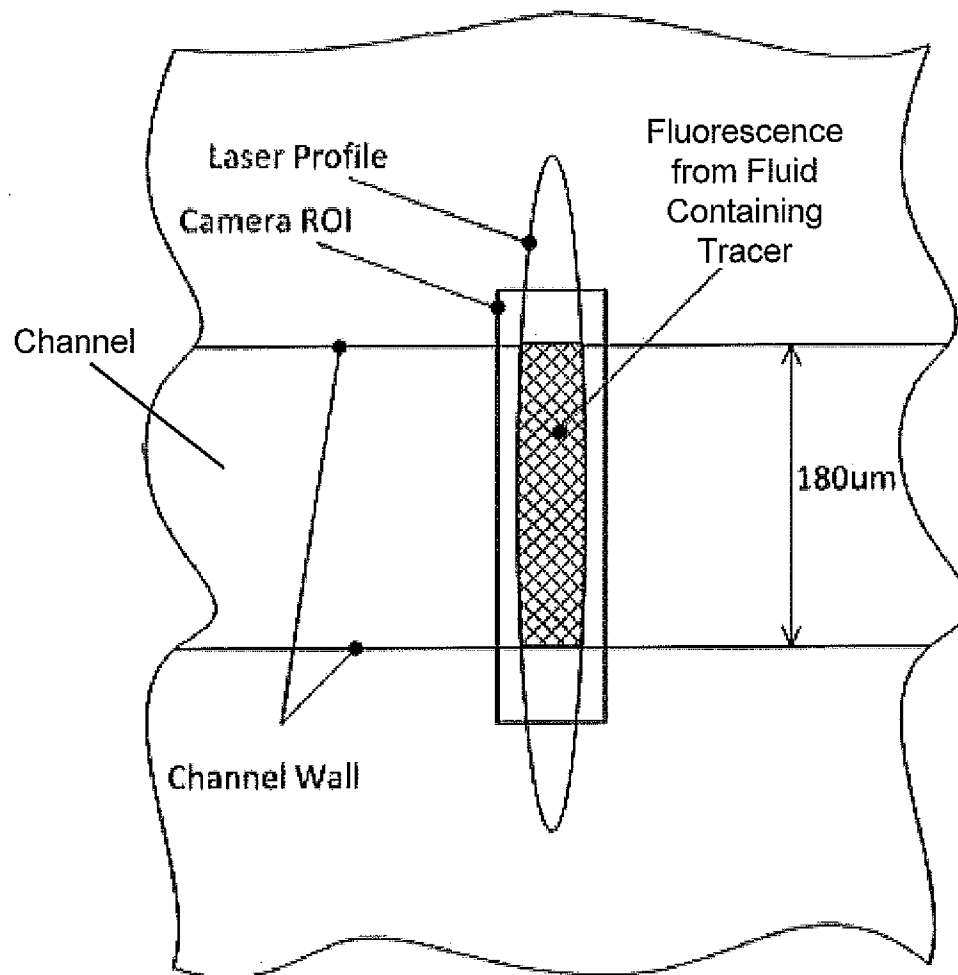


Fig. 102

Fig. 103

Fluidic Architecture and Protocol

Assay Protocol

Prime

Sample

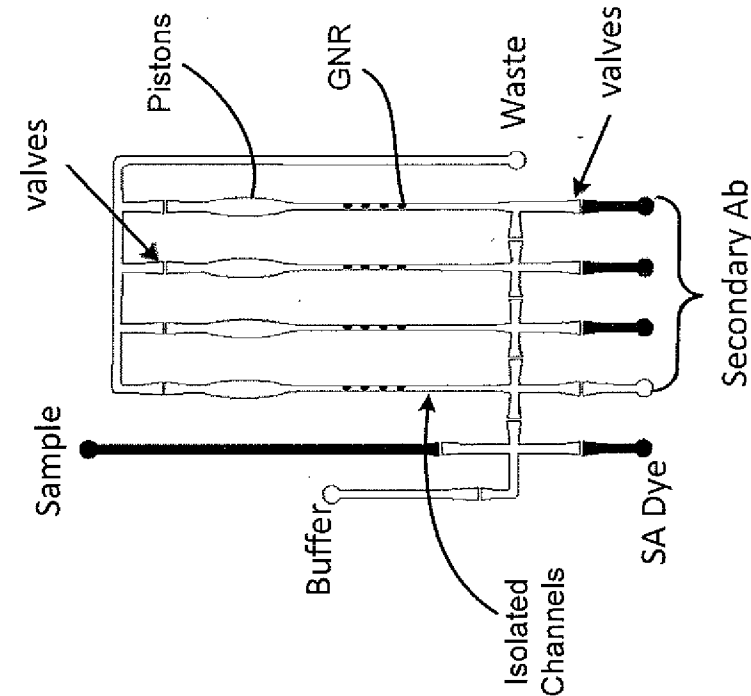
Wash

Secondary

Wash

Dye

Wash



Feature Volumes	Value (uL)
Sample	20
Diluent	20
Buffer	150
Circuit	1.8
Piston (single) Displacement	0.18
Piston (4) displacement	0.72
GNR	0.001