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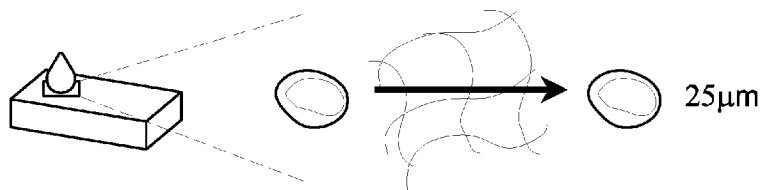


FIG. 1C

(57) Abstract: A paper-based microfluidic device includes a first layer of paper with pores having a first average pore size and a liquid-receiving area through which a liquid sample is received into the paper-based microfluidic device. A second layer of paper, with pores of a second average pore size, is stacked below the first layer of paper and has a channel with a starting end positioned at least in part in an overlapping manner with the liquid-receiving area. The channel has a terminating end extending laterally at a pre-determined wicking distance from the starting end. The first average pore size and the second average pore size cause a wicking effect in which at least some of the liquid sample flows along the channel at least a portion of the wicking distance between the start- ing end and the terminating end.



SEPARATION OF CELLS BASED ON SIZE AND AFFINITY
USING PAPER MICROFLUIDIC DEVICE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and benefit of U.S. Provisional Patent Application Serial No. 62/277,810, filed on January 12, 2016, which is hereby incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates generally to paper-based analytical and bioanalytical sensors for separation, detection, and quantification of cells from complex samples.

BACKGROUND OF THE INVENTION

[0003] Paper-based microfluidic devices have emerged as a platform that is capable of supporting the development of a number of useful analytical and bioanalytical sensors, the capabilities of which range from the detection of environmental contaminants to metabolites in blood plasma. Paper-based sensors often produce colorimetric results, which allow data to be interpreted rapidly at the point-of-use in a manner that is either qualitative (i.e., by eye) or quantitative through the use of simple readers. Utilizing paper as a substrate to develop analytical assays is beneficial because the infrastructure required to produce the analytical assays is minimal (e.g., a printer, heating element, and pipette), raw materials are inexpensive and ubiquitous (cents per sheet), and devices can be prototyped rapidly (within minutes from conception to use). By patterning paper with hydrophobic barriers, hydrophilic channels can be designed to control the wicking of fluids by capillary action. Complex, three-dimensional microfluidic networks can be fabricated from either stacking multiple layers of paper or folding a single layer of paper (i.e., origami). Simple design rules provide access to many different architectures of fluidic networks, which can facilitate the manufacture of devices that range from one-step, field-deployable diagnostic tools to sophisticated paper “machines.”

[0004] With the considerable interest in this field of research, a glaring oversight has been the lack of applications of paper-based microfluidic devices for the separation or detection of cells. This omission appears to be caused based on a perspective in which paper is viewed as a passive substrate, instead of being viewed as a component that is fundamental to the function of the microfluidic device. Consequently, paper has been applied only to the filtration of all cells from plasma or to the separation of misformed (i.e., sickled) red blood

cells, or as a scaffold for the study of cultures of mammalian cells. However, the ability to detect cells has significant value in applications related to, among others, personalized healthcare, monitoring of livestock, and determining the quality of food and water. These important capabilities are currently only available in established economies with centralized laboratories that are equipped with modern instrumentation and that include an educated workforce. Consequently, a significant percentage of the world's population – particularly those living in low-income and middle-income countries – have limited access to tools that could drastically improve the quality of life. Accordingly, paper has the potential to enable new classes of biological separations, analytical sensors, and point-of-use assays for underrepresented populations across the globe.

SUMMARY OF THE INVENTION

[0005] According to one aspect of the present disclosure, a three-dimensional (“3D”) paper-based microfluidic device is capable of supporting the development of analytical and bioanalytical sensors. The device allows for separation and/or quantification of the cells in whole human blood via size-exclusion determined by paper pore size and via affinity separation by biochemical functionalization of the paper, as described below in reference to the drawings. By way of example, the potential of the microfluidic device is directed to at least a future new low-cost platform for the identification of a critical hematological index (hematocrit), i.e., the ratio of packed red blood cell volume to total blood volume. Additional potential applications include personalized healthcare, monitoring of livestock, and determining the quality of food and water.

[0006] According to one aspect of the present disclosure, a paper-based microfluidic device, includes a first layer of paper with pores having a first average pore size, the first layer of paper having a liquid-receiving area through which a liquid sample is received into the paper-based microfluidic device. A second layer of paper is stacked below the first layer of paper, the second layer of paper having pores of a second average pore size. A channel is positioned within the second layer of paper and has a starting end positioned at least in part in an overlapping manner with the liquid-receiving area. The channel has a terminating end extending laterally at a predetermined wicking distance from the starting end. The first average pore size and the second average pore size cause a wicking effect in which at least some of the liquid sample flows along the channel at least a portion of the wicking distance between the starting end and the terminating end.

[0007] According to another aspect of the present disclosure, a method is directed to providing a paper-based microfluidic device and includes providing a first layer of paper with pores having a first average pore size, the first layer of paper having a liquid-receiving area through which a liquid sample is received into the paper-based microfluidic device. The method further includes stacking below the first layer of paper a second layer of paper having pores of a second average pore size, and positioning a channel within the second layer of paper. The channel has a starting end positioned at least in part in an overlapping manner with the liquid-receiving area of the first layer of paper, the channel having a terminating end extending laterally at a predetermined wicking distance from the starting end. The first average pore size and the second average pore size are selected such that, upon the receiving of the liquid sample, a wicking effect is caused in which at least some of the liquid sample flows along the channel at least a portion of the wicking distance between the starting end and the terminating end.

[0008] According to yet another aspect of the present disclosure, microfluidic device includes a sample-addition layer of a first porous material with pores having a first average pore size. The sample-addition layer has a liquid-receiving area through which a liquid sample is received into the microfluidic device. The microfluidic device further includes a sample-splitting layer located adjacent to the sample-addition layer, the sample-splitting layer having a first aperture for receiving a first portion of the liquid sample and a second aperture for receiving a second portion of the liquid sample. The microfluidic device also includes a separation membrane located adjacent to the first aperture of the sample-splitting layer, the separation membrane receiving only the portion of the liquid sample from the first aperture. The microfluidic device also includes a readout layer of a second porous material located adjacent the separation membrane, the second porous material having pores with a second average pore size that is different than the first average pore size. The readout layer has a first channel configured to receive in a starting end the first portion of the liquid sample from the sample-splitting layer, via the separation membrane. The first channel has a terminating end extending laterally at a predetermined wicking distance from the starting end, the first portion of the liquid sample flowing at least in part along the wicking distance to indicate a first value of the liquid sample. The readout layer further has a second channel positioned adjacent to the first channel for receiving the second portion of the liquid sample, the second channel including a stored reagent that reacts with the second portion of the liquid sample to indicate a second value of the liquid sample.

[0009] Additional aspects of the present invention will be apparent to those of ordinary skill in the art in view of the detailed description of various embodiments, which is made with reference to the drawings, a brief description of which is provided below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] FIG. 1A is an illustration showing selection of paper pore sizes for preventing migration of cells.

[0011] FIG. 1B is an illustration showing a broad range of cell sizes.

[0012] FIG. 1C is an illustration showing selection of paper pore sizes for permitting migration of cells.

[0013] FIG. 2A is an illustration of a disassembled 2-layer microfluidic device.

[0014] FIG. 2B is an illustration of the microfluidic device of FIG. 2A shown in assembled form and indicating different hematocrits.

[0015] FIG. 3A is an illustration showing the effect of paper pore size on the performance of a plurality of paper-based hematocrit assays.

[0016] FIG. 3B is an illustration showing the effect of a threshold pore size on the performance of a paper-based hematocrit assay.

[0017] FIG. 4A is an illustration showing separation of large granulocytes from small lymphocytes.

[0018] FIG. 4B is an illustration showing separation of somatic cells from bacteria.

[0019] FIG. 5 is an illustrating showing separation of cells in paper based on partitioning, with erythrocytes (but not leukocytes) binding to paper that has been grafted with dextran.

[0020] FIG. 6A is an illustration showing an integrated paper-based microfluidic device for separating multiple biological species from a single sample.

[0021] FIG. 6B is an illustration showing another integrated paper-based microfluidic device having multiple adhesive areas per layer for separating multiple biological species.

[0022] FIG. 7 is a graphical representation of various hematocrit percentages for samples of whole blood using microfluidic devices treated with sodium chloride and ethylenediaminetetraacetic acid (EDTA).

[0023] FIG. 8 is a graphical representation of various hematocrit percentages for samples of whole blood using microfluidic devices treated with EDTA on two different sections of the microfluidic devices.

[0024] FIG. 9 is a graphical representation of various hematocrit percentages for samples of isolated red blood cells in Alsever's solution.

[0025] FIG. 10 is a graphical representation of various hematocrit percentages for samples of whole blood using microfluidic devices treated with sodium chloride with and without EDTA.

[0026] FIG. 11 is a graphical representation comparing differences in performance between isolated red blood cells (RBCs) and whole blood.

[0027] FIG. 12 is a graphical representation illustrating a comparison of performance of isolated RBCs on microfluidic devices that are treated and are not treated with sodium chloride.

[0028] FIG. 13A is a perspective view illustration of a disassembled multi-layer microfluidic device.

[0029] FIG. 13B is top view illustration of the microfluidic device of FIG. 13A shown in assembled form.

[0030] FIG. 13C is back view illustration of the microfluidic device of FIG. 13A shown in assembled form.

[0031] FIG. 14A illustrates the microfluidic device of FIG. 13A with a first visual readout of a hematocrit ("Hct") and hemoglobin ("Hb") amounts.

[0032] FIG. 14B illustrates the microfluidic device of FIG. 13A with a second visual readout of the Hct and Hb amounts.

[0033] FIG. 14C illustrates the microfluidic device of FIG. 13A with a third visual readout of the Hct and Hb amounts.

[0034] While the invention is susceptible to various modifications and alternative forms, specific embodiments have been shown by way of example in the drawings and will be described in detail herein. It should be understood, however, that the inventions are not intended to be limited to the particular forms disclosed. Rather, the aspects disclosed herein cover all modifications, equivalents, and alternatives falling within the spirit and scope of the invention as defined by the appended claims.

DETAILED DESCRIPTION

[0035] While aspects of the present disclosure are susceptible of embodiment in many different forms, there is shown in the drawings and will herein be described in detail some embodiments with the understanding that the present disclosure is to be considered as an exemplification of the principles of the embodiments and is not intended to limit the broad aspect of the inventions to the embodiments illustrated. For purposes of the present detailed description, the singular includes the plural and vice versa (unless specifically disclaimed);

the words “and” and “or” shall be both conjunctive and disjunctive; the word “all” means “any and all”; the word “any” means “any and all”; and the word “including” means “including without limitation.”

[0036] Paper-based microfluidic devices are useful in the separation and quantification of cells in human blood based on two principles: (i) size-exclusion, in which a paper is selected with an ideal pore size to permit or restrict the flow of cells through a device; and (ii) affinity separation, in which a paper is biochemically functionalized to capture a specific cell of interest based on its expression of surface markers. The microfluidic devices described below in reference to the drawings are based on these two principles and bring cell-counting capabilities directly into hands of users at a point-of-need, with the potential to revolutionize diagnostics in a manner similar to, for example, the introduction of the home pregnancy test.

[0037] The microfluidic devices include paper-based analytical and bioanalytical sensors with features that focus at least in part on separation, detection, and quantification of cells from complex samples. The manufacturing of the microfluidic devices provides simple, yet functional, devices from layers of paper and tape, for example. In other examples, instead of or in addition to paper, microfluidic device include any porous materials configured or selected with the proper pore size. The microfluidic devices (i) are designed to allow the incorporation of a number of fluidic operations into paper-based analytical sensors, and (ii) are capable of controlling (i.e., permitting or impeding) the wicking of cells based on the pore sizes of the papers and through modifications of the chemical properties of the paper fibers through simple chemical functionalization reactions. A further benefit of the microfluidic devices is the ability to manufacture paper-based analytical sensors reproducibly and in large volumes, e.g., hundreds of millions of devices per year per test. Additional benefits of the microfluidic devices include (i) environmental monitoring of toxic or contaminating species, (ii) separations of complex mixtures of biological and non-biological matter, (iii) the study of interactions between microbes and hosts, and (iv) the development of diagnostic assays that are designed specifically for use in the developing world and limited-resource settings.

[0038] Referring to FIGs. 1A-1C, the use of cells in paper for bioanalytical assays has thus far been limited to those applications where cells are either impregnated into paper (e.g., to study the effects of nutrient gradients on the growth of cells and models for tumor formation) or filtered by the paper (e.g., for the detection of sickled red blood cells or to perform blood-typing assays). In both examples, paper serves as a medium for the broad exclusion or filtration of cells. One factor that has limited the development of paper-based assays for the detection of cells is the choice of material. As illustrated in FIG. 1A, a

substrates selected – typically, Whatman chromatography paper Grade no. 1 – do not wick mammalian cells due to their small average pore size. The use of materials with small pore sizes in paper-based devices is historical, rather than an absolute requirement. Hundreds of different grades of paper are available commercially and provide different combinations of pore sizes (ca. 0.2–200 μm), pore size distributions, thicknesses, surface areas, and overall chemical composition (e.g., cellulose-based or polymer-based). Cells also span a similarly large range of sizes, as illustrated in FIG. 1B. It follows, as illustrated in FIG. 1C, that a grade of paper could be selected that permits the migration of cells through a paper microfluidic device based on size. The broad range of available pore sizes facilitates the use of stacks of paper as size-exclusion media that selectively separates and isolates cells.

[0039] Referring to FIGs. 2A and 2B, a number of papers are characterized for their ability to permit or restrict the wicking of erythrocytes (i.e., red blood cells). Focusing on erythrocytes as a model system offers three important benefits: (i) they are available as stabilized and relatively purified suspensions in buffer; (ii) they are available in whole blood; and (iii) the ability to quantify their concentration provides a critical hematological index, the hematocrit. The hematocrit is the volume fraction of packed erythrocytes in whole blood – the ratio of packed red blood cell (RBC) volume to the total blood volume. Aberrant hematocrit values are related to diseases that are common in the developing world (e.g., anemia and dehydration). By way of example, in a healthy adult, the hematocrit is typically in the range of about 36%-48% for women and 42%-52% for men. Deviations from these ranges are typically indicative of diseases, such as anemia, which is indicated in lower percentages in patients, and dehydration, which is indicated at higher percentages in patients. Currently, measurements of the hematocrit require either a hematology analyzer or a centrifuge. Neither instrument is readily available in limited-resource settings, but their analytical capabilities are greatly needed.

[0040] For example, as illustrated in FIG. 2A, the properties of paper are used to measure the hematocrit using a distance X erythrocytes wicked laterally within a paper-based microfluidic device 100. The microfluidic device 100 includes a first (top) layer of paper 102 with a liquid-receiving area 104 through which a liquid sample 106 is received into the microfluidic device 100. The first layer of paper 102 has a first average pore size that functions as a filter and, for example, restricts flow of white cells.

[0041] The microfluidic device 100 further includes a second (bottom) layer of paper 108 that is stacked below the first layer of paper 102. The second layer of paper 108 has pores of a second average pore size and includes a channel 110 with a thickness T . The channel 110

has a starting end 112 positioned at least in part in an overlapping manner with the liquid-receiving area 104 of the first layer of paper 102. The channel 110 has a terminating end 114 that extends laterally at a predetermined wicking distance XT from the starting end 112. The dimensions of the channel 110, including the wicking distance XT and the channel thickness, are selected based on one or more paper characteristics, such as average paper pore size, paper pore size distribution, the paper porosity, paper bubble point, and/or paper flow rate. According to one example, each layer 102, 108 is a cellulose-based paper, such as Ahlstrom chromatography paper. Optionally, the second layer of paper 108 is pretreated with ethylenediaminetetraacetic acid (EDTA) and/or sodium chloride (NaCl) to promote lateral flow of RBCs through the paper matrix formed by the microfluidic device 100.

[0042] As illustrated in FIG. 2B, the first average pore size of the first layer of paper 102 and the second average pore size of the second layer of paper 108 cause a wicking effect in which at least some of the liquid sample 106 flows along the channel 110 at least a portion of the wicking distance X between the starting end 112 and the terminating end 114. For example, the liquid sample 106 travels a first wicking distance X1 if the hematocrit percentage is approximately 55%, a second wicking distance X2 if the hematocrit percentage is approximately 60%, a third wicking distance X3 if the hematocrit percentage is approximately 45%, and a fourth wicking distance X4 if the hematocrit percentage is approximately 35%. Accordingly, the average pore size of the layers of paper 102, 108 is selected such that the liquid sample 106 fills the channel 110 a shorter wicking distance X if (a) the liquid sample 106 has a higher concentration of the cells of interest (e.g., RBCs) than if (b) the liquid sample 106 has a lower concentration of the cells of interest.

[0043] The channel 110 has a thicker initial area, which extends approximately the same distance as the second wicking distance X2, and a narrower secondary area that extends to the end of the total wicking distance XT. The thicker initial area is much thicker than the general thickness T of the channel 110 in the secondary area (as illustrated in FIG. 2A). The thicker initial area permits the wicking of higher hematocrits (e.g., >60%) laterally by having a wider (or thicker) initial channel area. The initial wide area provides sufficient area for a high concentration of cells to migrate through the channel 110 with minimal aggregation, while not wicking beyond the initial wide area. However, the thinner secondary area of thickness T is provided to obtain resolved wicking distances for lower hematocrits (e.g., 30%-55%). According to one example, the initial wide area is approximately 5 millimeters thick by 10 millimeters long, and the thinner secondary area of thickness T is approximately 2 millimeters thick by 40 millimeters long.

[0044] The hematocrit percentage is optionally indicated via one or more flow indicators 116 that indicate certain percentage marks (e.g., 60, 50, 40, and 30), in a thermometer-styled readout. According to the embodiment of FIGs. 2A and 2B, the flow indicators 116 are positioned on the second layer of paper 108. However, in other embodiments, the flow indicators 116 are positioned on the first layer of paper 102, or are positioned on both layers of paper 102, 108. Additionally, in other embodiments the flow indicators 116 are configured to indicate any cell concentration of a liquid sample (not just hematocrit percentages).

[0045] The layers of paper 102, 108 are stacked in direct contact with each other or are stacked in close proximity with each other. For example, the second layer of paper 108 is optionally separated from the first layer of paper 102 by an air gap to facilitate ease of wicking flow of the liquid sample 106. In another example, an intermediate layer is interposed between the layers of paper 102, 108 to facilitate the attachment of the layers of paper 102, 108 to each other. For example, the intermediate layer is a removable adhesive that facilitates the temporary affixing of the layers 102, 108 for ease of assembly and/or disassembly of the microfluidic device 100.

[0046] This exemplary approach relies on the obstruction of pores within the plane of the paper, which occurs in proportion to the concentration of erythrocytes that is applied to the device. That is, the pores within a layer of paper are easier to obstruct with relatively large numbers of erythrocytes than with a lower concentration of cells. As a result of a bottlenecking effect, high hematocrits stop migrating sooner than low hematocrits, and the inherent red color of the erythrocytes provides a label-free indication of signal transduction. The wicking distances X measured in paper-based devices correlate to hematocrits, which are optionally measured using standard techniques, and the wicking distance X that RBCs wick laterally is proportional to the hematocrit as measured in the flow channel 110. According to one test, the sample test duration (or incubation period) is approximately 30 minutes.

[0047] In accordance with the illustrated embodiment, the first layer of paper 102 extends only near the liquid-receiving area 104. However, in other embodiments, the first layer of paper 102 extends away from the liquid-receiving area 104. For example, in one alternative embodiment, the first layer of paper 102 is similar or identical in length to the second layer of paper 108.

[0048] In accordance with another alternative embodiment, the microfluidic device 100 includes a laminate layer 120 affixed to a top surface of the channel 110 to prevent or minimize flow of liquid away from the area near the channel 110, such as flow towards the

first layer of paper 102. Additionally or alternatively, a laminate layer 122 is affixed to a bottom surface of the second layer of paper 108.

[0049] Referring to FIG. 3A, the effect of paper pore size on the performance of a paper-based hematocrit assay illustrates that small pore sizes restrict the transport of RBCs within a device, while larger pore sizes allow the RBC to migrate freely. Specifically, and by way of example only, a first microfluidic device 200 with a first average pore size of about 6 micrometers (μm) restrict the flow of RBCs to a first wicking distance Y1, a second microfluidic device 202 with a second average pore size of about 10 micrometers (μm) restrict the flow of RBCs to a second wicking distance Y2, a third microfluidic device 204 with a third average pore size of about 15 micrometers (μm) restrict the flow of RBCs to a third wicking distance Y3, and a fourth microfluidic device 206 with a fourth average pore size of about 25 micrometers (μm) restrict the flow of RBCs to a fourth wicking distance Y4. As the average pore size increases, so does the respective wicking distance.

[0050] Referring to FIG. 3B, in an alternative embodiment and by way of a further example, the third average pore size of about 15 micrometers (μm) is a threshold pore size that designates a maximum limit beyond which the performance level remains approximately the same for the same hematocrit assay. Accordingly, in this alternative embodiment the performance remains the same for both the third average pore size of about 15 micrometers (μm) and for the fourth average pore size of about 25 micrometers (μm). In other words, the third and fourth wicking distances Y3 and Y4 are approximately the same.

[0051] Referring to FIG. 4A, separations of cells from complex media suggest the ability to fractionate populations of leukocytes contained in the buffy coat of blood into its different components (e.g., neutrophils and lymphocytes). The capability to, for example, separate neutrophils and lymphocytes provides an important hematological index related to leukocyte counts that is useful in assessing susceptibility to infections or undesirable side-effects caused by drug treatments. Thus, based on a size-exclusion feature in which differences in cell size is a determinative factor, a microfluidic device 300 has paper layers with pore sizes selected to separate large granulocytes 302 from small lymphocytes 304. The microfluidic device 300 prevents the large granulocytes 302 from passing through the microfluidic device 300, while the small lymphocytes 304 are permitted to pass through the microfluidic device 300.

[0052] Referring to FIG. 4B, the use of microfluidic paper-based devices is also expected to address the important topic of livestock health, which is related to food quality, by performing analytical assessments of the quality of milk from cows and goats. In such separations – related to spoilage or mastitis – the background components of milk (i.e.,

colloids of casein and milk fat globules) are highly heterogeneous and present a unique matrix from which contaminating cells (i.e., somatic cells and bacteria) are separated. For example, a microfluidic device 310 has paper layers with pore sizes to separate somatic cells 312 from bacteria 314.

[0053] Referring to FIG. 5, a microfluidic device 400 include a first layer of paper 402, with a polymer material 403 grafted onto the paper, and a second layer of paper 404. Erythrocytes 406 – the major component of whole blood – are expected to present a significant source of interference for separations in which whole blood is the sample. If fractionation based on size is insufficient, additional and/or alternatives approaches are used to improve the quality of the separations. For example, in reference to differences in adhesive properties that exist between surfaces of erythrocytes 406 and leukocytes 408, an inexpensive polymer of glucose 403 (e.g., dextran) is to cause the aggregation of erythrocytes. Erythrocytes 406 – and not leukocytes 408 – partition preferentially into dextran-rich aqueous phases. Accordingly, based on this feature, the dextran material 403 that is grafted onto the first layer of paper 402 as a vehicle via which contaminating erythrocytes 406 are specifically removed from paper-based separation media.

[0054] The polymer material 403 can be any adhesive material that causes cells of a first type (e.g., erythrocytes 406) from a liquid sample to bind to the first layer of paper 402. Additionally, the average pore size of the first layer of paper 402 causes cells of a second type (e.g., leukocytes 408) to flow through a first liquid-receiving area 410 of the microfluidic device to a second liquid-receiving area 412. The second liquid-receiving area 412 is positioned at least in part in an overlapping manner with the first liquid-receiving area 410. The average pore size of the second layer of paper 404 causes the cells of the second type to wick through the second layer of paper 404.

[0055] In another exemplary approach, simple centrifugation methods (e.g., an egg-beater centrifuge) are used to stratify cells by density prior to introducing the fraction containing the least dense cells – rich in leukocytes – into paper-based devices. In yet another exemplary approach, a reagent (e.g., saponin) causes the selective hemolysis of erythrocytes. This approach likely requires devices to undergo rigorous washing to remove contaminants (e.g., hemoglobin) that are released by lysis.

[0056] Referring to FIG. 6A, a comprehensive fractionation of cells from whole blood is achieved with a 3D microfluidic device 500 having multiple layers of stacked paper. The 3D microfluidic device 500 separates multiple biological species from a single liquid sample 502 based on the several layers of paper 504-508, with each layer being a different grade of paper

with different pore sizes. Specifically, a first layer of paper 504 has an average pore size of about 20 micrometers (μm), a second layer of paper 505 has an average pore size of about 15 micrometers (μm), a third layer of paper 506 has an average pore size of about 6 micrometers (μm), a fourth layer of paper 507 has an average pore size of about 0.2 micrometers (μm), and a fifth layer of paper 508 includes a blot that does not include any cells, except potentially very small bacteria.

[0057] The microfluidic device 500 is assembled and the liquid sample 502 is added to a liquid-receiving area 510. The layers of paper 504-508 are stacked in an overlapping manner with respect to each other. Then, the microfluidic device 500 is disassembled and the layers of paper 504-508 are isolated to examine the separated cells. After disassembly, and by way of example, the first layer of paper 504 has retained only large white blood cells 512, the second layer of paper 505 has retained only small white blood cells 513, the third layer of paper 506 has retained only red blood cells 514, the fourth layer of paper 507 has retained only bacteria 515, and the fifth layer of paper 508 has retained plasma 516. In other words, each layer of paper 504-508 has an average pore size that is smaller than an average diameter of the respective cells of interest from the liquid sample 502. For example, the first layer of paper 504 has an average pore size – of about 20 micrometers (μm) – that is smaller than an average diameter of the large white blood cells 512.

[0058] Specific cell types and purified plasma is separated and stored within the layers of the 3D microfluidic device, which enables downstream and/or off-site analysis of samples of blood. In addition to using these separations in limited-resource settings, similar paper-based 3D microfluidic devices also enable: (i) purification of viruses from cell culture supernatants, and (ii) subsequent culture of microorganisms that have been separated using paper-based devices.

[0059] According to the illustrated embodiment, the average pore size decreases from the top layer 504 to the bottom layer 508. Based on specific applications, in accordance with other embodiments, the average pore size is the same in at least two of the plurality of layers 504-508 and/or the average pore size increases from the top layer 504 to the bottom layer 508.

[0060] Referring to FIG. 6B, a 3D microfluidic device 600 has multiple layers of stacked papers for separating multiple biological species from a single liquid sample 602, based on several layers of paper 604-608. A blot 610 collects any remaining cells and/or fragments from the sample 602. The microfluidic device 600 is generally similar to the microfluidic

device 500 illustrated in FIG 6A, except that one or more of the paper layers 604-608 include a plurality of adhesive areas treated with the same or different adhesive materials.

[0061] By way of example, one or more of the paper layers 604-608 is treated with a cocktail of antibodies to separate and detect cells from the sample 602. For example, a second paper layer 605 is treated with a cocktail of antibodies including anti-CD71 and anti-CD47, and a third paper layer 606 is treated with a single type of antibodies – anti-CD34. The second and third paper layers 605, 606 are treated in a single area (similar to the layers of the microfluidic device 500). In contrast, each of a fourth layer 607 and a fifth layer 608 is treated with antibodies in multiple areas 1-4. For example, the fourth layer 607 is treated with anti-CD15 in area 1, anti-CD177 in area 2, anti-CD193 in area 3, and anti-siglec8 in area 4. The fifth layer 608 is treated with anti-CD3 in area 1, anti-CD14 in area 2, anti-CD16 in area 3, and anti-CD19 in area 4.

[0062] According to one example, in response to adding the sample 602 to the assembled layers of the microfluidic device 600, cells are separated and detected as follows. Reticulocytes and erythrocytes are separated and detected in the second layer 605, rare stem cells are recovered in the third layer 606, granulocytes are recovered in area 1 of the fourth layer 607, neutrophils are recovered in area 2 of the fourth layer 607, basophils are recovered in area 3 of the fourth layer 607, eosinophils are recovered in area 4 of the fourth layer 607, T lymphocytes are recovered in area 1 of the fifth layer 608, monocytes are recovered in area 2 of the fifth layer 608, NK cells are recovered in area 3 of the fifth layer 608, B lymphocytes are recovered in area 4 of the fifth layer 608, and other cells and/or fragments are recovered in the blot 610.

[0063] Referring to FIG. 7, the graphical representation shows various hematocrit percentages for samples of whole blood using microfluidic devices treated with sodium chloride and ethylenediaminetetraacetic acid (EDTA). The solid line is a linear fit of the data series ($R^2 = 0.985$). Each data point is the average of five replicates and the error bars are standard error of the mean.

[0064] Referring to FIG. 8, the graphical representation shows various hematocrit percentages for samples of whole blood using microfluidic devices treated with EDTA on two different sections of the microfluidic devices (i.e., top layer or bottom layer). The solid line is the linear fit of the data series. Each data point is the average of three replicates and the error bars are standard error of the mean.

[0065] Referring to FIG. 9, the graphical representation shows various hematocrit percentages for samples of isolated red blood cells in Alsever's solution. The solid line is a

linear fit of the data series ($R^2 = 0.987$). Each data point is the average of five replicates and the error bars are standard error of the mean.

[0066] Referring to FIG. 10, the graphical representation shows various hematocrit percentages for samples of whole blood using microfluidic devices treated with sodium chloride with and without EDTA. The EDTA was treated on a lateral channel of the microfluidic device. Each data point for the EDTA-treated microfluidic-device data series is the average of three replicates and the error bars are standard error of the mean. Each data point for the data series of the microfluidic devices without EDTA treatment is the average of five replicates and the error bars are standard error of the mean.

[0067] Referring to FIG. 11, the graphical representation compares differences in performance between isolated RBCs and whole blood. Every data point for each data series is the average of five replicates and the error bars are standard error of the mean.

[0068] Referring to FIG. 12, the graphical representation shows a comparison of performance of isolated RBCs on microfluidic devices that are treated and that are not treated with sodium chloride. Every data point for each data series is the average of five replicates and the error bars are standard error of the mean.

[0069] Referring generally to FIGs. 13A-13C, an alternative embodiment illustrates a microfluidic device 700 for simultaneously determining both a hematocrit amount and a hemoglobin amount from a liquid (e.g., blood) sample 706. Independently, hematocrit and hemoglobin measurements provide clinicians with important information about a patient's health status. The microfluidic device 700 is beneficial at least because these two values can be provided simultaneously at the bedside or at a point-of-care. The assay implemented by the microfluidic device 700 facilitates the use of paper in multi-step, autonomous analyses of blood. Accordingly, the microfluidic device 700 provides one example of simultaneous detection of multiple hematological indices and other soluble markers of health (e.g., creatinine and glucose) using inexpensive and disposable paper-based devices.

[0070] Referring specifically to FIG. 13A, the microfluidic device 700 includes a sample-addition layer 702 with a liquid-receiving area 704 through which the blood sample 706 is received into the microfluidic device 700. The sample-addition layer 702 is made from a porous material (e.g., paper) having a selected average pore size as described above in reference, for example, to the first layer of paper 102.

[0071] The microfluidic device 700 further includes a readout layer 708 that is stacked below, but not adjacent to, the sample-addition layer 702. The readout layer 708 is made from a porous material (e.g., paper) having a selected average pore size, for example, as

described above in reference to the second layer of paper 108. The readout layer 708 includes a first channel 710 with a thickness T that has a starting end 712 positioned at least in part in an overlapping manner with the liquid-receiving area 704. The first channel 710 further has a terminating end 714 that extends laterally at a predetermined wicking distance XT from the starting end 712 in an X direction.

[0072] The readout layer 708 further has a second channel 711 that is adjacent to the first channel 710 and that is intended to receive a different type of cells from the liquid sample 706 than the type of cells received in the first channel 710. For example, the microfluidic device 700 is configured such that the first channel 710 indicates a hematocrit percentage in the liquid sample 706 and the second channel 711 indicates the hemoglobin level in the liquid sample 706. Although the second channel 711 is illustrated in FIG. 13A as a generally circular area, its shape and size can vary based on specific requirements. For example, according to an alternative embodiment, the second channel 711 is similar or identical in size and/or shape to the first channel 710.

[0073] The microfluidic device 700 further includes a sample-splitting layer 705 that is located between the sample-addition layer 702 and the readout layer 708. The sample-splitting layer 705 includes a first aperture area 705A through which at least some of the liquid sample 706 flows towards the first channel 710, and a second aperture area 705B through which at least some of the liquid sample 706 flows towards the second channel 711. According to an alternative embodiment, the aperture areas 705A, 705B are configured in the form of channels.

[0074] The microfluidic device 700 also includes a plasma separation membrane 707 that is located between the sample-splitting layer 705 and the readout layer 708. The membrane 707, according to this example, is configured with a size and shape that extends only between the second aperture area 705B of the sample-splitting layer 705 (which is above) and the second channel 711 of the readout layer 708 (which is below). In other words, the membrane 707 does not extend or act as a barrier between the first aperture area 705A of the sample-splitting layer 705 and the first channel 710 of the readout layer 708.

[0075] As such, according to one specific example, the membrane 707 filters cell debris from plasma remaining in the second aperture area 705B, to allow hemoglobin to be detected by a colorimetric reaction. Optionally, the pores of each of the sample-addition layer 702, the readout layer 708, the sample-splitting layer 705, and/or the membrane 707 are selected in accordance with the description provided above in reference to one or more of FIGs. 1A-12

(e.g., to cause a wicking effect through one or more of the first channel 710, the second channel 711, the first aperture 705A, and/or the second aperture 705B).

[0076] Referring specifically to FIGs. 13B and 13C, the microfluidic device 700 indicates a visual measurement based on the liquid sample 706. For example, a blood sample 706 is added to the microfluidic device 700 in the liquid-receiving area 704 where the blood sample 706 is allowed to saturate the sample-addition layer 702. The received blood sample 706 is split uniformly into the two aperture areas 705A, 705B. In the second aperture area 705B, a reagent (e.g., saponin) is stored to lyse all red blood cells. The plasma separation membrane 707 filters cell debris from the remaining plasma to allow hemoglobin to be detected by a colorimetric reaction (e.g., Drabkin's reagent stored on the microfluidic device 700).

[0077] The color formed by the colorimetric reaction is proportional to an amount of hemoglobin ("Hb"). For example, the reaction proceeds from a red color (indicative of low Hb) to a blue color (indicative of high Hb). As such, the reaction provides a visual readout of the level of Hb for a user.

[0078] The first aperture area 705A of the sample-splitting layer 705 is saturated with a fixed volume of blood, which allows the hematocrit ("Hct") assay to proceed in the first channel 710 of the readout layer 708. As previously discussed, the Hct assay requires the addition of a known volume of blood.

[0079] Referring to FIGs. 14A-14C, experimental data demonstrates that the amount detected Hb correlates with the measured hematocrit Hct. Specifically, samples of blood with red blood cells ("RBC") containing more cells at a higher Hct value have more hemoglobin Hb. For example, FIG. 14A shows that a relatively high 60% Hct value (as indicated by the traveled distance X1 in the first channel 710) correlates to a relatively high Hb amount (as indicated by a "blue" color in the second channel 711). FIG. 14B shows that a relatively medium 45% Hct value (as indicated by the traveled distance X2 in the first channel 710) correlates to a relatively medium Hb amount (as indicated by a changed color in the second channel 711). FIG. 14C shows that a relatively low 20% Hct value (as indicated by the traveled distance X3 in the first channel 710) correlates to a relatively low Hb amount (as indicated by a "red" color in the second channel 711).

[0080] According to alternative features of the microfluidic device described above, other markers for a multiplexed blood assay include one or more analytes of a blood metabolite panel. For example, the analytes include at least one of glucose, total protein, alkaline phosphatase, creatinine, and blood urea nitrogen (BUN).

[0081] Each of these embodiments and obvious variations thereof is contemplated as falling within the spirit and scope of the claimed invention, which is set forth in the following claims. Moreover, the present concepts expressly include any and all combinations and subcombinations of the preceding elements and aspects.

CLAIMS

What is claimed is:

1. A paper-based microfluidic device, comprising:
 - a first layer of paper with pores having a first average pore size, the first layer of paper having a liquid-receiving area through which a liquid sample is received into the paper-based microfluidic device;
 - a second layer of paper stacked below the first layer of paper, the second layer of paper having pores of a second average pore size; and
 - a channel positioned within the second layer of paper and having a starting end positioned at least in part in an overlapping manner with the liquid-receiving area, the channel having a terminating end extending laterally at a predetermined wicking distance from the starting end, the first average pore size and the second average pore size causing a wicking effect in which at least some of the liquid sample flows along the channel at least a portion of the wicking distance between the starting end and the terminating end.
2. The paper-based microfluidic device of claim 1, further including flow indicators indicative of the wicking distance traveled by the liquid sample along the channel.
3. The paper-based microfluidic device of claim 2, wherein the flow indicators are on the first layer of paper or on the second layer of paper.
4. The paper-based microfluidic device of claim 2, wherein the flow indicators are indicative of a cell concentration of the liquid sample.
5. The paper-based microfluidic device of claim 1, wherein the second layer of paper is stacked in contact with the first layer of paper.
6. The paper-based microfluidic device of claim 1, wherein the second layer of paper is stacked in close proximity to the first layer of paper, the second layer of paper being separated from the first layer of paper by an air gap.
7. The paper-based microfluidic device of claim 1, wherein the first average pore size and the second average pore size are selected such that the liquid sample fills the channel a

shorter wicking distance if (a) the liquid sample has a higher concentration of the cells of interest than if (b) the liquid sample has a lower concentration of the cells of interest.

8. The paper-based microfluidic device of claim 1, wherein both the first average pore size and the second average pore size are the same size.
9. The paper-based microfluidic device of claim 1, wherein the first average pore size is smaller than the second average pore size.
10. The paper-based microfluidic device of claim 1, wherein the first average pore size is larger than the second average pore size.
11. The paper-based microfluidic device of claim 1, wherein the first layer of paper extends only near the liquid-receiving area.
12. The paper-based microfluidic device of claim 1, wherein the first layer of paper and the second layer of paper are removably affixed to each other.
13. The paper-based microfluidic device of claim 1, wherein the first layer of paper and the second layer of paper are temporarily affixed to each other for a sample test duration.
14. The paper-based microfluidic device of claim 13, wherein a sample test duration is approximately 30 minutes.
15. The paper-based microfluidic device of claim 1, wherein dimensions of the channel, including the wicking distance and a channel thickness, are selected based on one or more paper characteristics including average paper pore size, paper pore size distribution, paper porosity, paper bubble point, and paper flow rate.
16. The paper-based microfluidic device of claim 1, wherein the second average pore size is smaller than an average diameter of cells of interest from the liquid sample.
17. The paper-based microfluidic device of claim 1, further comprising one or more additional layers of paper, including a third layer of paper, the third layer of paper being

stacked at least in part in an overlapping manner with and below the second layer of paper, the third layer of paper having pores of a third average pore size, the first average pore size being larger than the second average pore size, the second average pore size being larger than the third average pore size.

18. The paper-based microfluidic device of claim 1, further comprising a laminate layer affixed to a top surface of the channel.

19. The paper-based microfluidic device of claim 1, further comprising a laminate layer affixed to a bottom surface of the second layer of paper.

20. The paper-based microfluidic device of claim 1, wherein at least one of the first layer of paper and the second layer of paper is a cellulose-based paper.

21. A method for providing a paper-based microfluidic device, the method comprising:
providing a first layer of paper with pores having a first average pore size, the first layer of paper having a liquid-receiving area through which a liquid sample is received into the paper-based microfluidic device;

stacking below the first layer of paper a second layer of paper having pores of a second average pore size; and

positioning a channel within the second layer of paper, the channel having a starting end positioned at least in part in an overlapping manner with the liquid-receiving area of the first layer of paper, the channel having a terminating end extending laterally at a predetermined wicking distance from the starting end;

wherein, the first average pore size and the second average pore size are selected such that, upon the receiving of the liquid sample, a wicking effect is caused in which at least some of the liquid sample flows along the channel at least a portion of the wicking distance between the starting end and the terminating end.

22. The method of claim 21, further comprising providing flow indicators indicative of the wicking distance traveled by the liquid sample along the channel.

23. The method of claim 22, further comprising stacking the second layer of paper in contact with or in close proximity to the first layer of paper.

24. The method of claim 21, further comprising selecting the first average pore size and the second average pore size such that the liquid sample fills the channel a shorter wicking distance if (a) the liquid sample has a higher concentration of the cells of interest than if (b) the liquid sample has a lower concentration of the cells of interest.

25. The method of claim 21, further comprising affixing a laminate layer to at least one of a top surface of the channel or a bottom surface of the second layer of paper.

26. A paper-based microfluidic device with multiple layers of paper for separation of cells based on pore size, the microfluidic device comprising:

a first layer of paper with pores having a first average pore size and including a first liquid-receiving area through which a liquid sample is received into the microfluidic device, the first average pore size causing cells of a first size from the liquid sample to wick through the first layer of paper; and

a second layer of paper stacked below the first layer of paper and having pores of a second average pore size that is smaller than the first average pore size, the second layer of paper including a second liquid-receiving area that is positioned at least in part in an overlapping manner with the first liquid-receiving area and through which a remaining portion of the liquid sample is received, the second average pore size causing cells of a second size from the liquid sample to wick through the second layer of paper, the cells of the second size being smaller than the cells of the first size.

27. A paper-based microfluidic device with multiple layers of paper for separation of cells based on cell affinity, the microfluidic device comprising:

a first layer of paper with pores having a first average pore size and including a first liquid-receiving area through which a liquid sample is received into the microfluidic device, the first layer of paper including one or more areas having at least one adhesive material that causes cells of a first type from the liquid sample to bind to the first layer of paper, the first average pore size causing cells of a second type from the liquid sample to flow through the first liquid-receiving area; and

a second layer of paper stacked below the first layer of paper and having pores of a second average pore size, the second layer of paper including a second liquid-receiving area that is positioned at least in part in an overlapping manner with the first liquid-receiving area

and through which the cells of the second type are received from the first liquid-receiving area, the second average pore size causing the cells of the second type from the liquid sample to wick through the second layer of paper.

28. A microfluidic device, comprising:

- a sample-addition layer of a first porous material with pores having a first average pore size, the sample-addition layer having a liquid-receiving area through which a liquid sample is received into the microfluidic device;

- a sample-splitting layer located adjacent to the sample-addition layer, the sample-splitting layer having a first aperture for receiving a first portion of the liquid sample and a second aperture for receiving a second portion of the liquid sample;

- a separation membrane located adjacent to the second aperture of the sample-splitting layer, the separation membrane receiving only the second portion of the liquid sample from the second aperture; and

- a readout layer of a second porous material located adjacent the separation membrane, the second porous material having pores with a second average pore size that is different than the first average pore size, the readout layer having

- a first channel configured to receive in a starting end the first portion of the liquid sample from the sample-splitting layer, via the separation membrane, the first channel having a terminating end extending laterally at a predetermined wicking distance from the starting end, the first portion of the liquid sample flowing at least in part along the wicking distance to indicate a first value of the liquid sample, and

- a second channel being positioned adjacent to the first channel for receiving the second portion of the liquid sample, the second channel including a stored reagent that reacts with the second portion of the liquid sample to indicate a second value of the liquid sample.

29. The microfluidic device of claim 28, wherein the liquid sample is blood, the first value being indicative of a hematocrit percentage in the blood, the second value being indicative of a hemoglobin value in the blood.

30. The microfluidic device of claim 28, wherein the first portion and the second portion of the liquid sample are split uniformly between the first aperture and the second aperture of the sample-splitting layer.
31. The microfluidic device of claim 28, wherein the separation membrane is a third porous material with pores having a third average pore size that filters cell debris from the liquid sample.
32. The microfluidic device of claim 28, wherein at least one of the first porous material and the second porous material is a paper-based material.
33. The microfluidic device of claim 28, wherein the first value and the second value of the liquid sample are indicated simultaneously.

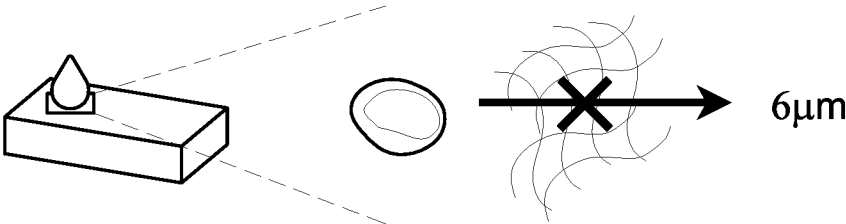


FIG. 1A

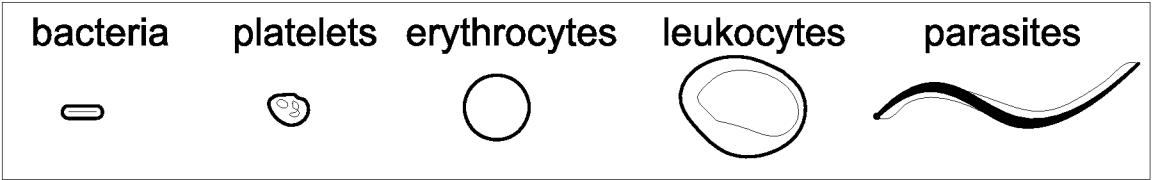


FIG. 1B

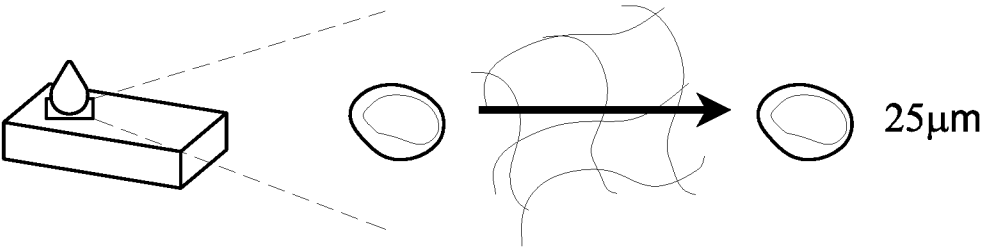
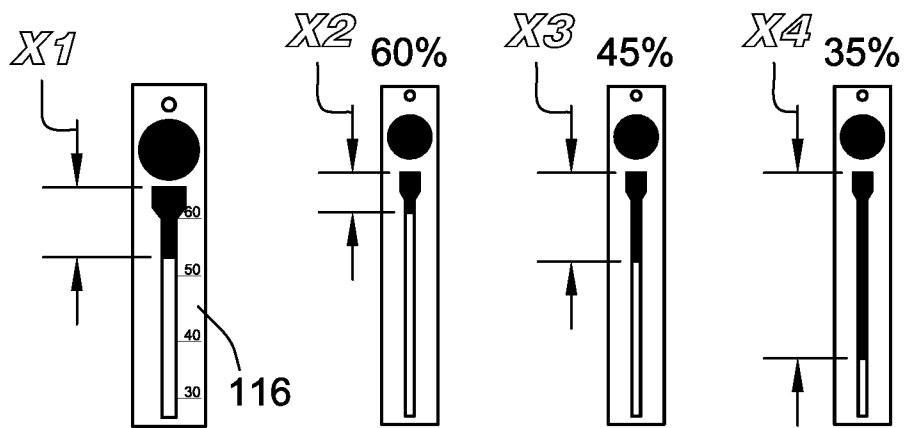
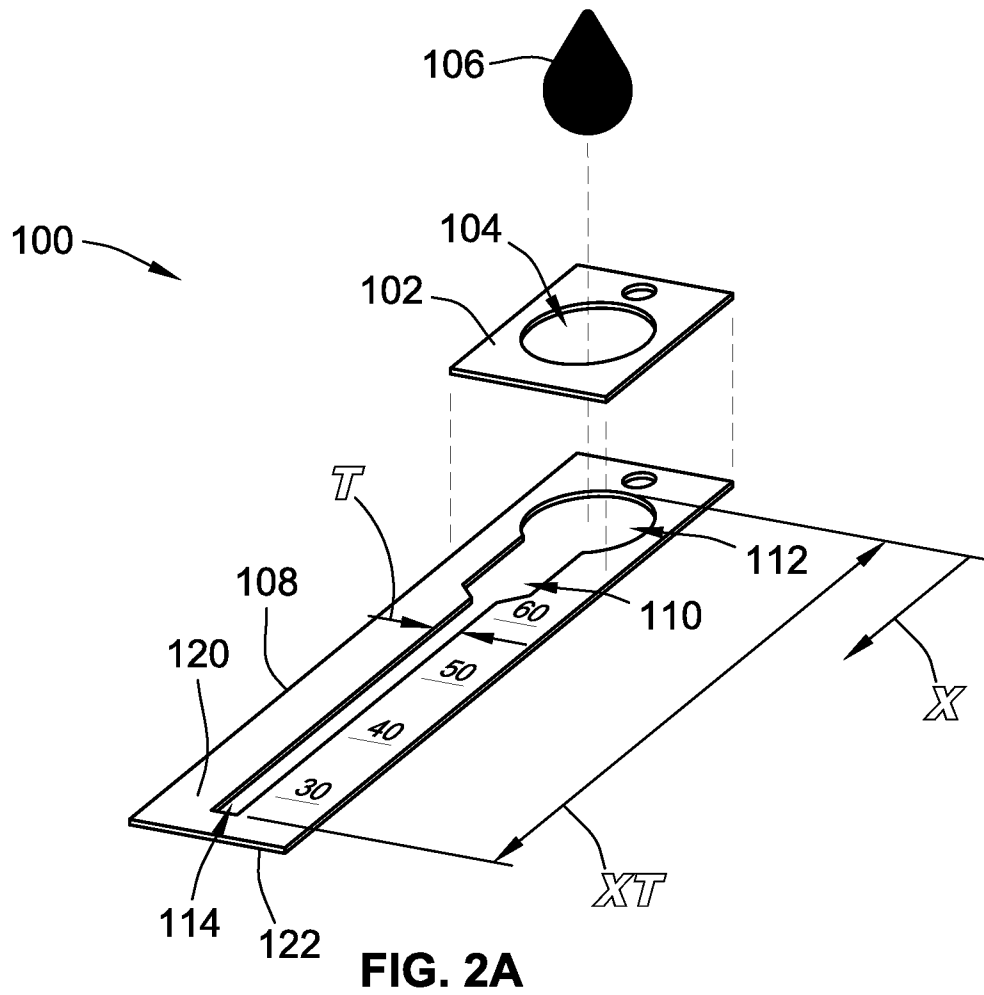
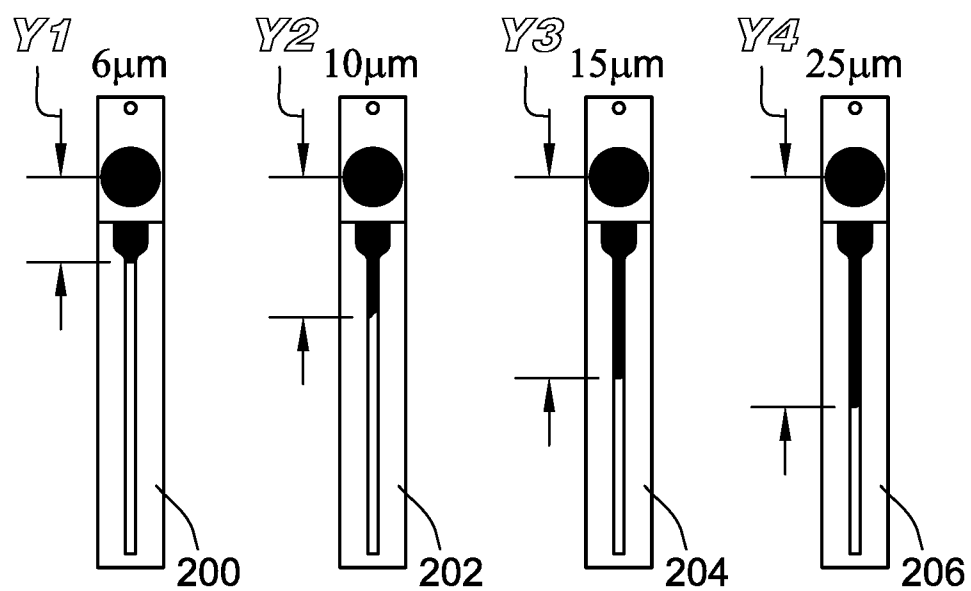
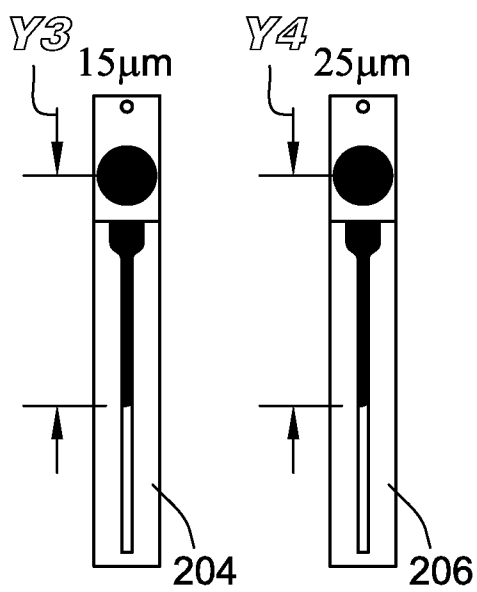


FIG. 1C



**FIG. 3A****FIG. 3B**

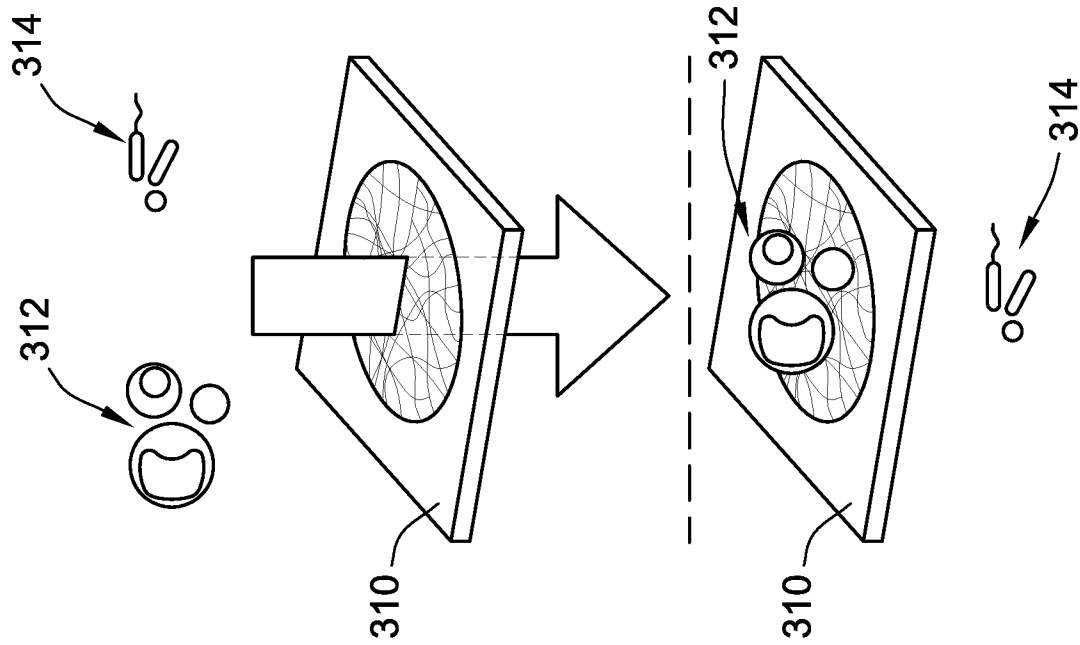


FIG. 4B

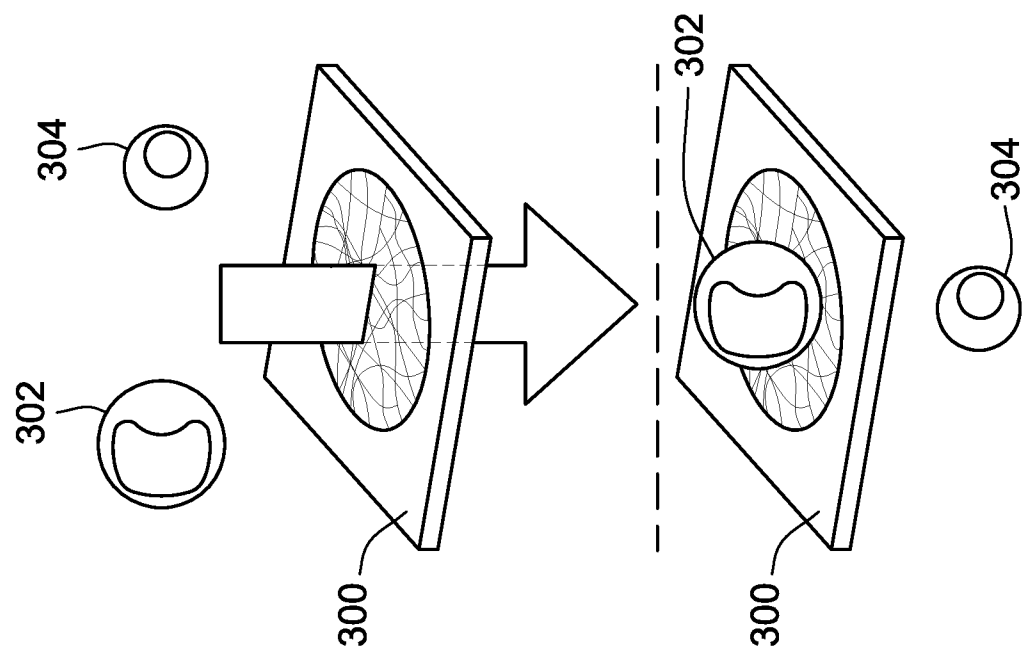


FIG. 4A

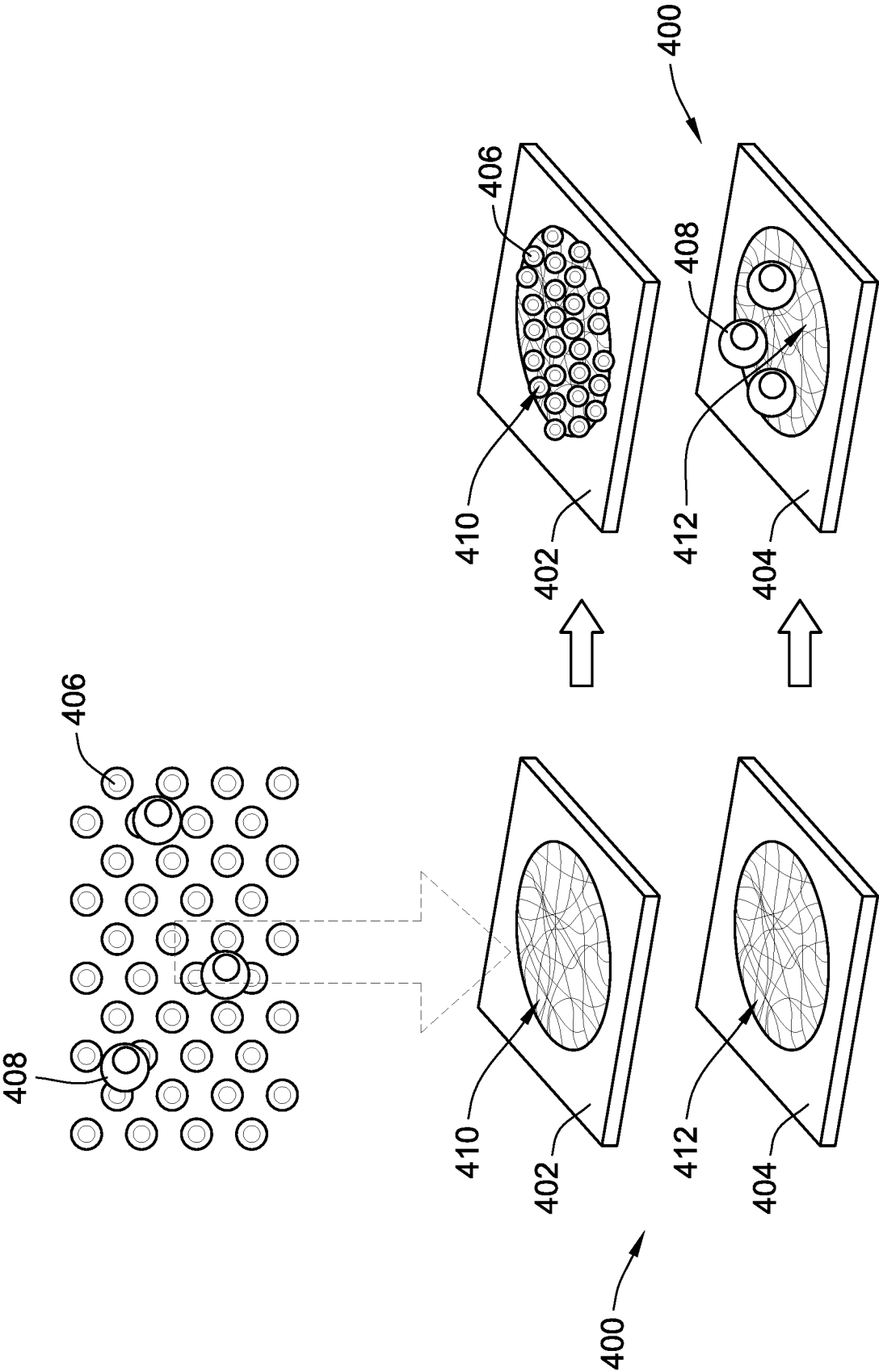


FIG. 5

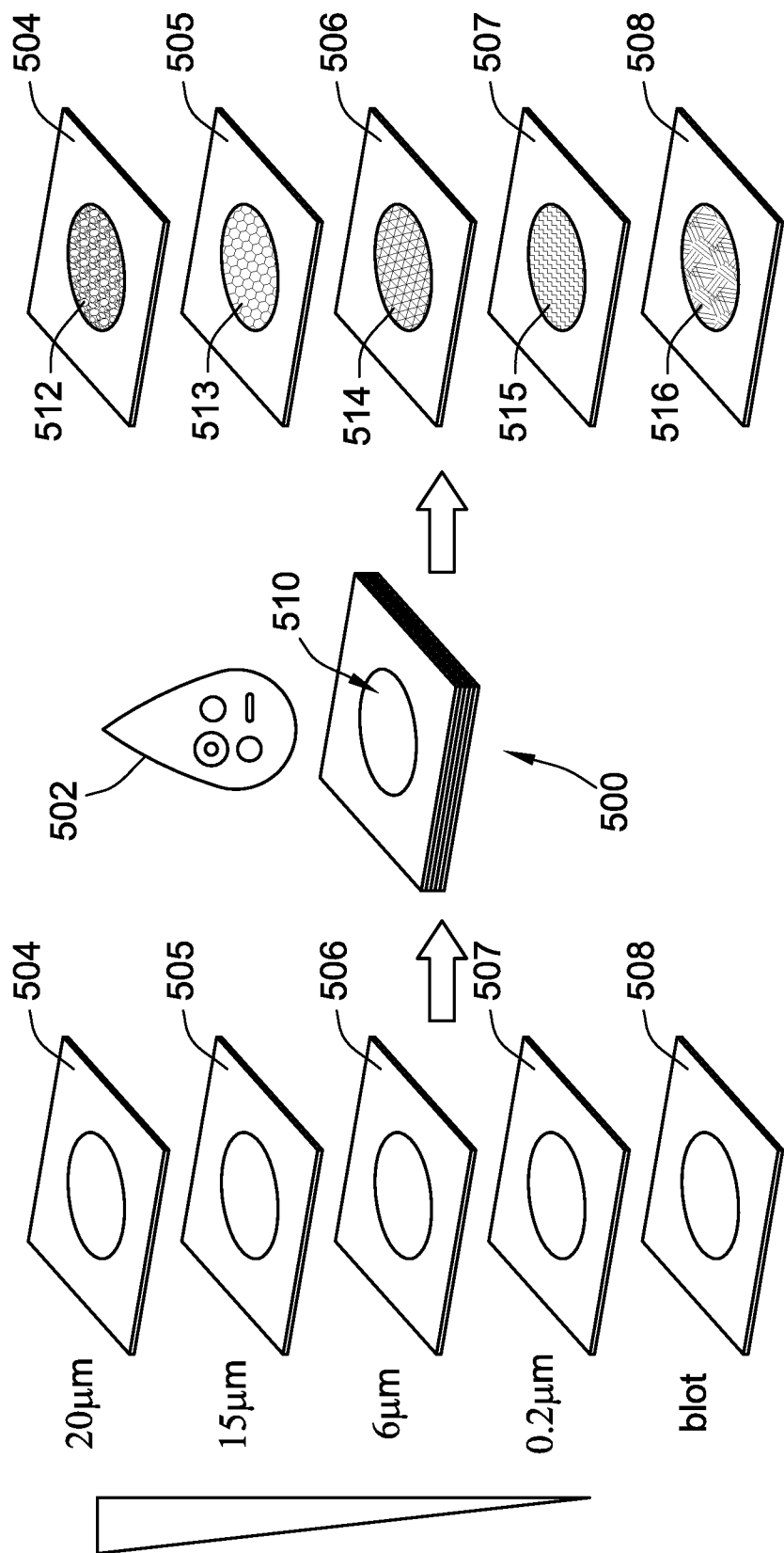
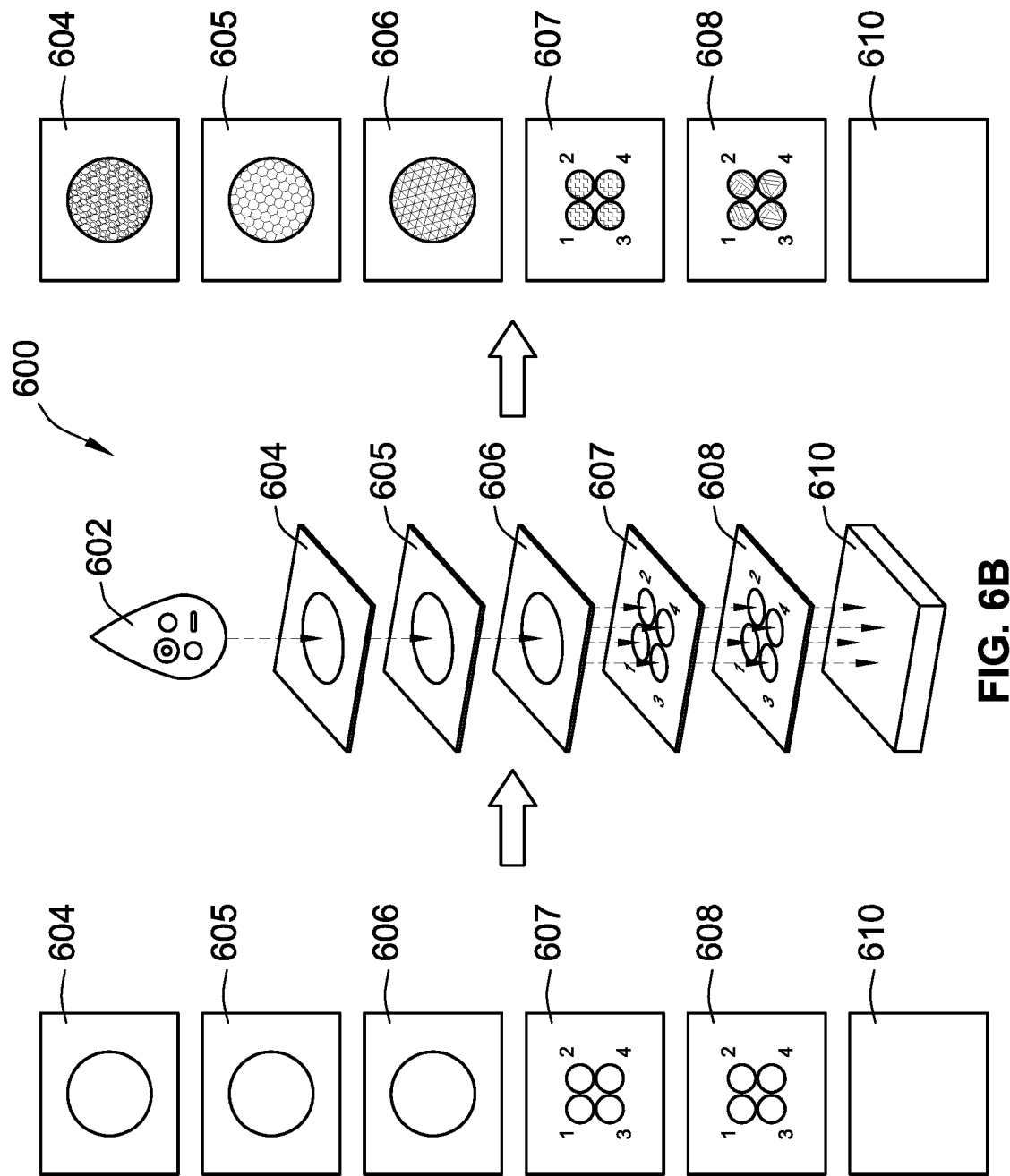
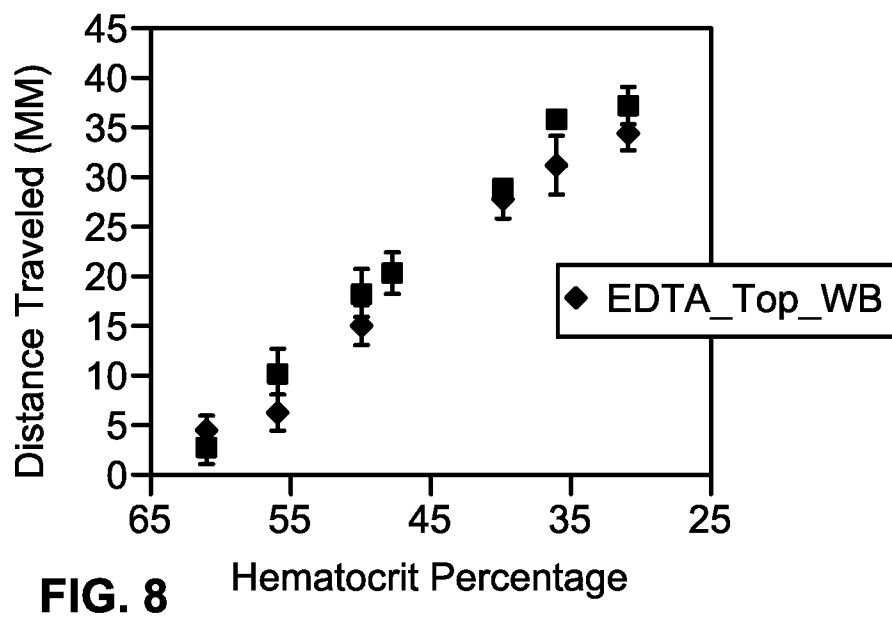
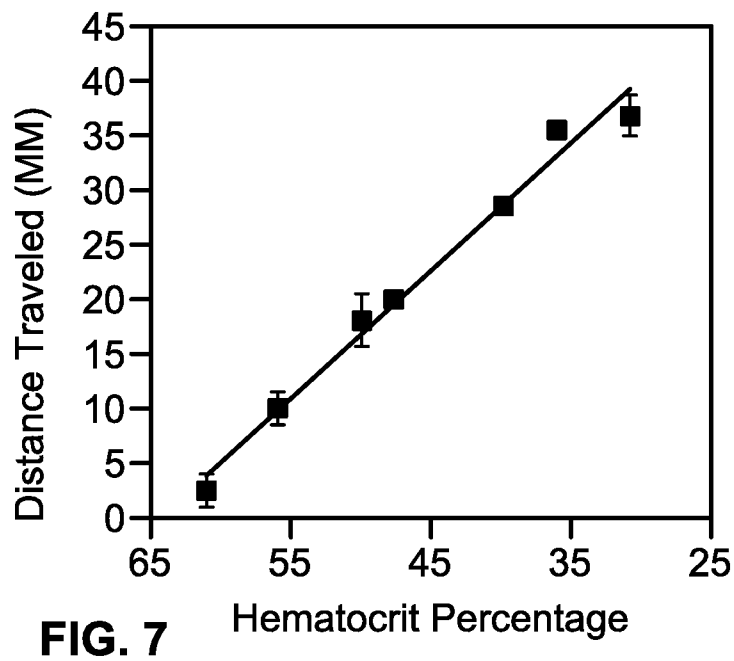
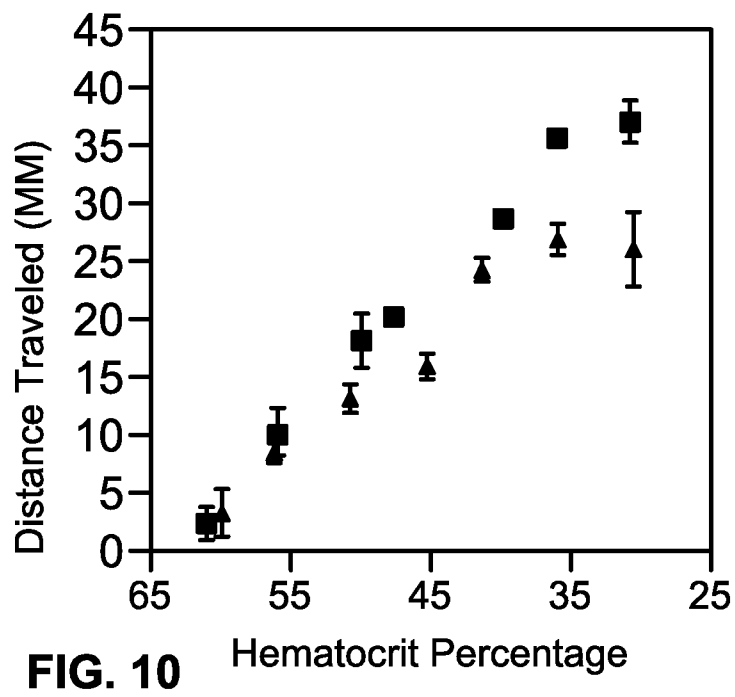
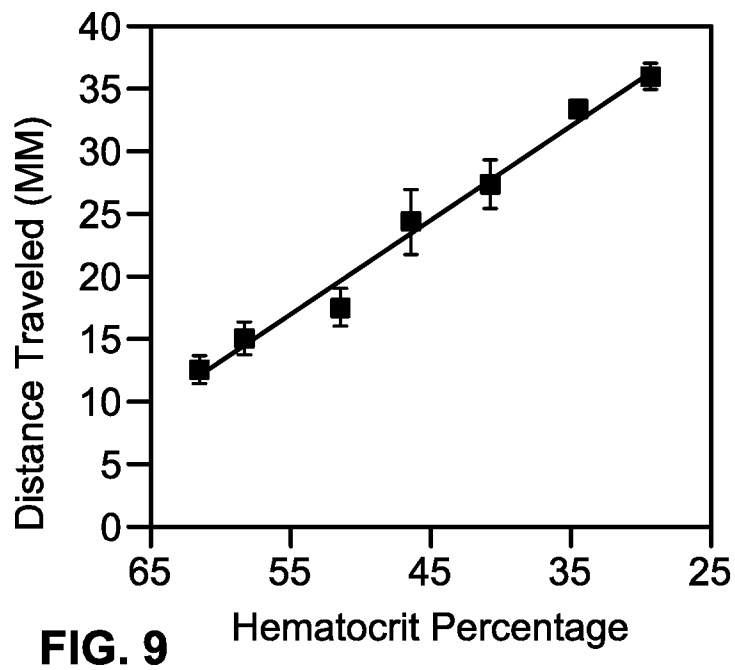


FIG. 6A







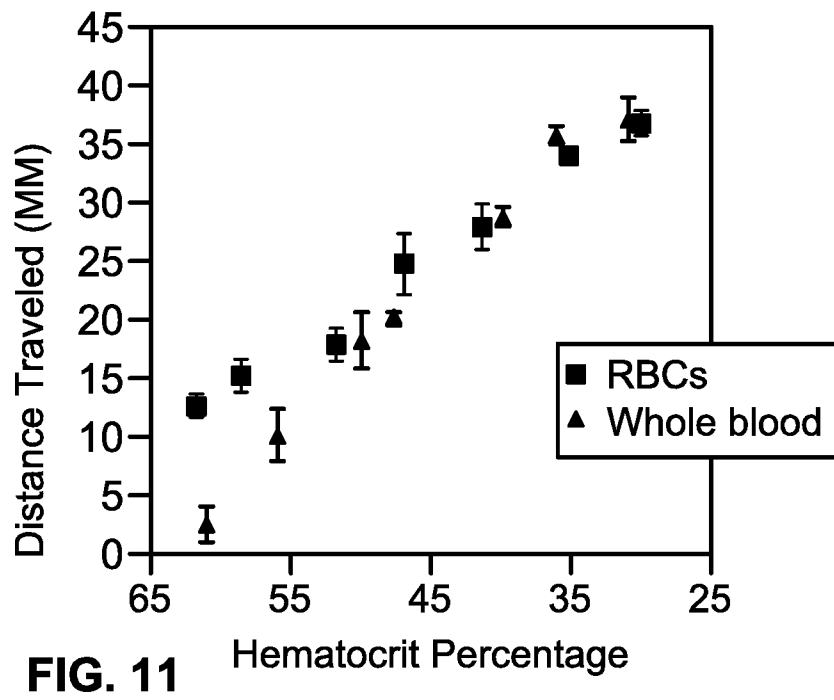


FIG. 11 Hematocrit Percentage

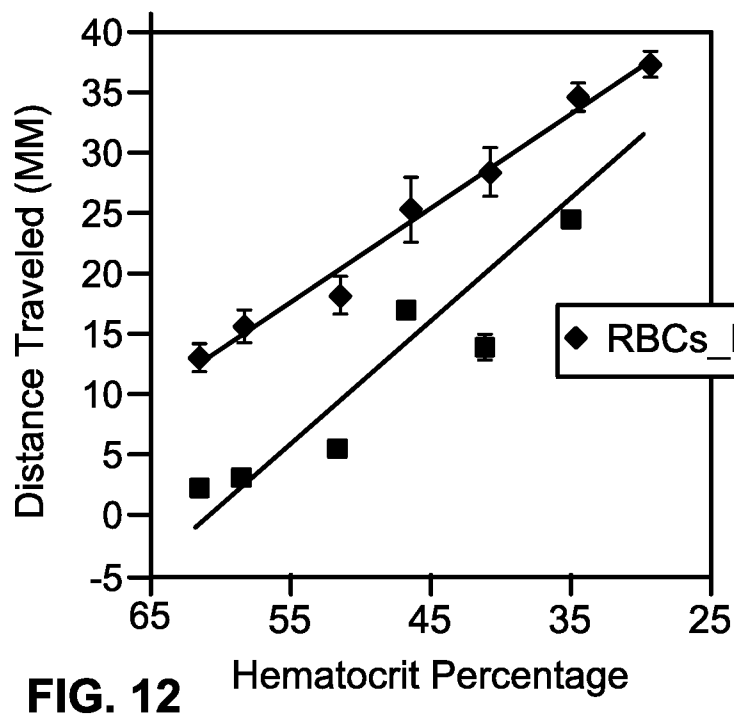
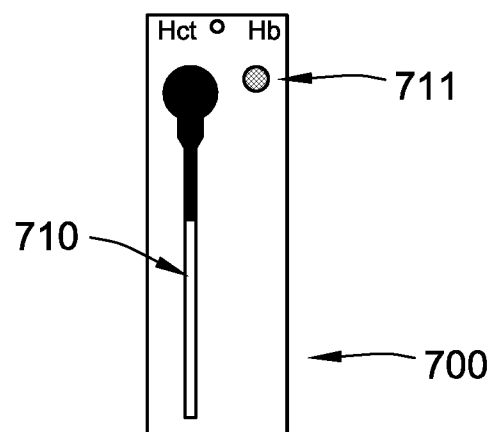
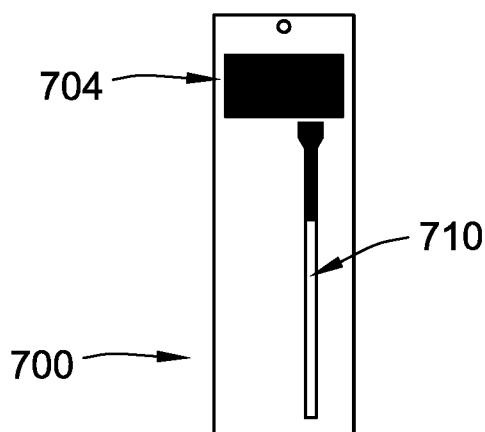
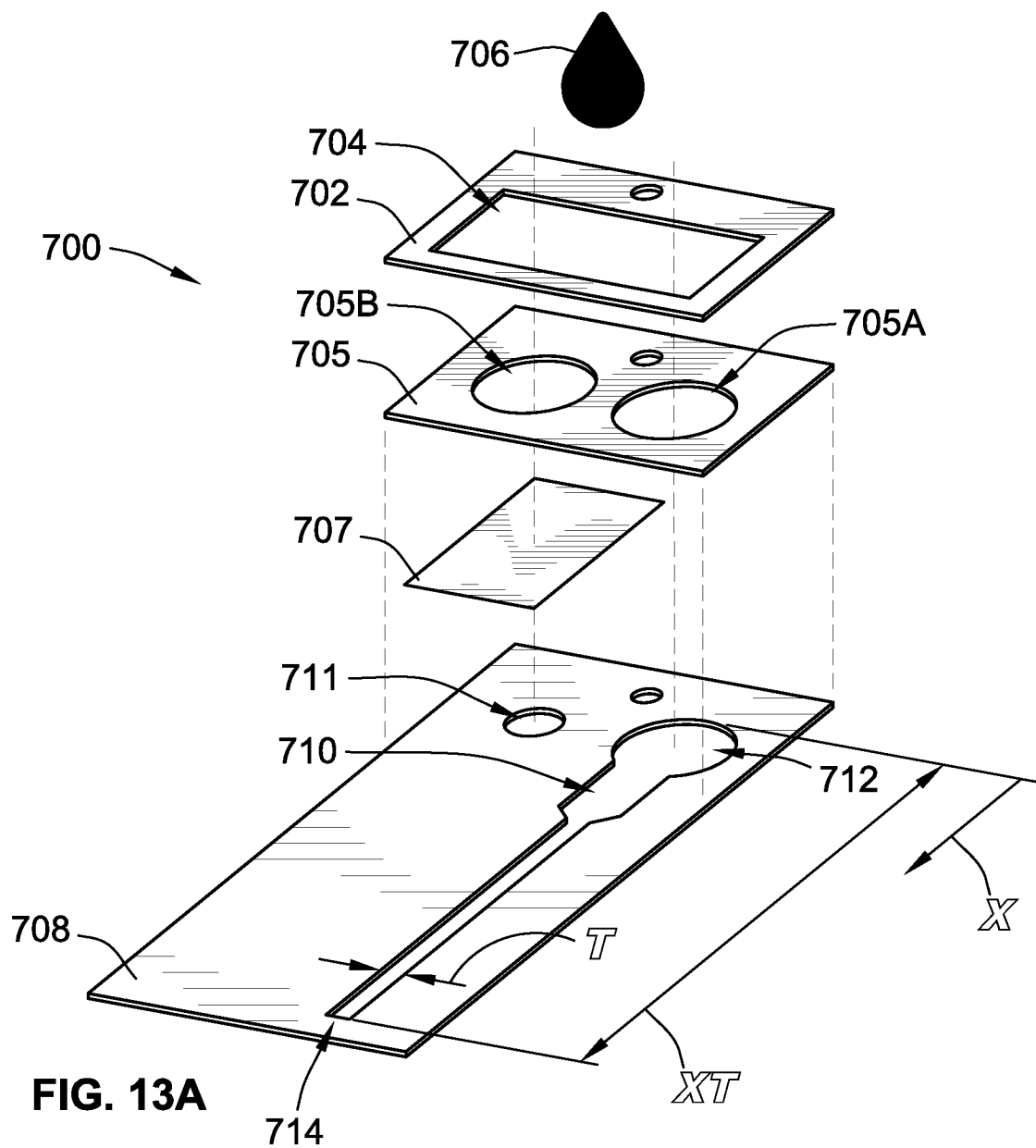
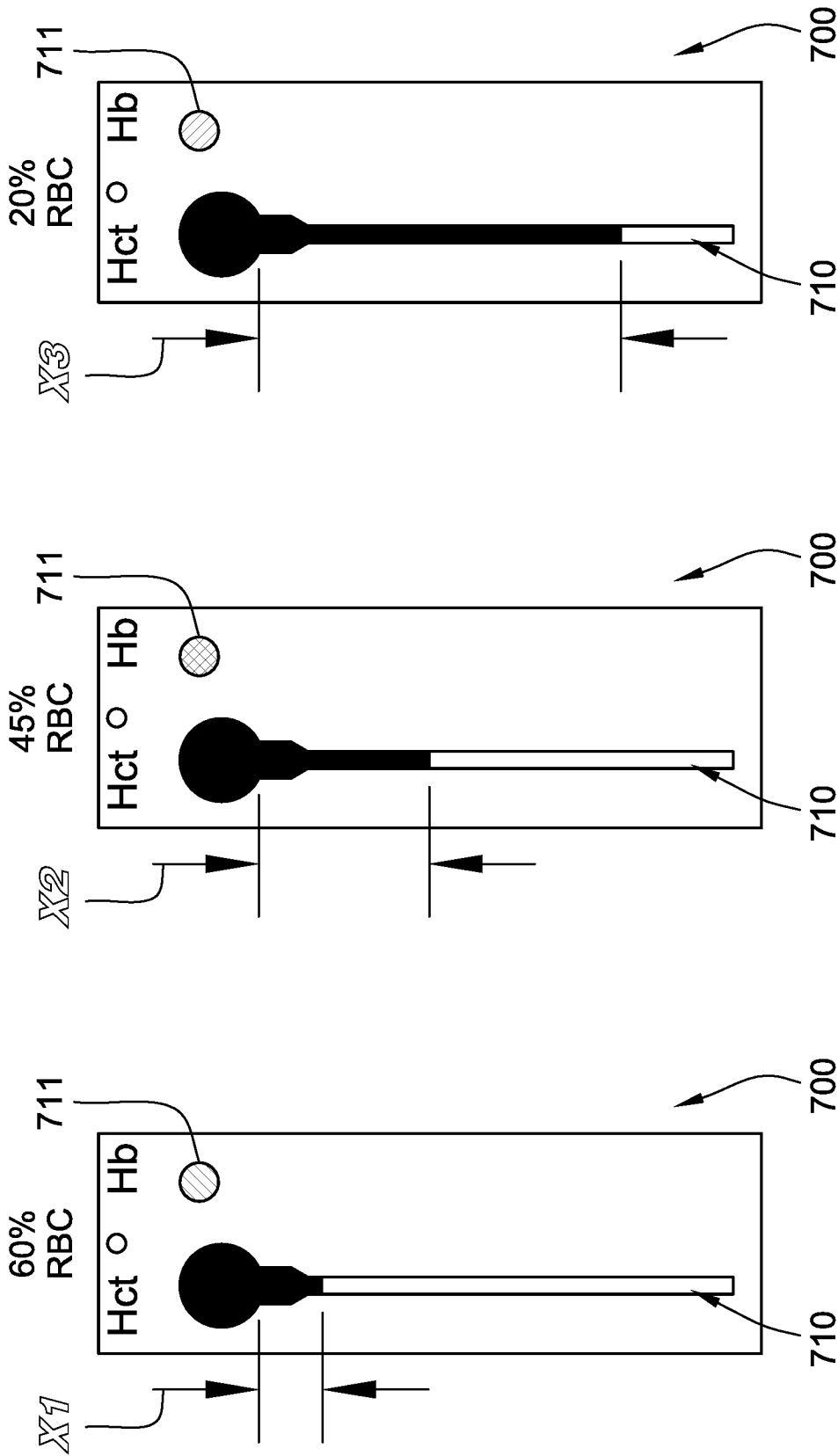


FIG. 12 Hematocrit Percentage





INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/13065

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 21/75, G01N 31/22, G01N 33/52 (2017.01)

CPC - A61M 1/34, A61M 1/341, A61M 1/3417, B01L 3/502753, B01L 3/502761

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2013/0267005 A1 (KARTALOV et al) 10 October 2013 (10.10.2013) fig 1, para [0032], [0037]-[0038], [0043], [0051], [0053], [0059]	1-10, 15-17, 21-24, 26
Y		11-14, 18-20, 25
Y	US 2015/0266023 A1 (FUCHIWAKI et al) 24 September 2015 (24.09.2015) fig 1, para [0073], [0148]	11, 19, 25
Y	US 2013/0331666 A1 (MILLER) 12 December 2013 (12.12.2013) para [0046]	12-14
Y	US 2014/0065647 A1 (CATE et al) 26 June 2014 (26.06.2014) fig 1D, 4, para [0027]	18
Y	US 2012/0322086 A1 (GARNIER et al) 20 December 2012 (20.12.2012) para [0019]	20
A	US 2010/00267065 A1 (GEIGER et al) 21 October 2010 (21.10.2010) entire document	1-26
A	US 2008/0038759 A1 (KEREN et al) 14 February 2008 (14.02.2008) entire document	1-26
A	US 2011/0111517 A1 (SIEGEL et al) 12 May 2011 (12.05.2011) entire document	1-26
A	US 5,087,556 A (ERTINGHAUSEN) 11 February 1992 (11.02.1992) entire document	1-26

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

27 April 2017

Date of mailing of the international search report

18 MAY 2017

Name and mailing address of the ISA/US

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Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/13065

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-26, directed to a paper-based microfluidic device and methods.

Group II: Claim 27, directed to a paper-based microfluidic device with multiple layers of paper for separation of cells based on cell affinity.

Group III: Claims 28-33, directed to a microfluidic device

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1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-26

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Continuation of Box III: Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

SPECIAL TECHNICAL FEATURES

The inventions of Group I-III each recite a special technical feature above and beyond the shared technical feature and device. In particular:

The special technical feature of Group I is a channel positioned within the second layer of paper and having a starting end positioned at least in part in an overlapping manner with the liquid-receiving area, the channel having a terminating end extending laterally at a predetermined wicking distance from the starting end, the first average pore size and the second average pore size causing a wicking effect in which at least some of the liquid sample flows along the channel at least a portion of the wicking distance between the starting end and the terminating end.; not required by the inventions of Groups II-III.

The special technical feature of Group II is a the first layer of paper including one or more areas having at least one adhesive material that causes cells of a first type from the liquid sample to bind to the first layer of paper, not required by the inventions of Groups I and III.

The special technical feature of Group III is a sample-splitting layer located adjacent to the sample-addition layer, the sample-splitting layer having a first aperture for receiving a first portion of the liquid sample and a second aperture for receiving a second portion of the liquid sample; and a separation membrane located adjacent to the second aperture of the sample-splitting layer, the separation membrane receiving only the second portion of the liquid sample from the second aperture, not required by the inventions of Groups I and II.

COMMON TECHNICAL FEATURES

Groups I-III share the technical features of a paper-based microfluidic device, comprising:

a first layer of paper with pores having a first average pore size, the first layer of paper having a liquid-receiving area through which a liquid sample is received into the paper-based microfluidic device;

a second layer of paper stacked below the first layer of paper, the second layer of paper having pores of a second average pore size. However, these shared technical features fail to make a contribution over the prior art of US 2013/0267005 A1 to Kartalov, et al., which teaches a paper-based microfluidic device (abstract, fig 1, 3, para [0059] describes chip as being made of paper), comprising:

a first layer (60, fig 1) of paper (para [0059] describes chip as made of paper) with pores having a first average pore size (fig 1, para [0038], [0053]), the first layer of paper having a liquid-receiving area (50) through which a liquid sample is received into the paper-based microfluidic device (fig 1, para [0037]);

a second layer (70, fig 1) of paper (para [0059] describes chip as made of paper) stacked below the first layer of paper (para [0032]), the second layer of paper having pores of a second average pore size (fig 1, para [0038]).

As the common features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Groups I-III lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.