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(54) **METHODS FOR QUALITATIVE AND QUANTITATIVE ANALYSIS OF CELLS AND RELATED OPTICAL BIO-DISC SYSTEMS**

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ation No. 60/386,073, filed on Oct. 19, 2001. Provisional application No. 60/386,071, filed on Oct. 26, 2001. Provisional application No. 60/344,977, filed on Nov. 7, 2001. Provisional application No. 60/338,679, filed on Nov. 13, 2001. Provisional application No. 60/334,131, filed on Nov. 30, 2001. Provisional application No. 60/355,644, filed on Feb. 5, 2002. Provisional application No. 60/358,479, filed on Feb. 19, 2002.

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(52) **U.S. Cl.** **435/7.2**

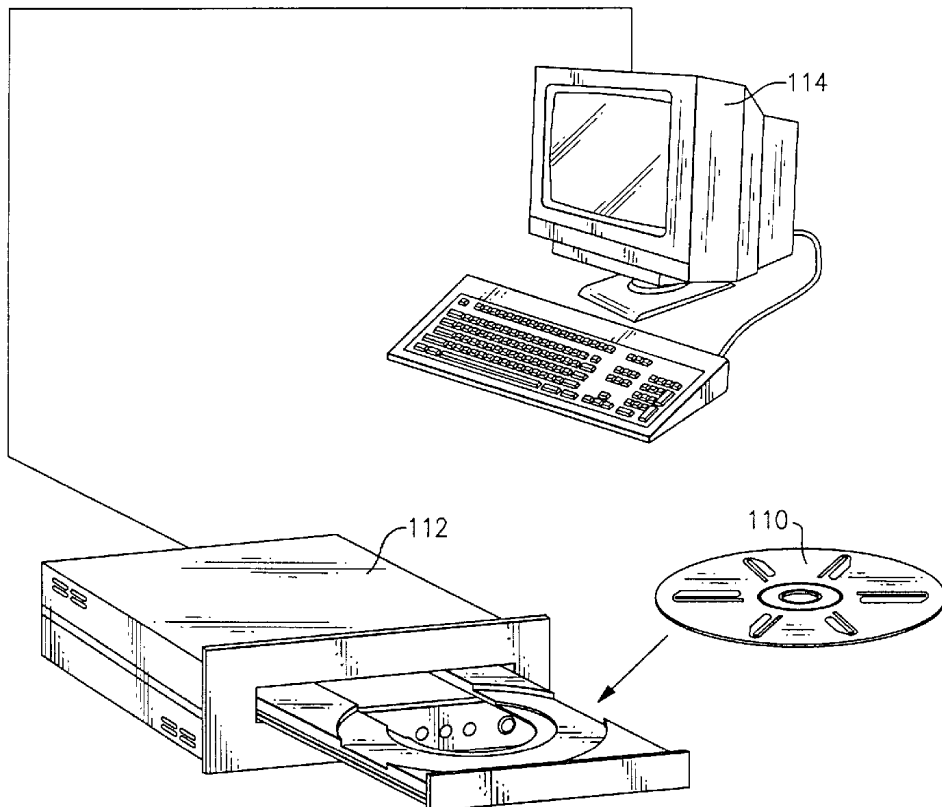
(21) Appl. No.: **10/230,959**
(22) Filed: **Aug. 29, 2002**

(57) **ABSTRACT**

Related U.S. Application Data

(63) Continuation-in-part of application No. 09/988,728, filed on Nov. 20, 2001.
(60) Provisional application No. 60/315,937, filed on Aug. 30, 2001. Provisional application No. 60/328,246, filed on Oct. 10, 2001. Provisional application No. 60/386,072, filed on Oct. 19, 2001. Provisional appli-

A clinical diagnostic assay is performed on an optical bio disc and is read with a disc drive. Methods and apparatus for detecting and quantifying specific blood cell analytes in biological samples using optical bio disc is disclosed. The method for determining the quantity of a specific type of blood cell in a biological sample includes binding an antibody to a capture zone on the disc, providing a sample to the capture zone, remove portions of the sample that are not bound in the capture zone, and counting bound cells. Also described is method and apparatus for performing a cluster designation count with an optical disc and disc drive and method for making an optical assay disc for performing such cluster designation count.



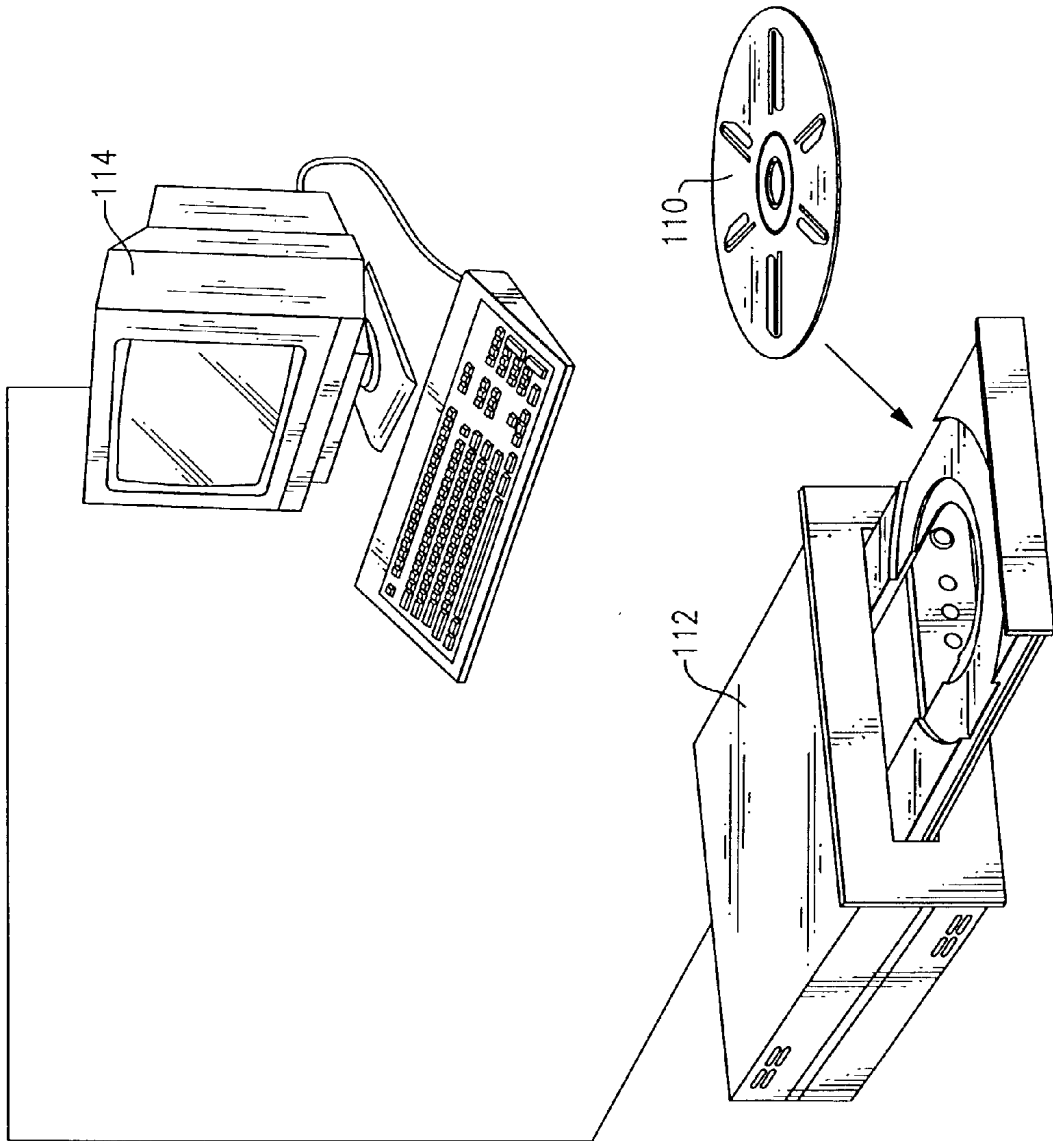
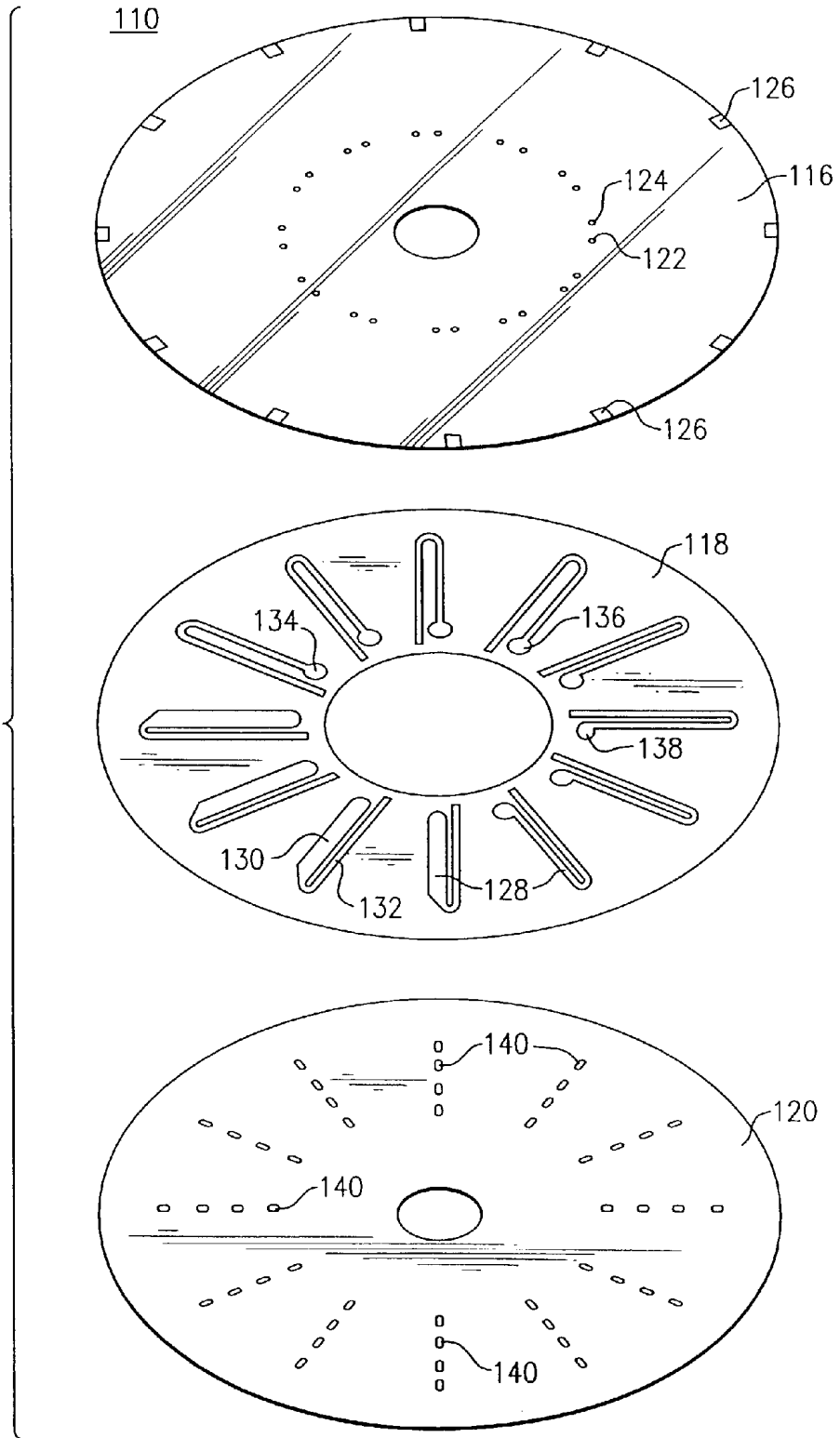


FIG. 1

FIG.2



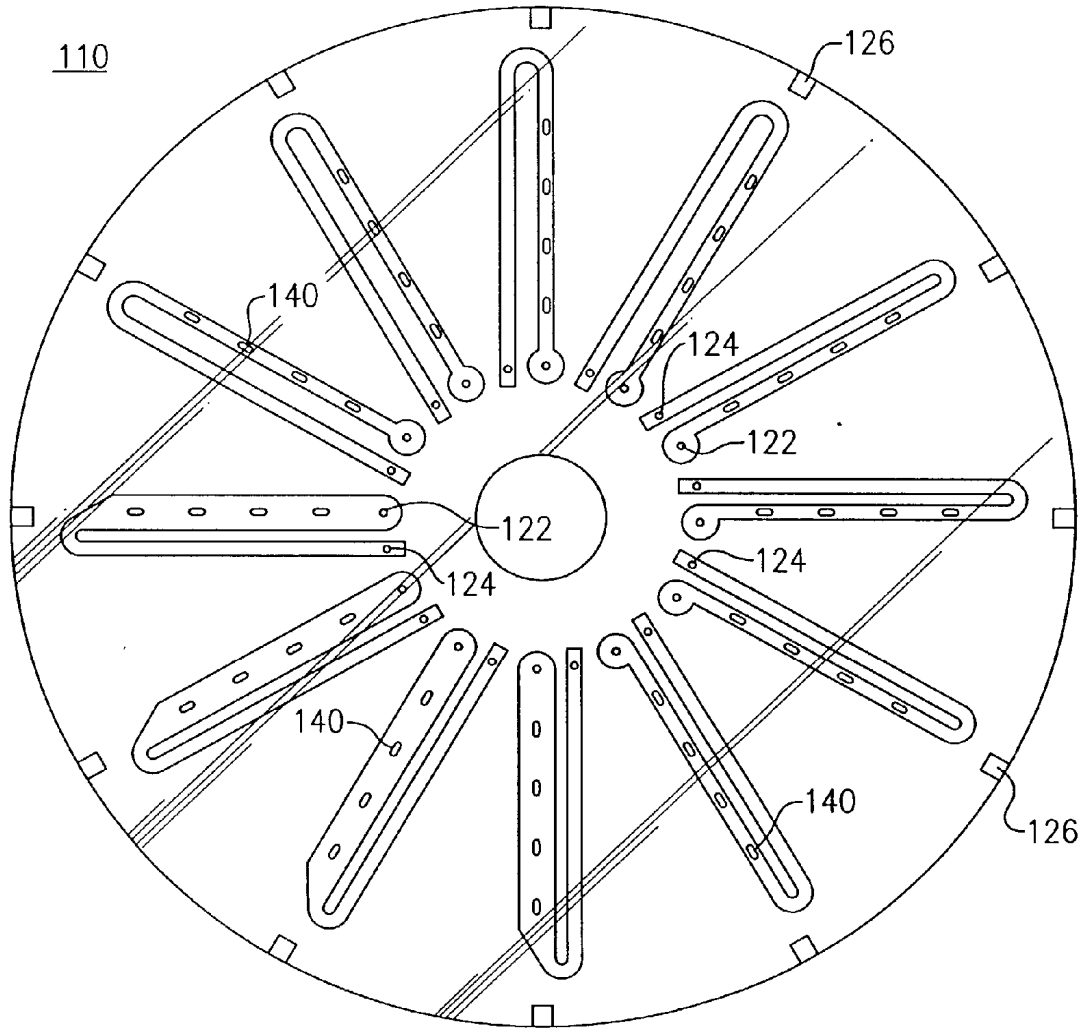


FIG.3

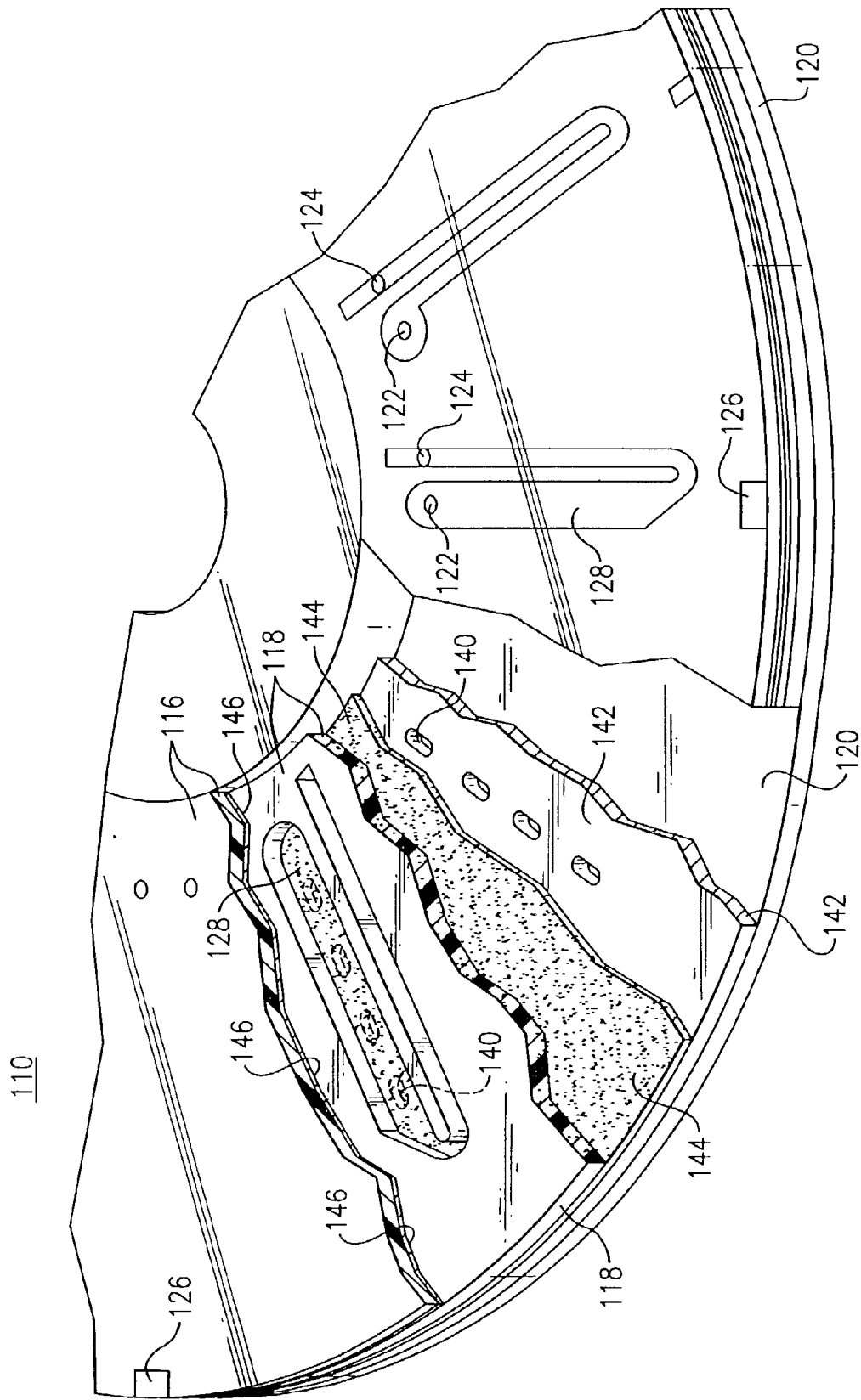
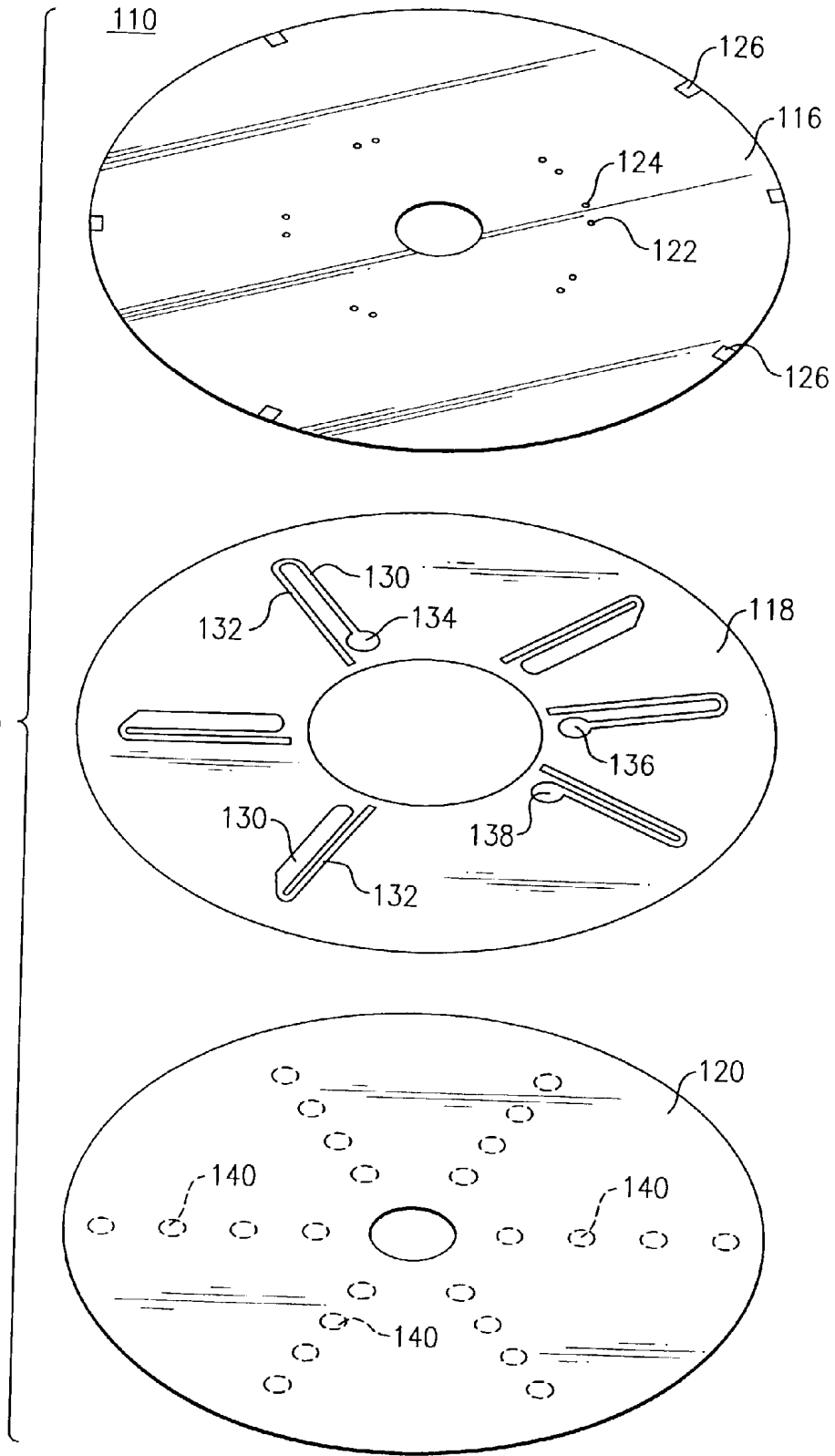


FIG. 4

FIG. 5



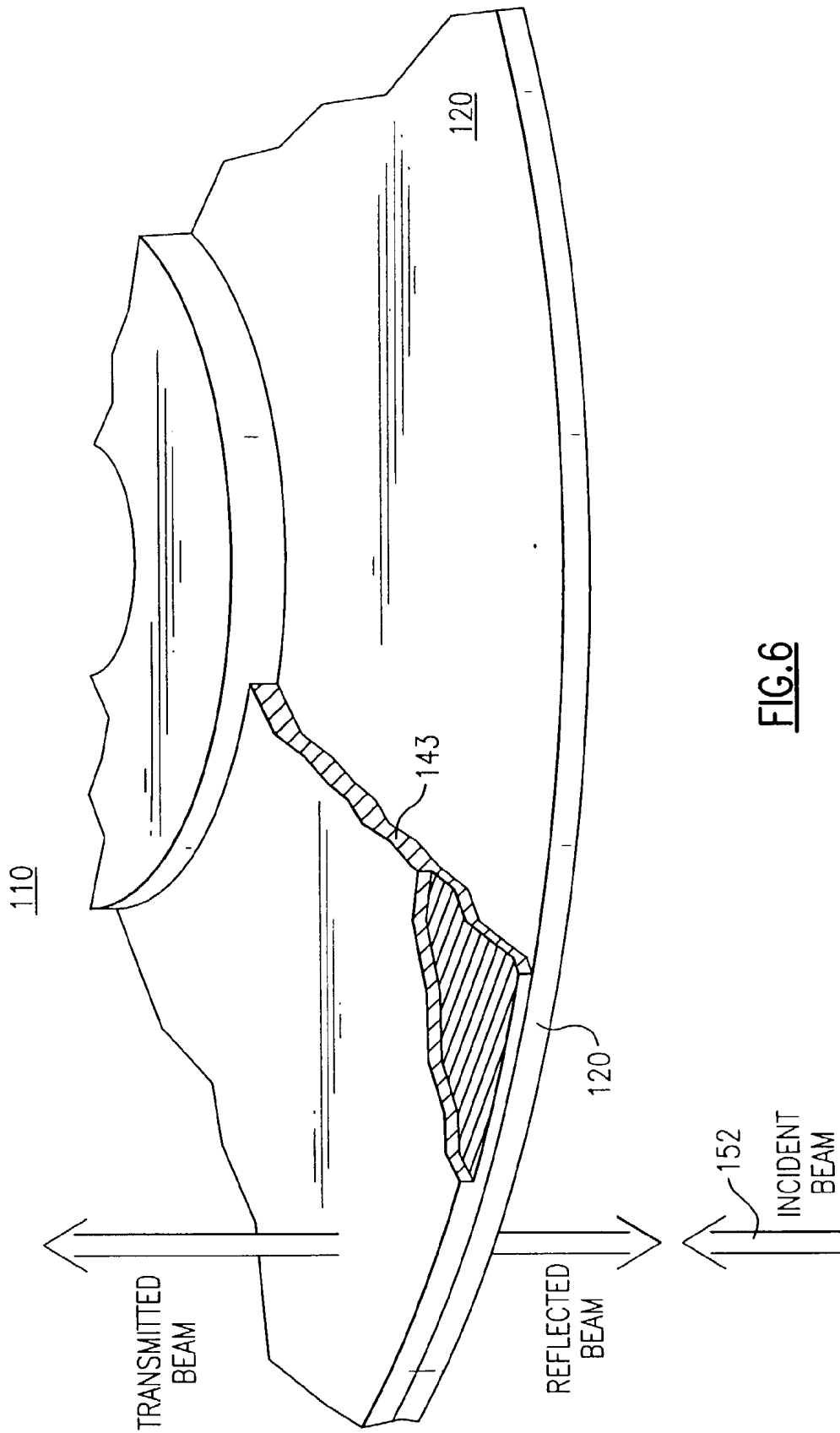


FIG.6

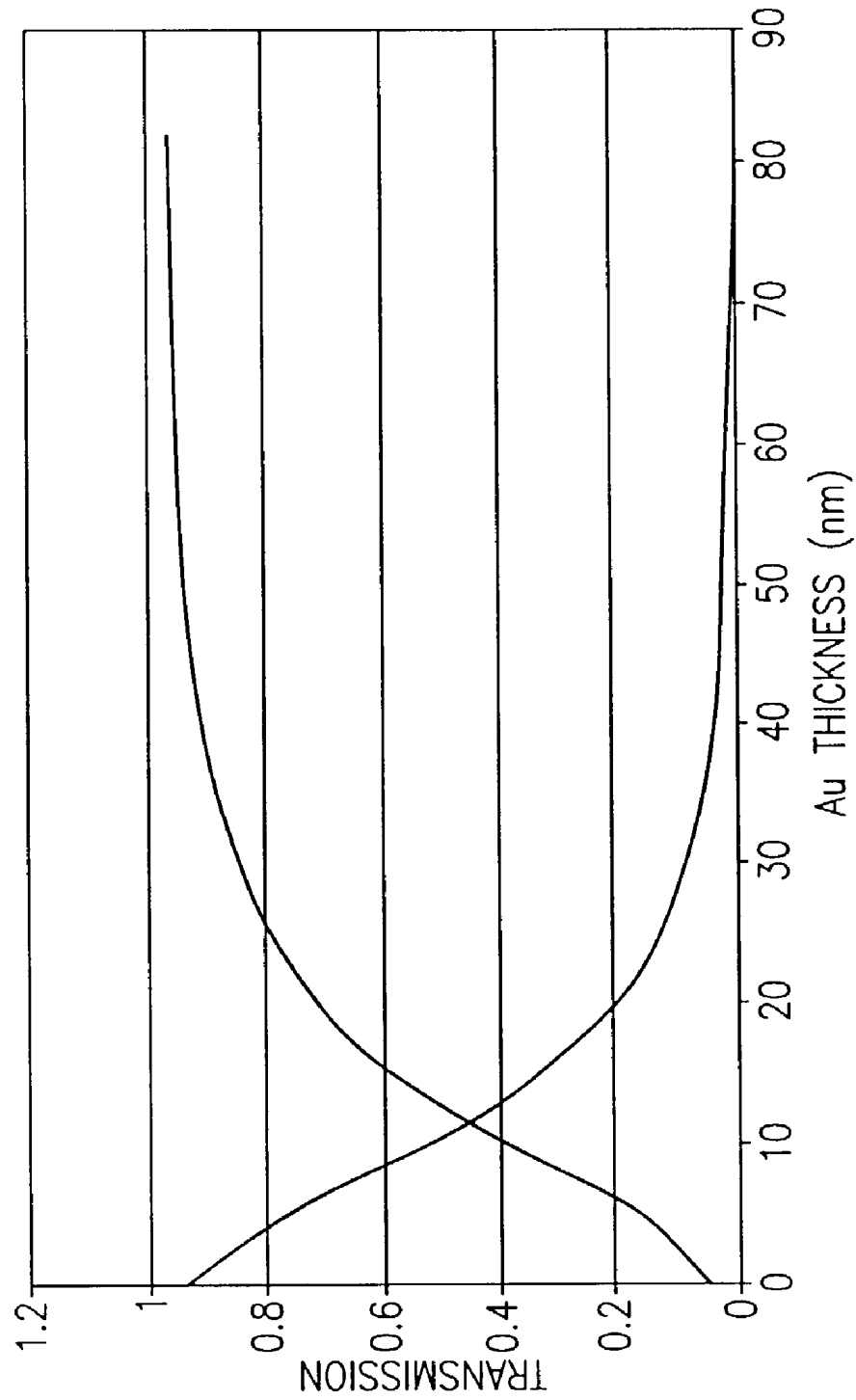


FIG. 7

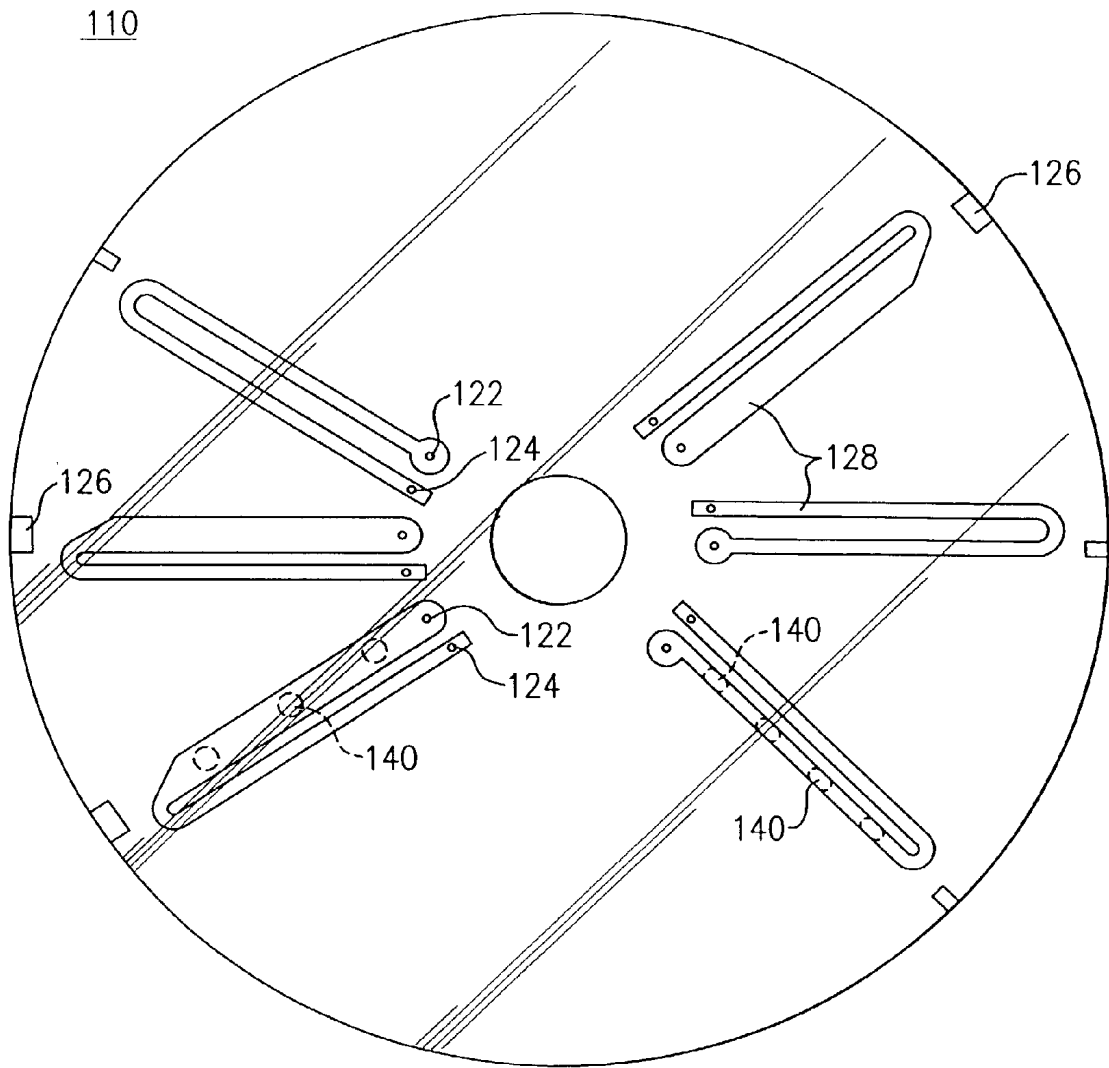


FIG.8

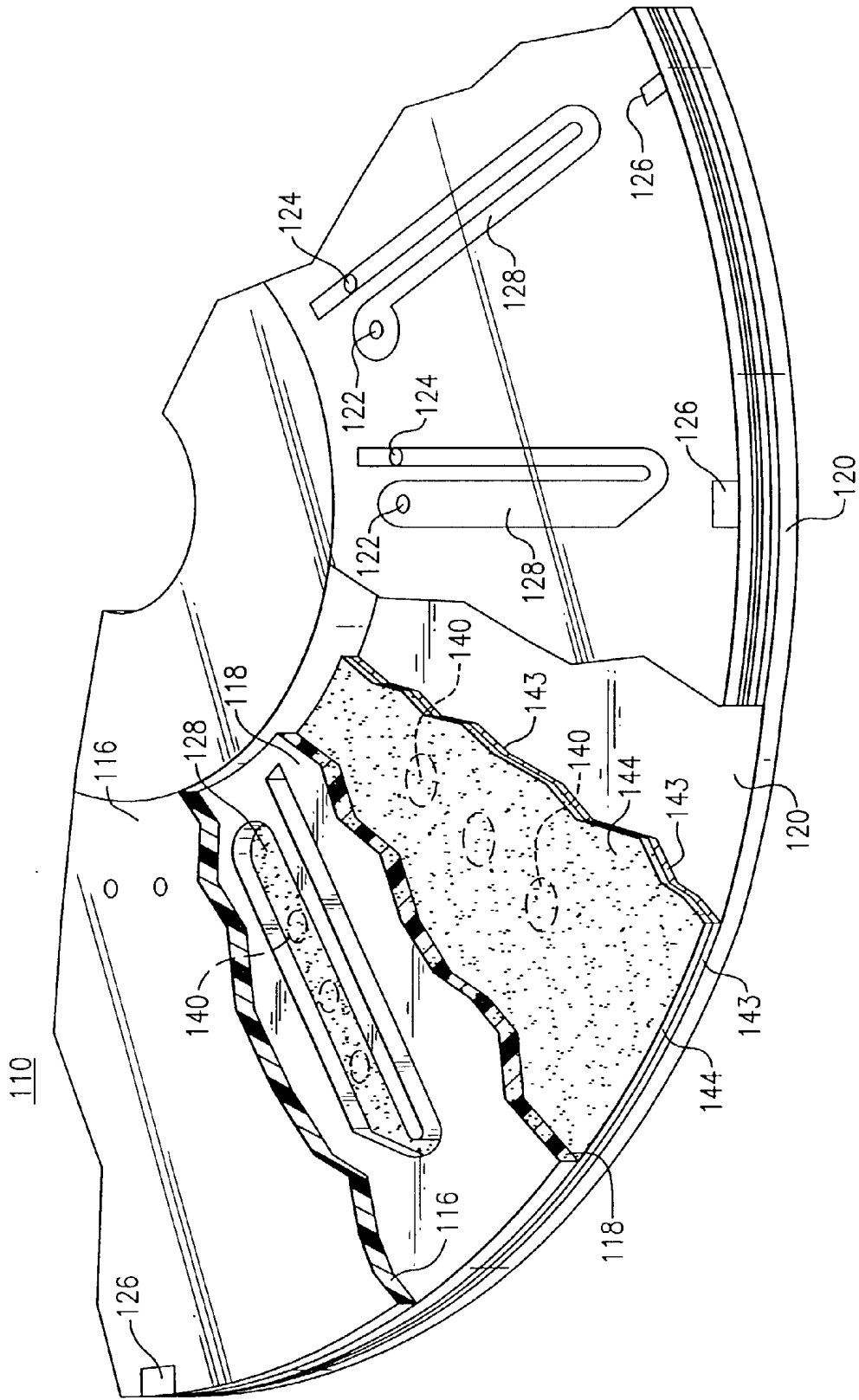


FIG. 9

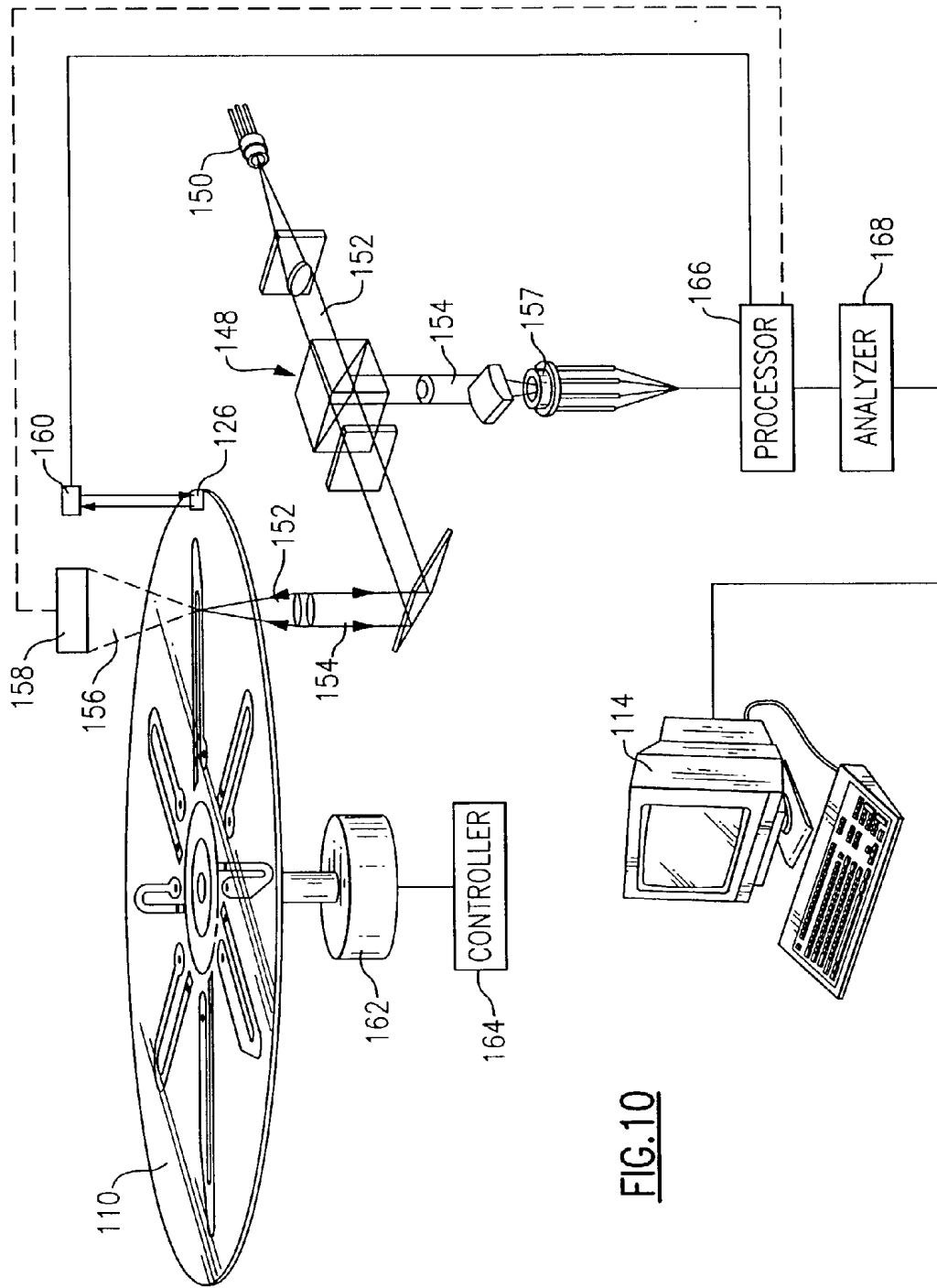


FIG. 10

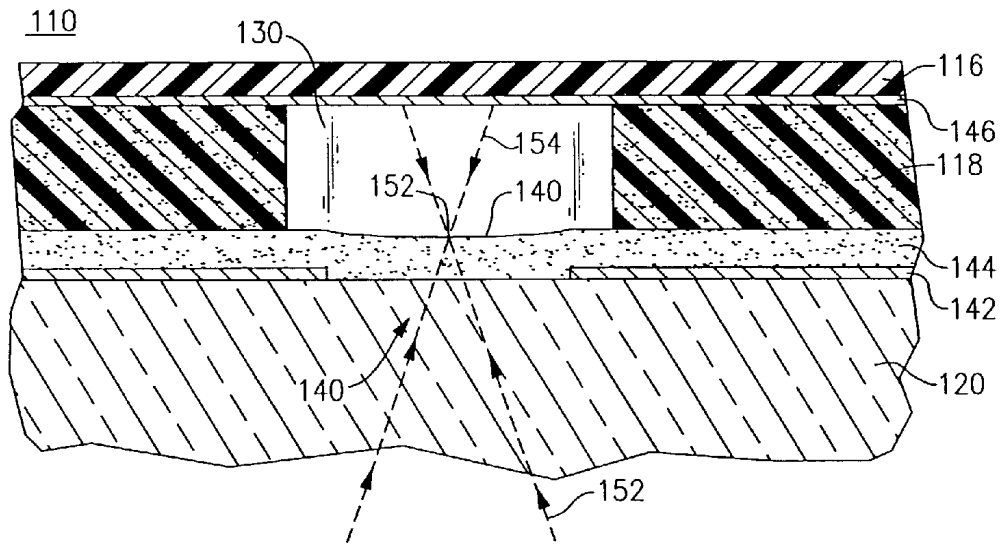


FIG.11

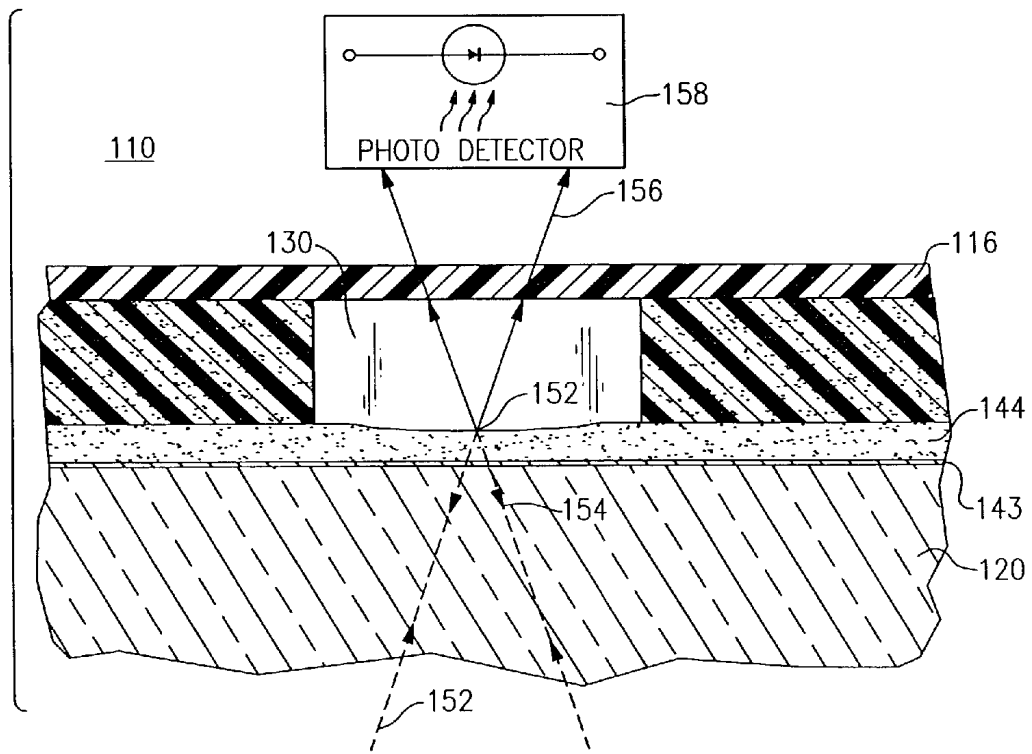


FIG.12

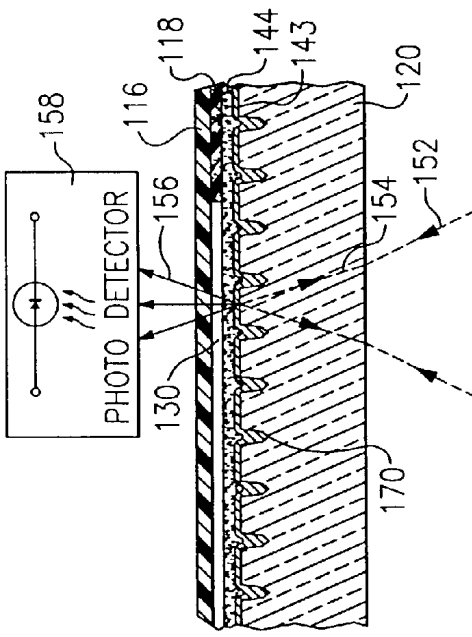


FIG. 14

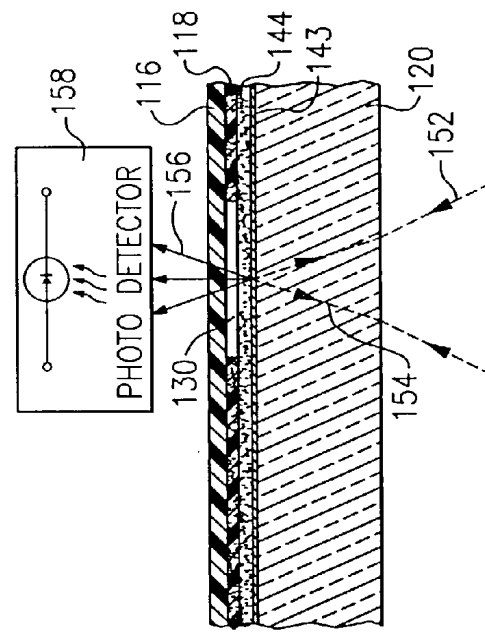


FIG. 16

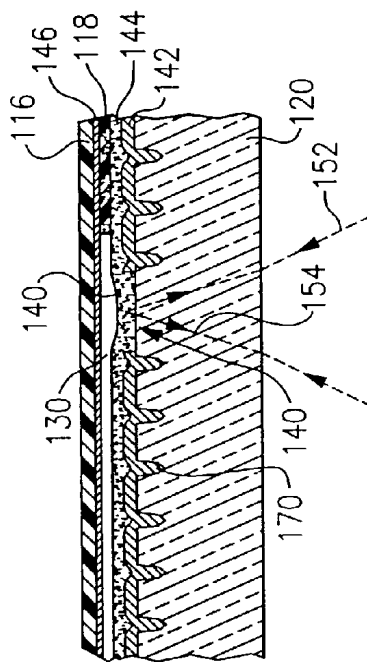


FIG. 13

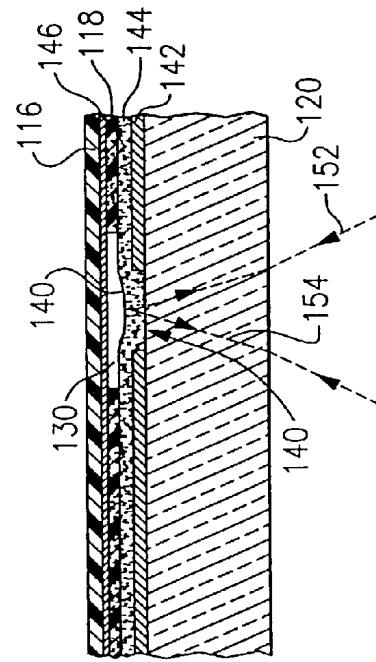
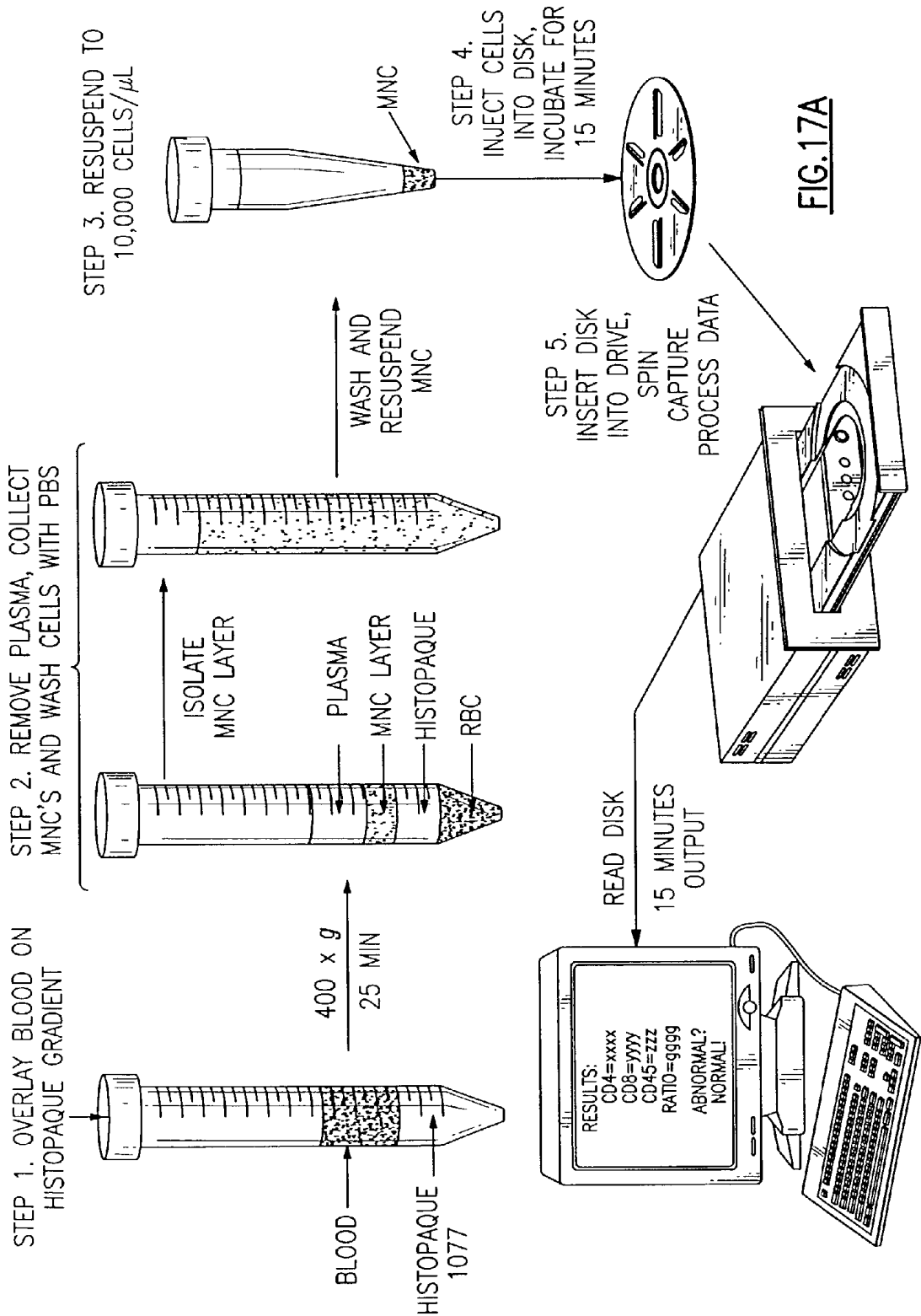


FIG. 15



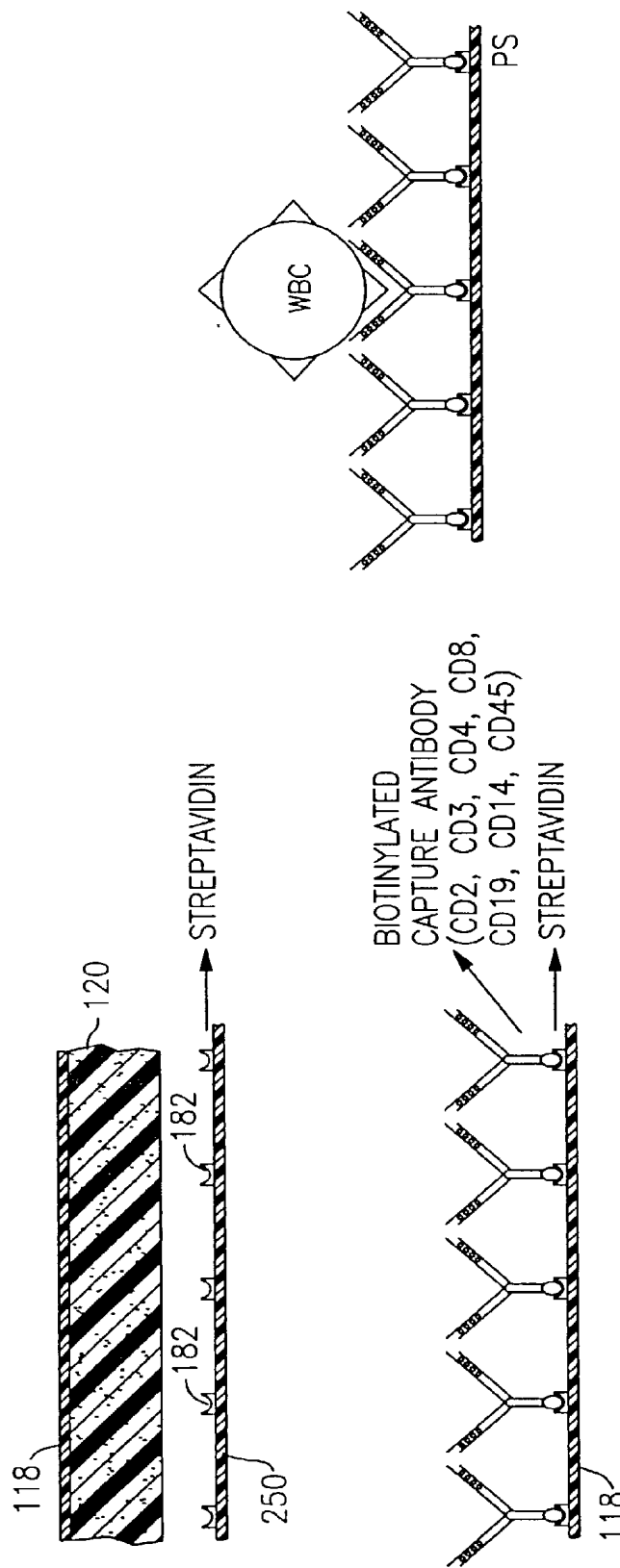


FIG.17B

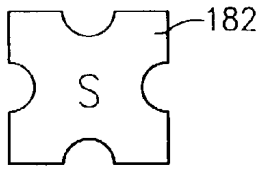


FIG. 18A

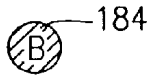


FIG. 18B

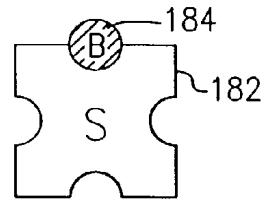


FIG. 18C

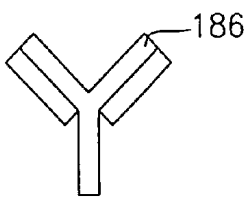


FIG. 18D

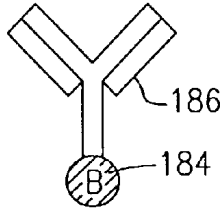


FIG. 18E

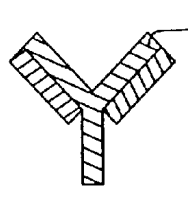


FIG. 18F

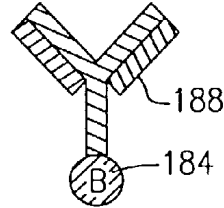


FIG. 18G

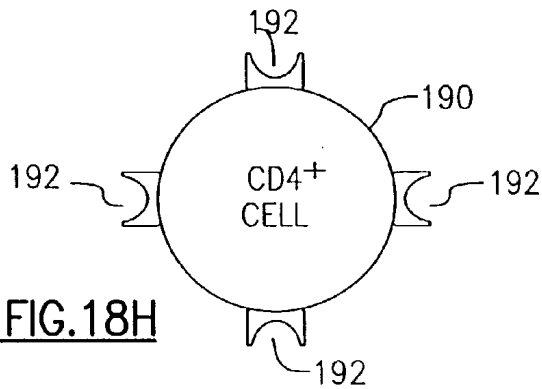


FIG. 18H

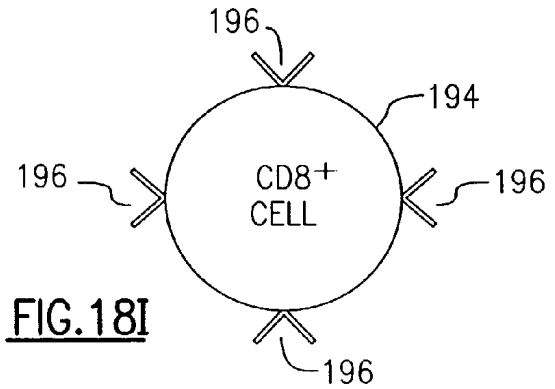


FIG. 18I

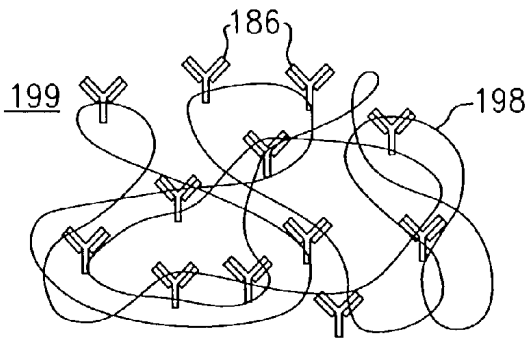


FIG. 18J

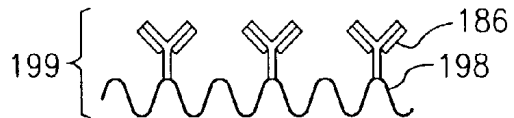


FIG. 18K

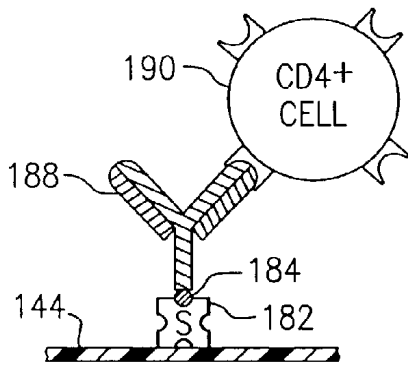


FIG. 19A

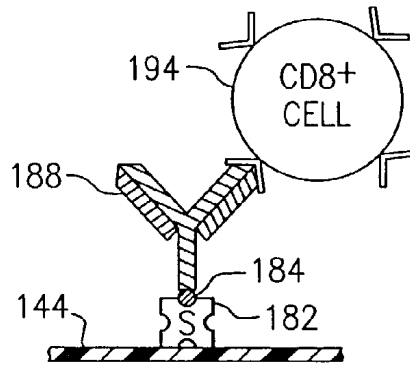


FIG. 19B

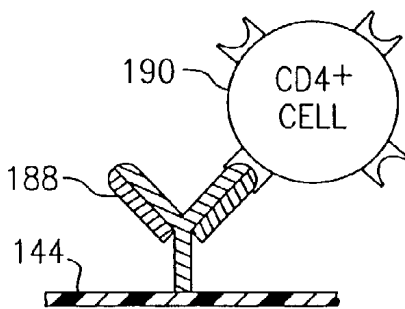


FIG. 19C

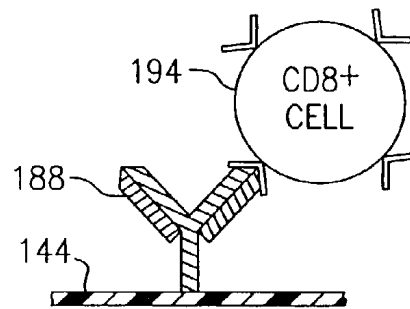


FIG. 19D

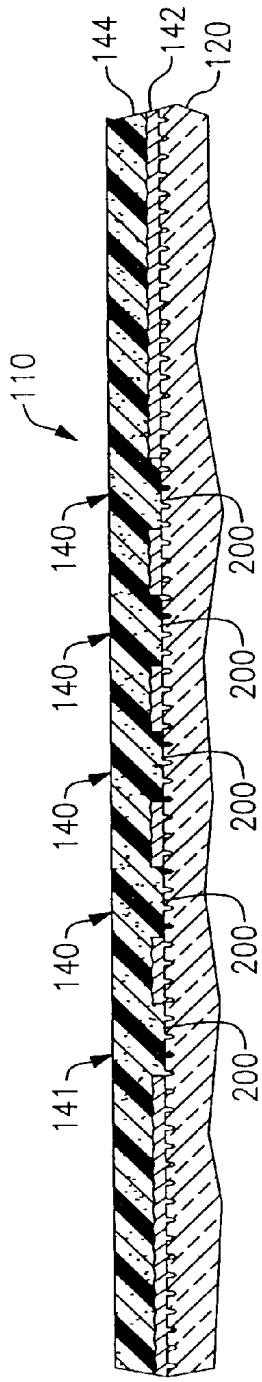


FIG. 20A

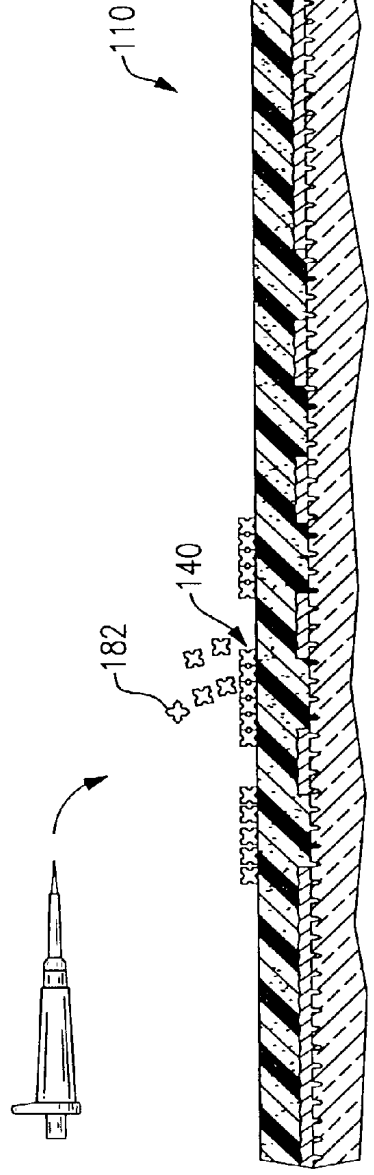


FIG. 20B

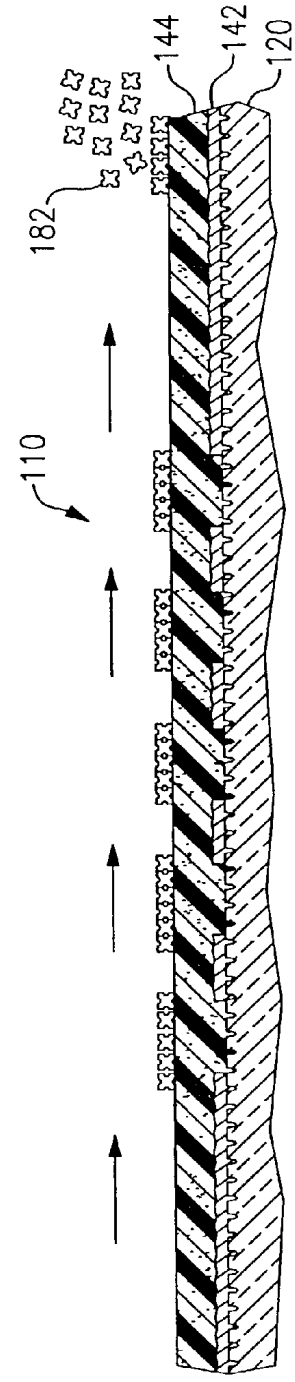


FIG. 20C

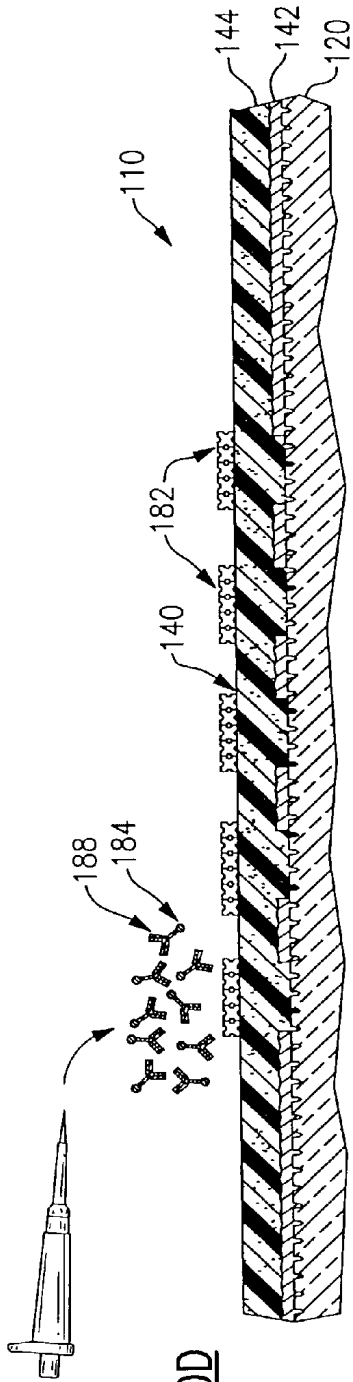


FIG. 20D

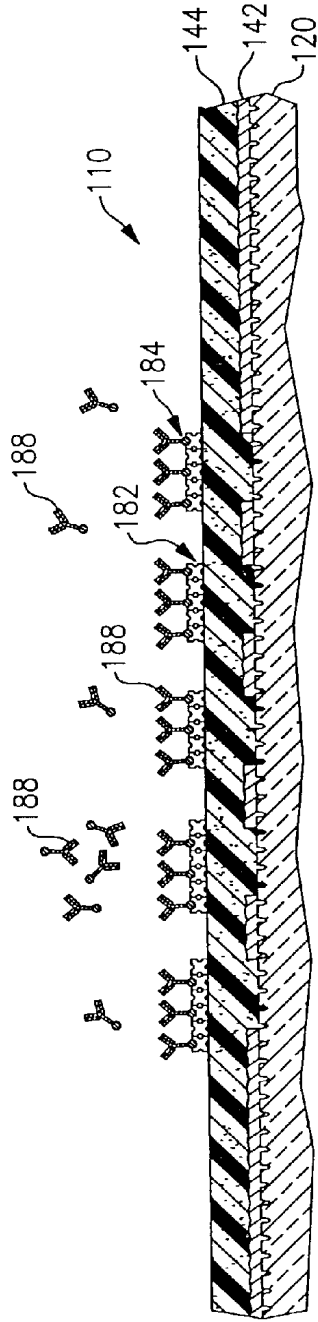


FIG. 20E

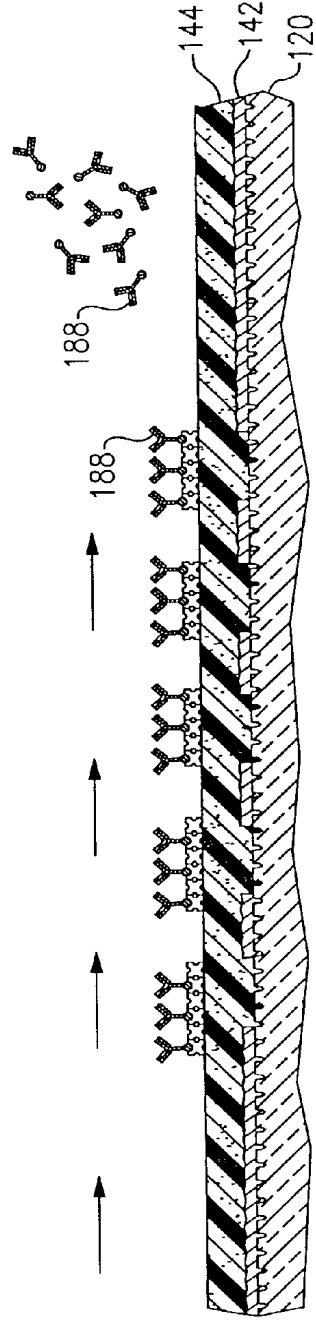


FIG. 20F

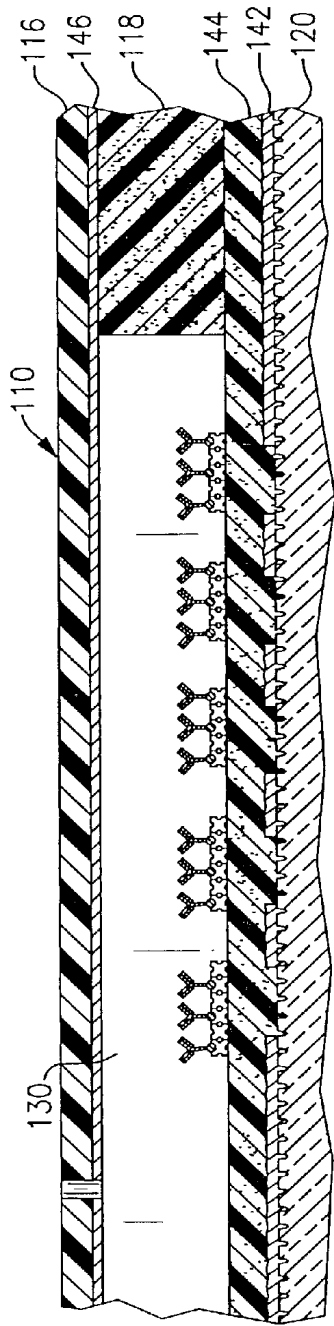


FIG. 20G

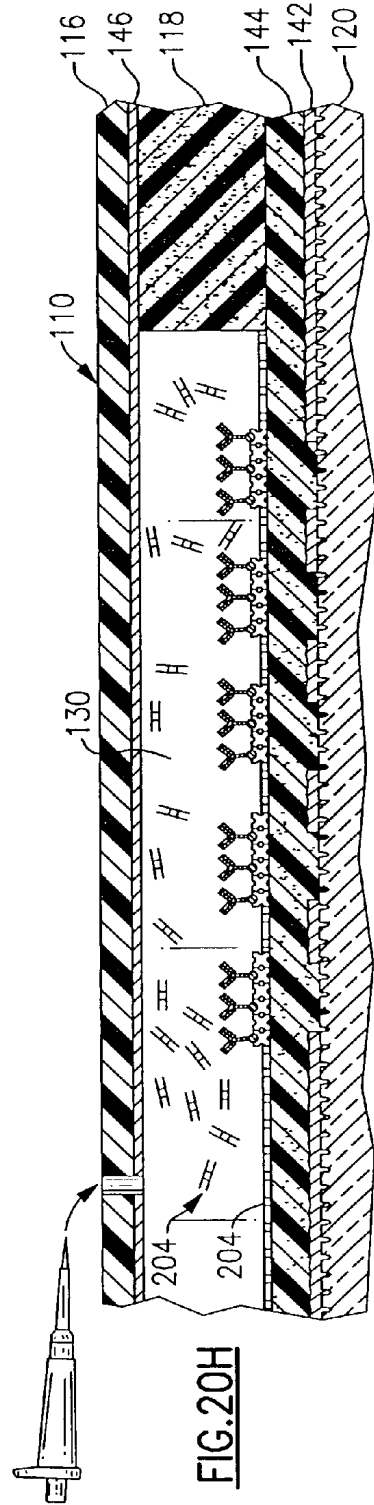


FIG. 20H

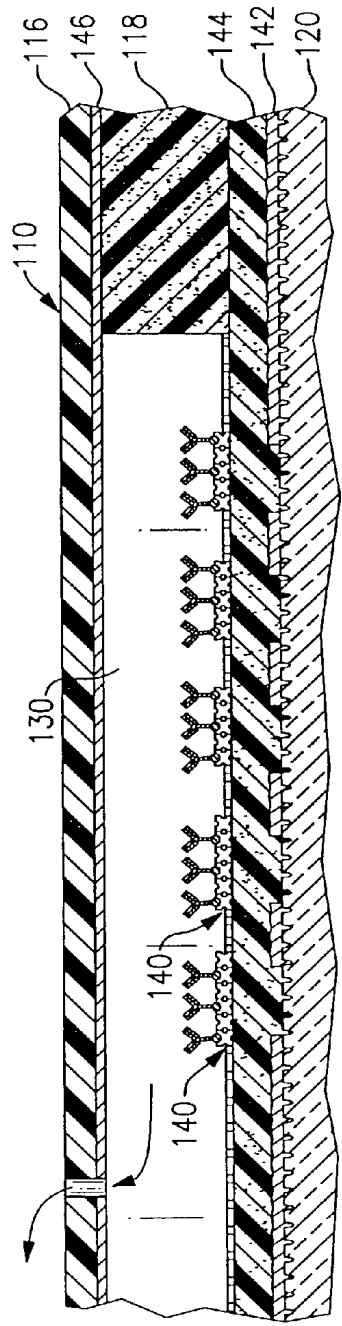


FIG. 20I

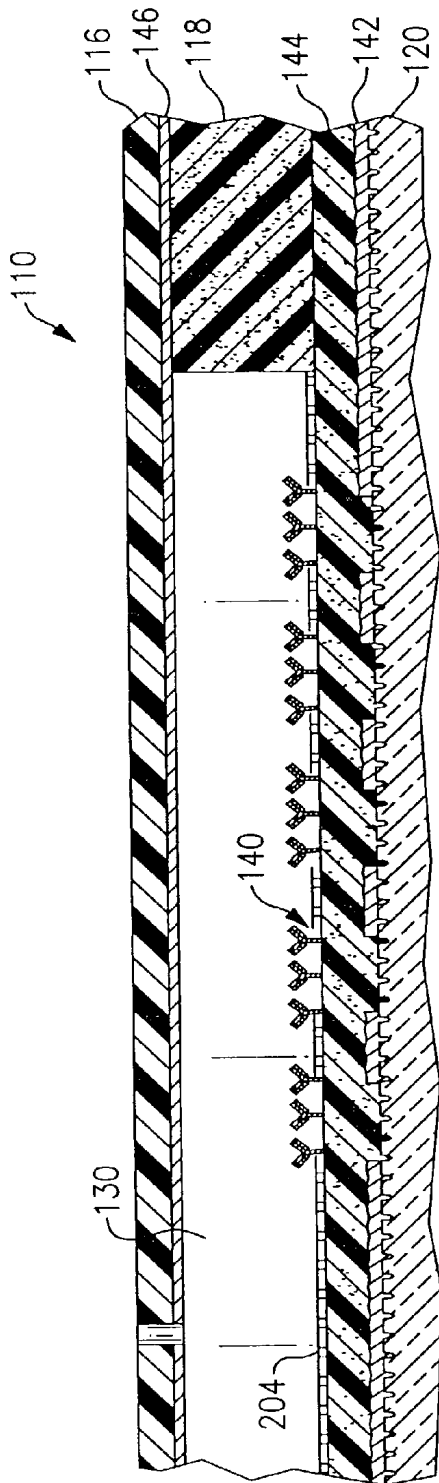


FIG. 21

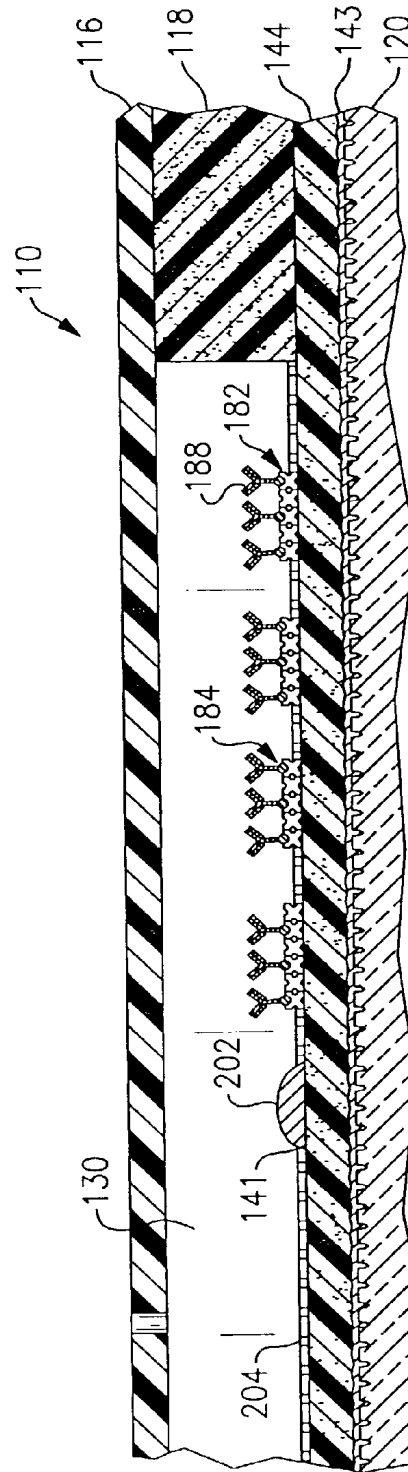


FIG. 22

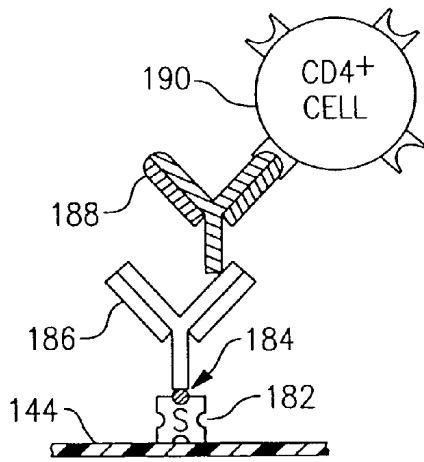


FIG. 23A

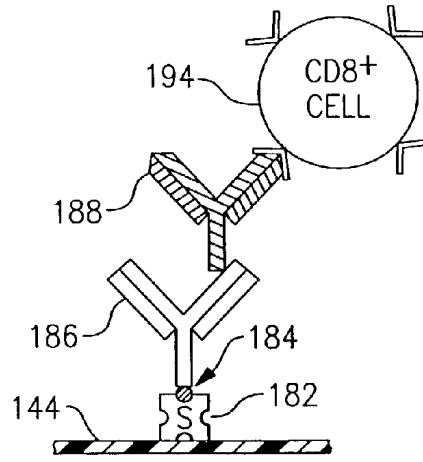


FIG. 23B

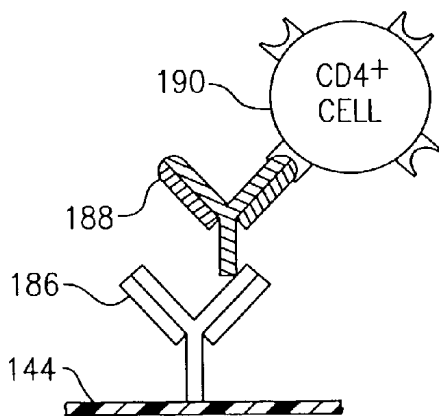


FIG. 23C

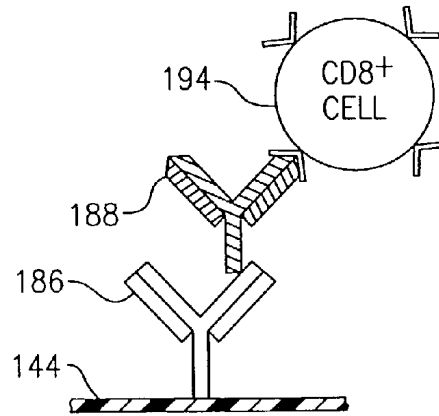


FIG. 23D

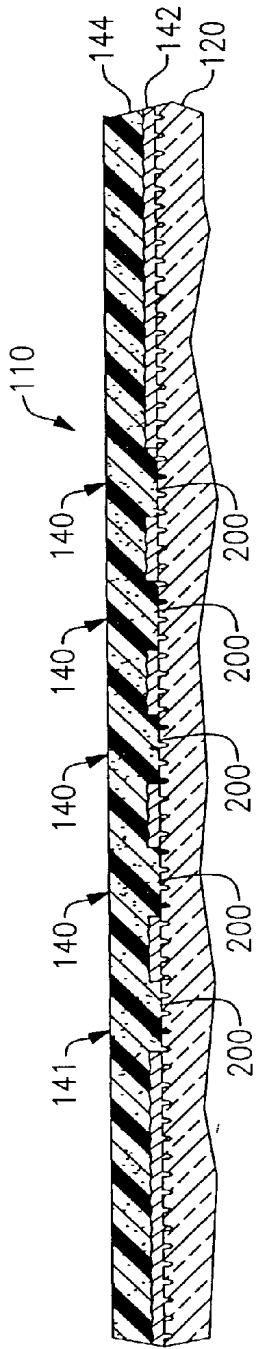


FIG. 24A

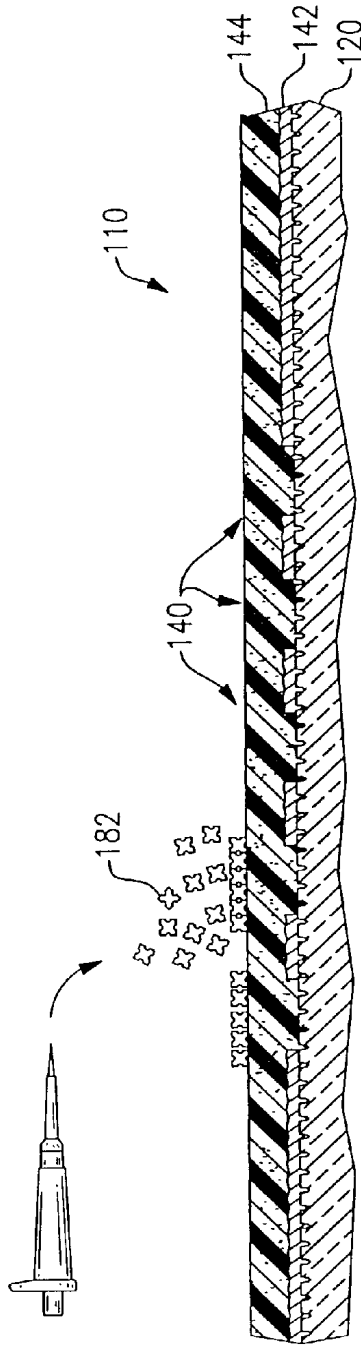


FIG. 24B

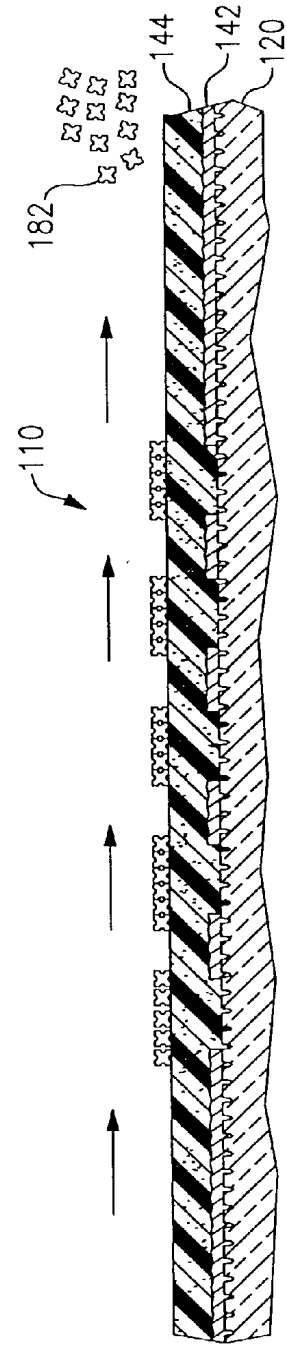


FIG. 24C

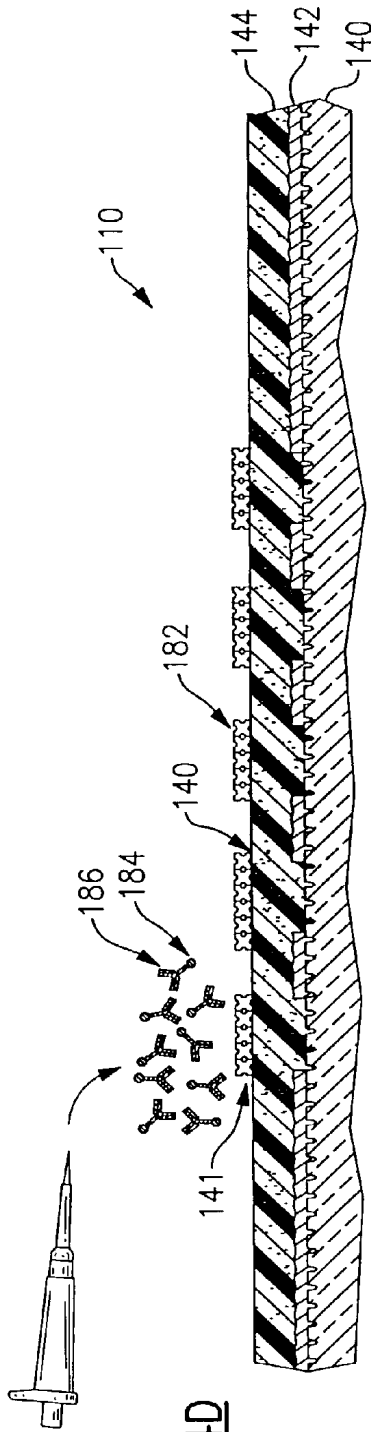


FIG. 24D

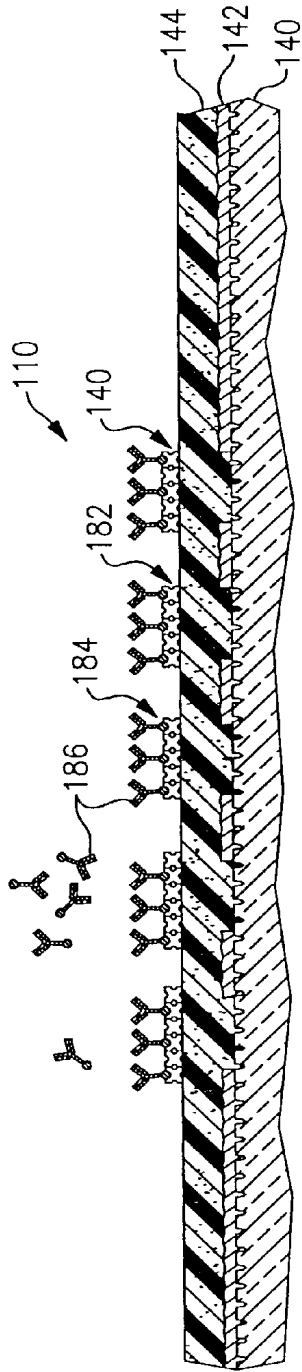


FIG. 24E

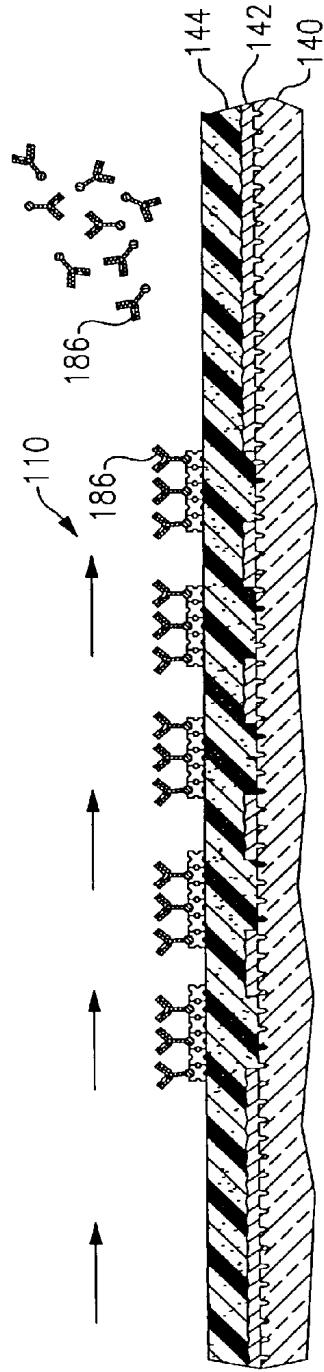
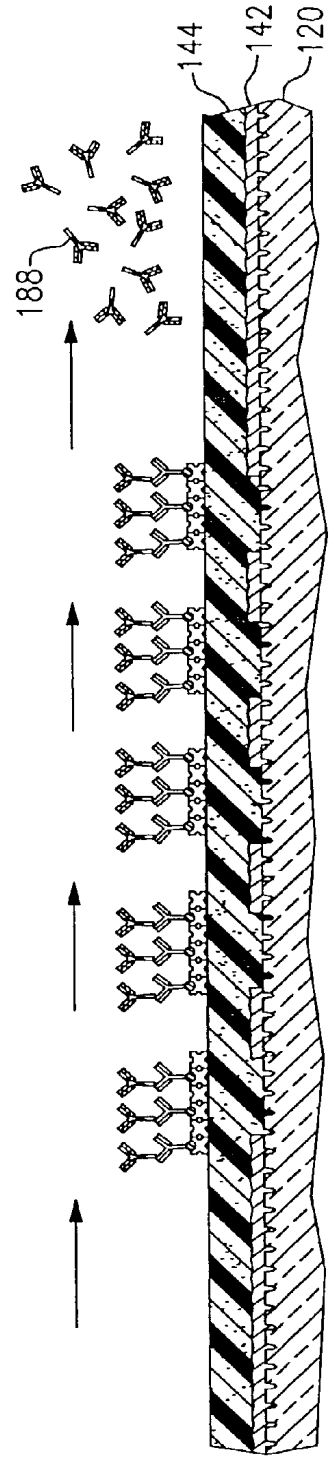
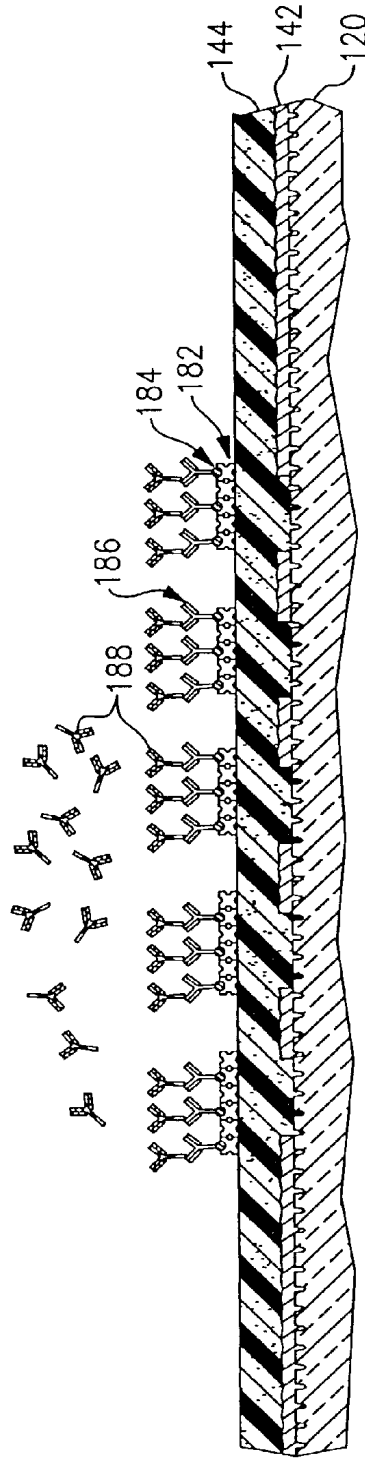
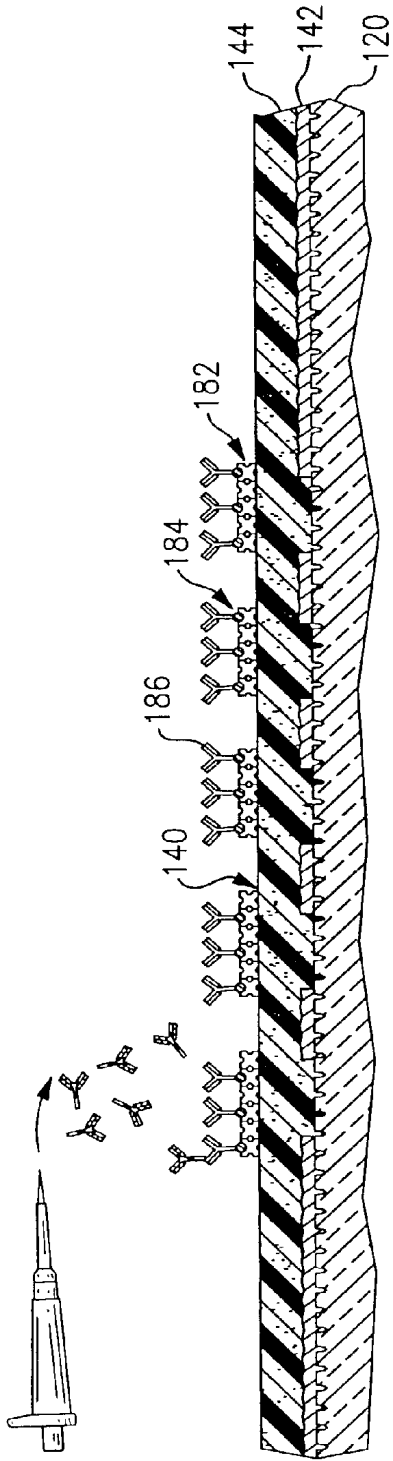


FIG. 24F



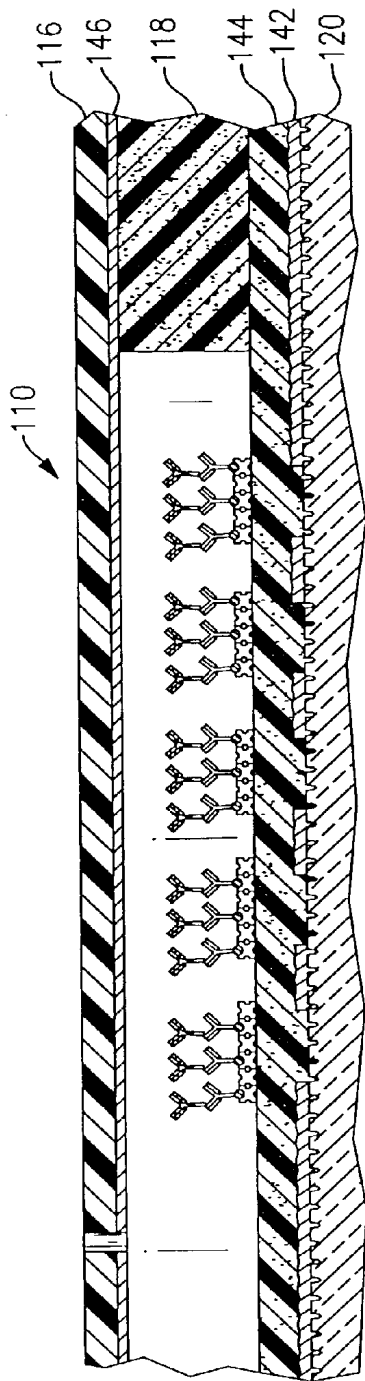


FIG. 24J

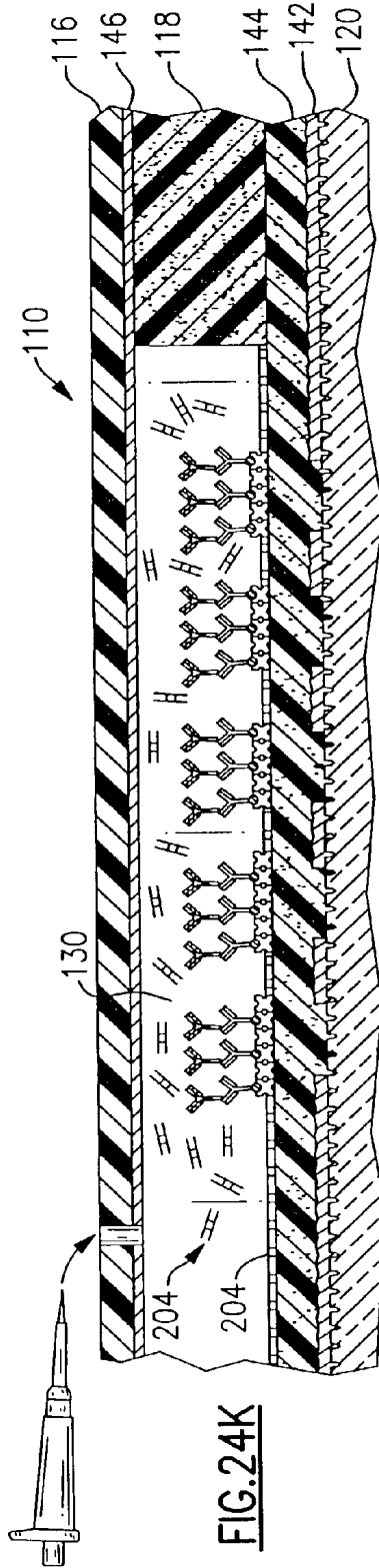


FIG. 24K

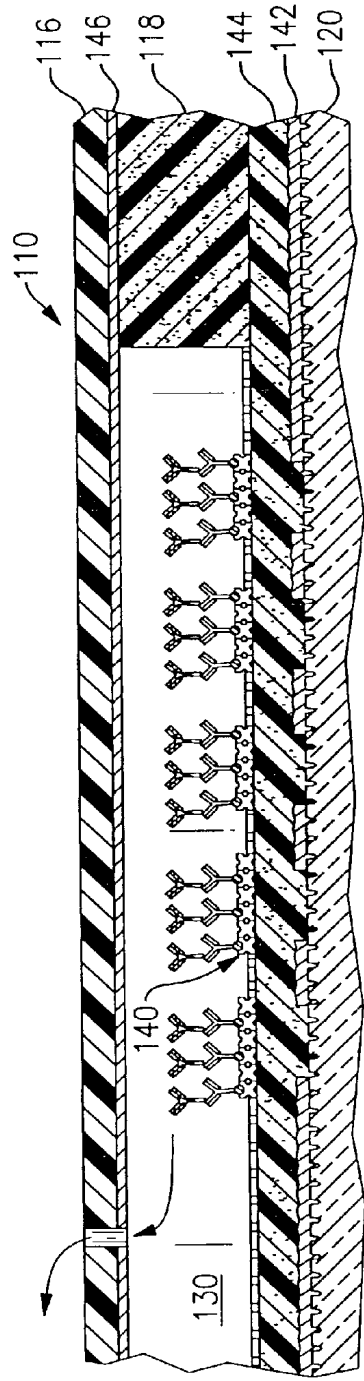


FIG. 24L

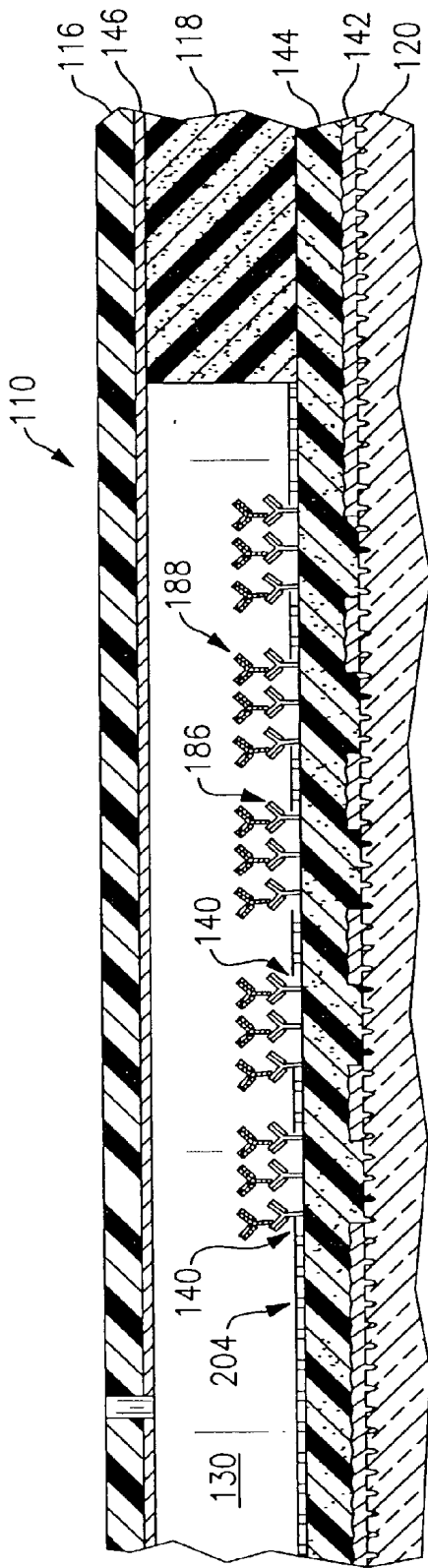


FIG. 25

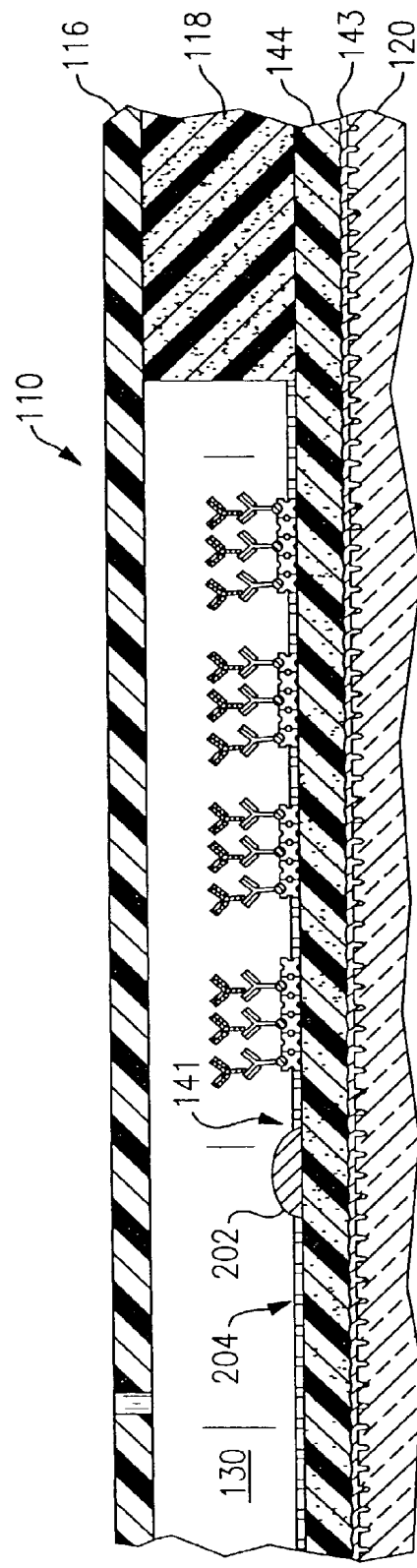


FIG. 26

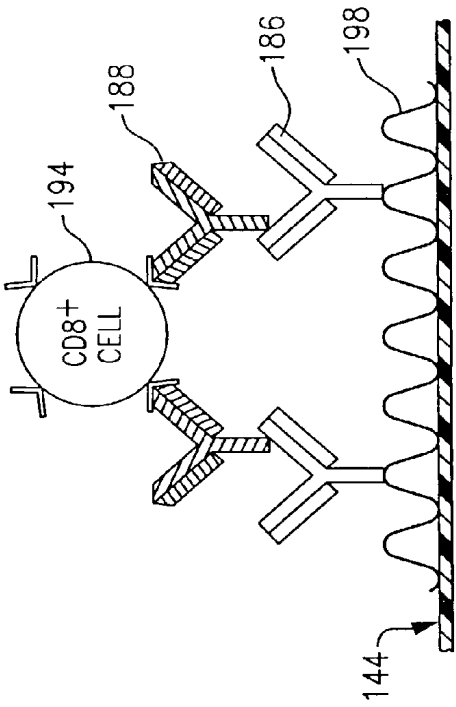


FIG. 27B

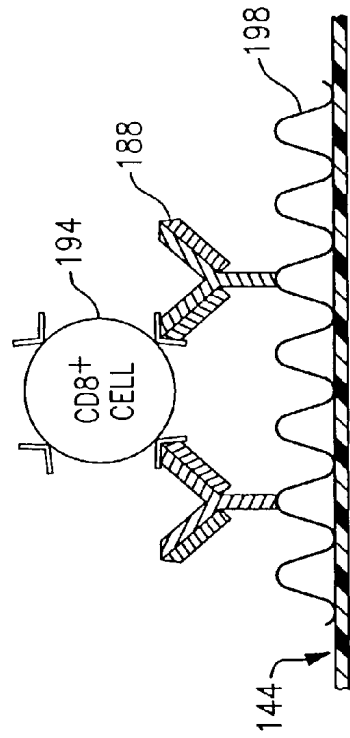


FIG. 27D

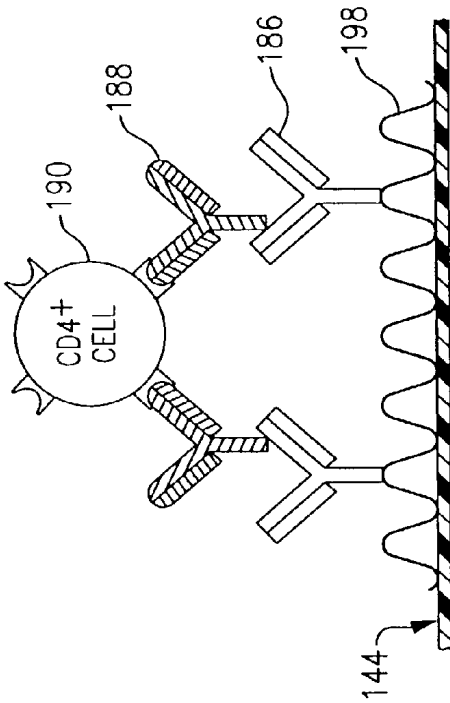


FIG. 27A

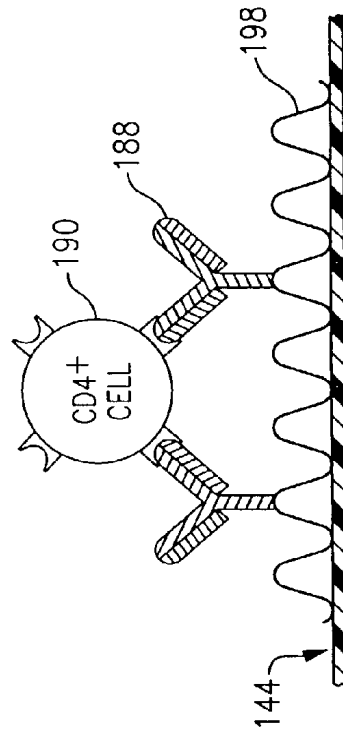
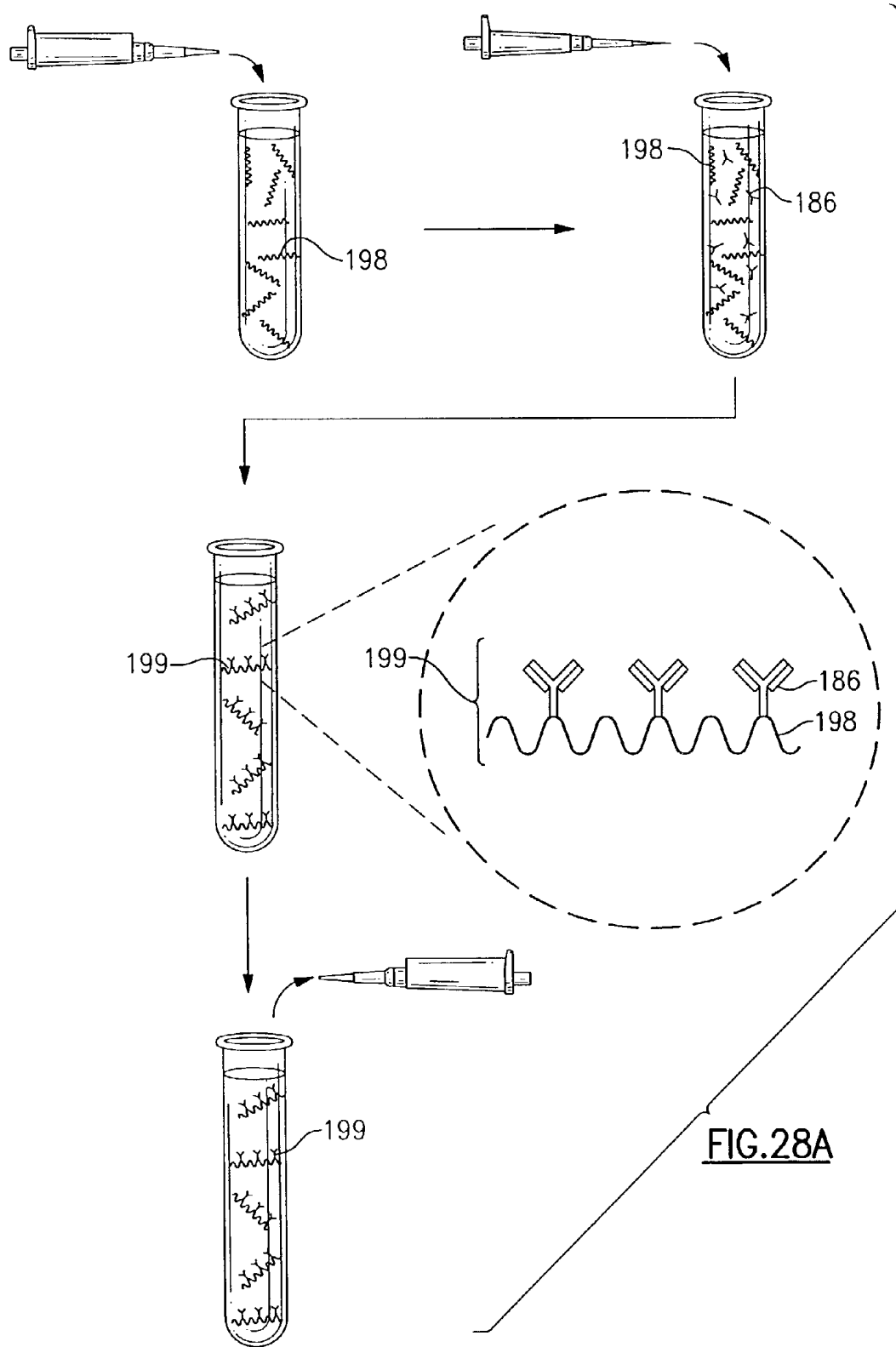


FIG. 27C



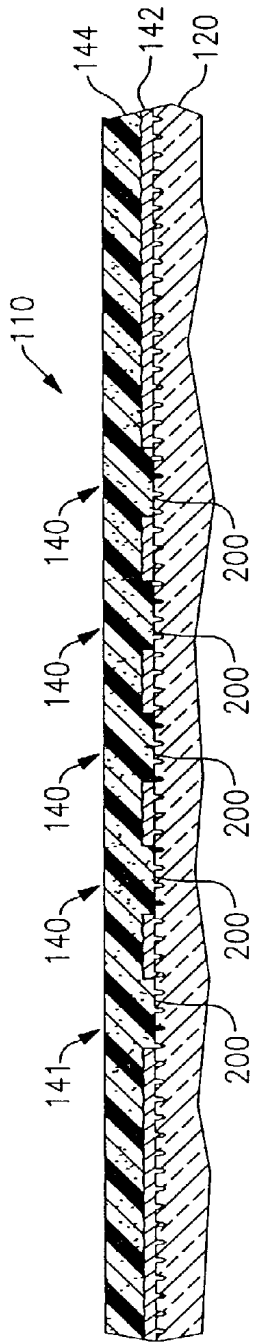


FIG. 28B

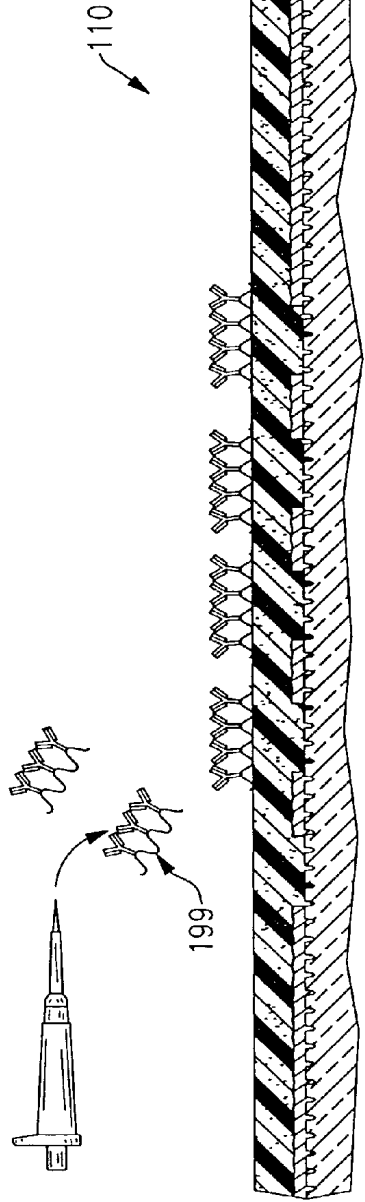


FIG. 28C

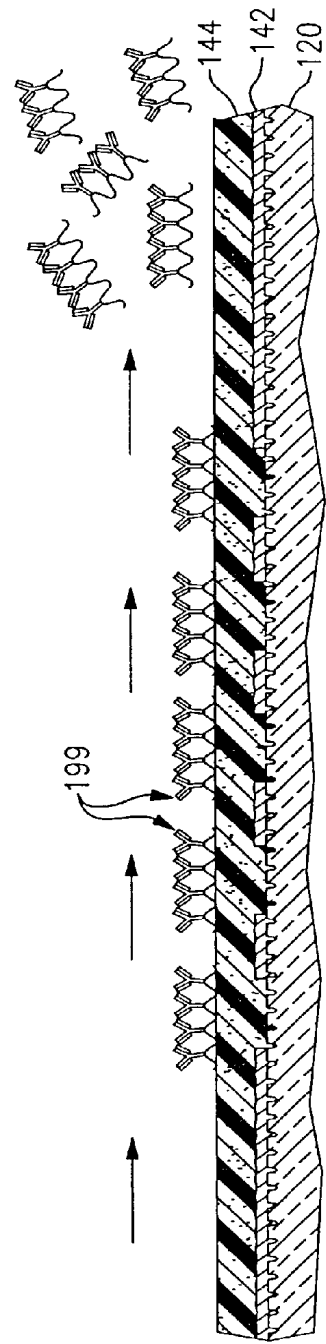


FIG. 28D

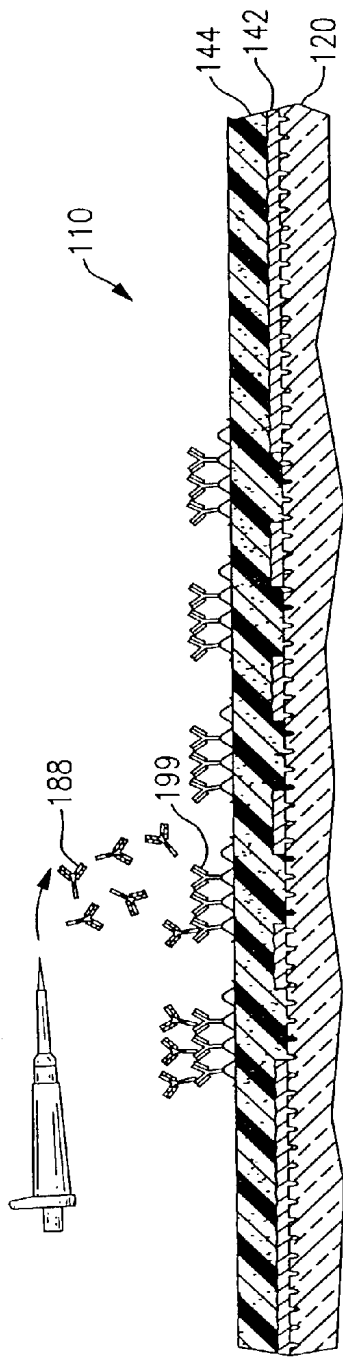


FIG. 28E

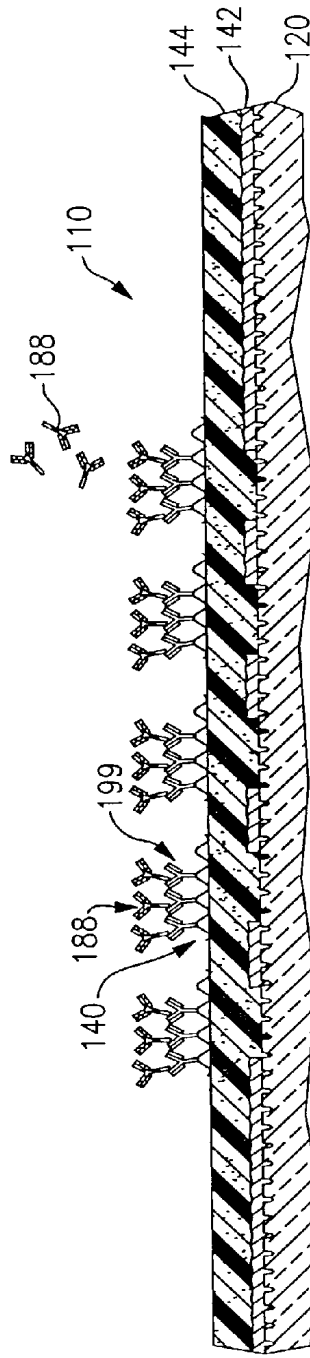


FIG. 28F

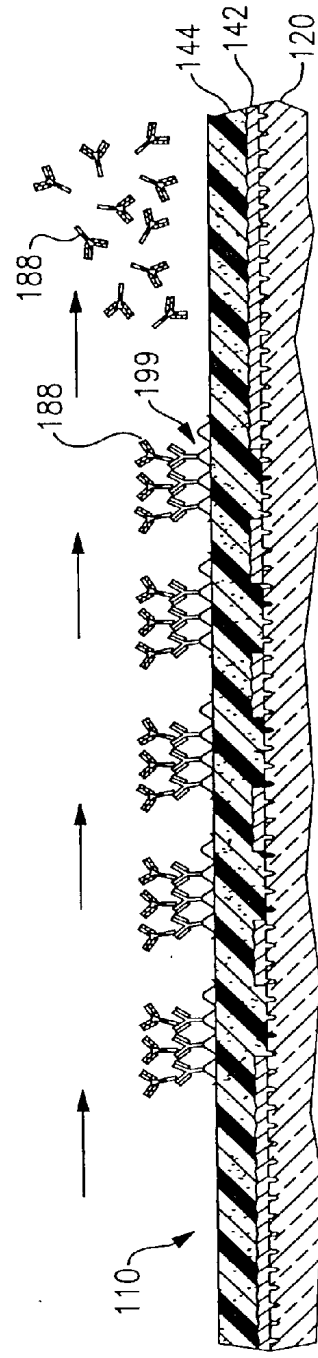


FIG. 28G

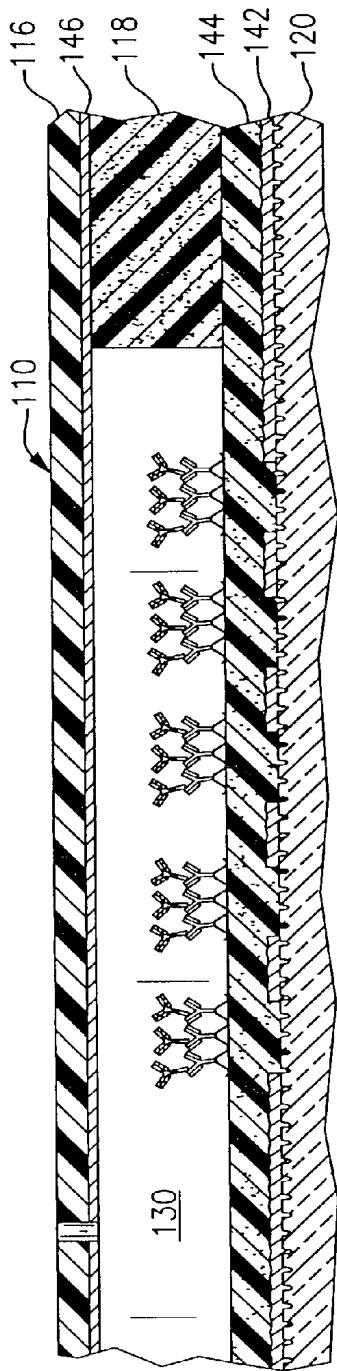


FIG. 28H

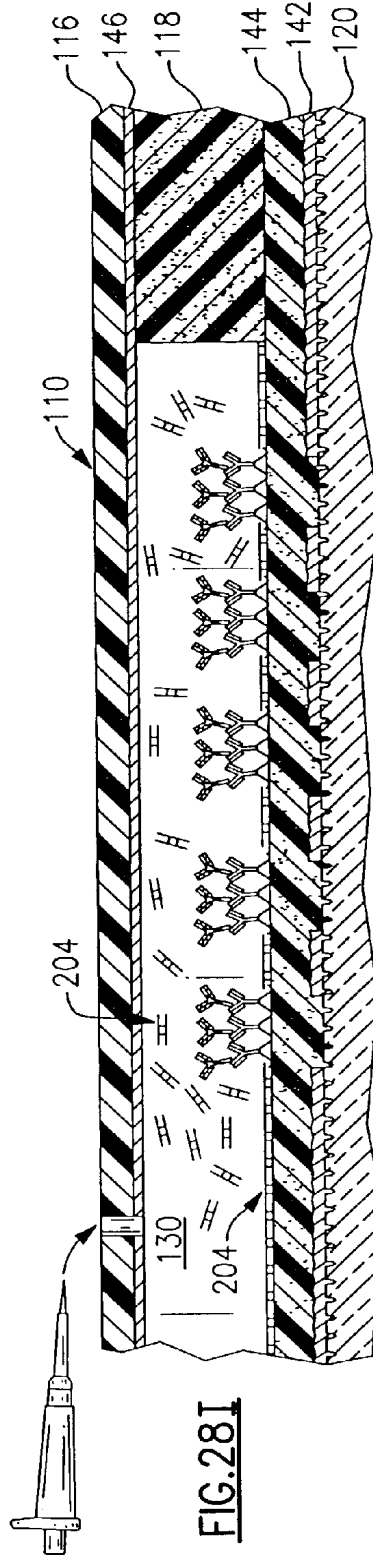


FIG. 28I

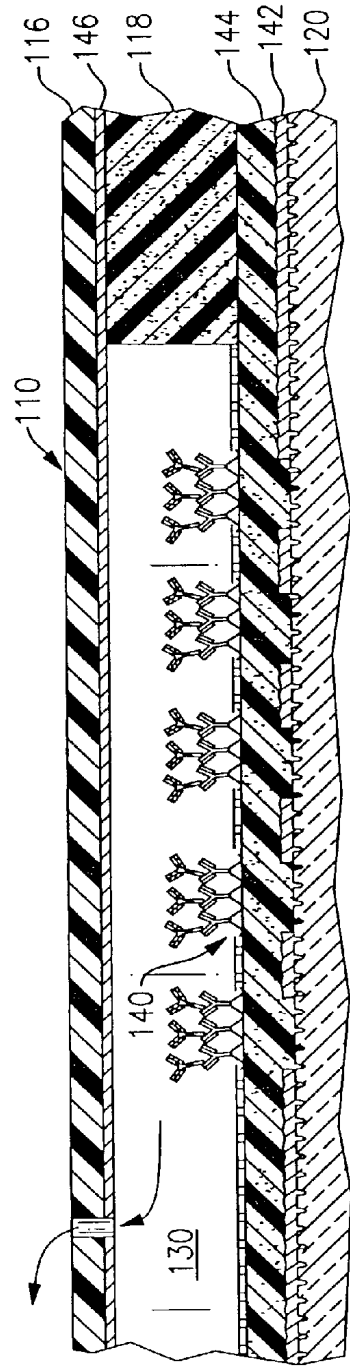


FIG. 28J

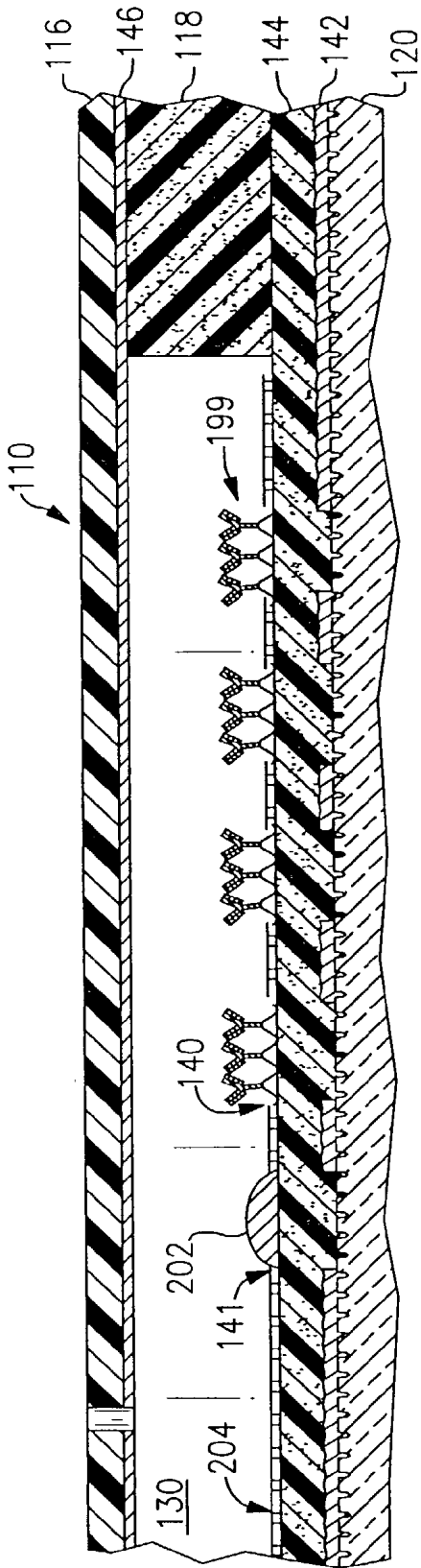


FIG. 29

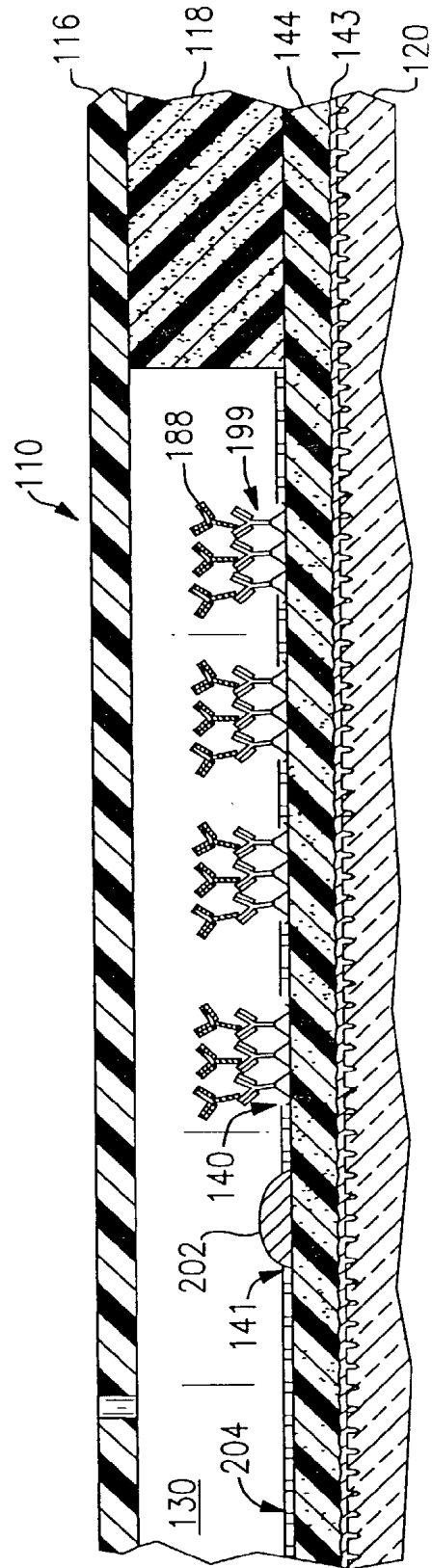


FIG. 30

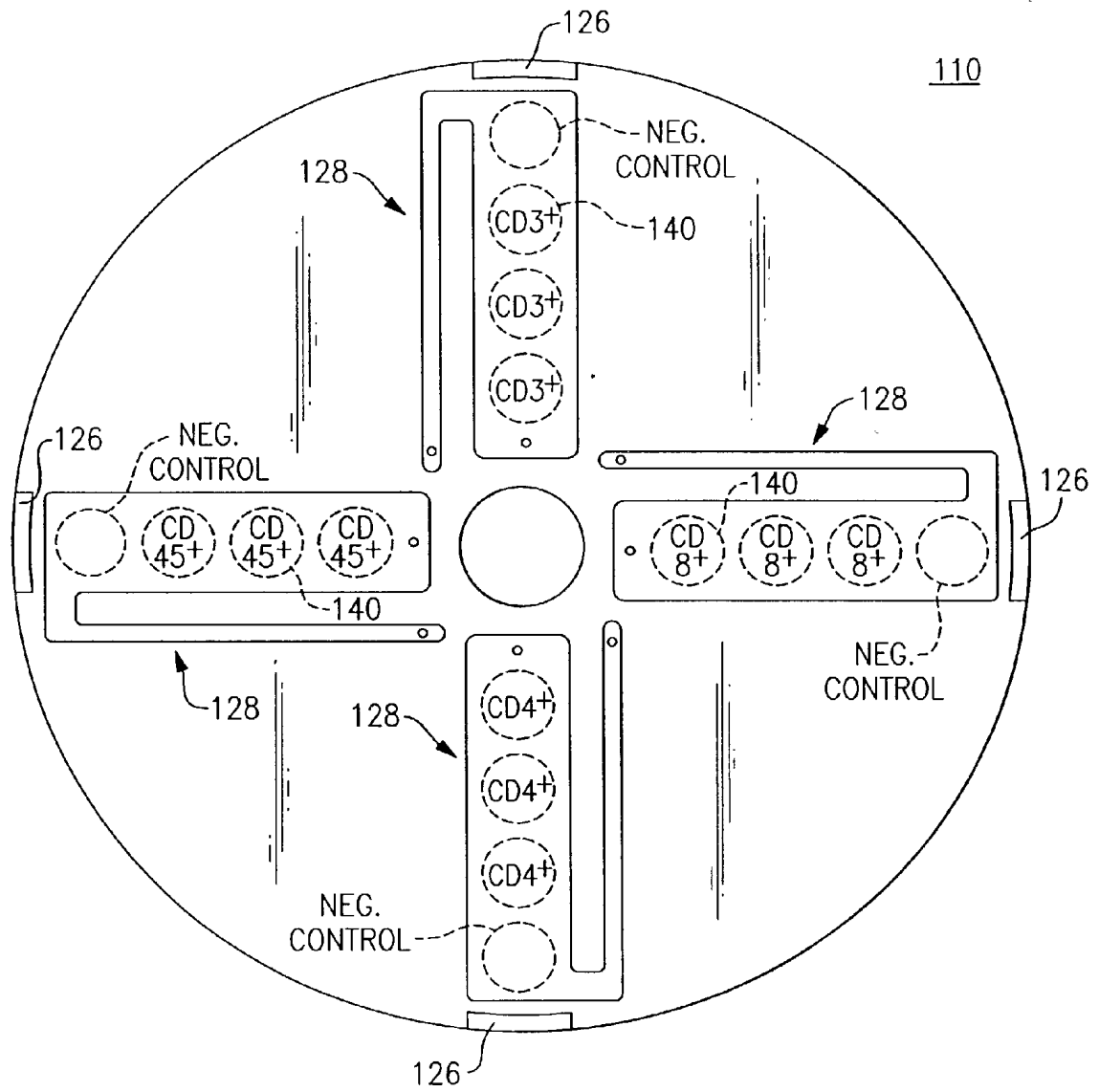


FIG.31A

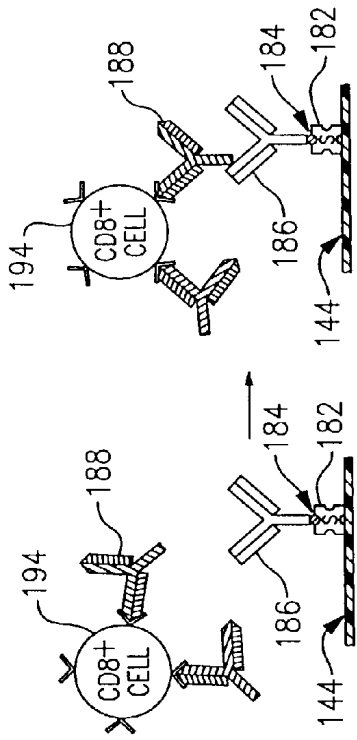


FIG. 31C

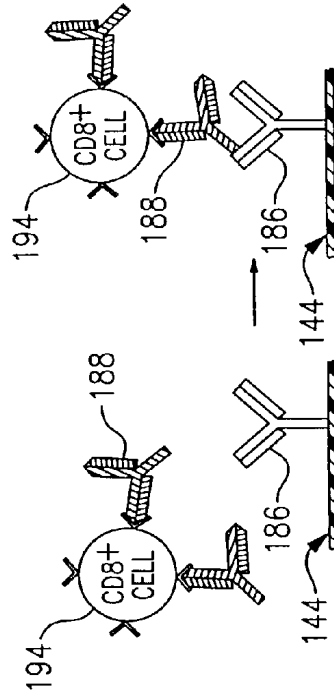


FIG. 31E

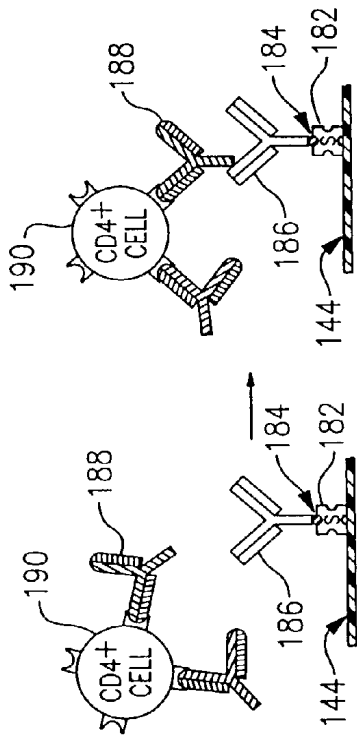


FIG. 31B

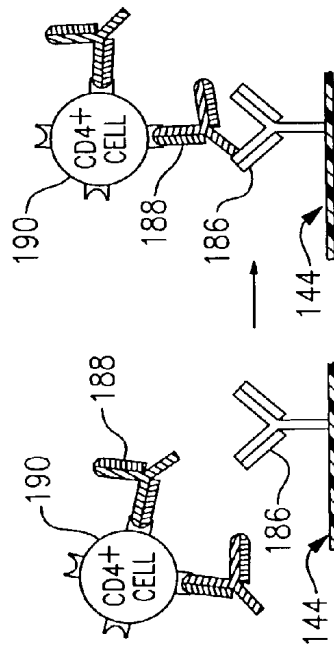


FIG. 31D

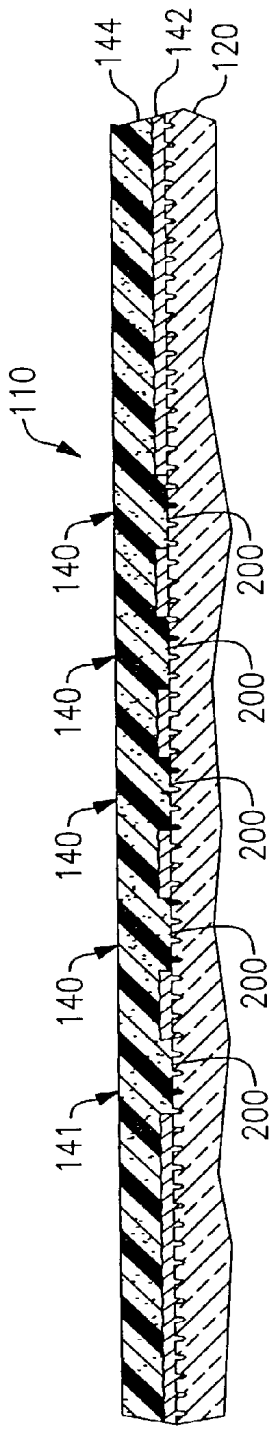


FIG. 32A

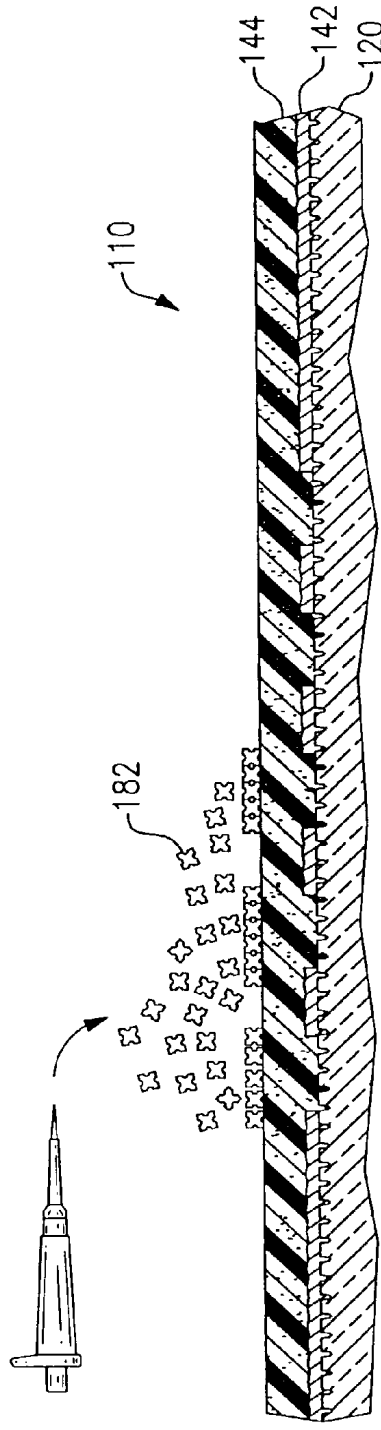


FIG. 32B

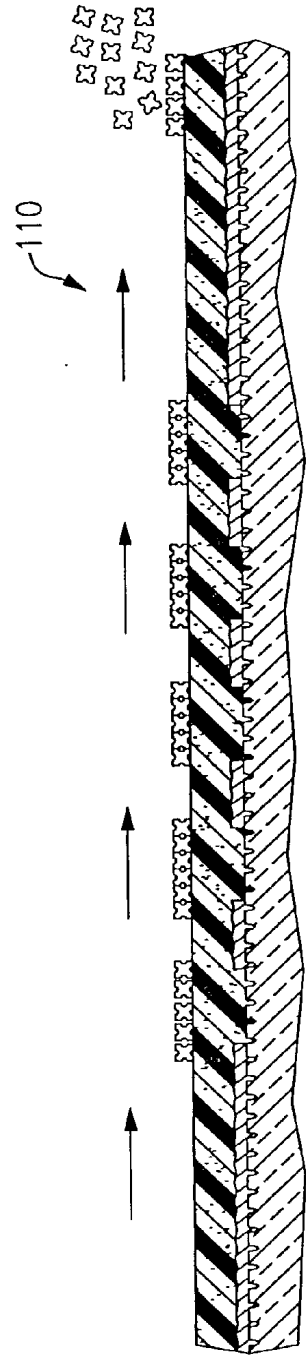


FIG. 32C

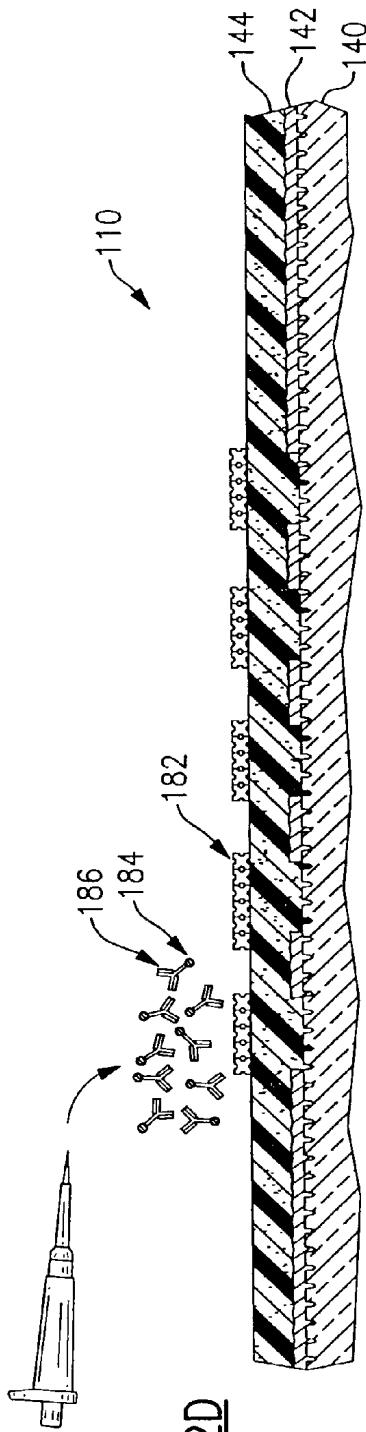


FIG. 32D

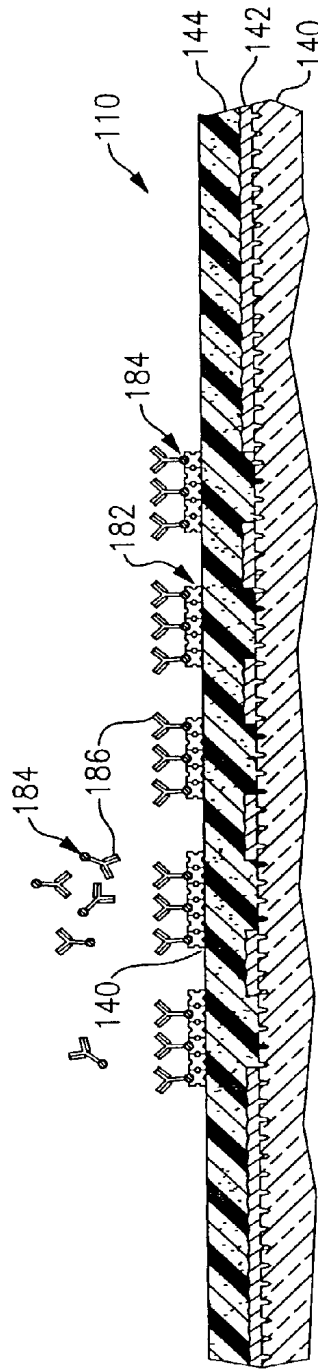


FIG. 32E

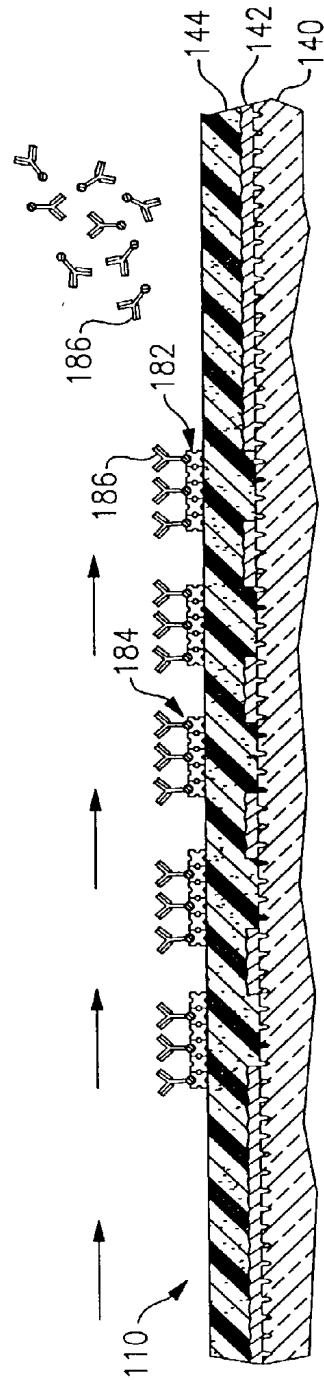


FIG. 32F

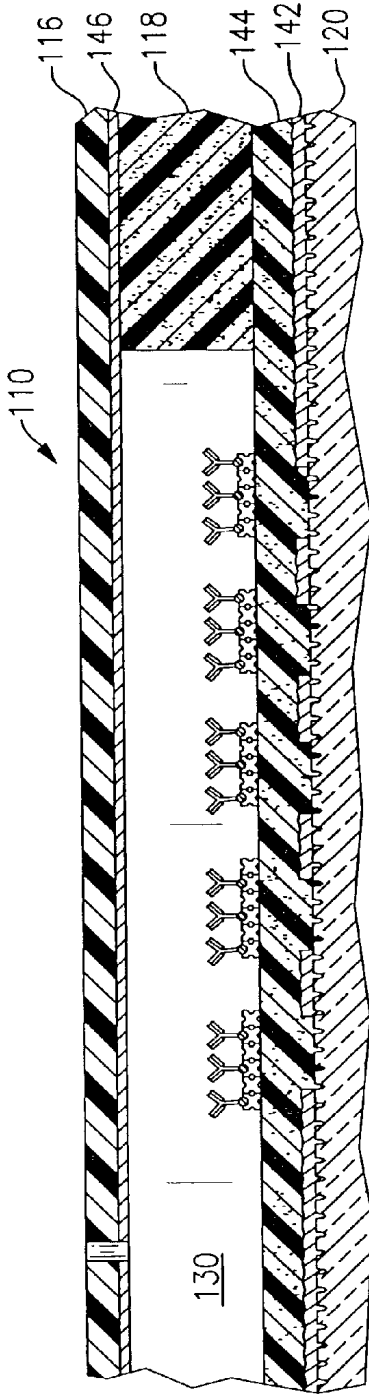


FIG. 32G

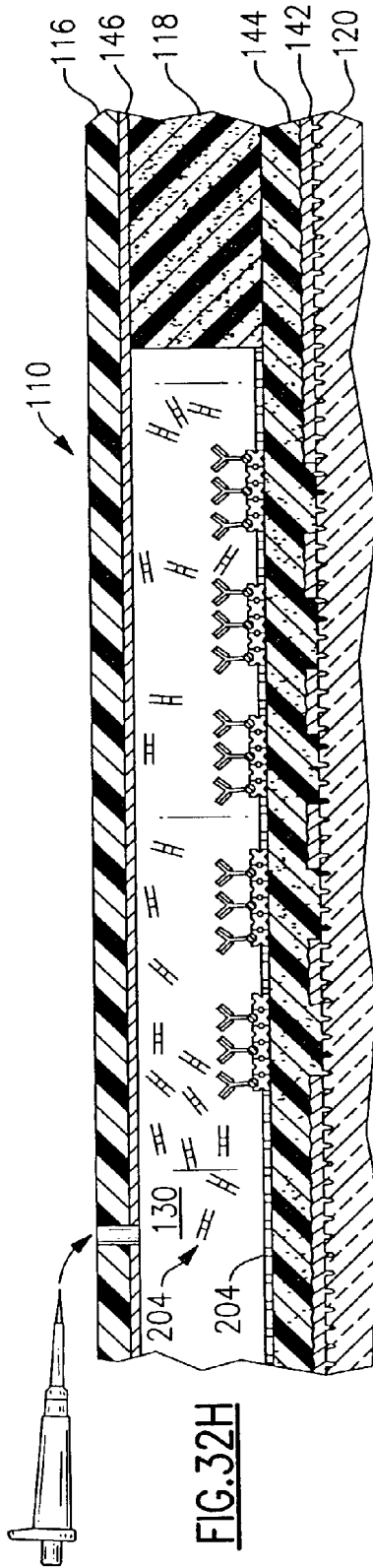


FIG. 32H

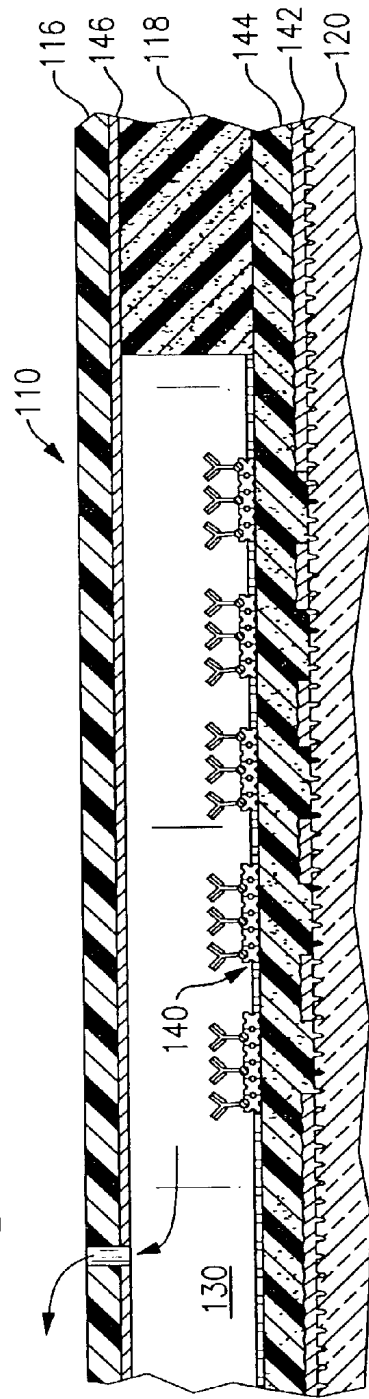


FIG. 32I

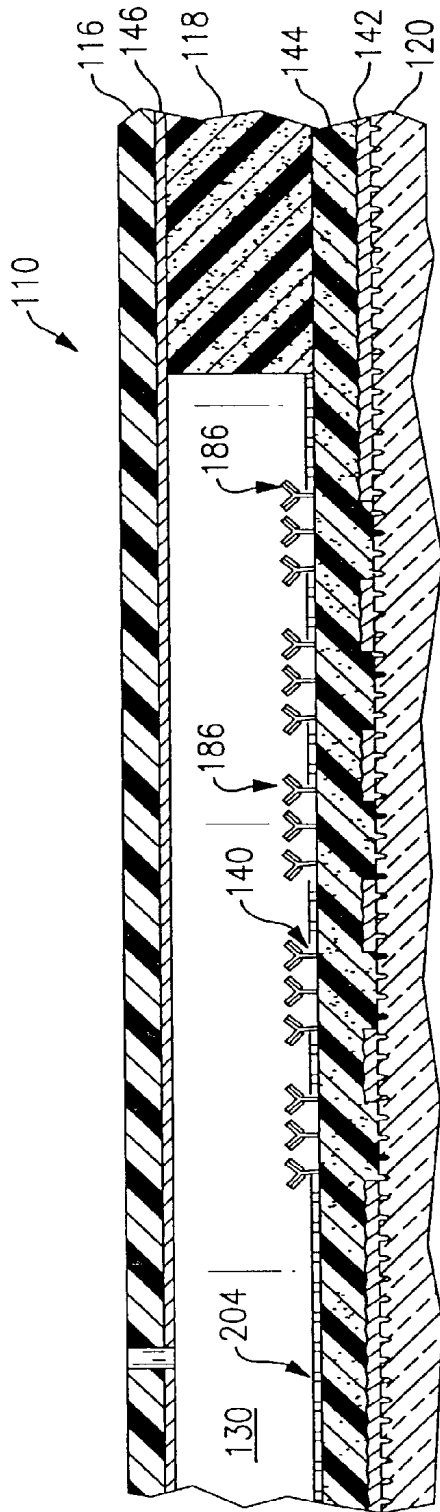


FIG. 33

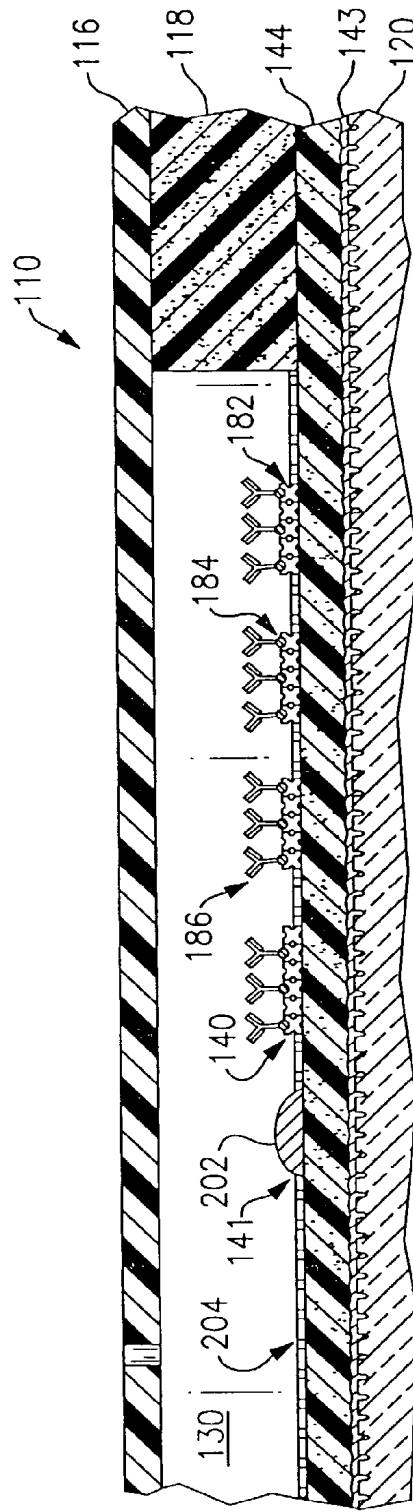


FIG. 34

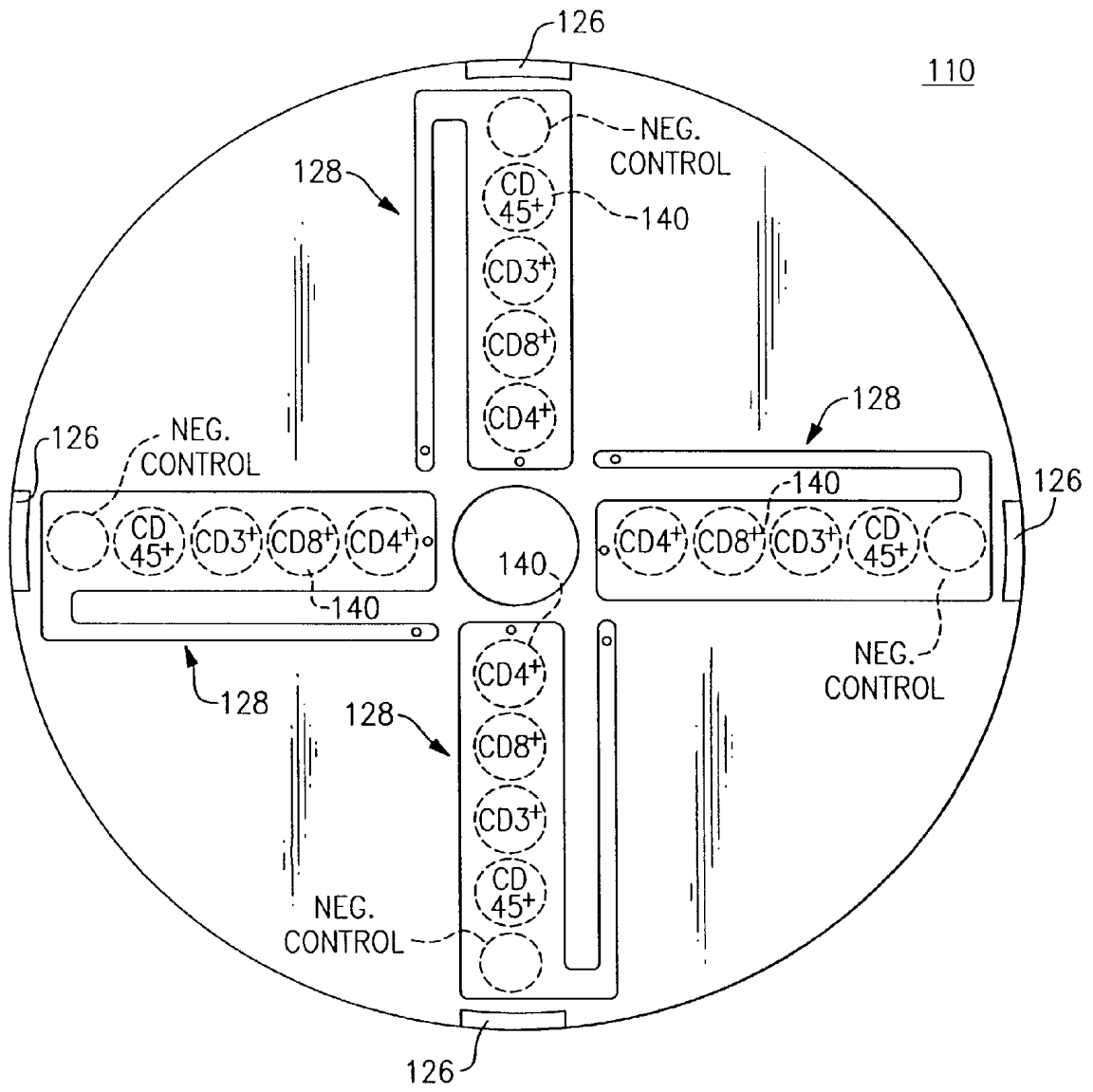
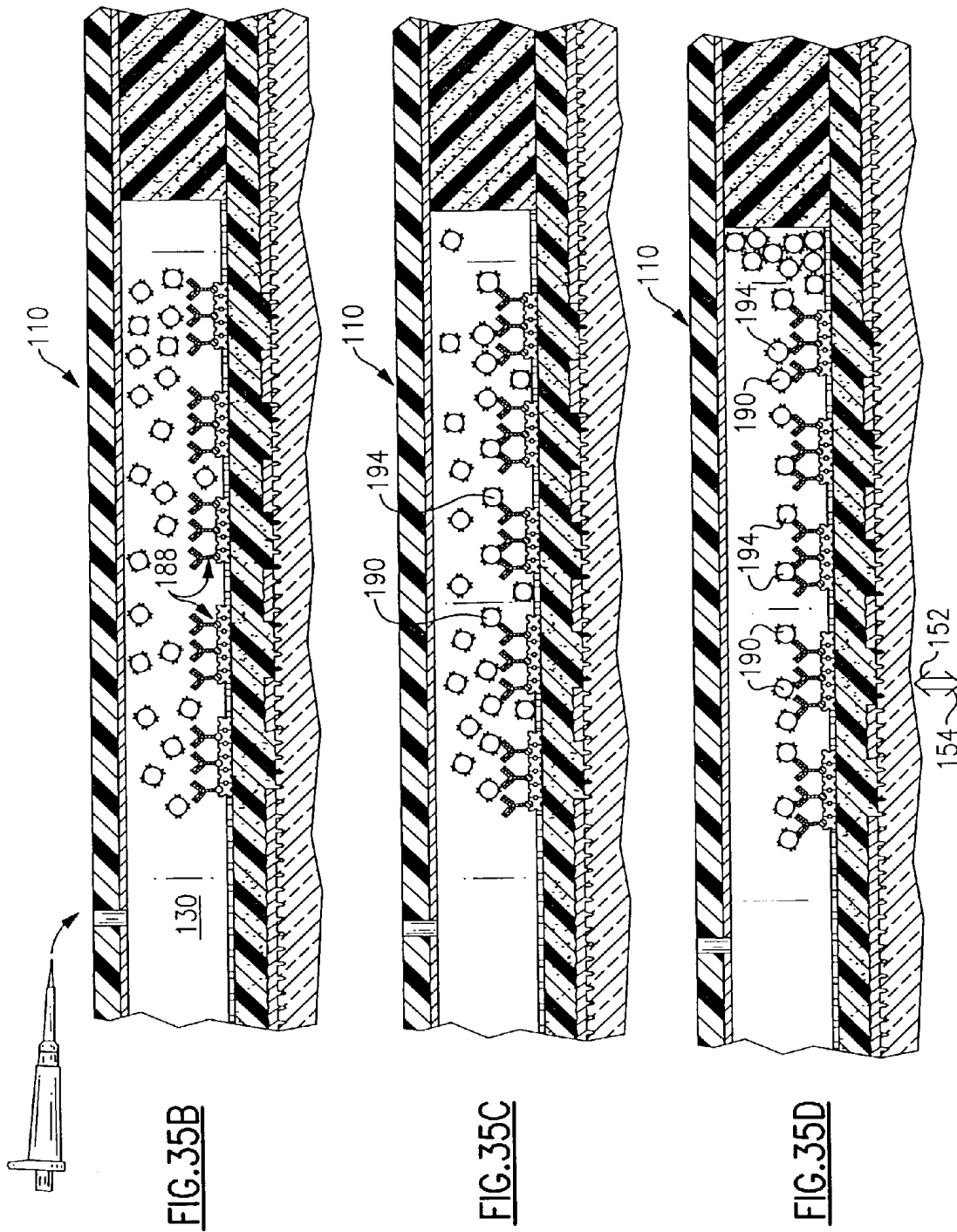


FIG.35A



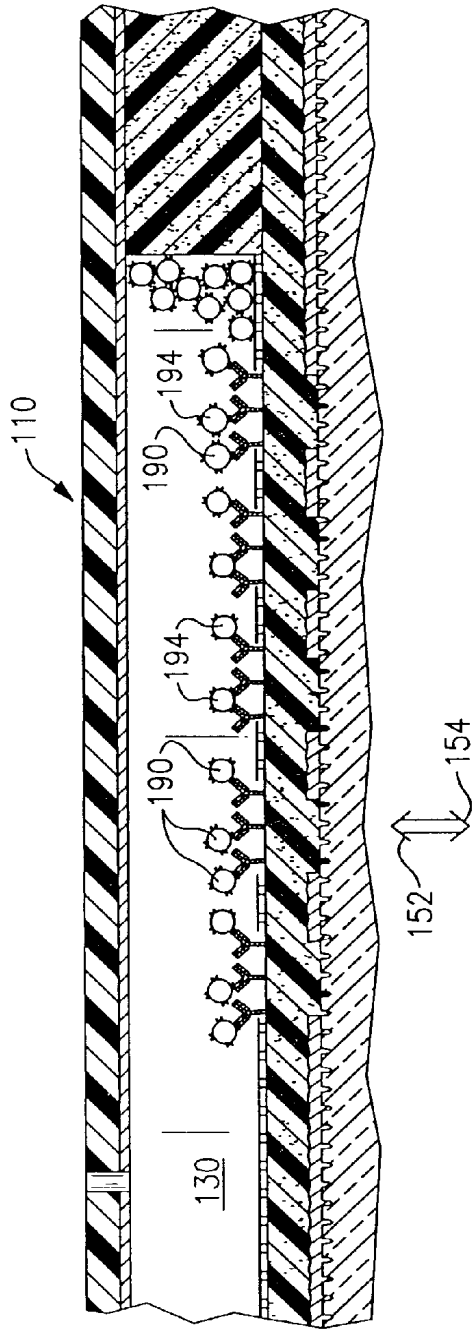


FIG. 36

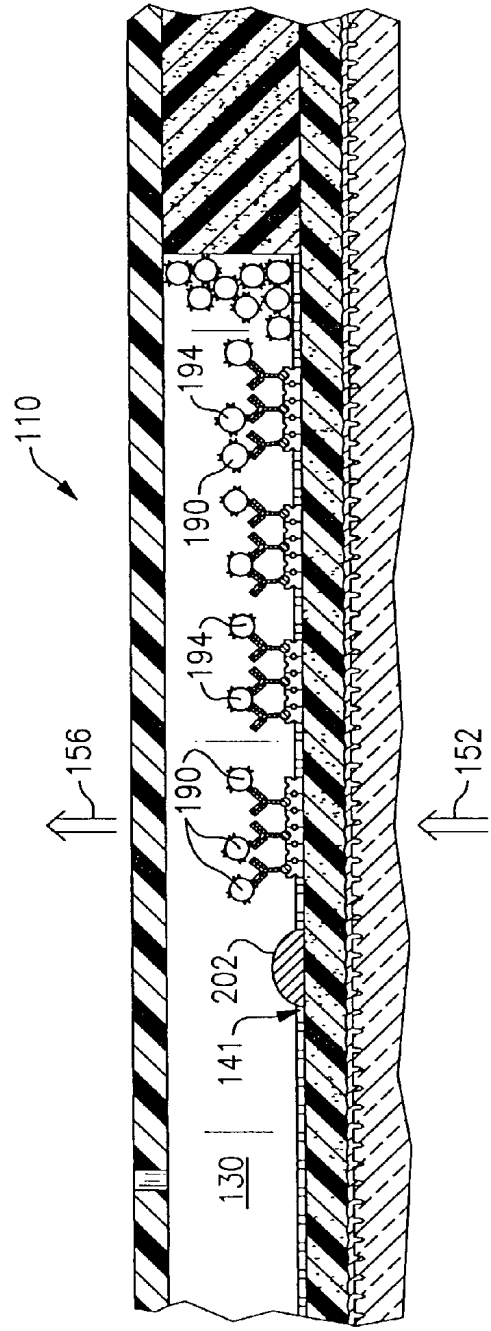
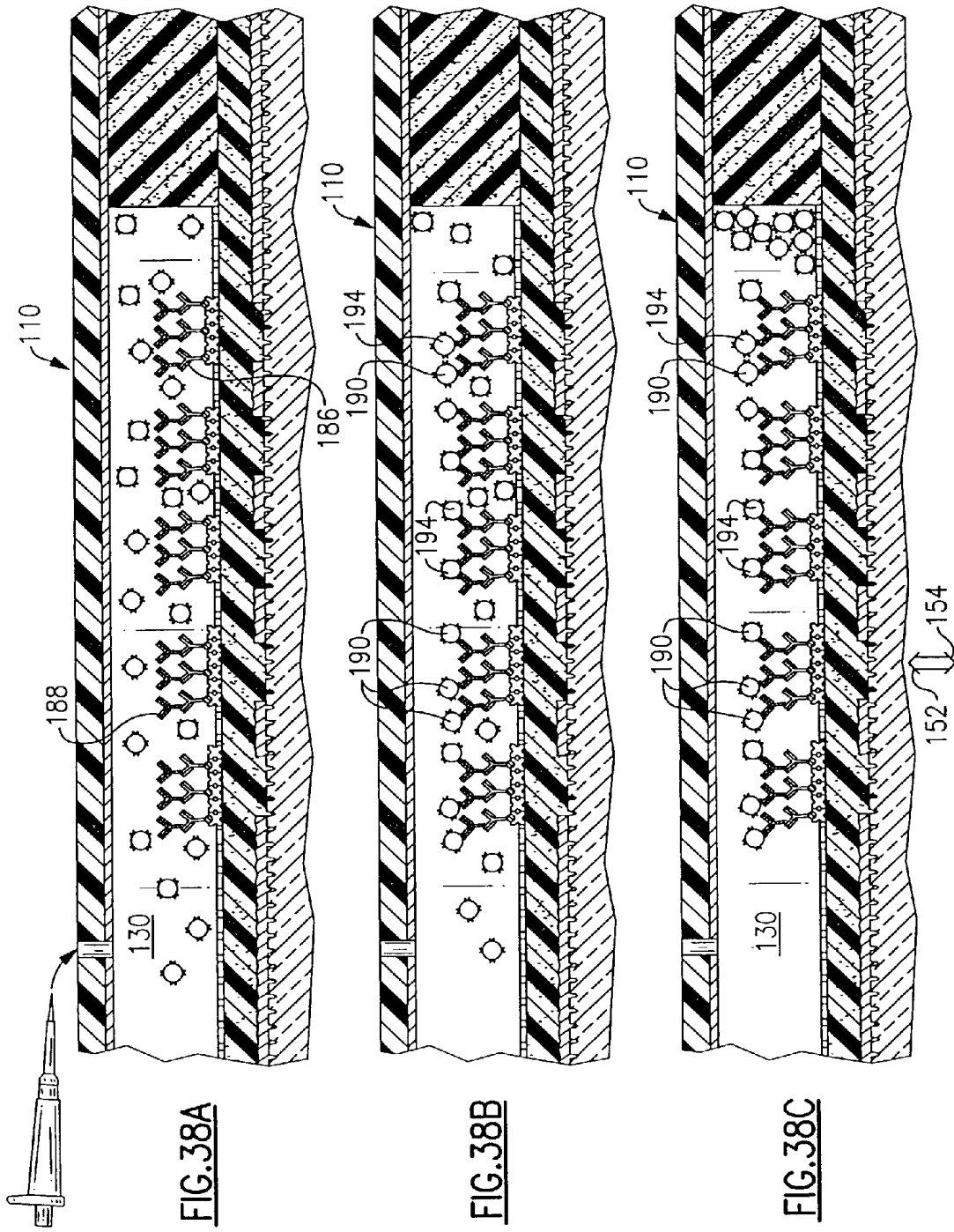


FIG. 37



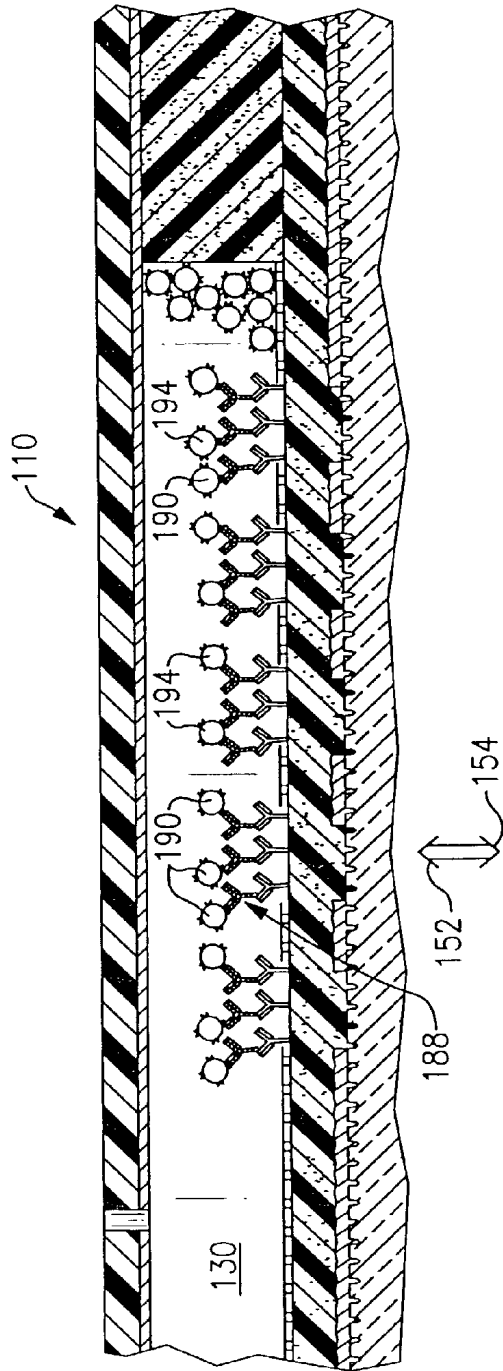


FIG. 39

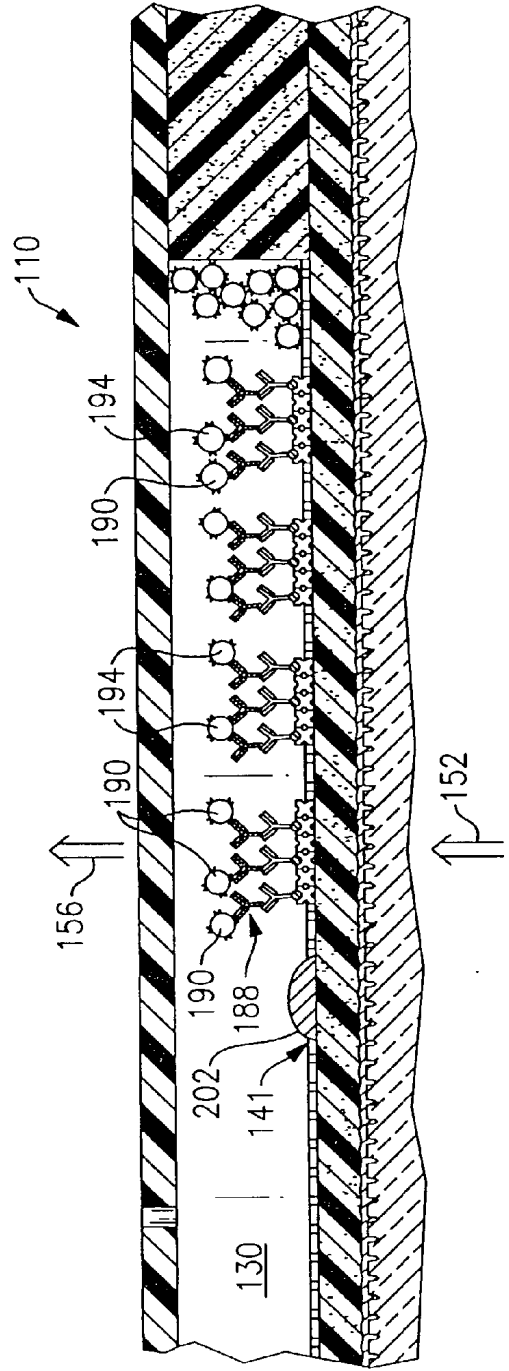
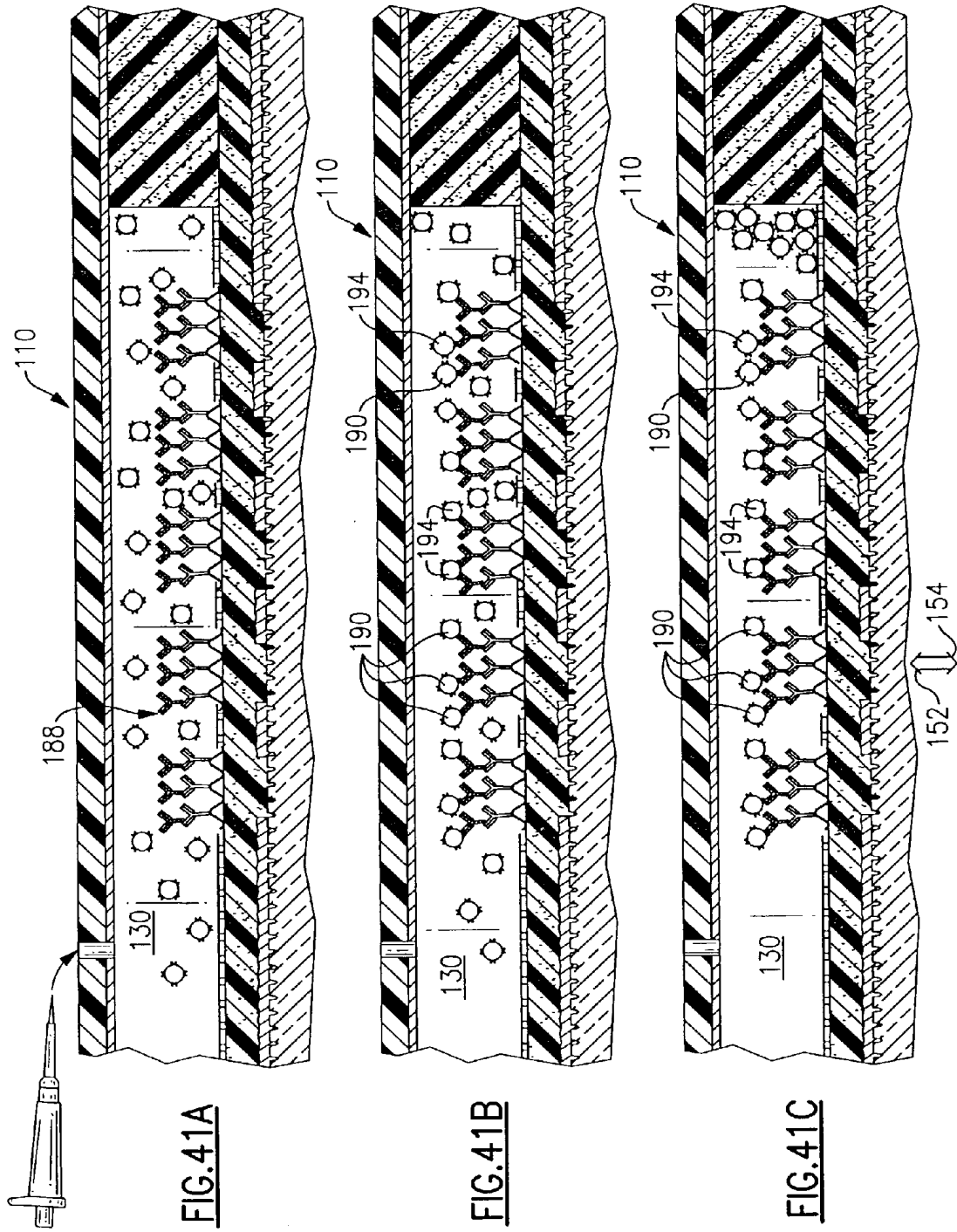


FIG. 40



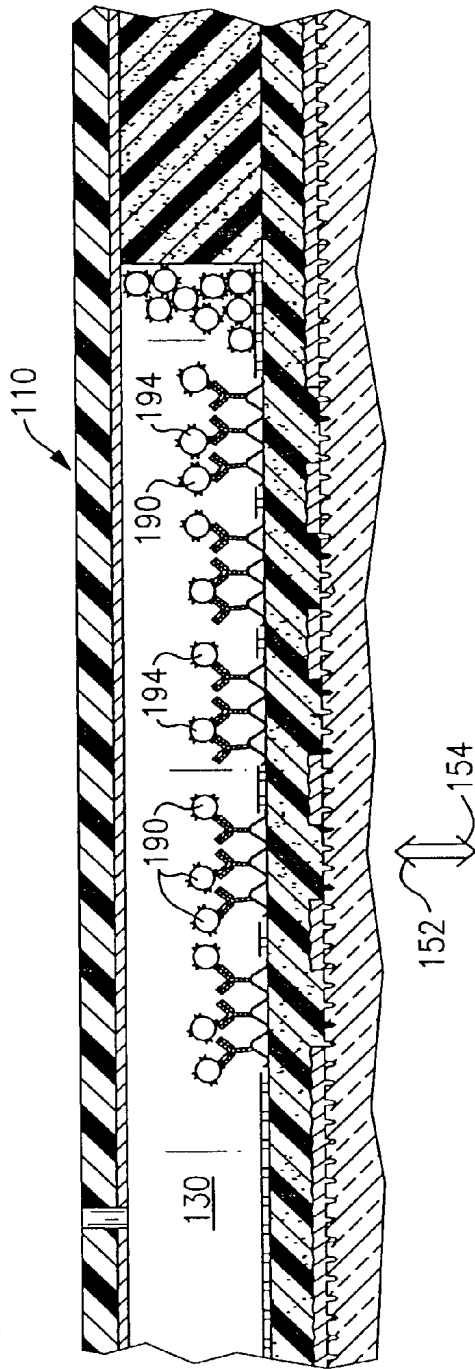


FIG. 42

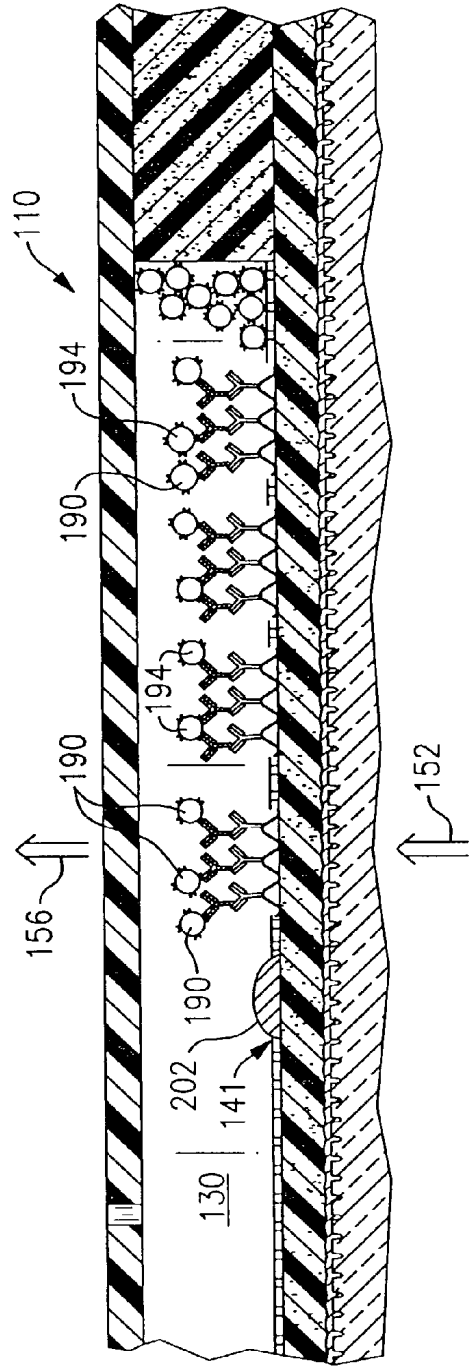


FIG. 43

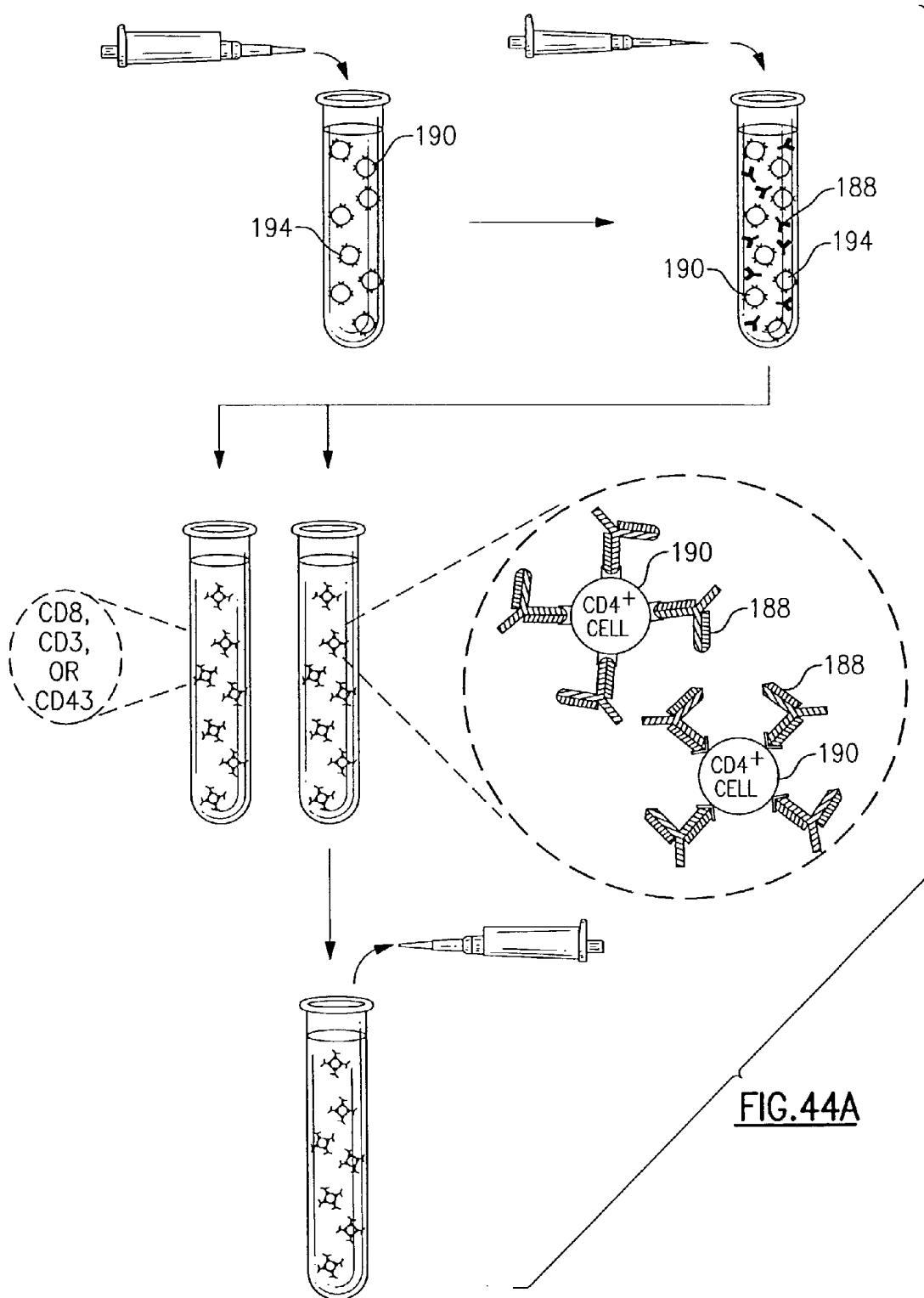


FIG.44A

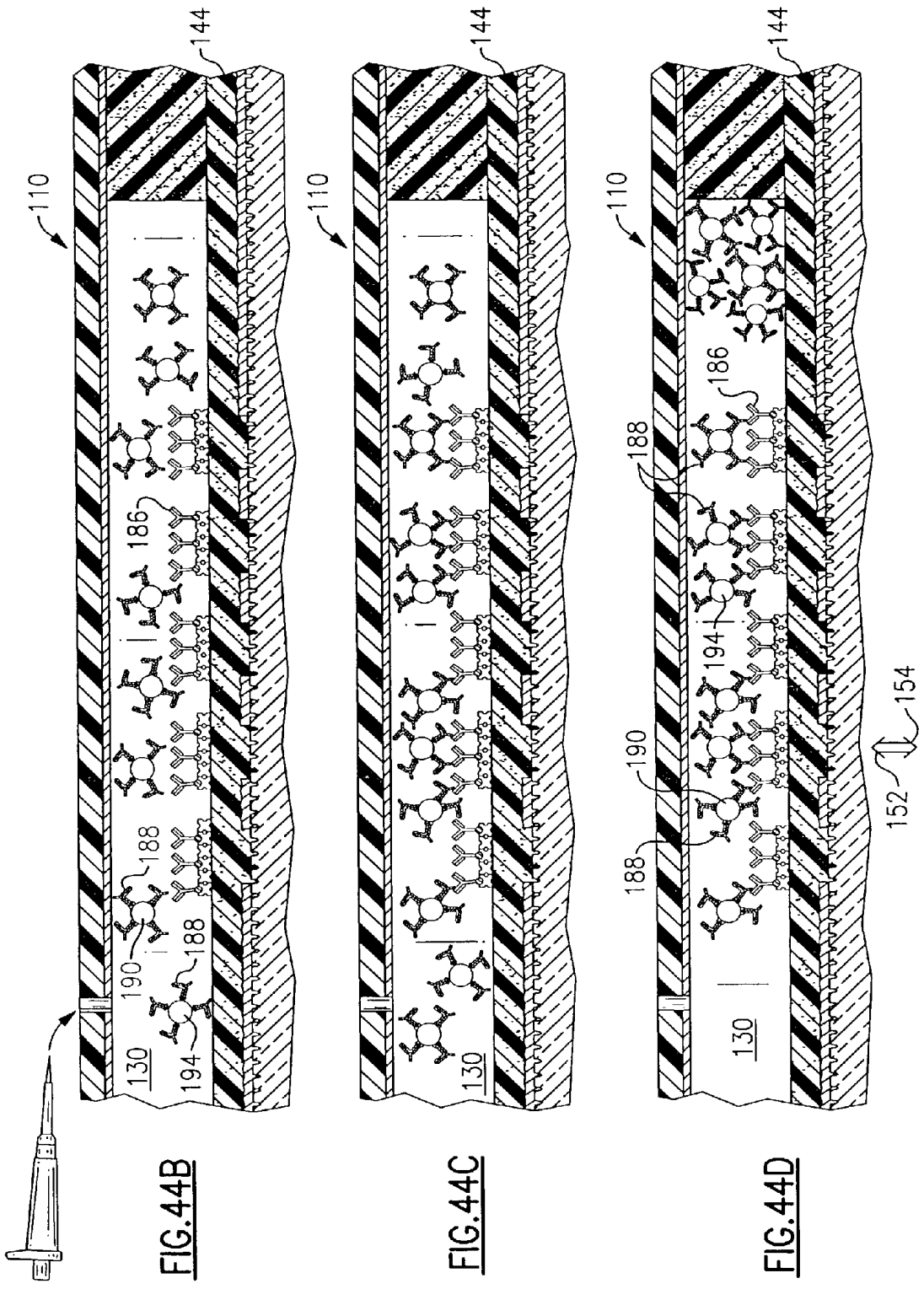


FIG. 44B

FIG. 44C

FIG. 44D

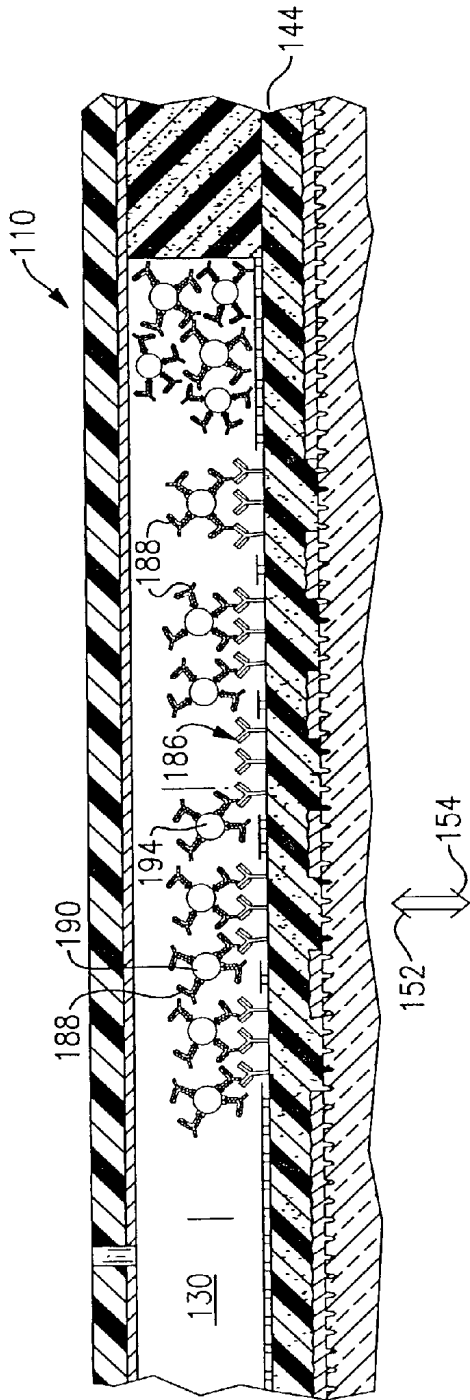


FIG. 45

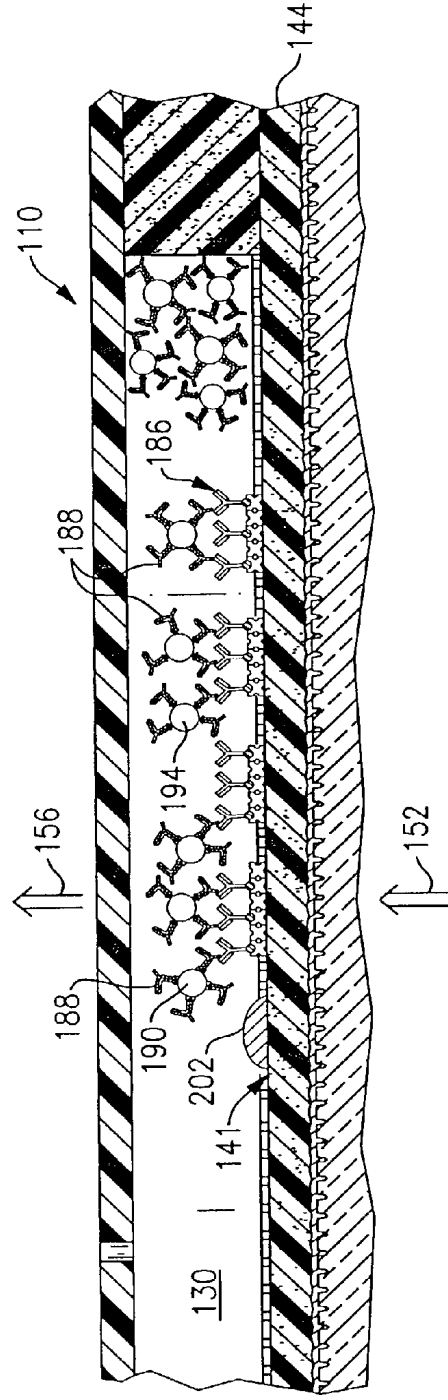


FIG. 46

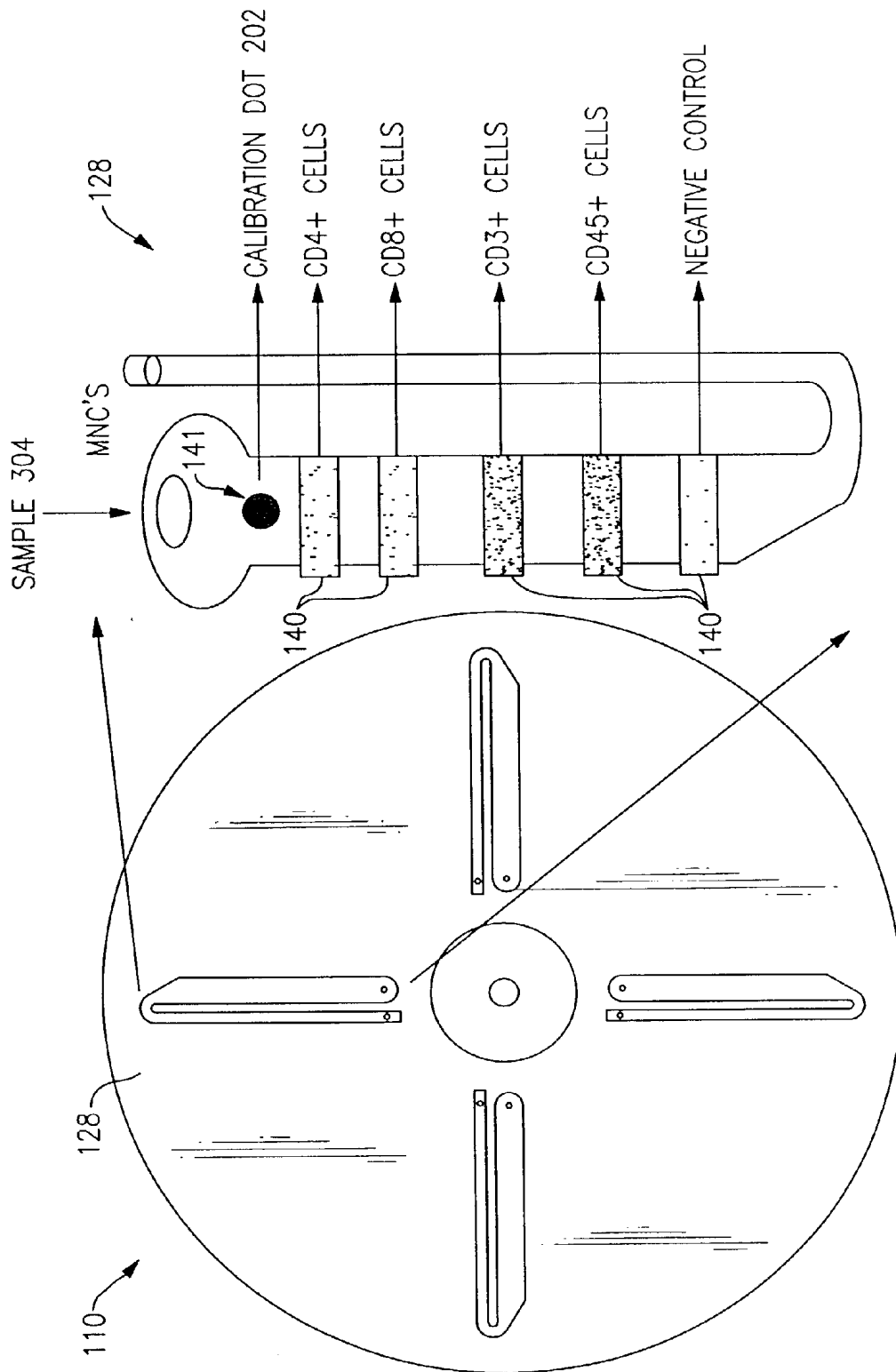


FIG.47

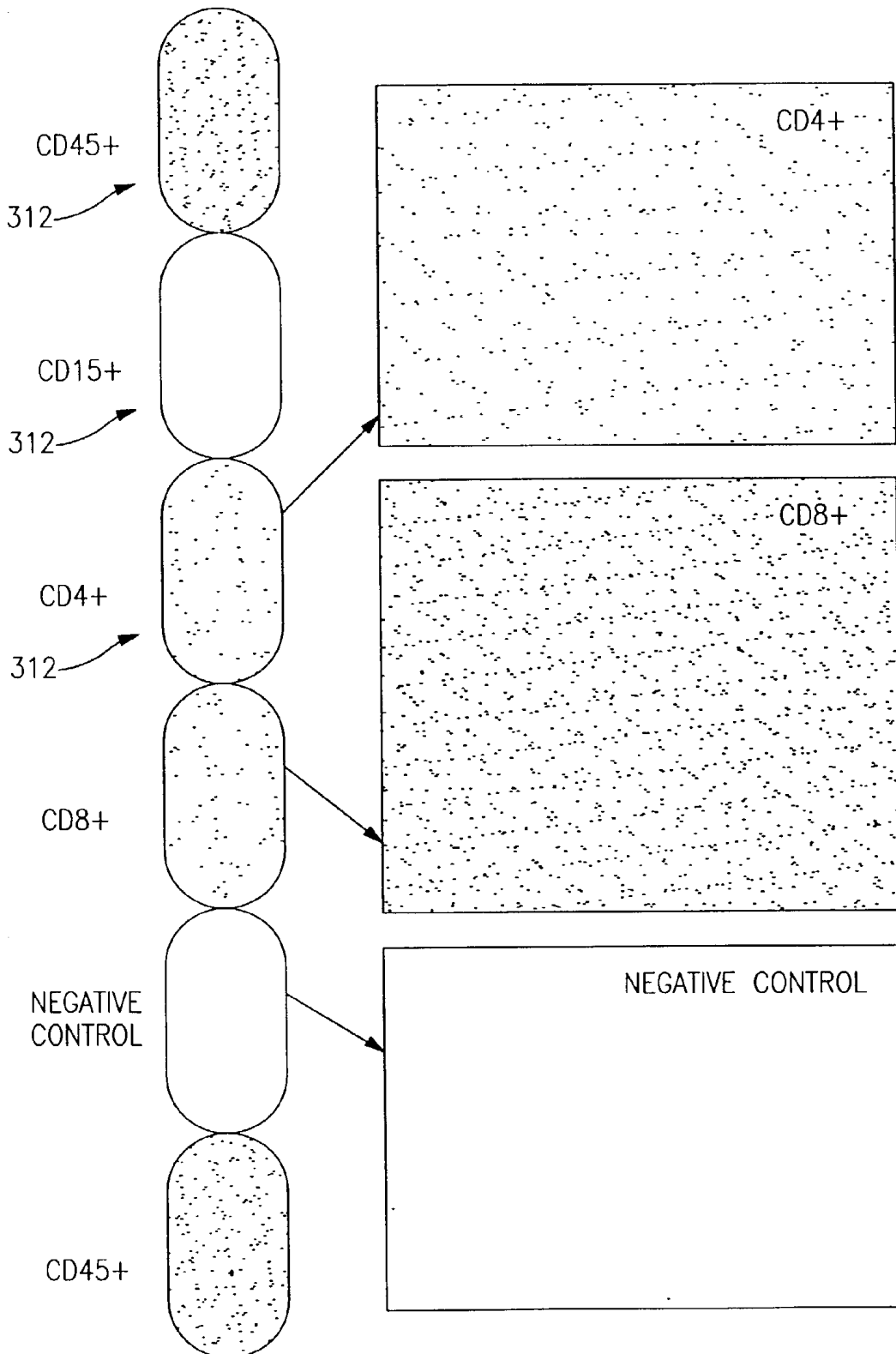


FIG.48A

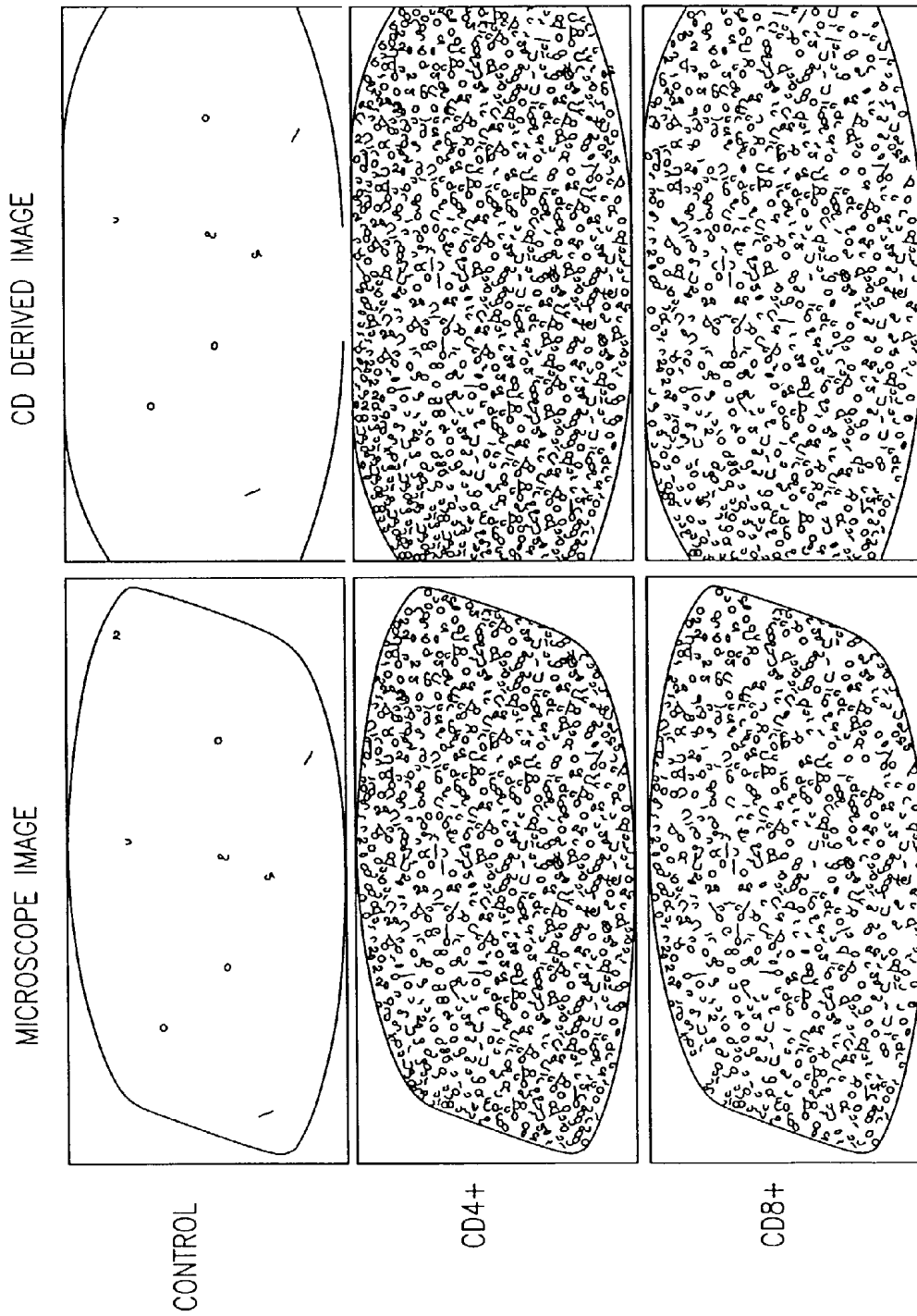
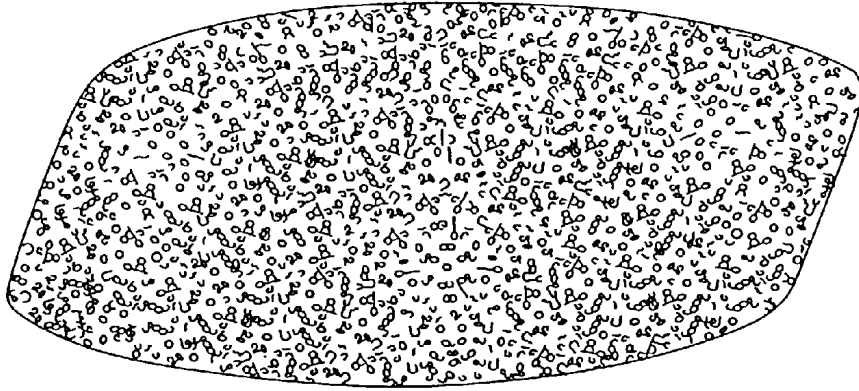


FIG. 48B

CD DERIVED IMAGE



MICROSCOPE IMAGE

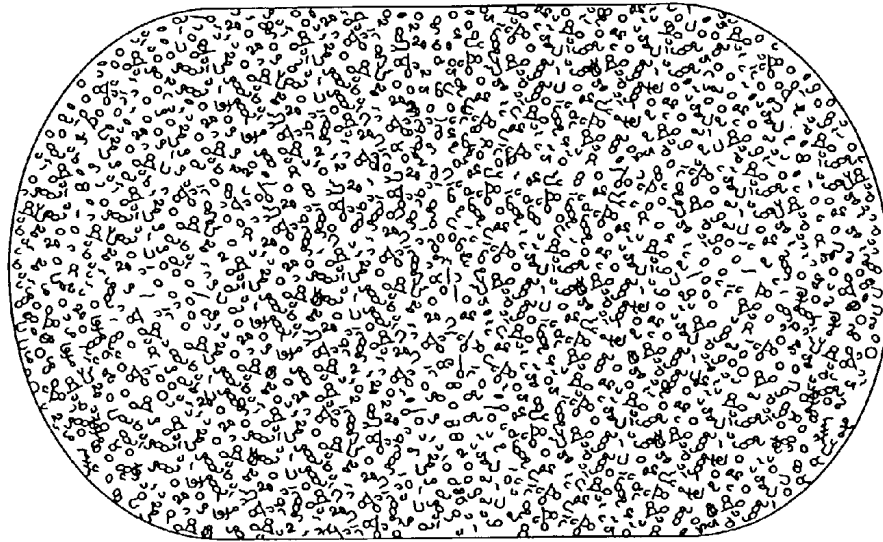


FIG. 49

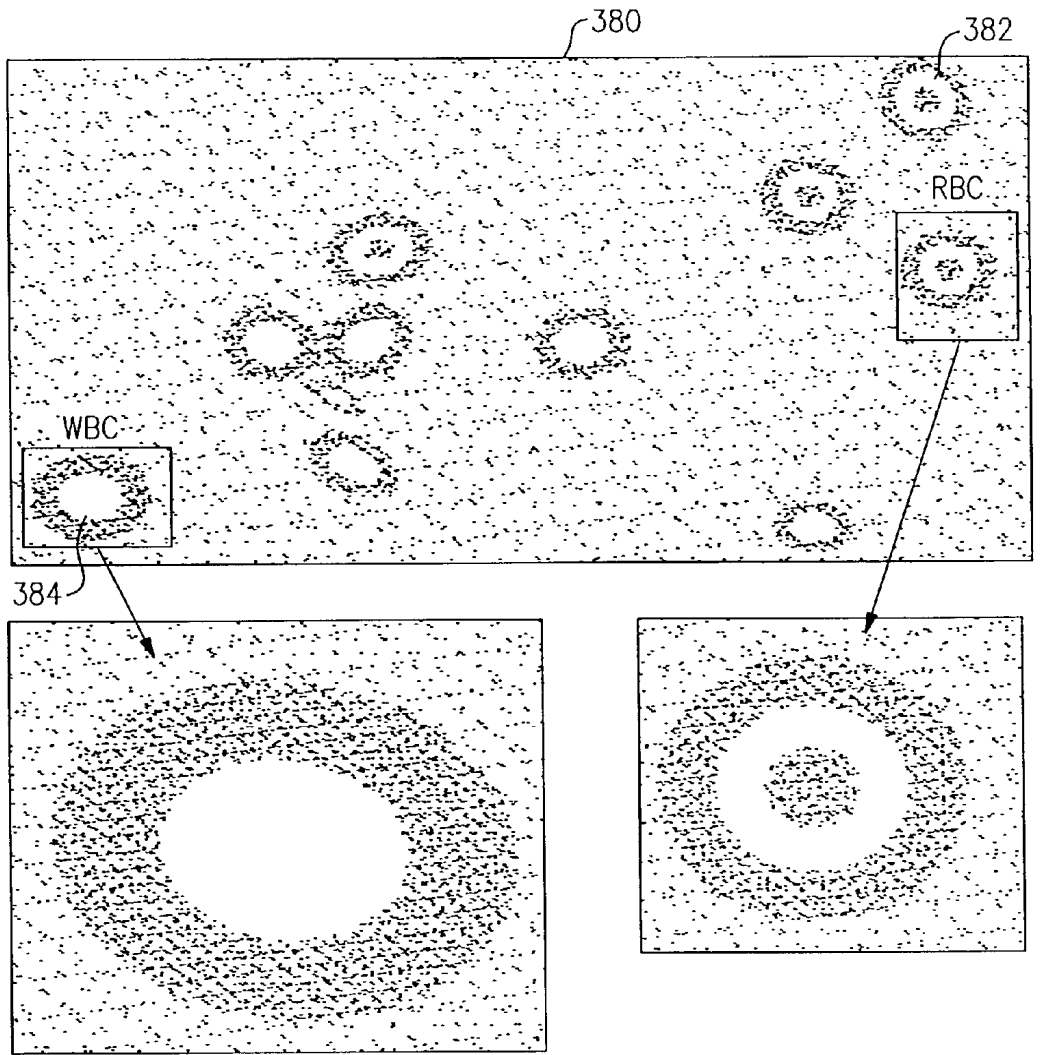


FIG.50

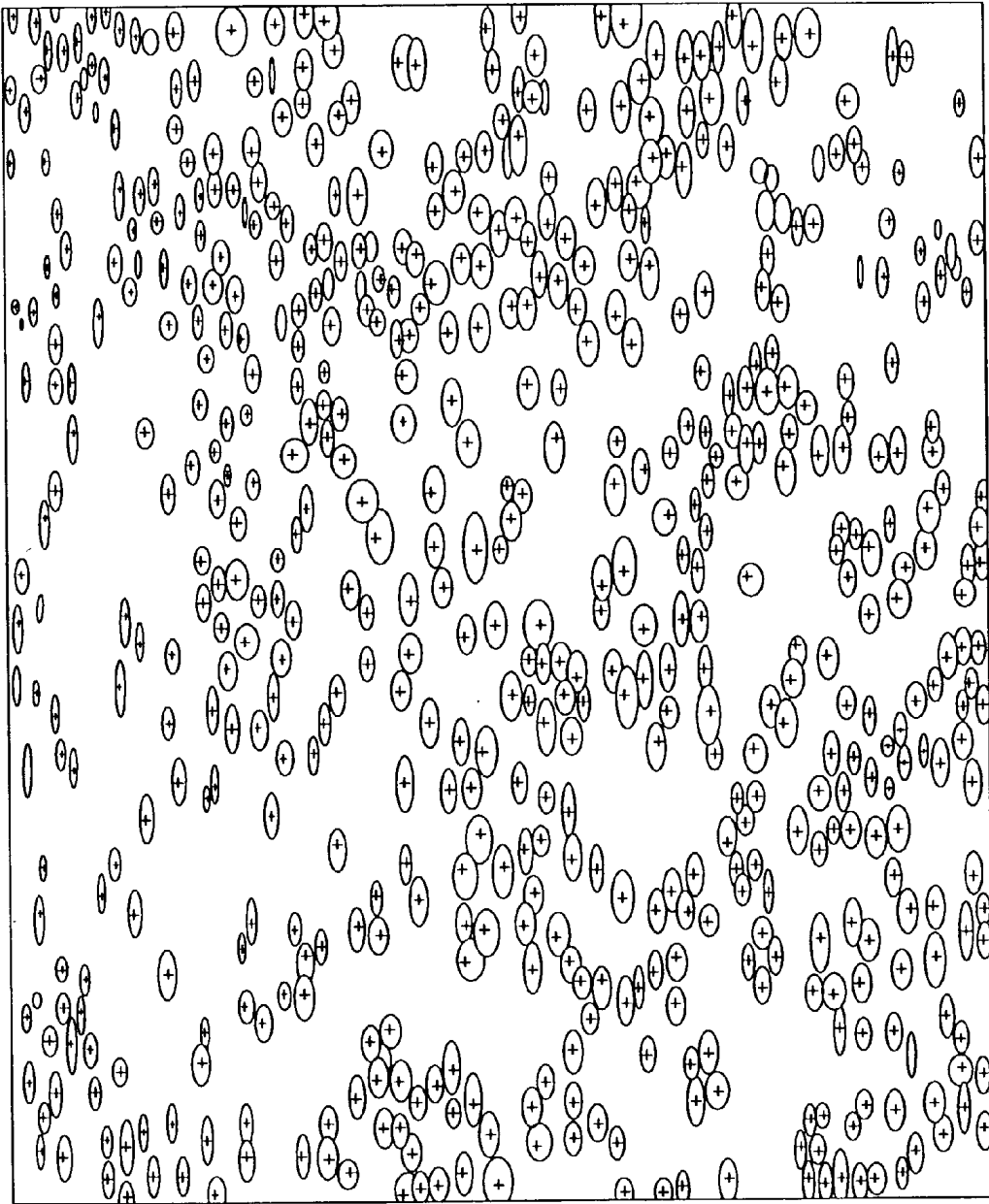


FIG. 51

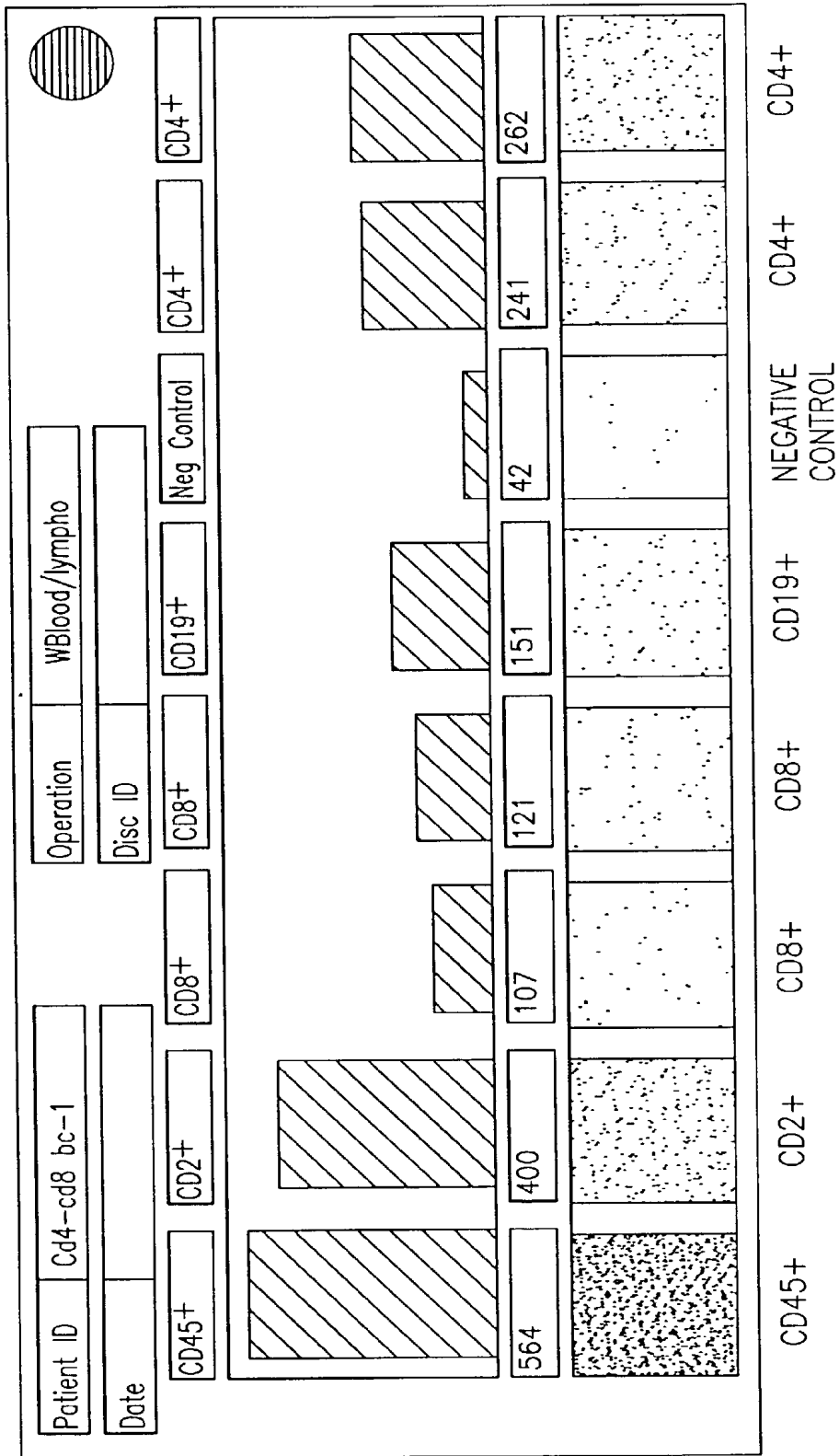


FIG.52

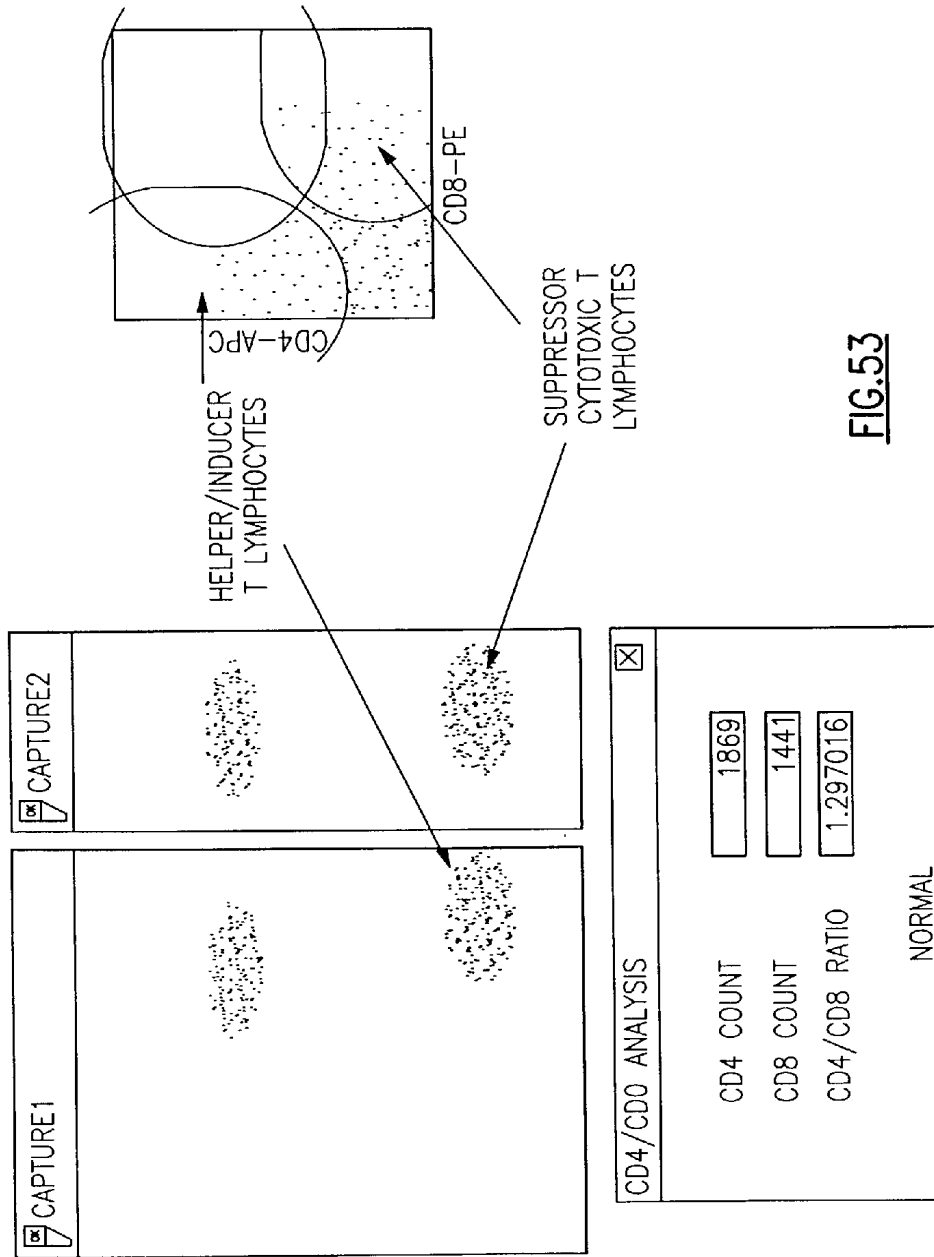


FIG.53

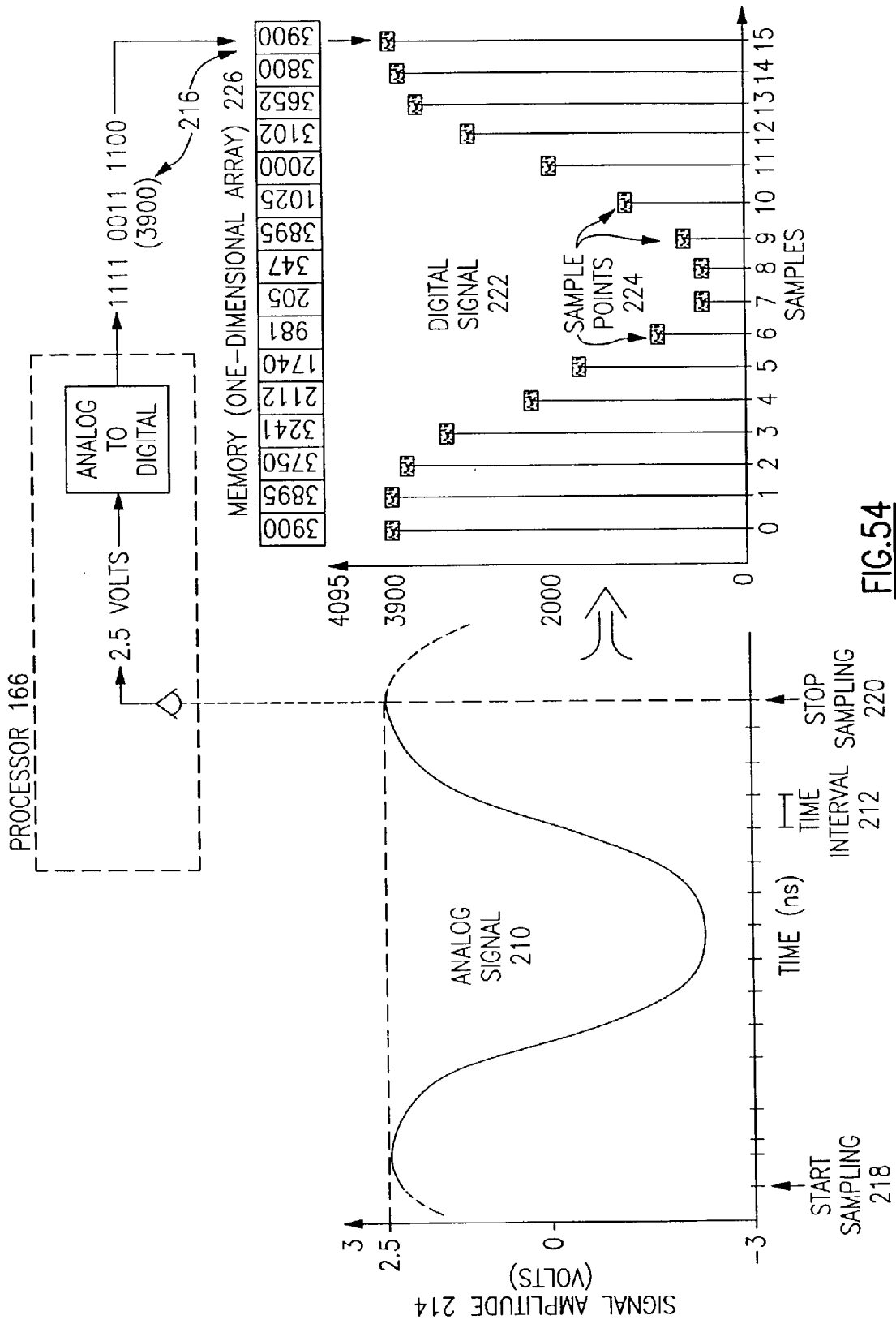


FIG. 54

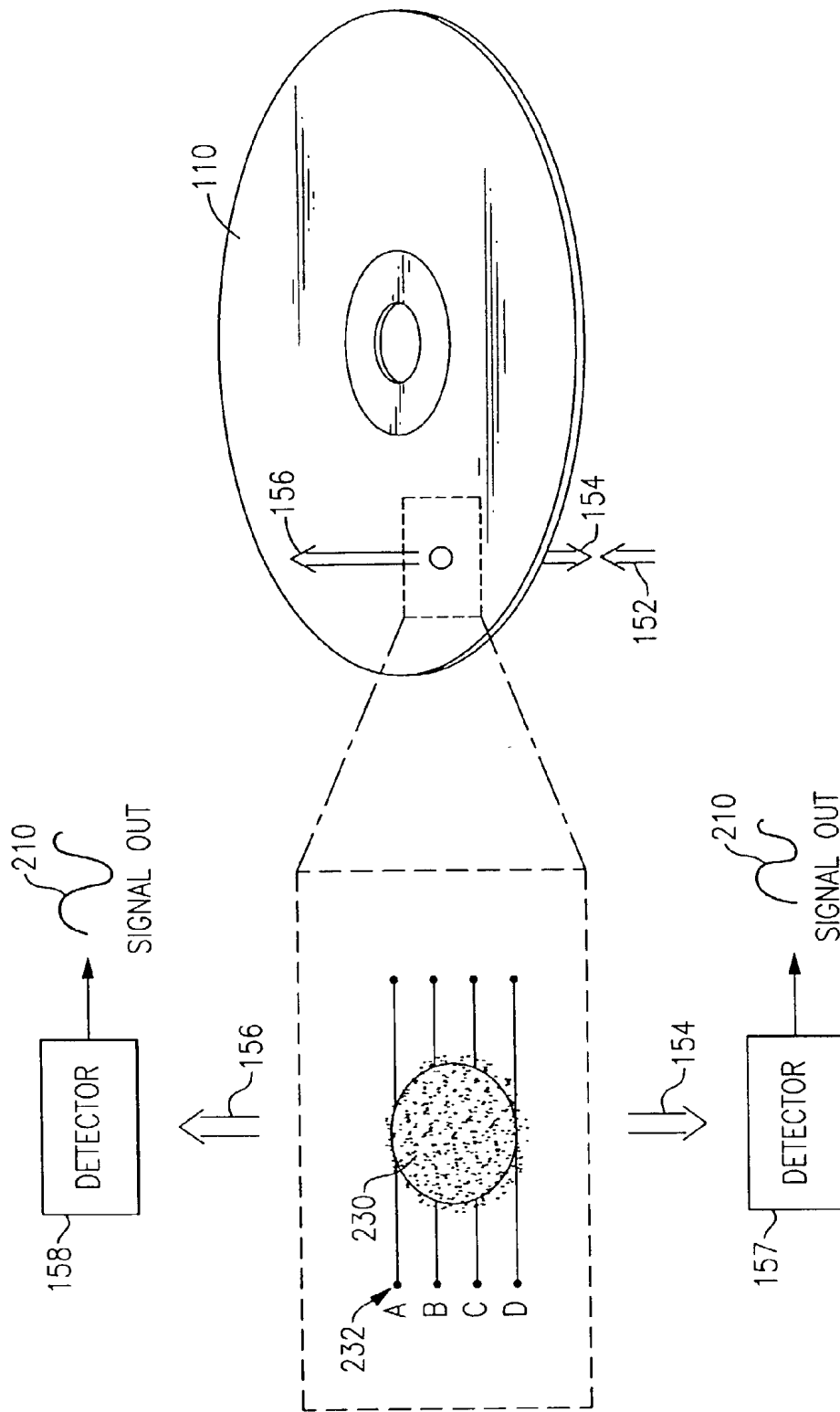


FIG.55

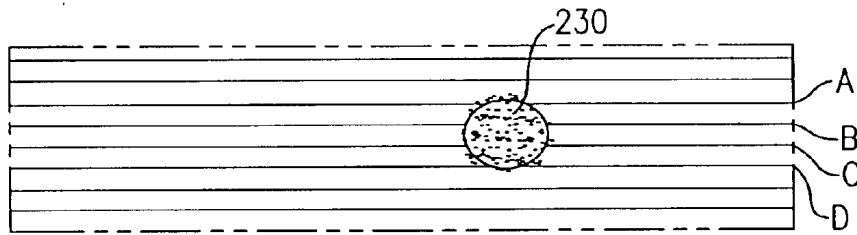


FIG.56A

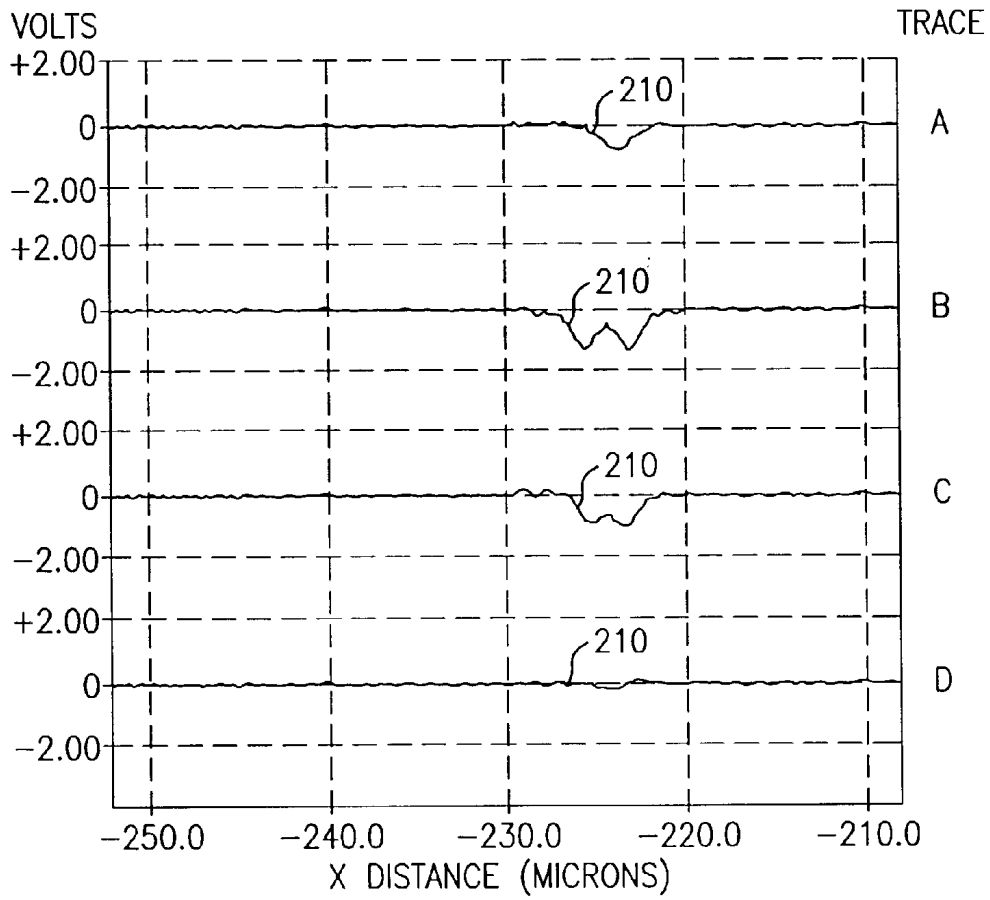


FIG.56B

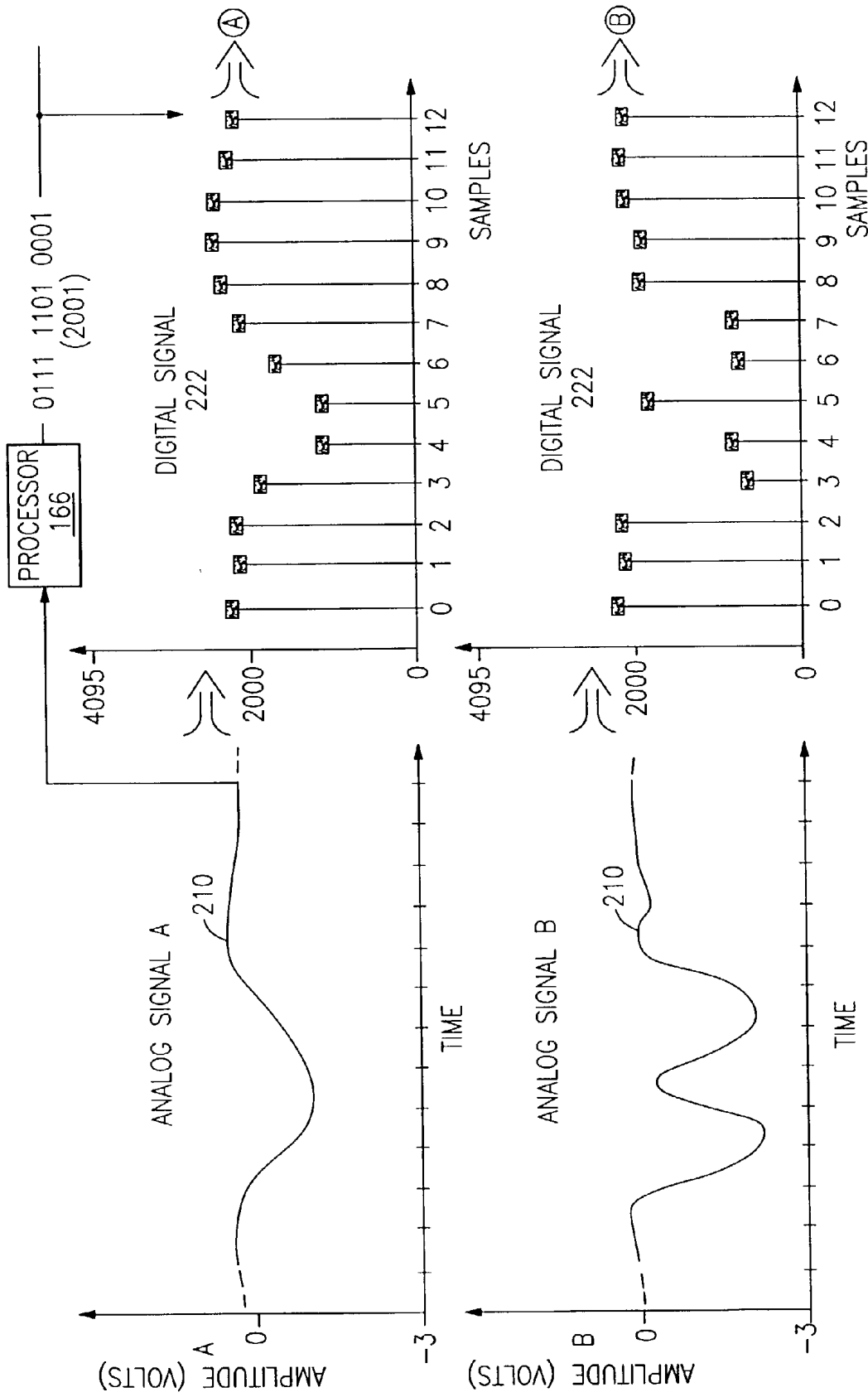


FIG.57A

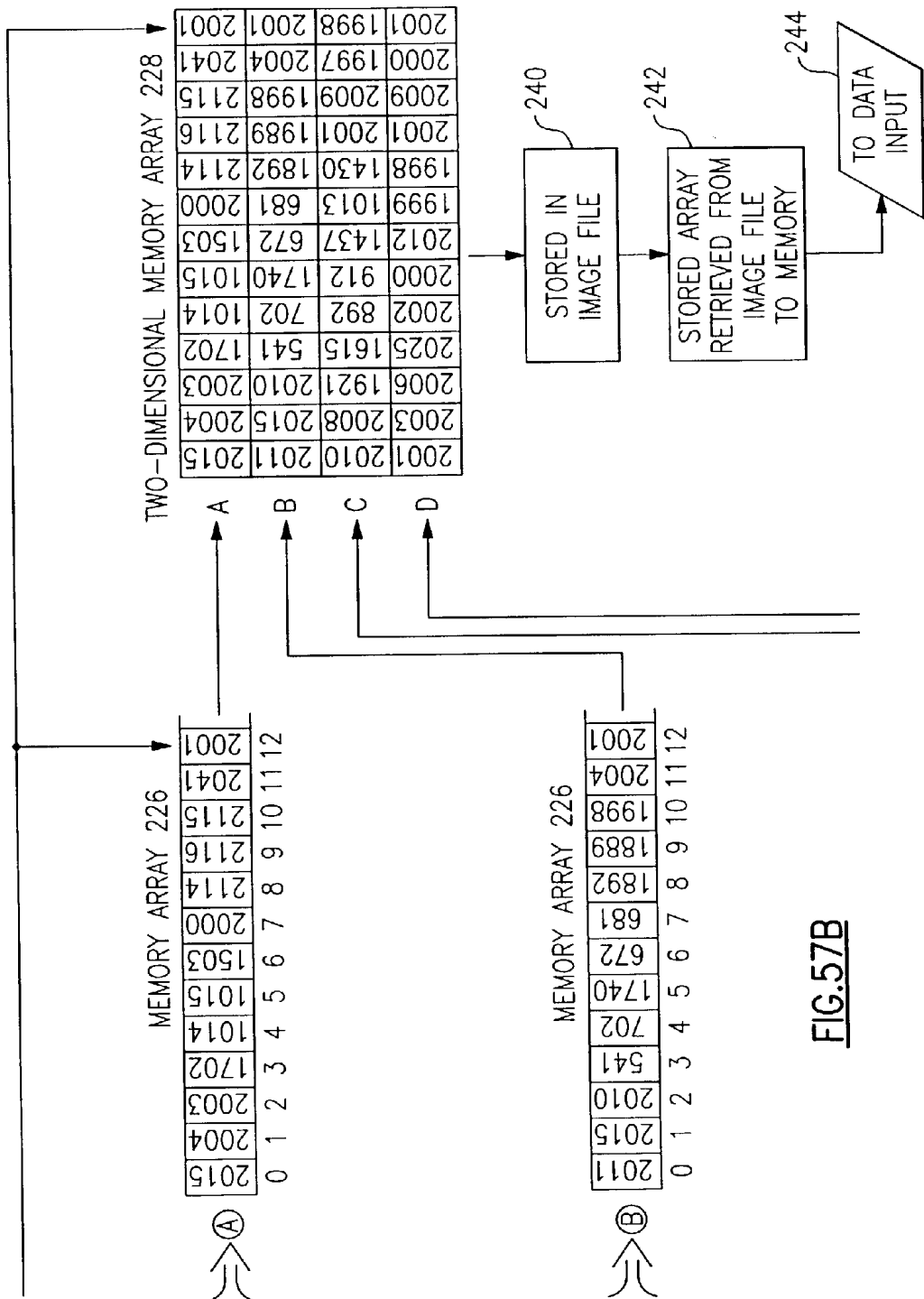


FIG.57B

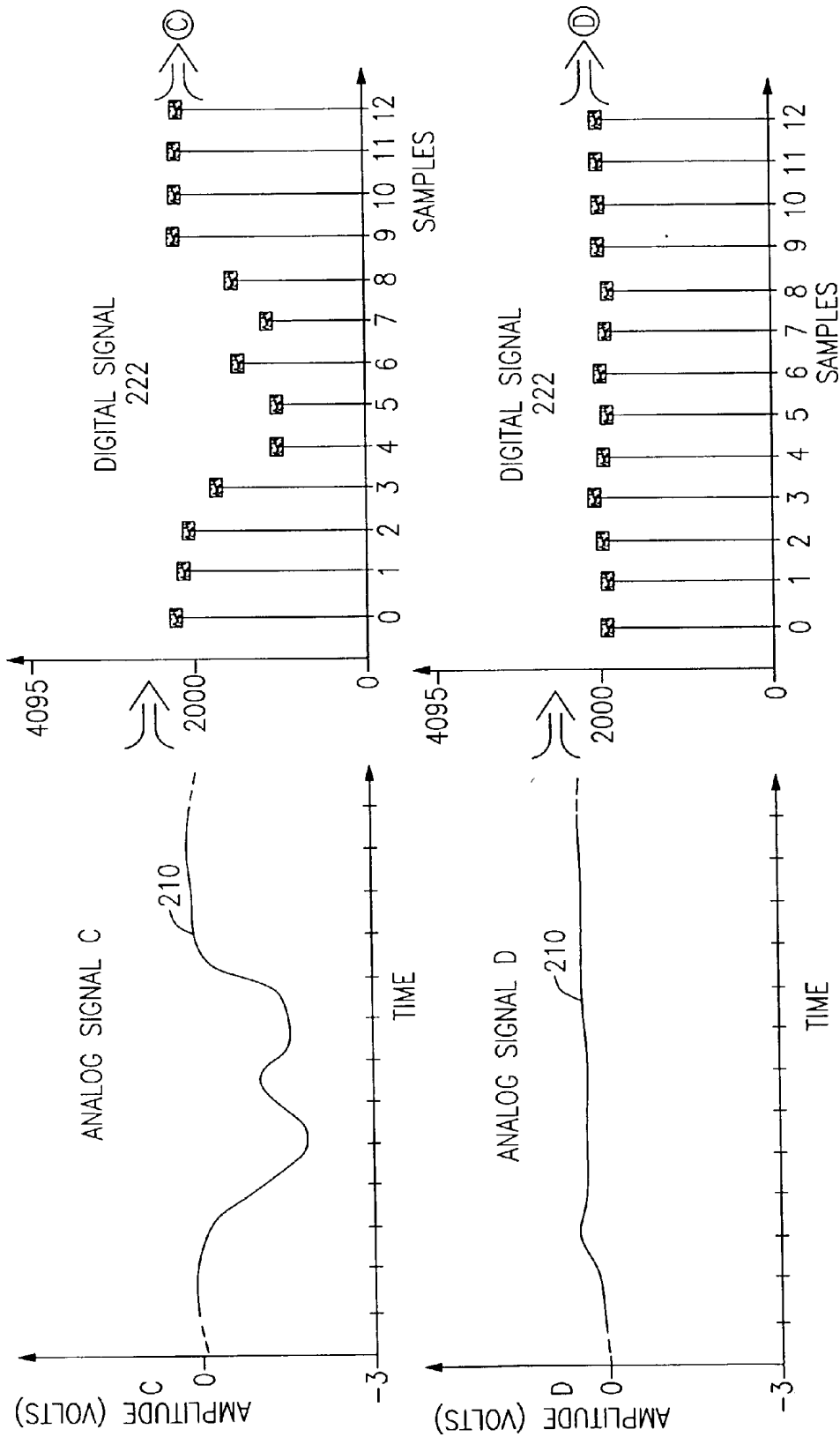


FIG.57C

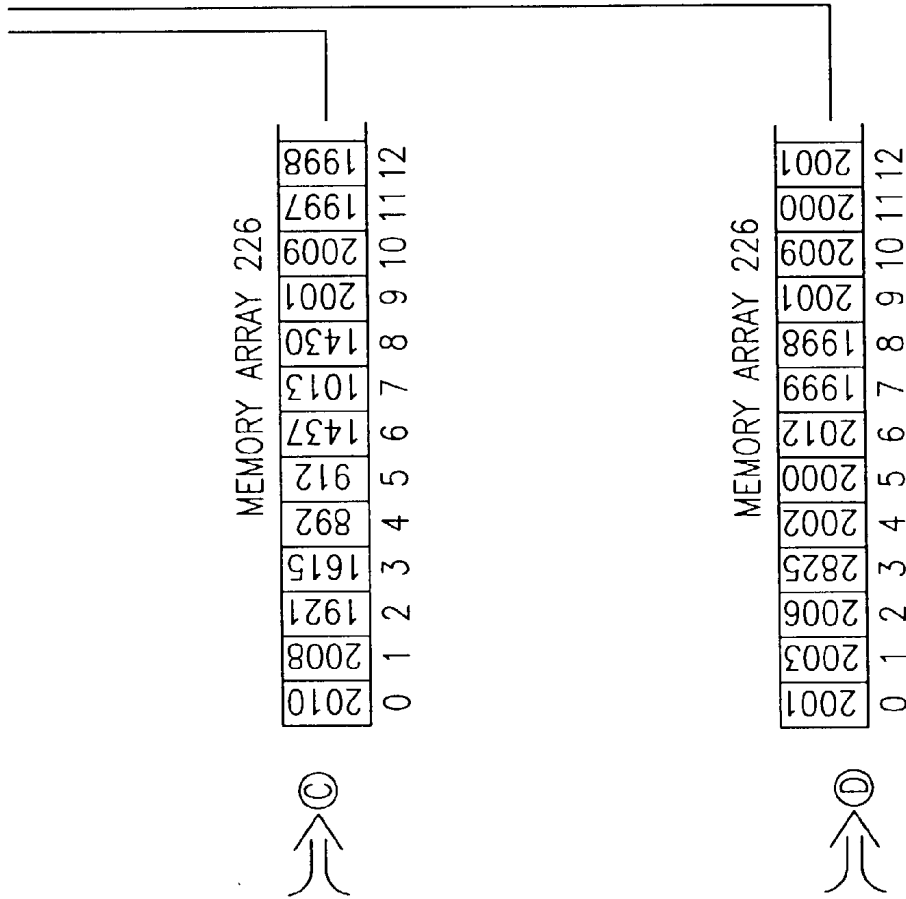


FIG.57D

FIG.57A	FIG.57B
FIG.57C	FIG.57D

FIG.57

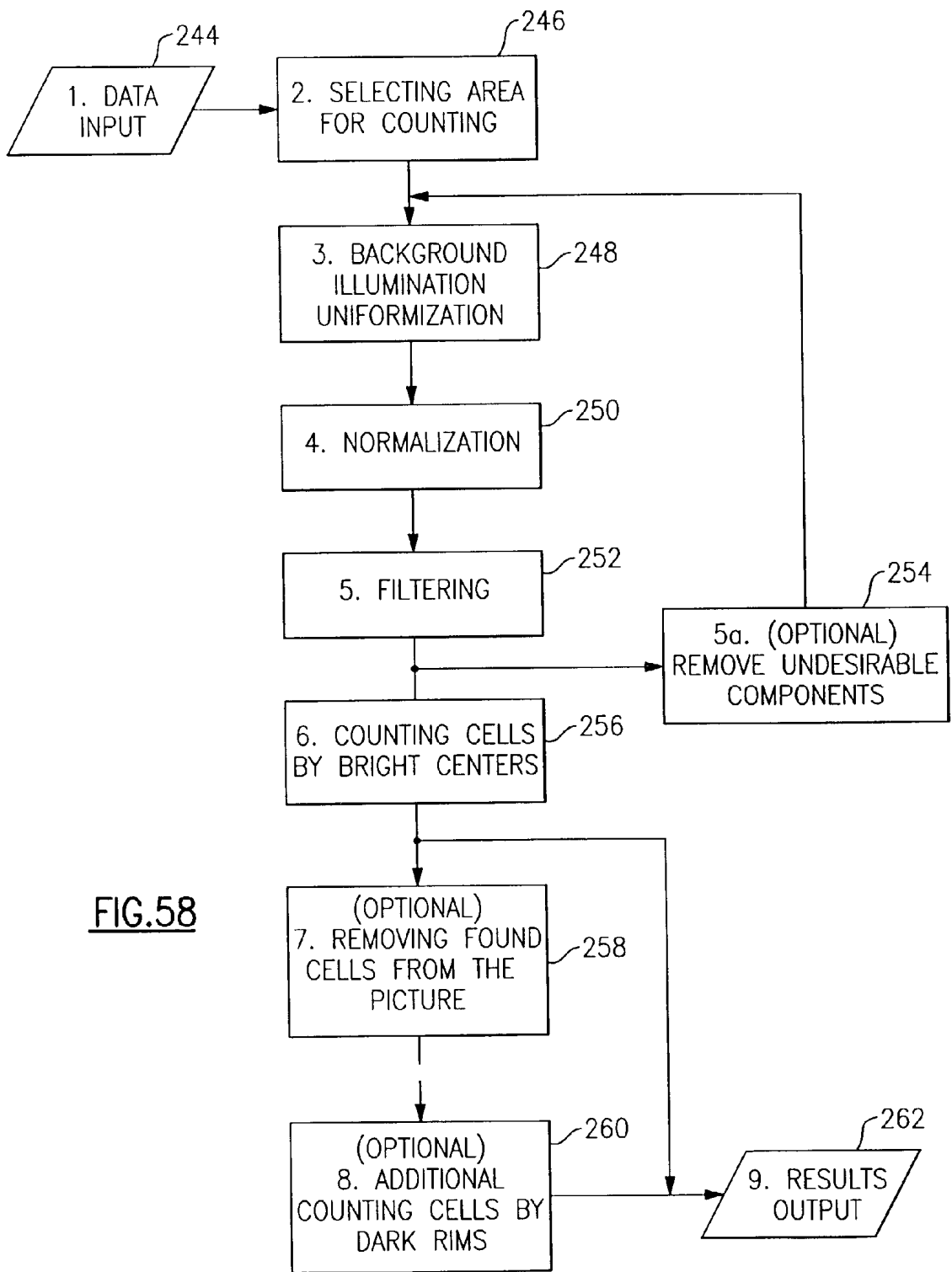


FIG.58

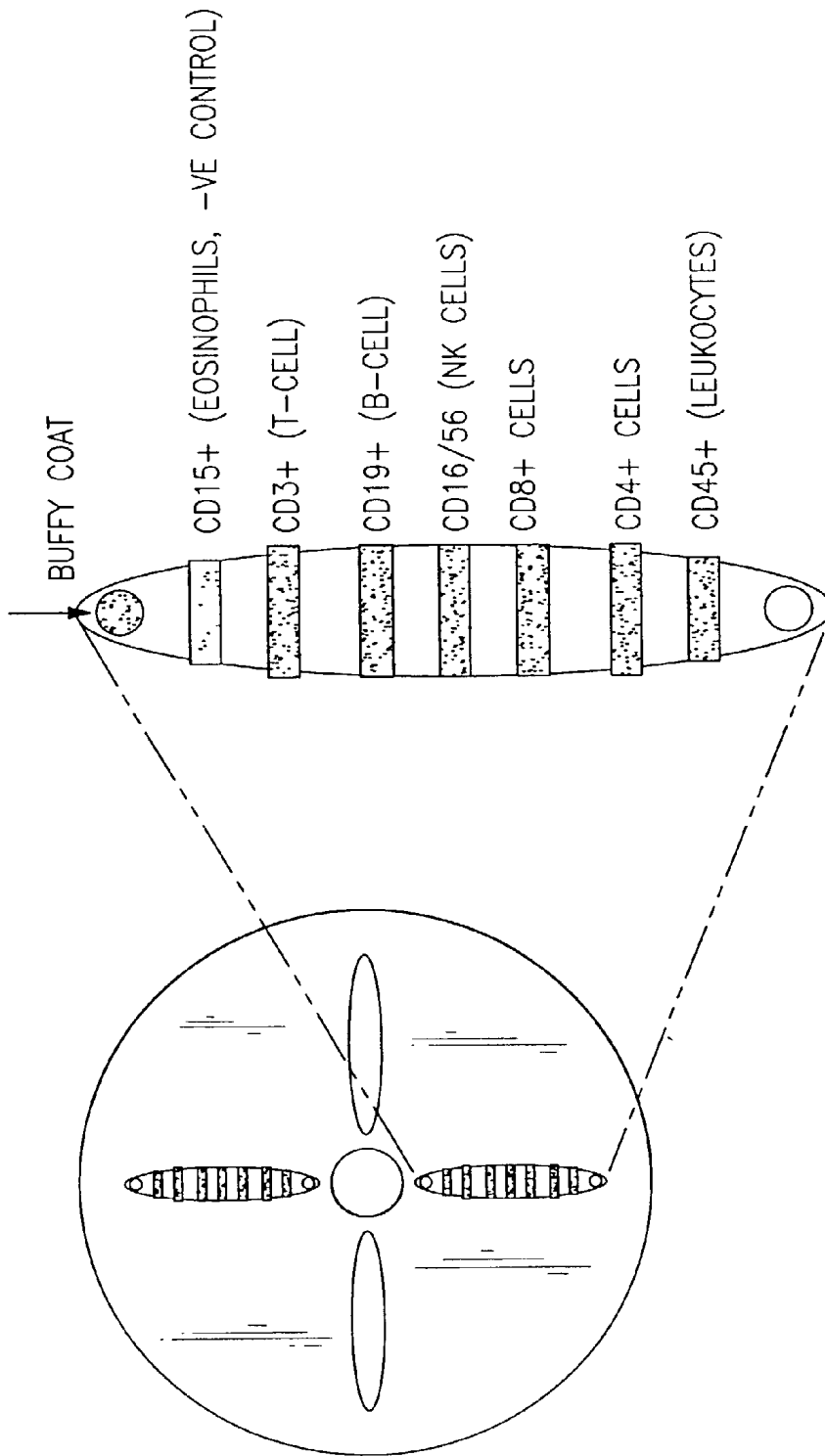


FIG.59

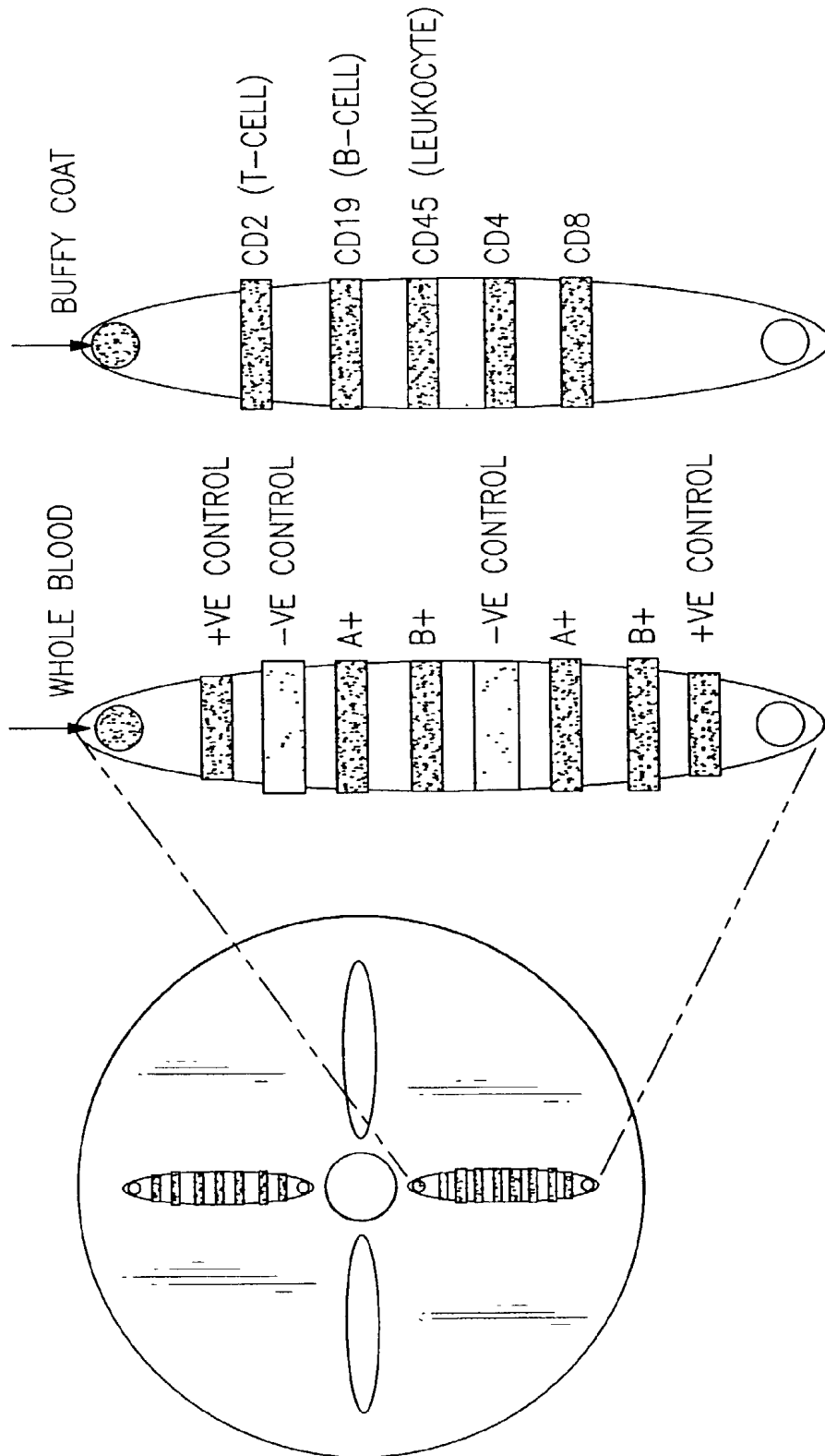


FIG. 60A

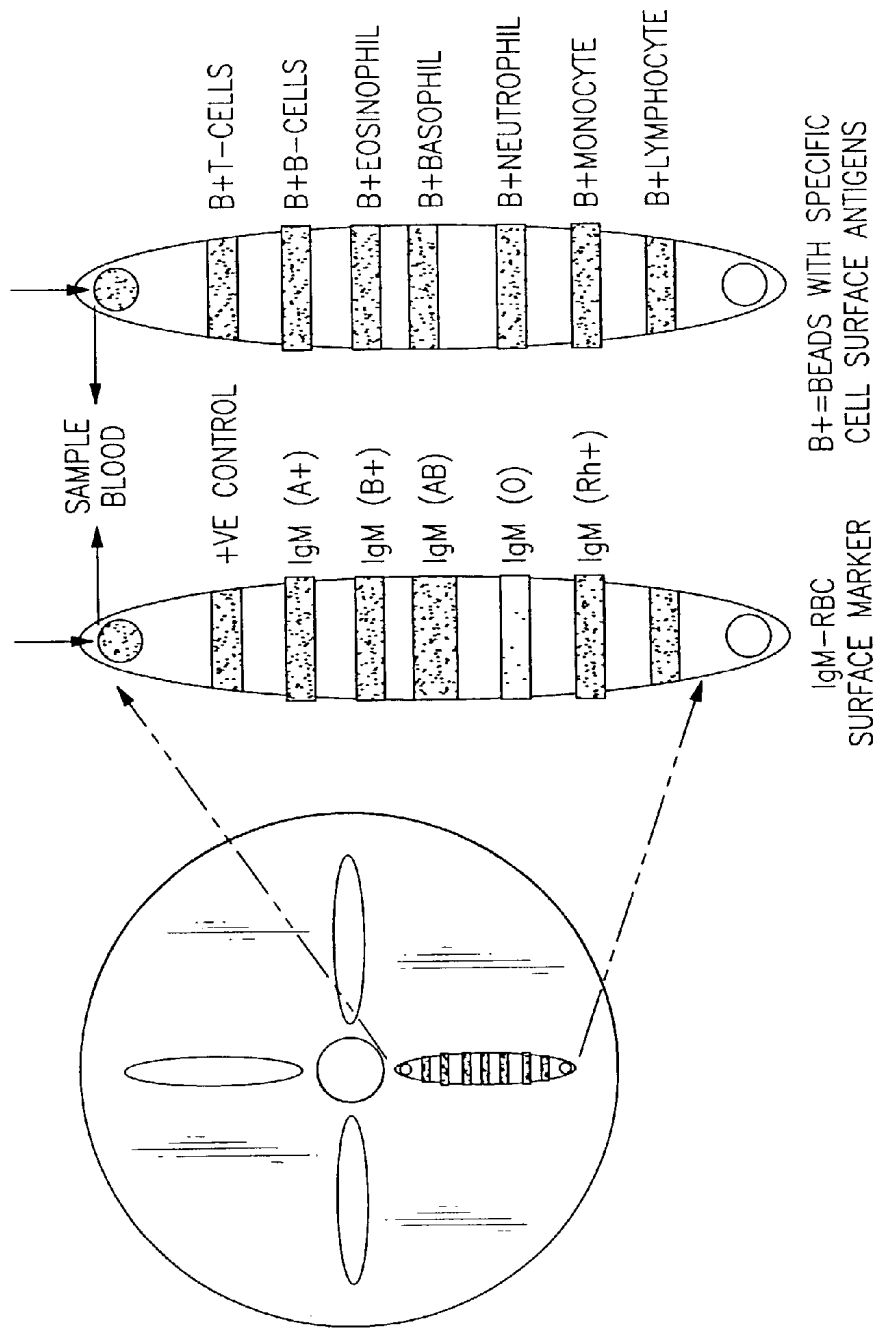


FIG.60B

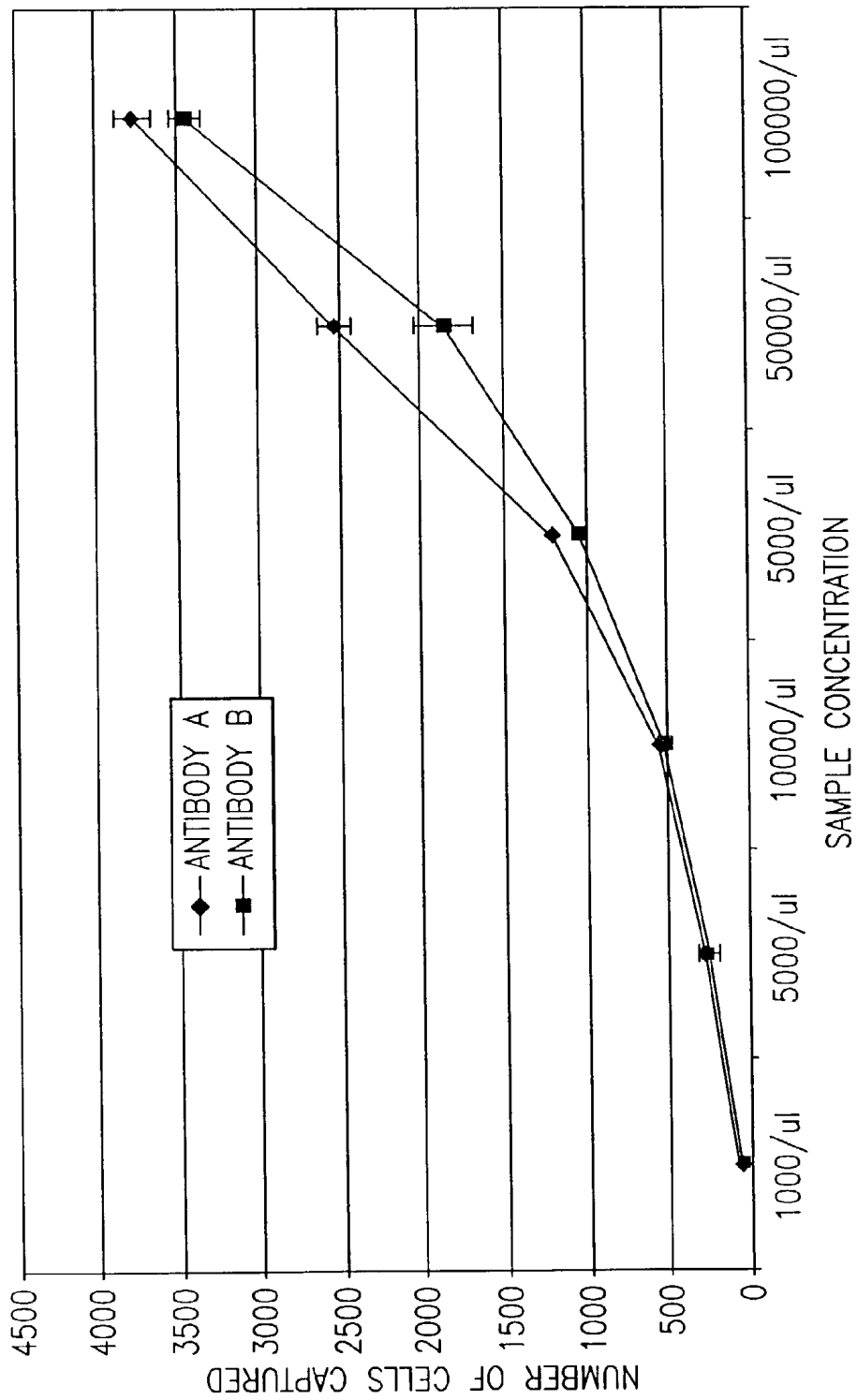


FIG.61

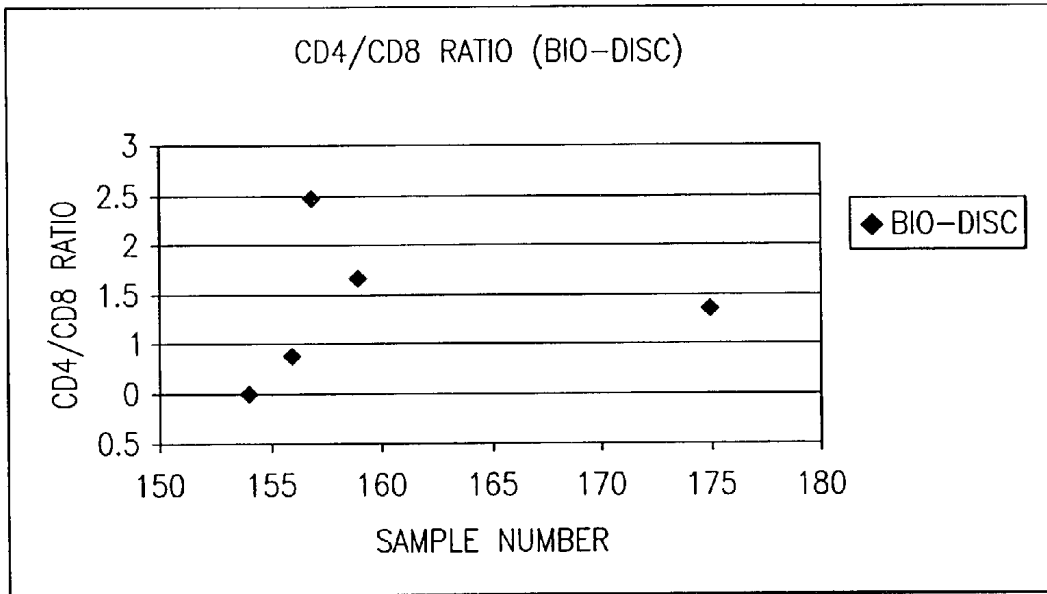


FIG.62A

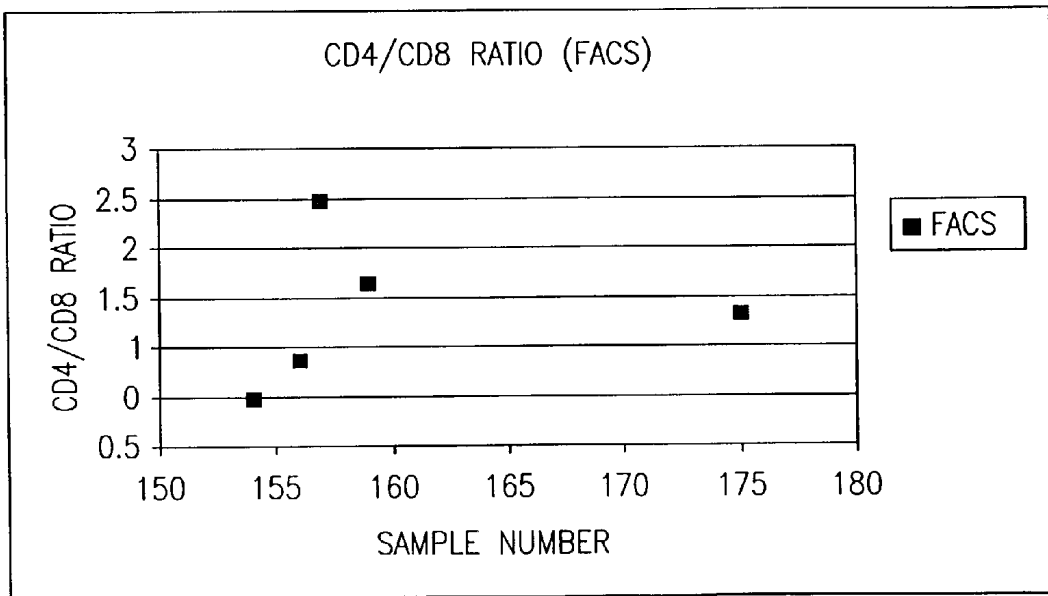


FIG.62B

METHODS FOR QUALITATIVE AND QUANTITATIVE ANALYSIS OF CELLS AND RELATED OPTICAL BIO-DISC SYSTEMS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part of U.S. application Ser. No. 09/988,728 filed Nov. 20, 2001.

[0002] This application also claims the benefit of priority from U.S. Provisional Application Serial No. 60/315,937 filed Aug. 30, 2001; No. 60/328,246 filed Oct. 10, 2001; No. 60/386,072 filed Oct. 19, 2001; No. 60/386,073 filed Oct. 19, 2001; No. 60/386,071 filed Oct. 26, 2001; No. 60/344,977 filed Nov. 7, 2001; No. 60/338,679 filed Nov. 13, 2001; No. 60/334,131 filed Nov. 30, 2001; No. 60/355,644 filed Feb. 5, 2002; and No. 60/358,479 filed Feb. 19, 2002.

[0003] These applications are all herein incorporated by reference in their entireties.

BACKGROUND OF THE INVENTION

[0004] 1. Field of the Invention

[0005] This invention relates in general to biological, chemical, cellular, and diagnostic assays and, in particular, to such assays conducted on cell populations derived from blood, hematological leukemias, lymphomas, bone marrow, and stem cells using optical bio-discs. More specifically, but without restriction to the particular embodiments hereinafter described in accordance with the best mode of practice, this invention relates to methods and apparatuses for detecting cellular analytes in biological samples using optical bio-discs.

[0006] 2. Background of the Invention

[0007] Blood cell counts are used during diagnosis, treatment, and follow-up to determine the health of the patient. Complete blood count (CBC) is a collection of tests including hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, platelet count, and white blood cell count. Blood count is the enumeration of the red corpuscles and the leukocytes per cu. mm. of whole blood.

[0008] White Blood Cell Count (WBC, leukocytes) is the total number of white blood cells in a standard sample of blood. In a normal healthy person, typically the WBC counts are 4000 to 10800 cells per microliter (μL). Factors such as exercise, stress, and disease can affect these values. A high WBC may indicate infection, leukemia, or tissue damage. There is increased risk of infection if it falls below 1000 cells per microliter.

[0009] Leukocyte differential testing is essential to gather information beyond that obtainable from the leukocyte count itself. Leukocyte differential count is used to evaluate newly suspected infection or fever (even if the CBC is normal), suspicion of a disorder associated with abnormalities, an abnormal leukocyte count, suspected leukemia, other abnormalities such as eosinophilia, monocytosis and basophilia. Repeated testing for leukocyte or leukocyte differential may be performed in the presence of severe leukopenia (e.g., secondary to drug therapy). During treatment, for e.g. chemotherapy or radiation therapy, blood counts are very

important to determine if the treatment is depleting healthy blood cells in addition to cancerous cells.

[0010] Differential leukocyte counts are determined by computerized cell counting equipment. The machine determines the total count and the percentages of the five major white cell types. In normal individuals, there are a majority of neutrophils (50-60%), followed by lymphocytes (20-40%), then monocytes (2-9%), with a few eosinophils (1-4%) and basophils (0.5-2%).

[0011] Within the category of lymphocytes, there are further lymphocytes and further sub-types of cells. For example, lymphocytes can be broadly divided into T-cells (thymus-derived lymphocytes) and B-cells (bursal-equivalent lymphocytes), which are largely responsible for cell-mediated and humoral immunity respectively. Although morphological characteristics have been used to classify groups within the leukocytes, morphology alone has proved inadequate in distinguishing the many functional capabilities of lymphocyte sub-types. To distinguish lymphocytes with various functions, techniques including analysis by resetting, immuno-fluorescence microscopy, enzyme histochemistry, and recently, monoclonal antibodies have been developed. T cells are distinguished by the presence of surface markers including two glycoproteins on their surface CD4 and CD8 (CD4+ T cells and CD8+ T cells). CD4+ T helper cells are involved in antibody-mediated immunity. They bind to antigen presented by B cells. And the result is development of clone of plasma cells secreting antibodies against antigenic material. T cells are also essential for cell-mediated immunity. CD4+ cells bind to antigen presented by antigen-presenting cells (APCs) like phagocytic macrophages and dendritic cells. The T cells then release lymphokines that attract other cells to the area. The result is inflammation, the accumulation of cells and molecules that attempt to wall off and destroy the antigenic material.

[0012] CD8+, cytotoxic/suppressor type cells secrete molecules that destroy the cell to which they have bound. This is a very useful function if the target cell is infected with a virus because the cell is usually destroyed before it can release a fresh crop of viruses which are able to infect other cells.

[0013] HIV and AIDS

[0014] Human immunodeficiency virus a retrovirus has high affinity for CD4+ T cells and therefore CD4 T cells are potent targets for the virus. Acquired immune deficiency syndrome (AIDS) provides a vivid and tragic illustration of the importance of CD4+ T cells in immunity. The human immunodeficiency virus (HIV) binds to CD4 molecules and thus invades and infects CD4+ T cells. As the disease progresses, the number of CD4+ T cells declines below its normal range of about 1000 per microliter (μl). One of the explanations may be the unceasing effort of the patient's CD8+ T cells to destroy the infected CD4+ cells.

[0015] When the number of CD4+ T cells in blood drop below 400 per microliter, the ability of the patient to mount an immune response declines dramatically. Not only patient becomes hypersusceptible to pathogens that invade the body, but also microorganisms, especially bacteria that normally inhabit our tissues without harming us. Eventually the patient dies of opportunistic infections like Candidiasis, Cytomegalovirus, Herpes simplex viruses, *Pneumocystis carinii*, pneumonia, Toxoplasmosis, Tuberculosis and others.

[0016] The estimation of CD4+ and CD8+ T-cell numbers and the ratio of CD4+/CD8+ T-cells is useful to assess the immune health of human patients with immune-compromised diseases. Individuals having AIDS, for example, shows the importance of CD4+ T-cells in immunity. As the disease progresses, the number of CD4+ T-cells declines below its normal range of about 1000 cells per μ l. As the patient's CD8+ T-cells destroy the infected CD4+ T-cells, uninfected CD4+ cells may undergo apoptosis. Thus, the ratio of CD4+ to CD8+ T-cells provides a diagnostic marker for the progression of the disease. The U.S. Public Health Service recommends that CD4+ levels be monitored every 3-6 months in all infected persons (40 million tests are done every year in 600 testing laboratories in the United States).

[0017] In addition to CD4 and CD8, there are many other cell surface antigens (e.g., CD3, CD16, CD19, CD45, CD56) which can be used to identify sub-types of lymphocytes. The ability to detect these cell surface antigens by antibody techniques has added a new dimension to diagnostic pathology, and a variety of techniques are available for the study of immunophenotypes of hematolymphoid disorders (e.g., AIDS, leukemias, and lymphomas). Conventional microimmunoassays such as radio-immunoassays (RIA), enzyme-immunoassay (EIA), fluorescence-immunoassay (FIA) use an isotope, an enzyme or a fluorescent substance to detect the presence or absence of corresponding analytes.

[0018] A number of therapeutic approaches are used to treat AIDS. The following approaches by themselves or in combinations with others are successfully used to treat the disease.

[0019] 1. Nucleoside analog reverse transcriptase inhibitors (NUKES), the first anti-HIV drug blocks reverse transcription that is required to synthesize viral DNA from RNA by providing "decoy" building blocks that interrupt the process (drugs like Zidovudine Retrovir AZT, Didanosine Videx, Zalcitabine Hivid, dideoxycytidine, Stavudine Zerit, Lamivudine Epivir, Zidovudine/Lamivudine Combivir, Abacavir Ziagen, Zidovudine/Lamivudine/Abacavir).

[0020] 2. Non-nucleoside reverse transcriptase inhibitors (NNRTIs or NON-NUKES), interrupt reverse transcription, by binding to the reverse transcriptase enzyme and restricting its activity (drugs like Nevirapine Viramune, Delavirdine Rescriptor, Efavirenz Sustiva).

[0021] 3. Protease inhibitors, block the action of protease, an enzyme that cuts HIV protein chains into specific proteins needed to assemble a new copy of the virus (drugs like Saquinavir Invirase, Ritonavir Norvir, Indinavir Crixivan, Nelfinavir Viracept, Saquinavi Fortovase, Amprenavir Agenerase, Saquinavir Invirase and others).

[0022] 4. Integrase inhibitors, block the action of integrase, an enzyme that inserts the viral DNA into the infected cell's DNA strands. No integrase inhibitors have been approved yet (Zintevir is in Phase I human trials).

[0023] 5. Attachment and fusion inhibitors, prevent the HIV virus from attaching to a cell. No fusion inhibitors have been approved yet (AMD-3100, Pentafusided, T 1249, PRO 452 and SC351125 are in phase I and II trials).

[0024] 6. Antisense drugs, these are a "mirror image" of part of the HIV genetic code that locks onto the virus to prevent it from functioning. One antisense drug, HGTV43 by Enzo is in Phase I trials.

[0025] 7. Immune Stimulators, use the body's chemical messengers to stimulate the immune response. Interleukin 2 (IL-2, Aldesleukin, Proleukin, Reticulose and Multikine and an inactivated virus preparation, HIV-1 Immunogen, is in Phase III trials.

[0026] Leukemia

[0027] Leukemia is a malignant disease that originates in a cell in the marrow. It is characterized by the uncontrolled growth of developing marrow cells. There are two major classifications of leukemia myelogenous or lymphocytic, which can each be acute or chronic. Acute leukemia progresses quickly and generally affects cells that are not yet fully developed. These immature cells therefore cannot fully carry out their normal functions. Chronic leukemia on the other hand progresses slowly and permits the growth of more mature and functional cells. Leukemia affects lymphoid cells (lymphocytic leukemia) or myeloid cells (myelogenous leukemia). Lymphocytic leukemia affects white blood cells or lymphocytes, where as myelogenous includes all other types of blood cells besides lymphocytes. The terms myelogenous or lymphocytic denote the cell type involved. There are four major types of leukemia's acute or chronic myelogenous leukemia and acute or chronic lymphocytic leukemia. The cause of leukemia is not known, but seems to be associated with a failure of the developing leukocytes to mature.

[0028] Normal, mature leukocytes cannot reproduce and are replaced by new cells generated in the bone marrow. Leukemic cells, in contrast, have the ability to reproduce but do not develop sufficiently to function properly. As leukemia progresses, the leukemic cells displace normal leukocytes, leaving the patient extremely vulnerable to infection. There are several forms of leukemia, both acute and chronic, which are classified according to the type of leukocyte affected. The major types of leukocytes involved in leukemia include lymphocytes and polymorphonuclear leukocytes.

[0029] There are two main forms of acute leukemia, acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML). ALL affects lymphocytes and occurs more often in children. AML affects the cells that form polymorphonuclear leukocytes and is more common in adults, although it can occur at any age. Acute leukemia is a rapidly progressing disease that results in the accumulation of immature, functionless cells in the marrow and blood. The marrow often can no longer produce enough normal red and white blood cells and platelets. Anemia, a deficiency of red cells, develops in virtually all leukemia patients. The lack of normal white cells impairs the body's ability to fight infections. A shortage of platelets results in bruising and easy bleeding.

[0030] Chronic leukemia is of two types, chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (CLL). CML affects immature polymorphonuclear leukocytes and usually occurs after 35 years of age. CLL affects lymphoid tissue and lymphatic cells and usually occurs in men over the age of 50. The prognosis for those with the chronic leukemias is largely dependent upon the age at which the disease occurs; as with AML, the symptoms can be controlled and life extended. Patients with CML are more likely to die as a result of leukemia than are those with CLL, because CML usually starts at an earlier age.

[0031] The specific diagnosis of leukemia requires a blood count and a bone marrow biopsy. Leukemia is confirmed by

the presence of large numbers of abnormal leukocytes in the blood and the typical leukemic cells in the bone marrow. With the chronic leukemias, the patient may be unaware of the disease, and a diagnosis is often made only when the patient is examined for another reason, such as during a routine checkup or before surgery.

[0032] The treatment of acute and chronic leukemia is often similar, but it is dependent on varying factors involved in each case. The aim of treatment is to suppress the reproduction of leukemic cells. Cytotoxic drugs, which prevent cell multiplication, are used for this purpose. The rapidly dividing leukemic cells are more susceptible to these drugs than are normal leukocytes. The treatment of the acute leukemias usually involves the use of several cytotoxic drugs together. Once the number of leukemic cells has been reduced, corticosteroids and only one or two cytotoxic drugs need be used to maintain the improvement. With the chronic leukemias, cytotoxic drugs and corticosteroids may also be used. In some cases, a blood transfusion may be necessary. The diagnosis of leukemia requires examination of the cells in blood or marrow. The aim of treatment is to bring about a complete remission. Complete remission means that there is no evidence of the disease and the patient returns to good health with normal blood and marrow cells. Relapse indicates a return of the leukemia cells and return of other signs and symptoms of the disease.

[0033] Most of the clinical findings in acute leukemia are due to bone marrow failure, which results from replacement of normal bone marrow elements by the malignant cell. Less common manifestations include direct organ infiltration (skin, gastrointestinal tract, meninges). Acute lymphoblastic leukemia (ALL) comprises 80% of the acute leukemias of childhood. The peak incidence is between 3 and 7 years of age. However, ALL is also seen in adults and comprises approximately 20% of adult acute leukemias. Acute myelogenous leukemia (AML; acute nonlymphocytic leukemia, "ANLL") is chiefly an adult disease.

[0034] Chronic lymphocytic leukemia (CLL) a B lymphocyte (rarely T lymphocytes) clonal malignancy. The disease is usually indolent, with slowly progressive accumulation of long-lived small lymphocytes. These cells are immunoincompetent. Chronic lymphocytic leukemia is manifested clinically by immunosuppression, bone marrow failure, and organ infiltration with lymphocytes.

[0035] An evaluation of the disease is done using a primary panel that assess the lineage assignment of the predominant blast population, followed by a secondary panel of mAbs characterizing the definite phenotype and maturational stage of the blast population depending on the results of the primary panel. Alternatively, a predetermined panel is selected for an immediate direct, "extensive" characterization of a wide range of immature and mature hematopoietic cells in the sample. These "extensive" characterizations may increase the sensitivity of the test when malignant cells are only in a minority, and also help to characterize the heterogeneity of pathological cells, as well as the concomitant disturbances in the maturation of other cellular lineages. In addition, this strategy offers a better control for consistency of staining results.

[0036] Leukemia Immunophenotyping

[0037] Surface markers in leukemia aid in identifying the tumor lineage for diagnostic and prognostic purposes. Com-

prehensive leukemia phenotyping begins with a review of the clinical history and morphology and a panel of markers are selected for each case. In most cases the lineage can be identified as T-cell, B-cell, or myeloid and a diagnosis, or differential diagnosis, can be made.

[0038] The aim of leukemia phenotyping is to identify the cell type of the neoplastic process. This phenotypic identification should outline the cell lineage and level of maturation, as an aid to the classification of the leukemia or lymphoma. Further, this phenotypic identification should assist in the determination as to whether the cell population is normal or abnormal and in the detection of a previously characterized population of cells in a sample for monitoring the disease remission, development or recurrence.

[0039] A Complete Blood Count (including White Blood Cell count) is then performed. Blood cell counts is an important index of the response of the disease to treatment. These counts are also important to learn the effects of drug treatment or radiation therapy. A blood count helps to determine if a drug is working. The actual counting of cells is usually done by expensive electronic counter that requires technical expertise to perform the test using the counter. The patterns of each cell type indicate if leukemia is present and the type of leukemia.

[0040] On average, there are about 4000 to 11,000 white blood cells per cubic millimeter of blood. If the total WBC count is over 11,000 cells/mm³, it is referred to as leukocytosis a normal response to infections of the body. However, the presence of excessive abnormal WBC (leukemic blasts) is referred to as leukemia. There are about 5 million red blood cells/mm³ in the blood. An abnormally low RBC may be a result of anemia. Anemia is indicative of leukemia.

[0041] Leukemia immunophenotyping is performed on blood or bone marrow specimens, however, other body fluids or tissues may be examined. Leukocytes obtained using RBC lysis method or density gradient isolation such as ficoll hypaque can be used. Where possible, a total leukocyte count and differential should be performed before processing, and the cell concentration adjusted accordingly.

[0042] Monoclonal Antibody Panels

[0043] Many laboratories use multicolor immunofluorescence detection, although, in some cases single color immunofluorescence may be adequate. Antibodies routinely included are: CD2, CD3, CD5, CD10, CD11c, CD14, CD19, CD20, CD22, CD23, CD25, CD45, CD103, FMC7, Heavy chains, Kappa and Lambda. If clinical or morphologic features suggest a "T" or "NK" lymphocyte disorder, then the following additional antibody combinations are also performed: CD3/CD4/CD8, CD7/CD5/HLA-DR, CD25/CD2/CD3, CD16/CD56/CD19, CD57/CD8/CD3, TCR alpha-beta/delta-gamma/CD3.

[0044] A number of current biological therapies are used to fight leukemia. For example, for leukemia, they have been effective in marrow replacement therapy and preventing rejection of donor bone marrow. Interferons are also been used in the treatment of chronic myelocytic leukemia (CML).

[0045] The presence of leukemic blast cells is also confirmed by examination of the marrow. Bone marrow fluid is withdrawn and examined under a microscope. Leukemic

blast cells are then be confirmed. If leukemia cells are found in the bone marrow sample, then test are then carried out to determine the extent of the disease. A spinal tap checks for leukemia cells in the fluid that fills the spaces in and around the brain and spinal cord, cerebrospinal fluid and x-rays are used to check the spread the disease to the chest.

[0046] Different chemotherapy drugs attack cancer cells in a different ways. Hence combinational chemotherapy treatments are usually given at the same time to maximize the effectiveness of chemotherapy.

[0047] Lymphoma

[0048] Traditionally, morphological characteristics have been used to classify cells by groups with functional similarities. Morphology alone, however, has proven inadequate in distinguishing the many functional capabilities of lymphocytes sub-sets (T cells, B cells and natural killer cells, etc. To distinguish lymphocytes with various functions, techniques including analysis by rosetting, immunofluorescence microscopy, enzyme histochemistry, and, recently flow cytometry with fluorescent labelled monoclonal signal antibodies has been developed. Lymphocytes can be broadly divided into T (thymus-derived) lymphocytes and B (bursal-equivalent) lymphocytes, which are responsible for cell mediated and humoral, immunity respectively.

[0049] Lymphoma is a general term for a group of malignant disorders that originate in lymphocytes, a type of white blood cell. Lymphocytes are distributed throughout the body but are concentrated in the marrow, lymph nodes, spleen, gastrointestinal tract and skin. These sites are the major organs that compose the lymphatic system.

[0050] Lymph nodes are very numerous and are located throughout the body from the scalp to the feet. They are connected by a special circulatory system called lymphatics. These vessels carry a fluid called the lymph in which lymphocytes are suspended as they move from node to node and eventually into the blood. The malignant transformation of a lymphocyte initiates a lymphoma. This occurs in a lymph node, usually, but may start in the stomach, intestines, skin or other organ. The malignant lymphocyte or lymphoma cell multiplies and the resulting cells accumulate, producing an enlarged lymph node or nodes, or a mass in another tissue, such as the stomach, skin or other site. In lymph nodes, the growing lymphoma cells crowd out the normal cells and produce abnormal cell patterns that can be identified and categorized in a biopsy specimen. Some lymphomas involve the marrow and may interfere with blood cell development leading to anemia and, in more severe cases, low white blood cell counts and platelet counts.

[0051] All lymphomas have in common the abnormal growth of malignant lymphocytes. The type of lymphoma, however, differs with the kind of lymphocyte affected and the pattern of lymph node involvement. Lymphomas are divided into three major types: low grade, intermediate grade, or high grade. These categories are based on the type of lymphoma cells present, the pattern of disruption of the lymph node, the immunophenotype of the lymphocytes (especially B cells versus T cells), and the nature of the chromosome abnormalities. Lymphomas belong to either Hodgkin or non-Hodgkin type. Hodgkin's Disease or Hodgkin's Lymphoma is a malignant growth of cells in the

lymph system. Hodgkin's Disease is the better-known form of lymphoma and the other lymphomas are grouped into what is called the Non-Hodgkin's lymphomas. However, in 1996 WHO recommended a classification system based on cell of origin (B vs. T) and the stage of differentiation (immature or precursor vs. mature or peripheral).

[0052] Hodgkin's disease usually arises in a lymph node or nodes. An immune system cell (lymphocyte) undergoes a malignant transformation, it multiplies, crowding out and impairing the function of the normal lymphocytes in the nodes and the lymph node enlarges. The malignant cell, which characterizes the disease, is the Reed-Sternberg cell and is derived from a lymphocyte. When the Reed-Sternberg cell is found in conjunction with other abnormal cell patterns in the lymph nodes characteristic of the disease, a diagnosis is made.

[0053] It is believed that Hodgkin's disease begins in one lymph node and the malignant cells travel through the lymphatic channels, a network of vessels that connect neighboring lymph nodes. In more advanced Hodgkin's disease, the lungs, liver, and bone marrow may be involved with tumor masses. The disease may affect these other tissues when the malignant cells spread from the lymph nodes through lymphatic vessels or blood vessels. Symptoms include swelling of lymph nodes, most often those on either side of the neck, but occasionally in the armpit, groin, or abdomen. Some other symptoms or signs include fatigue, recurrent fever, sweating at night, itching, pain in the back, legs or abdomen, bone pain, loss of appetite, and loss of weight.

[0054] A diagnosis of lymphoma is confirmed by biopsy. The tissue is studied for the presence, type, and arrangement of malignant cells. Tests are also done on the cells from the tissue to determine if they are lymphocytes and the type of lymphocyte they are. Additional tests are done to determine if the disease has spread through the lymph system or to other parts of the body. These tests may include additional biopsies, nuclear medicine, x-ray, or magnetic resonance imaging.

[0055] Lymphoma can be very difficult to detect. There are some symptoms for Non-Hodgkin's but they are not specific. Often a lymph node swells, especially in the upper body area. Diagnosis is initiated by taking a tissue biopsy. Tissue is microscopically examined for cancerous cells. A number of tests are done to evaluate lymphomas: examination of the lymph nodes, complete blood work for abnormal blood cell counts, blood chemistry, abnormal sedimentation rate, chest x-ray to view lymph nodes and to see if other organs are involved, computerized tomography (CT or CAT) scan or magnetic resonance imaging (MRI) scan of the chest, pelvis, and abdomen to determine the possible spread of the disease, a gallium scan to check for radioactive intake of gallium in the lymph system indicating swelling and ultimately disease, a bone marrow aspiration and biopsy to determine if the bone marrow has been affected by lymphoma, liquid bone marrow and a bone chip are extracted and examined under a microscope. The non-Hodgkin's Lymphoma types are of the following type: lymphoblastic lymphoma, small non-cleaved cell lymphoma (Burkitt's/Non-Burkitt's) and large cell lymphoma. Classification of lymphomas encompasses all the lymphoproliferative neoplasm. There are more than 20 clinicopathologic entities

described by the US National Cancer Institute (NCI) into the more clinically useful indolent or aggressive lymphomas.

[0056] Diagnosis and treatment for lymphomas depend on the cell type, pattern of lymph node involvement and extent of the disease. Early localized stages of the disease may be treated with radiation. Chemotherapy is the more frequent form of treatment for lymphomas since the disease, when diagnosed, is usually in multiple sites in the body. High-grade (aggressive) lymphomas are usually treated with several different chemotherapeutic drugs simultaneously.

[0057] Hodgkin's is medically diagnosed by taking a tissue sample (biopsy) and searching for the presence of Reed-Sternberg cells, a cell specific to Hodgkin's disease. A needle biopsy is sometimes used but a surgical biopsy, removal of a whole node, is preferred in getting enough tissue for a definite diagnosis.

[0058] Evaluation of the disease is typically performed by using a primary panel that assess the lineage assignment of the predominant blast population, followed by a secondary panel of monoclonal antibodies characterizing the definite phenotype and maturational stage of the blast population depending on the results of the primary panel. Alternatively, a predetermined panel is selected for an immediate direct, extensive characterization of a wide range of immature and mature hematopoietic cells in the sample. These extensive characterizations may increase the sensitivity of the test when malignant cells are only in a minority, and also help to characterize the heterogeneity of pathological cells, as well as the concomitant disturbances in the maturation of other cellular lineages. In addition, this strategy offers a better control for consistency of staining results.

[0059] Lymphoma Immunophenotyping

[0060] Surface markers in lymphoma aid in identifying the tumor lineage for diagnostic and prognostic purposes. Comprehensive leukemia/lymphoma phenotyping begins with a review of the clinical history and morphology and a panel of markers are selected for each case. In most cases the lineage can be identified as T cell, B cell, or myeloid and a diagnosis, or differential diagnosis, is made.

[0061] The aim of lymphoma phenotyping is to identify the cell type of the neoplastic process. This phenotypic identification should outline the cell lineage and level of maturation, as an aid to the classification of the lymphoma. Further, this phenotypic identification assists in the determination as to whether the cell population is normal or abnormal and in the detection of a previously characterized population of cells in a sample for monitoring the disease remission, development, or recurrence.

[0062] A complete blood count (including white blood cell count) is performed. Blood cell count is an important index of the response of the disease to treatment. These counts are also important to learn the effects of drug treatment or radiation therapy. The normal white cell counts are about 4000 to 11,000 per cubic millimeter in the blood. If the total WBC count is over 11,000 cells/mm³, it is referred to as leukocytosis a normal response to infections of the body. A blood count helps to determine if a drug is working. Traditionally, cell counts are performed by expensive electronic counters, like the FACS scanner, that require technical expertise to perform the test. The patterns of each cell type indicate if lymphoma is present and the type of lymphoma.

[0063] Monoclonal Antibody Panels

[0064] Many laboratories use multicolor immunofluorescence although in some cases single color immunofluorescence may be adequate. Antibodies routinely included are CD2, CD3, CD5, CD10, CD11c, CD14, CD19, CD20, CD22, CD23, CD25, CD45, CD103, FMC7, Heavy chains, Kappa, and Lambda. If clinical or morphologic features suggest a "T" or "NK" lymphocyte disorder, then the following additional antibody combinations are also performed: CD3/CD4/CD8, CD7/CD5/HLA-DR, CD25/CD2/CD3, CD16/CD56/CD19, CD57/CD8/CD3, TCR alpha-beta/delta-gamma/CD3.

[0065] A number of recently developed biologic therapies are used to fight lymphoma. For example, monoclonal antibodies (antibodies produced to eliminate a specific target cell) have been used in lymphomas.

[0066] The WBC differential count in patient with lymphoma is usually abnormal due to either increased leukocytes that may be due to two reasons, (a) Reactive—i.e. secondary to infection, neoplasia, or inflammation. Increased lymphocytes is common in children, (b) Neoplastic—i.e. leukemia's and related disorders or decreased leukocytes that may be due to (a) consumption or destruction (e.g. hypersplenism), (b) marrow failure (in which case there is usually a pancytopenia).

[0067] Neutrophils are important for fighting infection. When neutrophil numbers fall below 1000 cells per microliter the condition is called neutropenia. Lymphoma treatment can cause neutropenia. Obesity and smoking increase neutrophil count. Lymphocytes are divided into B (bone marrow matured) and T (thymus matured) lymphocytes. When the lymphocyte count falls below 1500 cells per microliter for adults or 3000 cells per microliter in children the condition is called lymphocytopenia. Lymphomas can cause lymphocytopenia. Platelets (thrombocytes) are cell-like particles that stop bleeding by gathering at a site where bleeding is occurring. They then activate and clump together to stop bleeding and promote clotting. Platelet counts increase during strenuous activity, if the patient has myeloproliferative disorders including infection, inflammation, malignancy, and if the spleen has been removed. The number of platelets in a standard sample of blood typically is 133,000 to 333,000 platelets per microliter. Thrombocytopenia occurs if the platelet count fall below 30,000, which results in abnormal bleeding. Counts below 5000 are considered life threatening. Increased platelets may be (a) Reactive—to bleeding, infection, or neoplasia, and (b) Primary—myeloproliferative disorders etc. Decreased platelets may be due to (a) Destructive—e.g. Immune Thrombocytopenia, or (b) Marrow failure—usually involves other blood cells as well (i.e. there is a pancytopenia). Since chemotherapy affects the production of blood cells, it is important to check the amount of various kinds of cells in the blood.

[0068] A CBC may be done by commercially available manual or electronic instruments that measure hemoglobin level, hematocrit, total leukocyte, and erythrocyte count. Variations may include a platelet count, a leukocyte differential count, and cellular indices. The hematology analyzers are fully automated and results are accurate for cell counts, types of cells in body fluids like CSF, pleural fluid, ascetic fluid, pericardial fluid and gastric aspiration.

[0069] The ability to detect cell-associated antigens by antibody techniques has added a new dimension to diagnostic pathology. A variety of techniques are available for the study of immunophenotypes of hematolymphoid disorders. However, further development of immunoassay methods utilizing an antibody-antigen reaction in a global detection method for a number of diseases including virus based disease such as acquired immune deficiency syndrome and T-Cell leukemia as well as various cancers, need to be developed. As would be apparent to one of skill in the art, the assay methods and optical bio-disc systems of the current invention may be used to perform such immunoassays.

[0070] Conventional microimmunoassays like radio-immunoassays (RIA), enzyme-immunoassay (EIA), fluorescence-immunoassay (FIA) use an isotope, an enzyme or a fluorescent substance in order to detect the presence or absence of corresponding antibodies or antigens, respectively, that react specifically therewith. However the above methods have limitations and disadvantages. RIA requires special installations, precautions, limited half-life and various other factors. Methods using enzyme or fluorescence substances as labels is measured by determining coloring or luminescence require sensitive, sophisticated instruments to detect the calorimetric or fluorescent reactions in addition to requiring several washing steps to remove excess, unbound, un-reacted reagents. Furthermore, application of the above methods of detection for cells particularly lymphocytes and cancer cells and the like specimens, needs improvement in technology for the preparation, detection and analysis in high efficiency.

[0071] A powerful tool developed around the use of fluorescent antibody specific for cell-surface antigens is the technique of fluorescence-activated cell sorting (FACS). This is a very reliable, fast and sensitive method. Flow cytometer is the most practical method that is automated and quantitative. The foremost requirement of a sample for flow cytometric analysis is that the sample is in a monodisperse suspension and labeling desired cells with fluorescent markers. However, it is very high-priced test and the whole system requires handling by a trained technician in a clinical analysis laboratory and an expensive instrument. Monoclonal antibodies are used as discrete probes and flow cytometry for objective quantification of large number of cells.

[0072] In addition the fundamental disadvantage is that the cells once analyzed are no longer available for repeated analysis or additional investigation for example microscopic examinations of rare event cells. A number of alternative technologies have been developed that have advantages and disadvantages over flow cytometer and all introduce their own specific problem.

[0073] Surface marker analysis is an important laboratory tool, which has been particularly very useful in studying leukemias, lymphomas and immunodeficiency diseases. Antibody-based micro array technologies certainly are the state-of-the art technique, particularly in clinical diagnostics, for identification of specific antigens in the samples including blood and tissue samples. Most diagnostic test require determination of only a limited panel of analytes (such as in of cancers, leukemia, lymphoma, thyroid disease, etc.). Therefore, the requirements by a miniaturized tech-

nology for only a very small amount of blood sample and the savings in time and cost of laboratory personnel, upon simultaneous measurement of all the clinically relevant parameters in a single test are likely to prove compelling attractive to hospital laboratories and point-of-care facilities due to its cost-effectiveness, labor effective and its simplicity.

[0074] As an alternative to prior art systems and methods for cell counting, we have developed a simple, inexpensive system for analyzing, detecting, and quantitating cells, in particular blood cells, inclusive of the parasites and pathogens that infest the blood and other biological fluids like CSF. Related information and signal processing methods and software have been developed to identify various blood cells, parasites, and pathogens.

[0075] As compared to prior methods and systems, we have developed a simple, miniaturized, ultra-sensitive, inexpensive system for cellular analysis. This system uses optical bio-discs, related detection assemblies, as well as information and signal processing methods and software.

SUMMARY OF THE INVENTION

[0076] It is a principal aspect of the present invention to provide for methods and apparatuses for detecting blood cell analytes in biological samples using optical bio-discs. The invention further relates to qualitative and quantitative methods for cell isolation and typing including immunophenotyping. The present invention may be advantageously employed in combination with any of the discs, assays, and systems disclosed in the following commonly assigned and co-pending patent applications: U.S. Provisional Application Serial No. 60/302,757 entitled "Clinical Diagnostic Optical Bio-Disc And Related Methods For Selection And Detection Of Lymphocytes Including Helper-Inducer/Suppressor-Cytotoxic Cells" filed Jul. 3, 2001; U.S. Provisional Application Serial No. 60/306,035 entitled "Quantitative and Qualitative Methods for Cell Isolation and Typing Including Immunophenotyping" filed Jul. 17, 2001; U.S. Provisional Application Serial No. 60/305,993 entitled "Capture Layer Assemblies and Optical Bio-Discs for Immunophenotyping" filed Jul. 17, 2001; U.S. Provisional Application Serial No. 60/306,592 entitled "Methods for Imaging Blood Cells, Blood-Borne Parasites and Pathogens, and Other Biological Matter Including Related Optical Bio-Discs and Drive Assemblies" filed Jul. 19, 2001; and U.S. Provisional Application Serial No. 60/307,263 entitled "Quantitative and Qualitative Methods for Cell Isolation and Typing Including Immunophenotyping" filed Jul. 23, 2001. All of these applications are herein incorporated by reference.

[0077] In another aspect, the invention is addressed at cell typing, including CD family of assays. CD4/CD8 assay, a generic homogeneous solid phase cell capture assay for rapid determination of absolute number of CD4+ and CD8+ T-lymphocytes and to obtain a ratio of CD4+/CD8+ lymphocytes in blood samples

[0078] It is also an aspect of the present invention to provide for systems and methods addressed at cell typing, including CD family of assays and cell surface markers in leukemic and lymphoma cancers. These assays are homogeneous solid phase cell capture assays for rapid determination of absolute number of various leukemia or CD specific surface markers in blood samples.

[0079] The present invention is further directed to methods for specific cell capture by off-site incubation of primary antibodies with samples and subsequent capture by surface-bound secondary antibodies. Optical bio-discs and supporting software and processing methods are further aspects of the present invention.

[0080] In yet another aspect, the present invention addresses systems and methods of cell isolation and typing based upon the principle of localized cell capture on specific locations on the disc. Several specific cell capture fields are created on disc by localized application of capture chemistries based upon monoclonal or polyclonal antibodies to particular lymphocyte (white blood cells) antigens.

[0081] The assay is performed in a bio-disc that includes a flow chamber having specific antibodies attached to the solid phase. The Helper-inducer/suppressor-cytotoxic test determines the absolute numbers of, for example, CD4+, CD8+, CD3+, CD19+ and CD45+ lymphocytes captured by specific antibodies in the capture zones using 7-15 ul (depending on the chamber depth) of mononuclear cells (MNC) isolated from the whole blood. Upon flooding the chamber with the MNC (30,000 cells/ul), cells expressing antigens or surface markers of interest like CD4+, CD8+, CD3+ and CD45+ cells are specifically captured in the capture zones on the disc. Alternatively, whole blood instead of MNCs may be directly assayed using the current cell capture methods. Also incorporated within the analysis chambers are defined negative control areas.

[0082] It is also an aspect of the invention to provide methods and apparatus for conducting an assay in association with an optical analysis disc to detect and count cells. A further aspect of the invention is to provide methods and apparatuses for conducting assays in association with an optical analysis disc to detect lymphocytes.

[0083] According to another aspect of the present invention, there is provided a method that includes providing a sample in or on a disc surface, the disc having encoded information which is readable by an optical reader. This information can be used to control the scanning of the reader relative to the disc.

[0084] The present test or assay can be performed in at least two ways. The first method is based upon the principle of optical imaging of blood cells in special channels located on the optical bio-disc. Approximately 5 to 20 microliters of whole blood is injected into specially designed channels on the disc. The images are analyzed with cell recognition software that identifies various red blood cells and leukocyte sub-types and generates a red blood cell and white cell differential count, respectively. The second method is based on specific cell capture using cell specific antibodies against a specific cell. In one particular embodiment thereof, antibodies are directed against lymphocytes (CD2, CD19), monocytes (CD14), and eosinophils (CD15), for example. These leukocyte sub-type specific antibodies are assembled/attached to a solid surface within a bio-disc that includes a flow chamber. In other embodiments, capture antibodies are directed against other cells having specific surface markers of interest such as, for example, CD3, CD4, CD8, and CD45.

[0085] Once the sample is loaded into the analysis chamber/s and allowed sufficient time to bind to their respective capture agents on the capture zones, the disc is spun in a disc

drive or any equivalent spinner at a pre-determined speed and time to remove unbound cells from the capture zones. After spinning, the disc is then loaded into the optical reader, and an incident beam of electromagnetic radiation from a radiation source is directed to the disc. The beam is scanned over the disc by rotating the disc about a central axis and by moving the incident beam in a direction radial to the axis. A beam of electromagnetic radiation either transmitted through or reflected from the disc is detected and analyzed to extract information characteristic of the sample.

[0086] Embodiments of the invention also include a disc with a substrate and cap spaced to form a chamber. A sample of material, such as blood with cells, is provided in the chamber. When the disc is rotated, the sample moves past capture zones. The capture zones include capture layers with antibodies or other specific binding partners that bind to antigens such as CD4 and CD8 that are cell surface markers on cell types of interest. Preferably one test can be used to image CD4 and CD8 and other analytes in a blood sample. According to another aspect of the present invention, there is provided a disc reader for directing light to target areas where the capture zones are located, and detecting transmitted or reflected light to identify and count captured cells. These CD4 and CD8 counts, and the ratio between them, are useful for monitoring conditions such as AIDS.

[0087] The test sample is preferably provided to a chamber within the disc. A single chamber preferably has multiple capture areas, each of which may have one or more antibodies. In one embodiment, a single channel has multiple capture zones, each with a different type of antibody, and may have capture zones that serve as control zones. These capture zones can be aligned along one or more radii of the disc. Detection methods include detecting transitions in the feature, or imaging the viewing window and using image recognition software to count captured cells. Counting may be direct, such as counting a desired cell; or indirect, such as counting a collection of desired and non-desired cells, counting non-desired cells, and subtracting to obtain a count of desired cells. The capture zone may have one or more layers of antibodies.

[0088] When a sample of cells is provided to the disc, the disc can be rotated in one or more stages to move the cells to the capture zones, then to move unbound cells away from the capture zones. The sample may be processed in other ways, e.g., incubated or heated with the light source that is used for detection. Microfluidics can be used to add stain or any other liquids that may be desired for on-disc processing of the sample. This processing is preferably specified in encoded information on the disc in information storage areas. The stored information may be advantageously employed to cause the drive and reader to rotate at desired speeds and for desired times with intermediate other steps, such as incubation.

[0089] Micro technologies are particularly valuable in clinical diagnostics for identification of cell types, parasites, pathogens, and other biological matter. The present invention utilizes micro technologies to perform differential white cell counts in whole blood on optical bio-discs. In addition, this invention is directed to imaging blood cells, performing differential white cell counts, and related processing methods and software.

[0090] Another test or assay according to the present invention may be performed in at least two ways. The first

method is based upon the principle of optical imaging of blood cells in special channels located on the optical bio-disc. Approximately 5 to 20 microliters of whole blood is injected into specially designed channels on the disc. The images are analyzed with cell recognition software that identifies the various leukocyte sub-types and generates a white cell differential count. The second method is based on specific cell capture using cell specific antibodies against specific cell. In this particular embodiment, antibodies are directed against lymphocytes (CD2, CD19), monocytes (CD14), and eosinophils (CD15), for example. As with the related assay discussed above, these leukocyte sub-type specific antibodies are assembled/attached to a solid surface within the bio-disc that includes a flow chamber.

[0091] A bio-disc drive assembly is employed to rotate the disc, read and process any encoded information stored on the disc, and analyze the cell capture zones in the flow chamber of the bio-disc. The bio-disc drive is provided with a motor for rotating the bio-disc, a controller for controlling the rate of rotation of the disc, a processor for processing return signals from the disc, and analyzer for analyzing the processed signals. The rotation rate is variable and may be closely controlled both as to speed, time of rotation, and direction of rotation. The bio-disc may also be utilized to write information to the bio-disc either before, during, or after the test material in the flow chamber and target zones is interrogated by the read beam of the drive and analyzed by the analyzer. The bio-disc may include encoded information for controlling the rotation of the disc, providing processing information specific to the type of immunotyping assay to be conducted and for displaying the results on a monitor associated with the bio-drive.

[0092] The differential cell count protocols in general and in particular differential white blood cell counting protocols are developed for CD, CD-R, or DVD formats, modified versions of these formats, and alternatives thereto. The read or interrogation beam of the drive detects the various cells in the analysis sample and generates images that can be analyzed with differential cell counter software.

[0093] Microscopic methods or sophisticated cell counters are essential to perform these tedious and laborious cell-counting assays. The present method uses optical bio-discs and related assemblies. Optical images of the various leukocyte sub-types free in the analysis chamber or those captured by a specific antibody method are generated and analyzed by cell recognition software programs that identify the various cellular elements in the blood or other body fluids by their light scattering properties. This return light is detected after the light/matter interaction between the incident beam and the sample of interest. The detected return

light signal is processed to provide discernable signal signatures or digital IDs. While prior art methods typically require preparation such as cell staining, RBC elimination, or other laborious protocols, embodiments of the present methods do not require any pre-processing of the sample. These methods include microscopic analysis or cell detection in a CD-type or optical disc reader using a top-detector, bottom-detector, event counter, or cell counter.

[0094] The following paragraphs provide a summary of the principal method steps according to certain specific preferred embodiments of the present invention directed to bio-disc manufacturing.

[0095] Disc Preparation: Gold reflective discs or transmissive discs are cleaned using an air gun to remove any dust particles. The disc is rinsed twice with iso-propanol, using the spin coater. A 2% polystyrene is spin coated on the disc to give a very thick coating throughout.

[0096] Deposition of Chemistry: One embodiment includes a three step deposition protocol that incubates: streptavidin, 30 minute incubated; biotinylated first antibody incubated for 60 minutes; and second capture antibody incubated for 30 minutes. All the steps are done at room temperature in a humidity chamber using stringent washing and drying steps between depositions.

[0097] Briefly, 1 μ l of 1 mg/ml streptavidin in phosphate buffered saline is layered over each window and incubated for 30 minutes. Excess streptavidin is rinsed off using distilled water and the disc is dried. Biotinylated IgG-dextran complex is prepared by combining equal volumes of biotinylated IgG (125 μ g/ml in PBS) and aldehyde-activated dextran (200 μ g/ml). Dextran-aldehyde biotinylated-IgG complex is layered over streptavidin in each capture window and incubated for 60 minutes or overnight in a refrigerator. Excess reagent is rinsed off and the disc spun-dry. Specific barcode capture patterns are created by layering capture antibodies on designated spots on the bio-disc slot. For a differential count, anti-neutrophil (CD128 or others), anti-lymphocyte (CD2, CD19, CD56, and others), anti-eosinophil (CD15), anti-monocyte (CD14), anti-basophil (CD63), and anti-platelets (CD32 and CD151) are layered in designated spot of each slot, for example. Tables 1, 2, and 3 below lists examples of variations of capture patterns for capture layer assembly. Incubate for 30 minutes or overnight in the refrigerator. Assemble the disc using a 25 μ m, 50 μ m, or 100 μ m (50 μ m channel requires twice the volume of sample as that needed for 25 μ m chamber), straight, U-shaped, or other channel formats and a clear cover disc (for use with a top detector) or reflective cover disc (for use with a bottom detector).

TABLE 1

Capture Layer Assembly and Variations I						
Window	1	2	3	4	5	6
1 st Layer (Active Layer)	Poly-Styrene	Poly-Styrene	Poly-styrene	Poly-Styrene	Poly-styrene	Poly-styrene
2 nd Layer		Strept-avidin	Strept-avidin	Strept-avidin	Strept-avidin	Strept-avidin
Secondary		B-anti-	B-anti-	B-anti-	B-anti-	B-anti-

TABLE 1-continued

Capture Layer Assembly and Variations I						
Window	1	2	3	4	5	6
Antibody		Mouse IgG + DCHO	Mouse IgG + DCHO	Mouse IgG + DCHO	Mouse IgG + DCHO	Mouse IgG + DCHO
Primary Antibody	Refer- ence Mark	Lymphocyte Specific Antibody	Neutrophil Specific antibody	Eosinophil Specific Antibody	Basophil Specific antibody	Monocyte Specific antibody

[0098] Proteins (e.g., antigens and antibodies) passively adsorb to the disc surface as a result of hydrophobic interactions between non-polar protein substructures and the disc surface. The rate and extent of adsorption of the proteins depends on a number of factors including ratio of surface area being coated to the volume of the coating solution, concentration of the substance being adsorbed, temperature, and time of adsorption. However, the robustness of the chemistry depends on the stability of the protein itself.

[0099] In comparison to red blood cells, leukocytes are much larger and may require a stable, robust chemistry for efficient cell capture. To achieve this on the bio-disc surface, we utilized the strong interaction of biotin and avidin. A bridge technique, in which a biotin-conjugated anti-mouse IgG (raised in sheep) coupled with aldehyde activated dextran was used as a bridge between the primary capture antibody and the streptavidin coated disc surface in one embodiment of the current invention. In addition or as an alternative to avidin-biotin cross linking, a poly-functional cross linking agent such as activated aldehyde dextran (DCHO) may be used to facilitate binding of antibodies

described below. Briefly, for example, 1 ul of 1 mg/ml streptavidin in phosphate buffered saline is layered over each window and incubated for 30 minutes. Excess streptavidin is rinsed off using distilled water and the disc is dried. IgG-dextran complex is prepared by combining equal volumes of biotinylated IgG (125 ug/ml in PBS) and activated dextran aldehyde (200 ug/ml). Dextran aldehyde-biotinylated IgG complex is layered over streptavidin in each capture window and incubated for 60 minutes or overnight in refrigerator. Excess reagent is rinsed off and the disc spun-dry. Specific capture zone patterns are created by layering capture antibodies on designated spots on the bio-disc slot. For Helper/inducer-suppressor/cytotoxic assay, anti-CD4, anti-CD8, and anti-CD3, and anti-CD45 are layered onto the secondary antibodies as designated below in Table 2. Incubate for 30 minutes or overnight in the refrigerator. Assemble the disc using a 25 um or 100 um "U"-shaped adhesive (3M) and a clear cover disc for use with the top detector or reflective cover disc for use with the bottom detector. Further details relating to the dual layer antibody capture method are discussed below in conjunction with FIGS. 35A-35D, 36, and 37.

TABLE 2

Capture Layer Assembly and Variations II						
Window	1	2	3	4	5	6
1 st Layer	Poly- styrene	Poly- styrene	Poly- styrene	Poly- styrene	Poly- styrene	Poly- styrene
2 nd Layer	Strept- avidin	Strept- avidin	Strept- avidin	Strept- avidin	Strept- avidin	Strept- avidin
Secondary Capture Antibodies	B-anti Mouse IgG + DCHO	B-anti Mouse IgG + DCHO	B-anti Mouse IgG + DCHO	B-anti Mouse IgG + DCHO	B-anti Mouse IgG + DCHO	B-anti Mouse IgG + DCHO
Primary Capture Antibodies		Anti- human CD4	Anti- human CD8	Anti-human CD3	Anti- human CD45	Anti-human CD15

onto the disc surface. A stable and strong bond between the capture layers and the disc surface enhances cell capture and may reduce antisera usage, reaction time, and non-specific binding.

[0100] In another embodiment of the current invention, specific cell capture on solid phase is done using a secondary capture antibody having specific affinity to a primary antibody. The secondary capture antibody is bound to the solid surface. Samples cells may incubated off-site with the primary antibody and injected into the assay chamber or the primary antibody layered over the secondary antibody, as

[0101] Alternatively, aliquots of MNC samples are incubated off-site with antibodies against cells of interest. For Helper/inducer-suppressor/cytotoxic assay, MNC are incubated with anti-CD4, anti-CD8, anti-CD3, anti-CD14 and anti-CD45 for 15 minutes for the interaction to be complete. The unbound antibody is removed by washing (centrifuge at 1000 rpm for 5 minutes). The washed complex is then injected into the chambers 25-100 um (created by 3M adhesive or related) that are layered with specific secondary capture antibodies like specific IgG. After incubation for 30 minutes, specifically captured cells are imaged with a top

TABLE 3-continued

Capture Layer Assembly and Variations III								
Antibody	IgG + DCHO	IgG + DCHO	IgG + DCHO	IgG + DCHO	IgG + DCHO	IgG + DCHO	IgG + DCHO	IgG + DCHO
Primary Capture Antibody	Reference Mark	anti- Human CD4	anti- Human CD4	anti- Human CD8	anti- Human CD8	anti- Human CD45	anti- Human CD19	
Pattern 6								
Channel	1	2	3	4	5	6	7	8
Primary Capture Antibody	Reference Mark	anti- Human CD4	anti- Human CD4	anti- Human CD8	anti- Human CD8	anti- Human CD45	anti- Human CD19	Blank

[0102] Disc Leak-Checking and Blocking Non Specific Binding of Undesired Cells: Since blood, a biohazardous material, is being analyzed, the discs are leak checked, as part of the quality control manufacturing aspects of the present invention, to make sure none of the chambers leak during spinning of the disc with the sample in situ. Each channel is preferably filled with StabilGuard, a commercially available blocking agent, and blocked for at an hour. The disc is spun at 5000 rpm for 5 minutes and inspected for leaks and disc stability. After checking for leaks, the disc is placed in a vacuum chamber for 24 hours. After vacuuming, the chambers filled with PBS buffer or empty are placed in a vacuum pouch and stored in refrigerator until use.

[0103] Isolation of Buffy-coat Layer from Whole Blood: Buffy coat is prepared by centrifuging defibrinated venous blood in a centrifuge tube for 25 minutes at 2800 rpm. The supernatant plasma is carefully removed with a fine pipette. Then pipette the underlying white layer that contains the leukocytes and the platelets. An alternate method to obtain the buffy coat from the blood without centrifugation is to allow the blood to sediment with sedimentation-enhancing agents such as fibrinogen, dextran, gum acacia, Ficoll, or methylcellulose. Boyum's reagent (methylcellulose and sodium metrizoate) is particularly suitable for obtaining leukocyte preparation without any red cell contamination. Alternatively the lymphocytes may be isolated from whole blood by positive or negative selection, or lysis methods.

[0104] Assay on Disc—Description of Base Technology: One preferred embodiment of the differential white cell count disc test includes three individual components, (1) base disc including the chemistry, (2) channel layer, and (3) cover disc.

[0105] Buffy coat or whole blood, preferably diluted in PBS, is injected into the disc chamber, the inlet and outlet ports of the chamber are sealed with tape and the disc is incubated for a desired time preferably at room temperature. For the first method, a given area (e.g., one millimeter square in area) on the disc is scanned using the standard 780 nm laser of the optical drive with the top or bottom detector. Related cell recognition software developed by assignee and disclosed in U.S. Provisional Application Serial No. 60/363,949 entitled "Methods for Differential Cell Counts Including Leukocytes and Use of Optical Bio-Disc for Performing Same" filed Mar. 12, 2002 and U.S. Provisional Application Serial No. 60/404,921 entitled "Methods For Differential

Cell Counts Including Related Apparatus and Software For Performing Same" filed Aug. 21, 2002, is automated to give a differential count from the captured image which is preferably equal to a millimeter square, for example. For the second method, the disc is scanned using the standard 780 nm laser to image the capture zone which may include lymphocytes, neutrophils, basophils, eosinophils, monocytes, and platelets. The cell recognition software developed by assignee is automated to perform the following routines: (a) centrifuge the disc to spin off excess unbound cells, (b) image an specific area or specific capture zones, and (c) data processing that includes counting the specifically captured cells in each capture zone and deriving the numbers of different sub-sets of leukocytes.

[0106] During the processing step, the recognition software reads across each capture zone and marks cells it encounters. Following processing data from each capture zone, the software displays the number of lymphocytes, neutrophils, basophils, eosinophils, monocytes, and platelets zones per micro liter volume of blood. The entire process takes about 10-15 minutes from inserting the disc into the optical drive to displaying to results of interest.

[0107] Related disclosure associated with the present invention is also presented in U.S. Provisional Application Serial No. 60/307,262 entitled "Capture Layer Assemblies and Optical Bio-Discs for Immunophenotyping" filed Jul. 23, 2001; U.S. Provisional Application Serial No. 60/307,264 entitled "Methods for Imaging Blood Cells, Blood-Borne Parasites and Pathogens, and Other Biological Matter Including Related Optical Bio-Discs and Drive Assemblies" filed Jul. 23, 2001; U.S. Provisional Application Serial No. 60/307,562 entitled "Optical Analysis Discs Including Fluidic Circuits for Optical Imaging and Quantitative Evaluation of Blood Cells Including Lymphocytes" filed Jul. 23, 2001; and U.S. Provisional Application Serial No. 60/307,487 entitled "Methods for Differential Cell Counts Including Leukocytes and Use of Optical Bio-Disc for Performing Same" filed Jul. 24, 2001, all of which are herein incorporated by reference.

[0108] The following paragraphs provided a summary of the principal elements of the disc specifications according to certain specific preferred embodiments of the present.

[0109] Tracking Design: In one preferred embodiment of the present invention, the disc is a forward Wobble Set

FDL21:13707 or FDL21:1270 coating with 300 nm of gold. On this reflective disc, oval data windows of size 2x1 mm are etched out by Lithography. "U" shaped channels are used to create chambers that are 25 μm in height. It takes about 7 μl of sample to fill the entire chamber including the inlet and outlet ports. A 4-window/4-channel format may be preferably used. However on the transmissive disc, no data windows are etched, and the entire disc is available for use.

[0110] Adhesive and Bonding: In one preferred embodiment, the adhesive or channel layer including the present "U" shaped fluidic circuits is made from Fraylock adhesive DBL 201 Rev C 3M94661. Alternatively straight channels are used to create the chambers.

[0111] Cover Disc: Clear disc, fully reflective with 48 sample inlets with a diameter of 0.040 inches location equidistant at radius 26 mm are used in one specific embodiment of the present disc assembly.

[0112] Data Capture and Processing: The data disc is scanned and read with the software at a preferred speed of X4 and a sample rate of 2.67 MHz using assignee's cell recognition software.

[0113] Software: The present invention further includes processing methods and related cell recognition and imaging software. This software is directed to conducting and displaying cell counts and differential cell counts. The present software may be stored on the optical bio-disc, in the optical disc drive reader device, or alternatively only accessible by the optical reader from a secured server. This server may be implemented in a computing network such as a Local Area Network (LAN), a Wide Area Network (WAN), or otherwise made available over the Internet under prescribed terms and conditions. Such distribution methods are disclosed in commonly assigned U.S. Provisional Application No. 60/246,824 entitled "Interactive Method and System for Analyzing Biological Samples and Processing Related Medical Information Using Specially Prepared Bio-Optical Disc, Optical Disc Drive, and Internet Connections" filed Nov. 8, 2000 and related U.S. patent application Ser. No. 09/986,078 entitled "Interactive System for Analyzing Biological Samples and Processing Related Information and the Use Thereof" filed Nov. 7, 2001, both of which are herein incorporated by reference in their entireties.

[0114] Materials: The materials employed to practice different preferred embodiments disclosed herein include a forward wobble gold metalized photo-resist disc, a transmissive gold metalized disc, pipettes and tips, spin coater, centrifuge, swing-out rotor, Vacutainer™ CPT tubes with an anti-coagulant such as sodium citrate or ethylene diamine tetra acetic acid (EDTA), humidity chamber, disc press, adhesive, cover disc, clear cover disc, tape or equivalent, vacuum apparatus, yellow tips, and vacuum chamber.

[0115] Reagents: The reagents employed in performing the cell counts according to certain methods of the present invention include phosphate buffered saline, isopropyl alcohol, distilled water, and StabilGuard.

[0116] It is an object of the present invention to overcome limitations in the known art. Another object of the present invention is to adapt a known optical disc system to perform differential white cell counts in whole blood on optical bio-discs. It is a further object of the present invention to image blood cells and perform differential white cell counts.

[0117] These and other objects and advantages of the present invention are achieved in an optical disc and drive system for performing a cluster designation count that includes an optical assay disc, a light source, a photo detector circuit of an optical disc drive, and a processor. The optical assay disc includes a substrate, an active layer disposed over the substrate, and a cap portion integrally attached to the active layer by an adhesive or channel member. The adhesive member has one or more portions removed therefrom thereby forming chambers in which one or more capture agents are immobilized. The capture agents, being immobilized on the active layer, and within the chambers, define discrete capture zones. The light source directs light to the disc at the capture zones. The photo detector circuit of the optical disc drive is configured to detect light reflected from, or transmitted through the disc and provide an information-carrying signal from an optical disc assembly. The processor is coupled to the photo detector circuit to obtain from the information-carrying signal, operational information used to operate the optical disc system and count items in the sample bound to the capture agents.

[0118] According to one particular aspect of this system, the processor includes image recognition software for detecting and imaging cells. In one embodiment, the photo detector is on the same side of the disc as the light source for detecting light reflected from the capture zones. In an alternate embodiment, the photo detector is on the opposite side of the disc as the light source for detecting light transmitted through the capture zones.

[0119] The present invention is also directed to a method of performing a cluster designation count with an optical disc and disc drive. The method includes the steps of providing a blood sample in a first tube containing a separation gradient, rotating the first tube at a time and speed sufficient to separate the blood sample into layers, resuspending a MNC layer that contains T-cells to form a MNC suspension, providing a sample of the MNC suspension on a disc surface that includes at least one capture zone containing at least one capture agent, loading the disc into an optical reader, rotating the disc, directing an incident beam of electromagnetic radiation to the capture zone, detecting a beam of electromagnetic radiation formed after interacting with the disc at the capture zone, converting the detected beam into an output signal, and analyzing the output signal to extract information relating to the number of cells captured at the capture zone. In one embodiment of this method, the optical disc is constructed with a reflective layer such that light directed to the capture zone and not striking a cell is reflected. In another embodiment of this method, the optical disc is constructed such that light directed to the capture zone and not striking a cell is transmitted through the optical disc. Other, related aspects pertaining to determining concentrations of cell populations in a sample is disclosed in commonly assigned and co-pending U.S. Provisional Application Serial No. 60/384,205 entitled "Optical Disc Systems For Determining The Concentration Of Cells or Particles In A Sample And Methods Relating Thereto" filed May 30, 2002. This application is herein incorporated by reference in its entirety.

[0120] According to another aspect of this method, the disc surface is coated with a first group of capture agents. In one embodiment of thereof, the capture agents are immo-

bilized on the disc surface by a cross-linking system. In an alternative embodiment, the capture agents are immobilized directly on the disc surface.

[0121] According to yet another aspect of this method, the capture agents define one or more discrete capture zones. In one particular embodiment thereof, the one or more capture zones are located within one or more chambers within the optical disc. In another embodiment of this method, the capture agents have a selective affinity for cell surface antigens. In an alternative embodiment, the capture agents are for binding with primary capture agents having a selective affinity for cell surface antigens. In a preferred embodiment, the cell surface antigens are independently selected from the CD family of antigens. In a more preferred embodiment, the cell surface antigens are selected from the group consisting of CD3, CD4, CD8, and CD45.

[0122] According to still another aspect of this method, the rotating includes rotating for a sufficient period of time at a sufficient speed so that the cells have an opportunity to bind with the capture agents. In an embodiment of this aspect of this method, the rotating further includes rotating for a sufficient period of time at a sufficient speed so that unbound cells are moved away from the capture zones. In a preferred embodiment of this aspect of this method, the rotating is done at a single speed.

[0123] The embodiments of the method according to these aspects of the present invention may advantageously further include the steps of directing the sample of MNC cells into proximity with the capture agents, incubating the cells in the presence of the capture agents, and allowing the cells to specifically bind to the capture agents. An embodiment of this method further includes the step of analyzing the number of cells captured to thereby determine a cell concentration in the sample. In one aspect of this embodiment, the analyzing includes detecting sufficiently large changes in the level of light reflected from or transmitted through the disc. In another aspect of this embodiment, the analyzing includes using image recognition to count the captured cells. In a preferred embodiment of this method, the image recognition distinguishes one type of white blood cell from another.

[0124] Another embodiment of this method further includes the steps of counting the captured cells in each of the capture zones and providing an output including cell counts. In an aspect of this embodiment, the output includes counts for CD4 cells and CD8 cells, and a ratio of CD4 to CD8 cells.

[0125] In accordance with yet another principal aspect of this invention, there is provided an alternate method of performing a cluster designation count. This alternate method includes the steps of (1) providing a blood sample in a tube containing a separation gradient, (2) rotating the tube at a time and speed sufficient to separate the blood sample into layers, (3) resuspending a MNC layer containing T-cells to form a MNC suspension, (4) adding a primary antibody to the MNC suspension to form a primary antibody-T-cell complex, (5) providing a sample of the primary antibody-T-cell complex on a disc surface that includes at least one capture zone containing at least one capture agent, (6) loading the disc into an optical reader, (7) directing an incident beam of electromagnetic radiation to the capture zone, (8) detecting a beam of electromagnetic radiation

formed after interacting with the disc at the capture zone, (9) converting the detected beam into an output signal, and (10) analyzing the output signal to extract information relating to the number of cells captured at the capture zone.

[0126] Similarly in one embodiment of this alternate method, the optical disc is constructed with a reflective layer such that light directed to the capture zone and not striking a cell is reflected. In another embodiment of this method, the optical disc is constructed such that light directed to the capture zone and not striking a cell is transmitted through the optical disc.

[0127] According to an aspect of this method, the disc surface is coated with a first group of capture agents. In one embodiment thereof, the capture agents are immobilized on the disc surface by a cross-linking system. In an alternative embodiment, the capture agents are immobilized directly on the disc surface.

[0128] According to yet another aspect of this method, the capture agents define one or more discrete capture zones. In one particular embodiment thereof, the one or more capture zones are located within one or more chambers within the optical disc. In another embodiment of this method, the capture agents are for binding with cell surface antigens. In an alternative embodiment, the capture agents are for binding with a second group of capture agents having a selective affinity for cell surface antigens. In a preferred embodiment, the cell surface antigens are independently selected from the CD family of antigens. In a more preferred embodiment, the cell surface antigens are selected from the group consisting of CD3, CD4, CD8, and CD45.

[0129] According to yet another aspect of this method, the rotating includes rotating for a sufficient period of time at a sufficient speed so that the cells have an opportunity to bind with the capture agents. In an embodiment of this aspect of this method, the rotating further includes rotating for a sufficient period of time at a sufficient speed so that unbound cells are moved away from the capture zones. In a preferred embodiment of this aspect of this method, the rotating is done at a single speed.

[0130] The embodiments of the method according to these aspects of the present invention may advantageously further include the steps of directing the sample of primary antibody-T-cell complex into proximity with the capture agents, incubating the complexes in the presence of the capture agents, and allowing the complexes to specifically bind to the capture agents. An embodiment of this method further includes the step of analyzing the number of complexes captured to thereby determine a cell concentration in the sample. In one aspect of this embodiment, the analyzing includes detecting sufficiently large changes in the level of light reflected from or transmitted through the disc. In another aspect of this embodiment, the analyzing includes using image recognition to count the captured complexes. In a preferred embodiment of this method, the image recognition distinguishes one type of white blood cell from another.

[0131] Another embodiment of this method further includes the steps of counting the captured cells in each of the capture zones and providing an output including cell counts. In an aspect of this embodiment, the output includes counts for CD4 cells and CD8 cells, and a ratio of CD4 to CD8 cells.

[0132] In any of the above methods, the tube may further contain an anticoagulant. Further, in many of the specific implementations and embodiments of these methods, the surface on which the capture agent is immobilized is internal to the disc and is bounded on opposite sides by a substrate and cap.

[0133] According to the manufacturing aspects of this invention, there is provided a method of making an optical assay disc for performing a cluster designation count. This method of making an optical assay disc includes the steps of providing a cross-linker in a tube, adding a capture agent to the tube, allowing the cross-linker and the capture agent to combine (forming a complex), providing a substrate, coating the substrate with an active layer, depositing the complex onto the active layer, and attaching a cap portion to the active layer using an adhesive member. In this embodiment, the cross-linker is aldehyde-activated dextran. The capture agents are for binding with cell surface antigens.

[0134] According to an aspect of this method, the depositing involves depositing the complexes at predefined locations, thereby forming capture zones. In one embodiment thereof, the attaching involves attaching a cap portion having a reflective layer such that light directed to the capture zone and not striking a cell is reflected. In an alternative embodiment thereof, the attaching involves attaching a cap portion having a semi-reflective layer such that light directed to the capture zone and not striking a cell is transmitted through the optical disc.

[0135] In an alternate embodiment, the capture agents include primary and secondary capture antibodies. The secondary antibodies are bound to the disc surface and have specific affinity for the primary capture antibodies. In this embodiment, the primary capture antibodies have a selective affinity for cell surface antigens of interest. In a preferred embodiment thereof, the cell surface antigens are selected from the CD family of antigens. In a more preferred embodiment, the cell surface antigens are selected from the group consisting of CD3, CD4, CD8, and CD45.

[0136] Also according to the manufacturing aspects of this invention, there is provided an alternate method of making an optical assay disc for performing a cluster designation count. This alternate method of making an optical assay disc includes the steps of providing a substrate, coating the substrate with an active layer, depositing a capture agent onto the active layer (forming a capture zone), incubating the substrate, rotating the substrate, and attaching a cap portion to the active layer using an adhesive member. In one particular embodiment of this method, the step of incubating involves incubating for a sufficient period of time, at a sufficient temperature to allow immobilization of the capture agent onto the active layer. In another embodiment of this method, the step of rotating involves rotating for a sufficient period of time at a sufficient speed so that non-immobilized capture agents are moved away from the capture zones.

[0137] In accordance with another aspect of this method, the capture agent is selected from the group consisting of IgG, biotinylated-IgG, anti-CD3 antibody, biotinylated-anti-CD3 antibody, anti-CD4 antibody, biotinylated-anti-CD4 antibody, anti-CD8 antibody, biotinylated-anti-CD8 antibody, anti-CD45 antibody, and biotinylated-anti-CD45 antibody. In one embodiment of this aspect, the capture agent is a primary capture agent. In an alternative embodiment, the

capture agent is a secondary capture. According to one aspect of this alternative embodiment, the method further includes the step of depositing a primary capture agent onto the secondary capture agent after the step of rotating. In another aspect of this alternative embodiment, the method further includes the steps of incubating the substrate and rotating the substrate following depositing the primary capture agent on the secondary capture agent.

[0138] According to yet another principal aspect of this invention, there is provided an optical assay disc for performing a cluster designation count. The optical assay disc includes a substrate, an active layer disposed over the substrate, a cap portion integrally attached to the active layer by an adhesive member (having one or more portions removed to form one or more chambers defined therebetween), and one or more capture agents immobilized on the active layer. The capture agents define discrete capture zones within the one or more chambers. In one embodiment of this assay disc, the capture agents are immobilized by a cross-linking system. In an alternative embodiment of this assay disc, the capture agents are immobilized by the active layer.

[0139] In one particular embodiment of this optical assay disc, the capture agents are antibodies having a selective affinity for cell surface antigens. In a preferred embodiment, the capture agents are selected from the group consisting of antibodies for CD3, CD4, CD8, and CD45. In another embodiment of this optical assay disc, the capture agents are antibodies having a selective affinity for primary antibodies that have a selective affinity for cell surface antigens. Similarly, in a preferred embodiment of this aspect of the optical assay disc, the primary antibodies are selected from the group consisting of antibodies for CD3, CD4, CD8, and CD45. In this embodiment of the optical assay disc, the primary capture agents are anti-human produced in mice, and the secondary capture agents are anti-mouse produced in goats.

[0140] Technical aspects related to the present invention are also in U.S. Provisional Application Serial No. 60/307,489 entitled "Optical Analysis Discs Including Microfluidic Circuits for Performing Cell Counts" filed Jul. 24, 2001; U.S. Provisional Application Serial No. 60/307,825 entitled "Methods for Reducing Non-Specific Binding of Cells on Optical Bio-Discs Utilizing Charged Matter Including Heparin, Plasma, or Poly-Lysine" filed Jul. 25, 2001; U.S. Provisional Application Serial No. 60/307,762 entitled "Methods for Reducing Non-Specific Binding of Cells on Optical Bio-Discs Utilizing Blocking Agents" filed Jul. 25, 2001; U.S. Provisional Application Serial No. 60/307,764 entitled "Methods for Reducing Bubbles in Fluidic Chambers Using Polyvinyl Alcohol and Related Techniques for Achieving Same in Optical Bio-Discs" filed Jul. 25, 2001; U.S. Provisional Application Serial No. 60/308,214 entitled "Sealing Methods for Containment of Hazardous Biological Materials within Optical Analysis Disc Assemblies" filed Jul. 27, 2001; and U.S. Provisional Application Serial No. 60/308,197 entitled "Methods for Calculating Qualitative and Quantitative Ratios of Helper/Inducer-Suppressor/Cytotoxic T-Lymphocytes Using Optical Bio-Disc Platform" filed Jul. 27, 2001, all of which are herein incorporated by reference in their entireties.

[0141] The methods and apparatus of the current invention have one or more advantages over the prior art which

include, but are not limited to, simple and quick on-disc processing without the necessity of an experienced technician to run the test, small sample volumes, use of inexpensive materials, and use of known optical disc formats and drive manufacturing. These and other features and advantages will be better understood by reference to the following detailed description when taken in conjunction with the accompanying drawing figures and technical examples.

BRIEF DESCRIPTION OF THE DRAWING

- [0142] Further objects of the present invention together with additional features contributing thereto and advantages accruing therefrom will be apparent from the following description of the preferred embodiments of the invention which are shown in the accompanying drawing figures with like reference numerals indicating like components throughout, wherein:
- [0143] **FIG. 1** is a pictorial representation of a bio-disc system according to the present invention;
- [0144] **FIG. 2** is an exploded perspective view of a reflective bio-disc as utilized in conjunction with the present invention;
- [0145] **FIG. 3** is a top plan view of the disc shown in **FIG. 2**;
- [0146] **FIG. 4** is a perspective view of the disc illustrated in **FIG. 2** with cut-away sections showing the different layers of the disc;
- [0147] **FIG. 5** is an exploded perspective view of a transmissive bio-disc as employed in conjunction with the present invention;
- [0148] **FIG. 6** is a perspective view representing the disc shown in **FIG. 5** with a cut-away section illustrating the functional aspects of a semi-reflective layer of the disc;
- [0149] **FIG. 7** is a graphical representation showing the relationship between thickness and transmission of a thin gold film;
- [0150] **FIG. 8** is a top plan view of the disc shown in **FIG. 5**;
- [0151] **FIG. 9** is a perspective view of the disc illustrated in **FIG. 5** with cut-away sections showing the different layers of the disc including the type of semi-reflective layer shown in **FIG. 6**;
- [0152] **FIG. 10** is a perspective and block diagram representation illustrating the system of **FIG. 1** in more detail;
- [0153] **FIG. 11** is a partial cross sectional view taken perpendicular to a radius of the reflective optical bio-disc illustrated in **FIGS. 2, 3, and 4** showing a flow channel formed therein;
- [0154] **FIG. 12** is a partial cross sectional view taken perpendicular to a radius of the transmissive optical bio-disc illustrated in **FIGS. 5, 8, and 9** showing a flow channel formed therein and a top detector;
- [0155] **FIG. 13** is a partial longitudinal cross sectional view of the reflective optical bio-disc shown in **FIGS. 2, 3, and 4** illustrating a wobble groove formed therein;
- [0156] **FIG. 14** is a partial longitudinal cross sectional view of the transmissive optical bio-disc illustrated in **FIGS. 5, 8, and 9** showing a wobble groove formed therein and a top detector;
- [0157] **FIG. 15** is a view similar to **FIG. 11** showing the entire thickness of the reflective disc and the initial refractive property thereof;
- [0158] **FIG. 16** is a view similar to **FIG. 12** showing the entire thickness of the transmissive disc and the initial refractive property thereof;
- [0159] **FIG. 17A** is a pictorial flow chart showing the analysis of a blood sample using methods of the invention;
- [0160] **FIG. 17B** is a pictorial detail view showing the attachment of antibodies to a white blood cell for use with the disc illustrated in **FIG. 17A**;
- [0161] **FIG. 18A** is a pictorial representation of streptavidin;
- [0162] **FIG. 18B** is a pictorial representation of biotin;
- [0163] **FIG. 18C** is a pictorial representation of the cross-linking system consisting of streptavidin and biotin;
- [0164] **FIG. 18D** is a pictorial representation of a secondary antibody;
- [0165] **FIG. 18E** is a pictorial representation of a biotinylated secondary antibody;
- [0166] **FIG. 18F** is a pictorial representation of a primary antibody;
- [0167] **FIG. 18G** is a pictorial representation of a biotinylated primary antibody;
- [0168] **FIG. 18H** is a pictorial representation of a CD4⁺ cell showing four CD4 surface antigens;
- [0169] **FIG. 18I** is a pictorial representation of a CD8⁺ cell showing four CD8 surface antigens;
- [0170] **FIG. 18J** is a pictorial representation showing secondary antibodies bound to aldehyde-activated dextran;
- [0171] **FIG. 18K** is a cross-sectional pictorial representation of **FIG. 18J**;
- [0172] **FIGS. 19A and 19B** are pictorial representations showing cell capture by a primary antibody that is bound to a substrate by a cross-linking system in a first implementation of the invention;
- [0173] **FIGS. 19C and 19D** are pictorial representations showing cell capture by a primary antibody that is directly bound to a substrate in a first implementation of the invention;
- [0174] **FIGS. 20A-20I** are cross-sectional side views showing embodiments of a first implementation of a method of depositing capture agents onto the capture zones of a reflective bio-disc using a cross-linking system according to the present invention;
- [0175] **FIG. 21** is an alternate embodiment of the reflective disc shown in **FIGS. 20A-20I** without use of the cross-linking system;

[0176] FIG. 22 shows the capture chemistries utilized in FIGS. 20A-20I as implemented in a transmissive disc format;

[0177] FIGS. 23A and 23B are pictorial representations showing cell capture by a primary antibody that is bound to a secondary antibody, which is bound to a substrate by a cross-linking system in a second implementation of the invention;

[0178] FIGS. 23C and 23D are pictorial representations showing cell capture by a primary antibody that is bound to a secondary antibody, which is directly bound to a substrate in a second implementation of the invention;

[0179] FIGS. 24A-24L are cross-sectional side views showing embodiments of a second implementation of a method of depositing capture agents onto the capture zones of a reflective bio-disc using primary and secondary capture antibodies and a cross-linking system according to the present invention;

[0180] FIG. 25 is an alternate embodiment of the reflective disc shown in FIGS. 24A-24L without use of the cross-linking system;

[0181] FIG. 26 shows the capture chemistries utilized in FIGS. 24A-24L as implemented in a transmissive disc format;

[0182] FIGS. 27A and 27B are pictorial representations showing cell capture by a primary antibody that is bound to a secondary antibody, which is bound to a substrate by a strand of DCHO in a second implementation of the invention;

[0183] FIGS. 27C and 27D are pictorial representations showing cell capture by a primary antibody that is bound directly to a substrate by a strand of DCHO in a second implementation of the invention;

[0184] FIG. 28A is a pictorial flow diagram showing preparation of antibody-DCHO complexes;

[0185] FIGS. 28B-28J are cross-sectional side views showing embodiments of a second implementation of a method of depositing capture agents onto the capture zones of a reflective bio-disc using primary and secondary antibodies and a strand of DCHO;

[0186] FIG. 29 is an alternate embodiment of the reflective disc shown in FIGS. 28B-28J without use of secondary antibodies;

[0187] FIG. 30 shows the capture chemistries utilized in FIGS. 28B-28J as implemented in a transmissive disc format;

[0188] FIG. 31A is a top plan view of an optical bio-disc showing four fluidic circuits each having several capture zones for a specific cell surface marker and a negative control zone;

[0189] FIGS. 31B and 31C are pictorial representations showing capture of a primary antibody-cell complex by a secondary antibody that is bound to a substrate by a cross-linking system in a third implementation of the invention;

[0190] FIGS. 31D and 31E are pictorial representations showing capture of a primary antibody-cell complex by a

secondary antibody that is directly bound to a substrate in a third implementation of the invention;

[0191] FIGS. 32A-32I are cross-sectional side views showing embodiments of a third implementation of a method of depositing capture agents onto the capture zones of a reflective bio-disc using a cross-linking system;

[0192] FIG. 33 is an alternate embodiment of the reflective disc shown in FIGS. 32A-32I without use of the cross-linking system;

[0193] FIG. 34 shows the capture chemistries utilized in FIGS. 32A-32I as implemented in a transmissive disc format;

[0194] FIG. 35A is a top plan view of an optical bio-disc showing four fluidic circuits each having several capture zones for different cell surface markers and a negative control zone;

[0195] FIGS. 35B, 35C, and 35D are cross-sectional side views of a reflective optical bio-disc showing embodiments of a first implementation of a method of blood sample analysis using a cross-linking system;

[0196] FIG. 36 is an alternate embodiment of the reflective disc shown in FIGS. 35B, 35C, and 35D without use of the cross-linking system;

[0197] FIG. 37 shows the capture chemistries utilized in FIGS. 35B, 35C, and 35D as implemented in a transmissive disc format;

[0198] FIGS. 38A, 38B, and 38C are cross-sectional side views of a reflective optical bio-disc showing embodiments of a second implementation of a method of blood sample analysis using primary and secondary capture antibodies and a cross-linking system;

[0199] FIG. 39 is an alternate embodiment of the reflective disc shown in FIGS. 38A, 38B, and 38C without use of the cross-linking system;

[0200] FIG. 40 shows the capture chemistries utilized in FIGS. 38A, 38B, and 38C as implemented in a transmissive disc format;

[0201] FIGS. 41A, 41B, and 41C are cross-sectional side views of a reflective optical bio-disc showing embodiments of a third implementation of a method of blood sample analysis using primary and secondary antibodies and a strand of DCHO;

[0202] FIG. 42 is an alternate embodiment of the reflective disc shown in FIGS. 41A, 41B, and 41C without use of secondary antibodies;

[0203] FIG. 43 shows the capture chemistries utilized in FIGS. 41A, 41B, and 41C as implemented in a transmissive disc format;

[0204] FIG. 44A is a pictorial flow diagram showing preparation of primary antibody-cell complexes;

[0205] FIGS. 44B, 44C, and 44D are cross-sectional side views of a reflective optical bio-disc showing embodiments of a fourth implementation of a method of blood sample analysis using primary and secondary capture antibodies and a cross-linking system;

[0206] FIG. 45 is an alternate embodiment of the reflective disc shown in FIGS. 44B, 44C, and 44D without use of the cross-linking system;

[0207] FIG. 46 shows the capture chemistries utilized in FIGS. 44B, 44C, and 44D as implemented in a transmissive disc format;

[0208] FIG. 47 is a pictorial diagram of an optical disc having chambers to illustrate a bar code technique according to an embodiment of the present invention;

[0209] FIG. 48A is an illustration of results obtained from an assay using the bar code format according to an embodiment of the present invention;

[0210] FIG. 48B shows corresponding microscope and disc images for CD4, CD8, and control regions;

[0211] FIG. 49 shows a larger view of corresponding microscope and disc images to illustrate the results obtainable from the methods and apparatus of the present invention;

[0212] FIGS. 50 and 51 illustrate the use of image recognition according to an embodiment of the present invention;

[0213] FIG. 52 is illustrative screen shot of expected output from the bar code according to an embodiment of the present invention;

[0214] FIG. 53 shows a method for going from captured cells to usable output according to an embodiment of the present invention;

[0215] FIG. 54 is a pictorial graphical representation of the transformation of a sampled analog signal to a corresponding digital signal that is stored as a one-dimensional array;

[0216] FIG. 55 is a perspective view of an optical disc with an enlarged detailed view of an indicated section showing a captured white blood cell positioned relative to the tracks of the bio-disc yielding a signal-containing beam after interacting with an incident beam;

[0217] FIG. 56A is a graphical representation of a white blood cell positioned relative to the tracks of an optical bio-disc according to the present invention;

[0218] FIG. 56B is a series of signature traces derived from the white blood cell of FIG. 56A according to the present invention;

[0219] FIG. 57 is a graphical representation illustrating the relationship between FIGS. 57A, 57B, 57C, and 57D;

[0220] FIGS. 57A, 57B, 57C, and 57D, when taken together, form a pictorial graphical representation of transformation of the signature traces from FIG. 56B into digital signals that are stored as one-dimensional arrays and combined into a two-dimensional array for data input;

[0221] FIG. 58 is a logic flow chart depicting the principal steps for data evaluation according to processing methods and computational algorithms related to the present invention;

[0222] FIG. 59 is a pictorial representation showing an analysis chamber having different capture zones for analysis of white blood cells;

[0223] FIGS. 60A and 60B are pictorial representations showing analysis chambers having different capture zones for analysis of red and white blood cells;

[0224] FIG. 61 is a graph showing the number of CD4+ cells captured as a function of cell concentration in a CD marker assay using an optical bio-disc according to the present invention;

[0225] FIG. 62A is a scatter plot of data showing CD4/CD8 ratios from blood samples analyzed using the optical bio-disc of the present invention; and

[0226] FIG. 62B is a scatter plot of data showing CD4/CD8 ratios from the same blood samples of FIG. 62A analyzed using FACS analysis.

DETAILED DESCRIPTION OF THE INVENTION

[0227] The present invention is directed to disc drive systems, optical bio-discs, cellular assays and related cell counting methods, image processing techniques, and related software. Each of these aspects of the present invention is discussed below in further detail.

[0228] Aspects of the present invention relating to the disc drive systems, optical bio-discs, cellular assays and related cell counting methods, image processing techniques, and related software disclosed herein are also presented in U.S. Provisional Application Serial No. 60/338,679 entitled "Quantitative and Qualitative Methods for Cell Isolation and Typing Including Immunophenotyping" filed Nov. 13, 2001; U.S. Provisional Application Serial No. 60/332,001 entitled "Capture Layer Assemblies and Optical Bio-Discs for Immunophenotyping" filed Nov. 14, 2001; U.S. Provisional Application Serial No. 60/334,131 entitled "Methods for Calculating Qualitative and Quantitative Ratios of Helper/Inducer-Suppressor/Cytotoxic T-Lymphocytes Using Optical Bio-Disc Platform" filed Nov. 30, 2001; U.S. Provisional Application Serial No. 60/349,392 entitled "RBC Sieving Protocol Evaluations of Helper/Inducer-Suppressor/Cytotoxic T-Lymphocytes Using Whole Blood and Related Optical Bio-Disc" filed Jan. 17, 2002; U.S. Provisional Application Serial No. 60/349,449 entitled "RBC Lysis Protocol Evaluations of Helper/Inducer-Suppressor/Cytotoxic T-Lymphocytes Using Whole Blood and Related Optical Bio-Disc" filed Jan. 18, 2002; U.S. Provisional Application Serial No. 60/353,300 entitled "Methods for Differential Cell Counts Including Leukocytes and Use of Optical Bio-Disc for Performing Same" filed Jan. 31, 2002; U.S. Provisional Application Serial No. 60/355,644 entitled "Cluster Designation Assays Performed on Optical Bio-Disc Including Equi-Radial Analysis Zones" filed Feb. 5, 2002; U.S. Provisional Application Serial No. 60/358,479 entitled "Cluster Designation Assays Performed on Optical Bio-Disc Including Equi-Radial Analysis Zones and Related Systems and Methods" filed Feb. 19, 2002; U.S. Provisional Application Serial No. 60/363,949 entitled "Methods for Differential Cell Counts Including Leukocytes and Use of Optical Bio-Disc for Performing Same" filed Mar. 12, 2002; and U.S. Provisional Application Serial No. 60/404,921 entitled "Methods For Differential Cell Counts Including Related Apparatus and Software For Performing Same" filed Aug. 21, 2002, all of which are herein incorporated by reference.

[0229] Drive System and Related Discs

[0230] FIG. 1 is a perspective view of an optical bio-disc 110 according to the present invention as implemented to conduct the cell counts and differential cell counts disclosed herein. The present optical bio-disc 110 is shown in conjunction with an optical disc drive 112 and a display monitor 114. Further details relating to this type of disc drive and disc analysis system are disclosed in commonly assigned and co-pending U.S. patent application Ser. No. 10/008,156 entitled "Disc Drive System and Methods for Use with Bio-discs" filed Nov. 9, 2001 and U.S. patent application Ser. No. 10/043,688 entitled "Optical Disc Analysis System Including Related Methods For Biological and Medical Imaging" filed Jan. 10, 2002, both of which are herein incorporated by reference.

[0231] FIG. 2 is an exploded perspective view of the principal structural elements of one embodiment of the optical bio-disc 110. FIG. 2 is an example of a reflective zone optical bio-disc 110 (hereinafter "reflective disc") that may be used in the present invention. The principal structural elements include a cap portion 116, an adhesive member or channel layer 118, and a substrate 120. The cap portion 116 includes one or more inlet ports 122 and one or more vent ports 124. The cap portion 116 may be formed from polycarbonate and is preferably coated with a reflective surface 146 (FIG. 4) on the bottom thereof as viewed from the perspective of FIG. 2. In the preferred embodiment, trigger marks or markings 126 are included on the surface of the reflective layer 142 (FIG. 4). Trigger markings 126 may include a clear window in all three layers of the bio-disc, an opaque area, or a reflective or semi-reflective area encoded with information that sends data to a processor 166, as shown FIG. 10, that in turn interacts with the operative functions of the interrogation or incident beam 152, FIGS. 6 and 10.

[0232] The second element shown in FIG. 2 is an adhesive member or channel layer 118 having fluidic circuits 128 or U-channels formed therein. The fluidic circuits 128 are formed by stamping or cutting the membrane to remove plastic film and form the shapes as indicated. Each of the fluidic circuits 128 includes a flow channel 130 and a return channel 132. Some of the fluidic circuits 128 illustrated in FIG. 2 include a mixing chamber 134. Two different types of mixing chambers 134 are illustrated. The first is a symmetric mixing chamber 136 that is symmetrically formed relative to the flow channel 130. The second is an off-set mixing chamber 138. The off-set mixing chamber 138 is formed to one side of the flow channel 130 as indicated.

[0233] The third element illustrated in FIG. 2 is a substrate 120 including target or capture zones 140. The substrate 120 is preferably made of polycarbonate and has a reflective layer 142 deposited on the top thereof, FIG. 4. The target zones 140 are formed by removing the reflective layer 142 in the indicated shape or alternatively in any desired shape. Alternatively, the target zone 140 may be formed by a masking technique that includes masking the target zone 140 area before applying the reflective layer 142. The reflective layer 142 may be formed from a metal such as aluminum or gold.

[0234] FIG. 3 is a top plan view of the optical bio-disc 110 illustrated in FIG. 2 with the reflective layer 142 on the cap

portion 116 shown as transparent to reveal the fluidic circuits 128, the target zones 140, and trigger markings 126 situated within the disc.

[0235] FIG. 4 is an enlarged perspective view of the reflective zone type optical bio-disc 110 according to one embodiment of the present invention. This view includes a portion of the various layers thereof, cut away to illustrate a partial sectional view of each principal layer, substrate, coating, or membrane. FIG. 4 shows the substrate 120 that is coated with the reflective layer 142. An active layer 144 is applied over the reflective layer 142. In the preferred embodiment, the active layer 144 may be formed from polystyrene. Alternatively, polycarbonate, gold, activated glass, modified glass, or modified polystyrene, for example, polystyrene-co-maleic anhydride, may be used. In addition, hydrogels can be used. Alternatively as illustrated in this embodiment, the plastic adhesive member 118 is applied over the active layer 144. The exposed section of the plastic adhesive member 118 illustrates the cut out or stamped U-shaped form that creates the fluidic circuits 128. The final principal structural layer in this reflective zone embodiment of the present bio-disc is the cap portion 116. The cap portion 116 includes the reflective surface 146 on the bottom thereof. The reflective surface 146 may be made from a metal such as aluminum or gold.

[0236] Referring now to FIG. 5, there is shown an exploded perspective view of the principal structural elements of a transmissive type of optical bio-disc 110 according to the present invention. The principal structural elements of the transmissive type of optical bio-disc 110 similarly include the cap portion 116, the adhesive or channel member 118, and the substrate 120 layer. The cap portion 116 includes one or more inlet ports 122 and one or more vent ports 124. The cap portion 116 may be formed from a polycarbonate layer. Optional trigger markings 126 may be included on the surface of a thin semi-reflective layer 143, as best illustrated in FIGS. 6 and 9. Trigger markings 126 may include a clear window in all three layers of the bio-disc, an opaque area, or a reflective or semi-reflective area encoded with information that sends data to the processor 166, FIG. 10, which in turn interacts with the operative functions of the interrogation beam 152, FIGS. 6 and 10.

[0237] The second element shown in FIG. 5 is the adhesive member or channel layer 118 having fluidic circuits 128 or U-channels formed therein. The fluidic circuits 128 are formed by stamping or cutting the membrane to remove plastic film and form the shapes as indicated. Each of the fluidic circuits 128 includes the flow channel 130 and the return channel 132. Some of the fluidic circuits 128 illustrated in FIG. 5 include the mixing chamber 134. Two different types of mixing chambers 134 are illustrated. The first is the symmetric mixing chamber 136 that is symmetrically formed relative to the flow channel 130. The second is the off-set mixing chamber 138. The off-set mixing chamber 138 is formed to one side of the flow channel 130 as indicated.

[0238] The third element illustrated in FIG. 5 is the substrate 120 which may include the target or capture zones 140. The substrate 120 is preferably made of polycarbonate and has the thin semi-reflective layer 143 deposited on the top thereof, FIG. 6. The semi-reflective layer 143 associated

with the substrate **120** of the disc **110** illustrated in **FIGS. 5 and 6** is significantly thinner than the reflective layer **142** on the substrate **120** of the reflective disc **110** illustrated in **FIGS. 2, 3 and 4**. The thinner semi-reflective layer **143** allows for some transmission of the interrogation beam **152** through the structural layers of the transmissive disc as shown in **FIGS. 6 and 12**. The thin semi-reflective layer **143** may be formed from a metal such as aluminum or gold.

[0239] **FIG. 6** is an enlarged perspective view of the substrate **120** and semi-reflective layer **143** of the transmissive embodiment of the optical bio-disc **110** illustrated in **FIG. 5**. The thin semi-reflective layer **143** may be made from a metal such as aluminum or gold. In the preferred embodiment, the thin semi-reflective layer **143** of the transmissive disc illustrated in **FIGS. 5 and 6** may range from 10-300 Å thick and does not exceed 400 Å. This thinner semi-reflective layer **143** allows a portion of the incident or interrogation beam **152** to penetrate and pass through the semi-reflective layer **143** to be detected by a top detector **158**, **FIGS. 10 and 12**, while some of the light is reflected or returned back along the incident path. As indicated below, Table 4 presents the reflective and transmissive characteristics of a gold film relative to the thickness of the film. The gold film layer is fully reflective at a thickness greater than 800 Å. While the threshold density for transmission of light through the gold film is approximately 400 Å.

[0240] In addition to Table 4, **FIG. 7** provides a graphical representation of the inverse relationship of the reflective and transmissive nature of the thin semi-reflective layer **143** based upon the thickness of the gold. Reflective and transmissive values used in the graph illustrated in **FIG. 7** are absolute values.

TABLE 4

Au film Reflection and Transmission (Absolute Values)			
Thickness (Angstroms)	Thickness (nm)	Reflectance	Transmittance
0	0	0.0505	0.9495
50	5	0.1683	0.7709
100	10	0.3981	0.5169
150	15	0.5873	0.3264
200	20	0.7142	0.2057
250	25	0.7959	0.1314
300	30	0.8488	0.0851
350	35	0.8836	0.0557
400	40	0.9067	0.0368
450	45	0.9222	0.0244
500	50	0.9328	0.0163
550	55	0.9399	0.0109
600	60	0.9448	0.0073
650	65	0.9482	0.0049
700	70	0.9505	0.0033
750	75	0.9520	0.0022
800	80	0.9531	0.0015

[0241] With reference next to **FIG. 8**, there is shown a top plan view of the transmissive type optical bio-disc **110** illustrated in **FIGS. 5 and 6** with the transparent cap portion **116** revealing the fluidic channels, the trigger markings **126**, and the target zones **140** as situated within the disc.

[0242] **FIG. 9** is an enlarged perspective view of the optical bio-disc **110** according to the transmissive disc embodiment of the present invention. The disc **110** is

illustrated with a portion of the various layers thereof cut away to show a partial sectional view of each principal layer, substrate, coating, or membrane. **FIG. 9** illustrates a transmissive disc format with the clear cap portion **116**, the thin semi-reflective layer **143** on the substrate **120**, and trigger markings **126**. In this embodiment, trigger markings **126** include opaque material placed on the top portion of the cap. Alternatively the trigger marking **126** may be formed by clear, non-reflective windows etched on the thin reflective layer **143** of the disc, or any mark that absorbs or does not reflect the signal coming from the trigger detector **160**, **FIG. 10**. **FIG. 9** also shows, the target zones **140** formed by marking the designated area in the indicated shape or alternatively in any desired shape. Markings to indicate target zone **140** may be made on the thin semi-reflective layer **143** on the substrate **120** or on the bottom portion of the substrate **120** (under the disc). Alternatively, the target zones **140** may be formed by a masking technique that includes masking the entire thin semi-reflective layer **143** except the target zones **140**. In this embodiment, target zones **140** may be created by silk screening ink onto the thin semi-reflective layer **143**. In the transmissive disc format illustrated in **FIGS. 5, 8, and 9**, the target zones **140** may alternatively be defined by address information encoded on the disc. In this embodiment, target zones **140** do not include a physically discernable edge boundary.

[0243] With continuing reference to **FIG. 9**, an active layer **144** is illustrated as applied over the thin semi-reflective layer **143**. In the preferred embodiment, the active layer **144** is a 40 to 200 μm thick layer of 2% polystyrene. Alternatively, polycarbonate, gold, activated glass, modified glass, or modified polystyrene, for example, polystyrene-co-maleic anhydride, may be used. In addition, hydrogels can be used. As illustrated in this embodiment, the plastic adhesive member **118** is applied over the active layer **144**. The exposed section of the plastic adhesive member **118** illustrates the cut out or stamped U-shaped form that creates the fluidic circuits **128**.

[0244] The final principal structural layer in this transmissive embodiment of the present bio-disc **110** is the clear, non-reflective cap portion **116** that includes inlet ports **122** and vent ports **124**.

[0245] Referring now to **FIG. 10**, there is a representation in perspective and block diagram illustrating optical components **148**, a light source **150** that produces the incident or interrogation beam **152**, a return beam **154**, and a transmitted beam **156**. In the case of the reflective bio-disc illustrated in **FIG. 4**, the return beam **154** is reflected from the reflective surface **146** of the cap portion **116** of the optical bio-disc **110**. In this reflective embodiment of the present optical bio-disc **110**, the return beam **154** is detected and analyzed for the presence of signal elements by a bottom detector **157**. In the transmissive bio-disc format, on the other hand, the transmitted beam **156** is detected, by a top detector **158**, and is also analyzed for the presence of signal elements. In the transmissive embodiment, a photo detector may be used as a top detector **158**.

[0246] **FIG. 10** also shows a hardware trigger mechanism that includes the trigger markings **126** on the disc and a trigger detector **160**. The hardware triggering mechanism is used in both reflective bio-discs (**FIG. 4**) and transmissive bio-discs (**FIG. 9**). The triggering mechanism allows the

processor 166 to collect data only when the interrogation beam 152 is on a respective target zone 140. Furthermore, in the transmissive bio-disc system, a software trigger may also be used. The software trigger uses the bottom detector to signal the processor 166 to collect data as soon as the interrogation beam 152 hits the edge of a respective target zone 140. FIG. 10 further illustrates a drive motor 162 and a controller 164 for controlling the rotation of the optical bio-disc 110. FIG. 10 also shows the processor 166 and analyzer 168 implemented in the alternative for processing the return beam 154 and transmitted beam 156 associated with the transmissive optical bio-disc.

[0247] As shown in FIG. 11, there is presented a partial cross sectional view of the reflective disc embodiment of the optical bio-disc 110 according to the present invention. FIG. 11 illustrates the substrate 120 and the reflective layer 142. As indicated above, the reflective layer 142 may be made from a material such as aluminum, gold or other suitable reflective material. In this embodiment, the top surface of the substrate 120 is smooth. FIG. 11 also shows the active layer 144 applied over the reflective layer 142. As also shown in FIG. 11, the target zone 140 is formed by removing an area or portion of the reflective layer 142 at a desired location or, alternatively, by masking the desired area prior to applying the reflective layer 142. As further illustrated in FIG. 11, the plastic adhesive member 118 is applied over the active layer 144. FIG. 11 also shows the cap portion 116 and the reflective surface 146 associated therewith. Thus when the cap portion 116 is applied to the plastic adhesive member 118 including the desired cutout shapes, flow channel 130 is thereby formed. As indicated by the arrowheads shown in FIG. 11, the path of the incident beam 152 is initially directed toward the substrate 120 from below the disc 110. The incident beam then focuses at a point proximate the reflective layer 142. Since this focusing takes place in the target zone 140 where a portion of the reflective layer 142 is absent, the incident continues along a path through the active layer 144 and into the flow channel 130. The incident beam 152 then continues upwardly traversing through the flow channel to eventually fall incident onto the reflective surface 146. At this point, the incident beam 152 is returned or reflected back along the incident path and thereby forms the return beam 154.

[0248] FIG. 12 is a partial cross sectional view of the transmissive embodiment of the bio-disc 110 according to the present invention. FIG. 12 illustrates a transmissive disc format with the clear cap portion 116 and the thin semi-reflective layer 143 on the substrate 120. FIG. 12 also shows the active layer 144 applied over the thin semi-reflective layer 143. In the preferred embodiment, the transmissive disc has the thin semi-reflective layer 143 is made from a metal such as aluminum or gold 10-300 Angstroms thick and does not exceed 400 Angstroms. This thin semi-reflective layer 143 allows a portion of the incident or interrogation beam 152, from the light source 150, FIG. 10, to penetrate and pass upwardly through the disc to be detected by a top detector 158, while some of the light is reflected back along the same path as the incident beam but in the opposite direction. In this arrangement, the return or reflected beam 154 is reflected from the semi-reflective layer 143. Thus in this manner, the return beam 154 does not enter into the flow channel 130. The reflected light or return beam 154 may be used for tracking the incident beam 152 on pre-recorded information tracks formed in or on the semi-reflective layer

143 as described in more detail in conjunction with FIGS. 13 and 14. In the disc embodiment illustrated in FIG. 12, a physically defined target zone 140 may or may not be present. Target zone 140 may be created by direct markings made on the thin semi-reflective layer 143 on the substrate 120. These markings may be formed using silk screening or any equivalent method. In the alternative embodiment where no physical indicia are employed to define a target zone (such as, for example, when encoded software addressing is utilized) the flow channel 130 in effect may be employed as a confined target area in which inspection of an investigational feature is conducted.

[0249] FIG. 13 is a cross sectional view taken across the tracks of the reflective disc embodiment of the bio-disc 110 according to the present invention. This view is taken longitudinally along a radius and flow channel of the disc. FIG. 13 includes the substrate 120 and the reflective layer 142. In this embodiment, the substrate 120 includes a series of grooves 170. The grooves 170 are in the form of a spiral extending from near the center of the disc toward the outer edge. The grooves 170 are implemented so that the interrogation beam 152 may track along the spiral grooves 170 on the disc. This type of groove 170 is known as a "wobble groove". A bottom portion having undulating or wavy sidewalls forms the groove 170, while a raised or elevated portion separates adjacent grooves 170 in the spiral. The reflective layer 142 applied over the grooves 170 in this embodiment is, as illustrated, conformal in nature. FIG. 13 also shows the active layer 144 applied over the reflective layer 142. As shown in FIG. 13, the target zone 140 is formed by removing an area or portion of the reflective layer 142 at a desired location or, alternatively, by masking the desired area prior to applying the reflective layer 142. As further illustrated in FIG. 13, the plastic adhesive member 118 is applied over the active layer 144. FIG. 13 also shows the cap portion 116 and the reflective surface 146 associated therewith. Thus, when the cap portion 116 is applied to the plastic adhesive member 118 including the desired cutout shapes, the flow channel 130 is thereby formed.

[0250] FIG. 14 is a cross sectional view taken across the tracks of the transmissive disc embodiment of the bio-disc 110 according to the present invention as described in FIG. 12, for example. This view is taken longitudinally along a radius and flow channel of the disc. FIG. 14 illustrates the substrate 120 and the thin semi-reflective layer 143. This thin semi-reflective layer 143 allows the incident or interrogation beam 152, from the light source 150, to penetrate and pass through the disc to be detected by the top detector 158, while some of the light is reflected back in the form of the return beam 154. The thickness of the thin semi-reflective layer 143 is determined by the minimum amount of reflected light required by the disc reader to maintain its tracking ability. The substrate 120 in this embodiment, like that discussed in FIG. 13, includes the series of grooves 170. The grooves 170 in this embodiment are also preferably in the form of a spiral extending from near the center of the disc toward the outer edge. The grooves 170 are implemented so that the interrogation beam 152 may track along the spiral. FIG. 14 also shows the active layer 144 applied over the thin semi-reflective layer 143. As further illustrated in FIG. 14, the plastic adhesive member or channel layer 118 is applied over the active layer 144. FIG. 14 also shows the cap portion 116 without a reflective surface 146. Thus, when the cap is applied to the plastic adhesive member 118 including the

desired cutout shapes, the flow channel **130** is thereby formed and a part of the incident beam **152** is allowed to pass therethrough substantially unreflected.

[0251] FIG. 15 is a view similar to FIG. 11 showing the entire thickness of the reflective disc and the initial refractive property thereof. FIG. 16 is a view similar to FIG. 12 showing the entire thickness of the transmissive disc and the initial refractive property thereof. Grooves **170** are not seen in FIGS. 15 and 16 since the sections are cut along the grooves **170**. FIGS. 15 and 16 show the presence of the narrow flow channel **130** that is situated perpendicular to the grooves **170** in these embodiments. FIGS. 13, 14, 15, and 16 show the entire thickness of the respective reflective and transmissive discs. In these figures, the incident beam **152** is illustrated initially interacting with the substrate **120** which has refractive properties that change the path of the incident beam as illustrated to provide focusing of the beam **152** on the reflective layer **142** or the thin semi-reflective layer **143**.

[0252] Cellular Assays

[0253] A generic homogeneous solid phase cell capture assay for the rapid determination of absolute number of CD4⁺ and CD8⁺ T-lymphocyte populations and ratio of CD4⁺/CD8⁺ lymphocytes in blood samples may be performed utilizing the methods of the invention. The test, which is run within small flow channels incorporated into a bio-disc, determines the number of CD4⁺, CD8⁺, CD2⁺, CD3⁺, CD19⁺, and CD45⁺ cells captured by the specific antibodies on the capture zones in 7-15 μ l of mononuclear cells (MNC) isolated from whole blood. The test is based upon the principle of localized cell capture on specific locations on the disc. Several specific cell capture zones are created on the disc by localized application of capture chemistries based upon monoclonal or polyclonal antibodies to particular blood cell surface antigens. Upon flooding the 25-100 μ l chambers with the MNC blood (10,000-30,000 cells/ μ l), cells expressing CD4, CD8, CD2, CD3, CD19, and CD45 antigens are captured in the capture zones within the disc. Also incorporated within the capture zones are defined negative control areas.

[0254] FIG. 17A is a pictorial flow chart showing the analysis of a blood sample. In step 1, blood (4-8 ml) is collected directly into a 4 or 8 ml Becton Dickinson CPT Vacutainer™ and an anticoagulant such as EDTA, acid citrate dextrose (ACD), or heparin. In an equivalent step of another embodiment of the invention, 3 ml of blood in anticoagulant is overlaid into a tube **172** containing a separation gradient **176** such as Histopaque® 1077. In any case, the blood sample **174** is preferably used within two hours of collection. The tube **172** containing the separation gradient **176** with blood sample **174** overlay is centrifuged at 400 \times g in a biohazard centrifuge with horizontal rotor and swing out buckets for 25 minutes at room temperature. After centrifugation, the plasma layer **178** is removed (step 2), leaving about 2 mm of plasma above the mononuclear cell (MNC) fraction **180**. The MNC layer **180** is collected and washed with phosphate buffer saline (PBS). Cells are pelleted by centrifugation at 300 RCFs (1200 rpm) for 10 minutes at room temperature to remove any remaining platelets. The supernatant is removed and the MNC pellet **180** is re-suspended in PBS by tapping the tube gently. The final pellet **180** is re-suspended (step 3) to a cell count of 10,000-30,000 cells/ μ l depending upon the height of the flow channel **130** of the bio-disc **110**.

[0255] The flow channel **130** of a bio-disc **110** is flooded with 7 μ l of the MNC suspension, and the inlet ports **122** and vent ports **124** (FIGS. 3 and 8) of the chamber are sealed with an adhesive tab or other suitable sealing member (step 4). The bio-disc **110** is incubated for 15 minutes at room temperature, and then scanned using a 780 nm laser in an optical drive **112** to image the capture field (step 5). It should be understood that if a transmissive bio-disc **110** is used, optical drive **112** optionally includes a top detector **158** (FIG. 10) to image the capture field. Software is preferably encoded on the disc to instruct the drive to automatically perform the following tasks: (a) centrifuge the disc to spin off excess unbound cells in one or more stages; (b) image specific capture windows on a display monitor **114**; and (c) process data. Data processing includes, but is not limited to, counting the specifically captured cells in each capture zone and deriving the ratio of CD4⁺/CD8⁺ or whichever ratio is programmed to be determined. Other desired ratios may be readily provided by alternative embodiments of the present invention.

[0256] As further illustrated in FIG. 17A, the present invention is directed to a method of performing a cluster designation count with an optical disc and disc drive system. The method includes the steps of providing a blood sample in a first tube containing a separation gradient, rotating the first tube at a time and speed sufficient to separate the blood sample into layers, resuspending a MNC layer that contains T-cells to form a MNC suspension, providing a sample of the MNC suspension on a disc surface that includes at least one capture zone containing at least one capture agent, loading the disc into an optical reader, rotating the disc, directing an incident beam of electromagnetic radiation to the capture zone, detecting a beam of electromagnetic radiation formed after interacting with the disc at the capture zone, converting the detected beam into an output signal, and analyzing the output signal to extract information relating to the number of cells captured at the capture zone. In one embodiment of this method, the optical disc is constructed with a reflective layer such that light directed to the capture zone and not striking a cell is reflected. In another embodiment of this method, the optical disc is constructed such that light directed to the capture zone and not striking a cell is transmitted through the optical disc.

[0257] During the analyzing/processing step, the software reads across each capture zone image and marks cell images as it encounters them. For example, following counting of the number of captured CD4⁺ and CD8⁺ cells, the software calculates the ratio of CD4⁺/CD8⁺ cells and displays both the absolute numbers of cells in CD4⁺, CD8⁺, CD3⁺ and CD45⁺ capture zones per microliter of whole blood and also the CD4⁺/CD8⁺ ratio. The entire process takes about 12 minutes from inserting the disc into the optical drive to obtaining the numbers and ratios. Other related aspects regarding quantitating specific cell populations in a sample is disclosed in commonly assigned and co-pending U.S. Provisional Application Serial No. 60/382,327 entitled "Methods For Calculating Specific Populations Of Cells Captured In An Optical Bio-Disc" filed May 22, 2002. This application is herein incorporated by reference in its entirety.

[0258] In one embodiment, the disc is a forward Wobble Set FDL21:13707 or FDL21:1270 CD-R disc coated with 300 nm of gold as the encoded information layer. On a reflective disc, viewing windows of size 2 \times 1 mm oval are

etched out of the reflective layer by known lithography techniques. In some designs of transmissive disc, no separate viewing windows are etched, and the entire disc is available for use. In one specific embodiment, the adhesive layer is Fraylock adhesive DBL 201 Rev C 3M94661. The cover is a clear disc with 48 sample inlets with a diameter of 0.040 inches located equidistantly at radius 26 mm. The data disc is scanned and read with the software at speed 4x and sample rate 2.67 MHz using CD4⁺/CD8⁺ counting software.

[0259] Referring to FIG. 17B, in one embodiment of the present invention, a thick layer of polystyrene 118 is formed over a substrate 120 and is (optionally) layered with streptavidin 182. Cell capture antibodies are attached to the streptavidin 182 through biotin. These antibodies can include biotinylated antibodies attached to Dextran-activated aldehyde coated over the streptavidin to create an ample number of binding sites for the capture antibody. This creates a strong capture chemistry that can specifically form robust bonds with white blood cells (WBCs).

[0260] FIGS. 18A, 18B, and 18C are pictorial representations of a cross-linking system used in an embodiment of the present invention. It should be understood that a cross-linking system involves one or more cross-linking agents, or conjugates, to cross-link one or more macromolecular moieties to another. A cross-link may be a covalent or non-covalent interaction between two macromolecular moieties, usually formed when two macromolecular free radicals combine. Chemical modifications or conjugation processes to achieve cross-links involve the reaction of one functional group with another, resulting in the formation of a bond. The creation of bioconjugate reagents with reactive or selectively reactive functional groups forms the basis for simple and reproducible cross-linking or tagging of target molecules (*"Bioconjugate Techniques,"* Greg T. Hermanson, Academic Press, San Diego, Calif., (1996)).

[0261] Cross-linking agents include, but are not limited to homobifunctional linkers, heterobifunctional linkers, and zero-length cross-linkers. Homobifunctional linkers are linkers with two reactive sites of the same functionality, such as glutaraldehyde. These reagents could tie one protein to another by covalently reacting with the same common groups on both molecules. Heterobifunctional conjugation reagents contain two different reactive groups that can couple to two different functional targets on proteins and other macromolecules. For example, one part of a cross-linker may contain an amine-reactive group, while another portion may consist of a sulfhydryl-reactive group. The result is the ability to direct the cross-linking reaction to selected parts of target molecules, thus garnering better control over the conjugation process. Zero-length cross-linkers mediate the conjugation of two molecules by forming a bond containing no additional atoms. Thus, one atom of a molecule is covalently attached to an atom of a second molecule with no intervening linker or spacer. One of ordinary skill in the art may refer to *"Bioconjugate Techniques,"* Greg T. Hermanson, Academic Press, San Diego, Calif., (1996), for a detailed description of cross-linking agents.

[0262] In the present invention, cross-linking agents are bound to the surface of a bio-disc to immobilize capture agents within the target zones. A preferred cross-linking

system is the heterobifunctional group consisting of biotin-streptavidin, i.e. biotinylated capture agents bound to an avidin-coupled substrate. FIG. 18A is a pictorial representation of streptavidin 182. Without limitation, streptavidin includes avidin, streptavidin, and modifications, thereof. As shown, the protein comprises four subunits, each of which contains one binding site for biotin (Hermanson). Streptavidin 182 can be coupled to plastics such as polystyrene by various chemistries. Ideally, streptavidin 182 is attached to the active layer 144 (FIGS. 4 and 9) of the bio-disc, binding essentially irreversibly to biotinylated sensing elements (e.g. antibodies).

[0263] FIG. 18B is a pictorial representation of biotin 184. Biotin (or vitamin H) is a naturally occurring growth factor present in small amounts within every cell. Biotin's interaction with the proteins avidin and streptavidin is among the strongest non-covalent affinities known. A biotin molecule 184 may be attached directly to a protein via its valeric acid side chain or derivitized with other organic components to create spacer arms and various reactive groups. Amines, carboxylates, sulfhydryls, and carbohydrate groups can be specifically targeted for biotinylation through the appropriate choice of biotin derivative (Hermanson). FIG. 18C is a pictorial representation of the cross-linking system consisting of biotin 184 interacting with streptavidin 182.

[0264] Implementations of the embodiments of the invention utilize capture agents to perform the assays described herein. It should be understood that a capture agent refers to any macromolecule for detecting an analyte. The capture agents of the invention include macromolecules preferentially selective, or having a selective binding affinity, for an analyte of interest. Capture agents include, but are not limited to, synthetic or biologically produced nucleic acid and synthetic or biologically produced proteins. Examples of capture agents that can be employed by this invention, include, but are not restricted to, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), oligonucleotides, polymerase chain reaction products, or a combination of these nucleotides (chimera), antibodies (monoclonal or polyclonal), cell membrane receptors, and anti-sera reactive with specific antigenic determinants (such as on viruses, cells, or other materials), drugs, peptides, co-factors, lectins, polysaccharides, cells, cellular membranes, and organelles. Preferably, capture agents of the invention are antibodies.

[0265] Antibodies include, but are not limited to polyclonal, monoclonal, and recombinantly created antibodies. Antibodies of the invention can be produced in vivo or in vitro. Methods for the production of antibodies are well known to those skilled in the art. For example, see *Antibody Production: Essential Techniques*, Peter Delves (Ed.), John Wiley & Son Ltd, ISBN: 0471970107 (1997), which is incorporated herein in its entirety by reference. Alternatively, antibodies may be obtained from commercial sources, e.g., Research Diagnostics Inc., Pleasant Hill Road, Flanders, N.J. 07836. Antibodies of the invention are not meant to be limited to antibodies of any one particular species; for example, antibodies of humans, mice, rats, and goats are all contemplated by the invention. Preferably, the primary antibodies of the invention are anti-human produced in mice, and the secondary antibodies of the invention are anti-mouse produced in goats.

[0266] The term "antibody" is also inclusive of any class or subclass of antibodies, as any or all antibody types may

be used to bind to cell surface antigens. The use of antibodies in the art of medical diagnostics is well known to those skilled in the art. For example, see *Diagnostic and Therapeutic Antibodies (Methods in Molecular Medicine)*, Andrew J. T. George and Catherine E. Urch (Eds.), Humana Press; ISBN: 0896037983 (2000) and *Antibodies in Diagnosis and Therapy: Technologies, Mechanisms and Clinical Data (Studies in Chemistry Series)*, Siegfried Matzku and Rolf A. Stahel (Eds.), Harwood Academic Pub.; ISBN: 9057023105 (1999), which are incorporated entirely herein by reference.

[0267] In at least some embodiments of the invention, a plurality of capture agents is used to detect analytes of interest. FIG. 18D is a pictorial representation of the IgG class of antibodies used in the methods of the invention as a secondary capture agent 186. It should be understood that secondary capture agents of the invention include, but are not limited to, agents having an affinity for another capture agent, which have an affinity for the analyte of interest. FIG. 18E shows the secondary capture agent IgG 186 bound to a biotin molecule 184 hereinafter referred to as biotinylated-IgG.

[0268] FIG. 18F is a pictorial representation of a primary capture agent 188. It should be understood that a primary capture agent 188 of the invention has a selective affinity for the analyte of interest. Preferably, a primary capture agent is an antibody having an affinity for leukocytes. More specifically, these antibodies are directed against lymphocytes (CD2, CD19), monocytes (CD14), eosinophils (CD15), and other cell surface markers of interest. FIG. 18G shows the primary capture agent 188 bound to a biotin molecule 184. In addition to CD4 and CD8, there are antibodies against many other cell surface antigens (e.g., CD3, CD16, CD19, CD45, CD56), which can be used to identify sub-types of lymphocytes.

[0269] FIG. 18H is a pictorial representation of a CD4⁺ T-cell 190. CD4⁺ T-cells bind to specific antigens expressed by antigen-presenting cells (APCs) such as phagocytic macrophages and dendritic cells, and release lymphokines that attract other immune system cells to the area. The result is inflammation, and the accumulation of cells and molecules that attempt to wall off and destroy the antigenic material. It should be understood that CD4⁺ T-cells have a multitude of antigens 192 over the entire cell surface. However, FIG. 18H shows the CD4⁺ T-cell 190 having four antigens 192 for illustrative purposes only.

[0270] FIG. 18I is a pictorial representation of a CD8⁺ T-cell 194. These T-cells secrete molecules that destroy the cell to which they have bound. This is important in fighting viral infections, as the CD8⁺ T-cells destroy the infected cells before they can release a fresh crop of viruses able to infect other cells. It should be understood that CD8⁺ T-cells have a multitude of antigens 196 over the entire cell surface. However, FIG. 18I shows the CD8⁺ T-cell 194 having four antigens 196 for illustrative purposes only.

[0271] FIG. 18J is a pictorial representation showing secondary antibodies 186 bound to a 3-Dimensional matrix of aldehyde-activated dextran (DCHO) 198, thereby forming a DCHO-antibody complex 199. Dextran is mainly a linear polysaccharide consisting of repeating units of D-glucose linked together by glycosidic bonds. Used extensively as a cross-linking agent, dextran is multivalent in nature,

which allows molecules to be attached at numerous sites along the polymer chain (Hermanson). For illustrative purposes only, antibodies 186 bound to dextran 198 will be depicted as shown in FIG. 18K.

[0272] Aspects relating the cellular assays of the present invention are also disclosed in U.S. Provisional Application Serial No. 60/308,176 entitled "Quantitative and Qualitative Methods for Characterizing Cancerous Blood Cells Including Leukemic Blood Samples Using Optical Bio-Disc Platform" filed Jul. 27, 2001; U.S. Provisional Application Serial No. 60/312,248 entitled "Methods for Quantitative and Qualitative Characterization of Cancerous Blood Cells Including Lymphoma Blood Samples Using Optical Bio-Disc Platform" filed Aug. 15, 2001; U.S. Provisional Application Serial No. 60/313,514 entitled "Methods for Specific Cell Capture by Off-Site Incubation of Primary Antibodies with Sample and Subsequent Capture by Surface-Bound Secondary Antibodies and Optical Bio-Disc Including Same" filed Aug. 20, 2001; U.S. Provisional Application Serial No. 60/313,715 entitled "RBC Lysis Protocol Evaluations of Helper/Inducer-Suppressor/Cytotoxic T-Lymphocytes Using Whole Blood and Related Optical Bio-Disc" filed Aug. 20, 2001; U.S. Provisional Application Serial No. 60/313,536 entitled "RBC Sieving Protocol Evaluations of Helper/Inducer-Suppressor/Cytotoxic T-Lymphocytes Using Whole Blood and Related Optical Bio-Disc" filed Aug. 20, 2001; and U.S. Provisional Application Serial No. 60/315,937 entitled "Quantitative and Qualitative Methods for Cell Isolation and Typing Including Immunophenotyping" filed Aug. 30, 2001, all of which are herein incorporated by reference.

[0273] Implementation I

[0274] FIGS. 19A-19D are pictorial representations of analyte capture in a first implementation of the invention. FIGS. 19A and 19B show capture of CD4⁺ T-cells 190 and CD8⁺ T-cells 194 by biotinylated primary antibodies (FIG. 18G). The antibodies 188 are immobilized on the active layer 144 of the bio-disc 110 (FIGS. 4 and 9) by the cross-linking agent streptavidin 182. FIGS. 19C and 19D show another embodiment of the same implementation of the invention without a cross-linking system. In this embodiment, primary antibodies 188 are immobilized directly on the active layer 144 of the bio-disc 110.

[0275] FIGS. 20A-20I are cross-sectional views showing construction of an embodiment of the first implementation of the invention. The first embodiment involves construction of a reflective disc utilizing a cross-linking system to immobilize the capture agents within the flow channels of the bio-disc. Referring to FIG. 20A, a light-transparent substrate 120, a reflective layer 142, and an active layer 144 of an optical bio-disc 110 are shown. Portions of reflective layer 142 are removed (or openings were created when deposited) to produce viewing windows 200 through which light can be directed at the locations of capture zones 140 where the antibodies are to be affixed. FIG. 20A shows five such capture zones 140, the first thereof indicated as capture zone 141. The active layer 144 is preferably polystyrene, which is spin-coated, or otherwise deposited by methods known in the art, over the reflective layer 142 to form a smooth surface with a thickness of about 40 to 300 microns. Streptavidin 182 is then deposited over each capture zone 140 and 141, and the disc 110 is incubated at room tem-

perature in a humidity chamber for 30 minutes (FIG. 20B). The disc 110 is washed to remove unbound streptavidin 182, and then spin-dried to completely remove moisture from the surface of the disc 110 (FIG. 20C). A reference mark or calibration dot 202 is deposited over the first capture zone 141 and biotinylated primary antibodies 188 are deposited over each successive capture zone 140 (FIGS. 20D and 20E). The disc 110 is then incubated at room temperature in a humidity chamber for 30 minutes. Unbound antibodies 188 are removed with a PBS wash, and the disc 110 is spin-dried to remove surface moisture (FIG. 20F). An adhesive member 118 is attached to the active layer 144 (FIG. 20G). A cap portion 116 (FIG. 2) may be formed from polycarbonate and is preferably coated with a reflective surface 146 on the bottom as best shown in FIG. 4. The cap portion 116 is integrally attached to adhesive member 118 thereby forming flow channels 130 within the disc (FIG. 20G). A blocking agent 204, such as StabilGuard®, is injected into each flow channel 130 to quickly and effectively block any remaining nonspecific binding sites of the active layer 144 (FIG. 20H). The disc 110 is incubated at room temperature in a humidity chamber for a predetermined time of preferably 30 minutes, and any remaining solution is completely removed via vacuum (FIG. 20I).

[0276] A second embodiment of the first implementation of the invention involves construction of a reflective disc 110 without the use of a cross-linking system to immobilize the capture agents within the flow channels 130 of the bio-disc 110. In this embodiment, a reference mark or calibration dot 202 is deposited over the first window 141 and non-biotinylated primary antibodies 188 (FIG. 18F) are deposited directly onto the active layer 144 (FIG. 20A) over each successive capture zone 140. The disc 110 is then incubated at room temperature in a humidity chamber for preferably 30 minutes (FIG. 20E). Unbound antibodies 188 are removed with a PBS wash, and the disc 110 is spin-dried to remove surface moisture (FIG. 20F). The adhesive member 118 is attached to the active layer 144. A cap portion 116 (FIG. 2) may be formed from polycarbonate and is preferably coated with a reflective surface 146 on the bottom as best shown in FIG. 4. The cap portion 116 is integrally attached to adhesive member 118 thereby forming flow channels 130 within the disc (FIG. 20G). A blocking agent 204, such as StabilGuard®, is injected into each flow channel 130 to quickly and effectively block any remaining nonspecific binding sites of the active layer 144 (FIG. 20H). The disc is incubated at room temperature in a humidity chamber for 30 minutes, and any remaining solution is completely removed via vacuum (FIG. 20I). FIG. 21 shows a cross-sectional view of the completed reflective bio-disc 110 without use of a cross-linking system.

[0277] A third embodiment of the first implementation of the invention involves construction of a transmissive disc 110 utilizing a cross-linking system to immobilize the capture agents within the flow channels 130 of the bio-disc 110. In this embodiment, the substrate 120, semi-reflective layer 143 without viewing windows 200 (FIG. 20A), and active layer 144 are presented as shown in FIG. 5. Deposition of streptavidin 182, biotinylated primary antibodies 188, reference dot 202, and blocking agent 204 are as described above, and as shown in FIGS. 20B-20H. A respective adhesive member 118 is attached to the active layer 144. An optically transparent cap portion 116 (FIG. 5) may be formed from polycarbonate. The cap portion 116 is inte-

grally attached to adhesive member 118 thereby forming flow channels 130 within the disc 110 (FIG. 20G). FIG. 22 shows a cross-sectional view of the completed transmissive bio-disc 110 utilizing a cross-linking system. It should be understood that a fourth embodiment of the first implementation may be constructed by those skilled in the art. The fourth embodiment involves construction of a transmissive disc 110 without the use of a cross-linking system to immobilize the capture agents within the flow channels of the bio-disc 110 (FIGS. 21 and 22).

[0278] Implementation II

[0279] FIGS. 23A-23D are pictorial representations of analyte capture in a second implementation of the invention. FIGS. 23A and 23B show capture of CD4⁺ T-cells 190 and CD8⁺ T-cells 194 by primary antibodies 188 (FIG. 18F). The primary antibodies 188 are bound to biotinylated secondary antibodies 186 (FIG. 18E) immobilized on the active layer 144 of the bio-disc 110 (FIGS. 4 and 9) by the cross-linking agent streptavidin 182. FIGS. 23C and 23D show another embodiment of the same implementation of the invention without a cross-linking system. In this embodiment, the secondary antibodies 186 are immobilized directly on the active layer 144 of the bio-disc 110.

[0280] FIGS. 24A-24L are cross-sectional views showing construction of an embodiment of the second implementation of the invention. The first embodiment involves construction of a reflective disc utilizing a cross-linking system to immobilize the capture agents within the flow channels 130 of the bio-disc 110. Referring to FIG. 24A, a light-transparent substrate 120, a reflective layer 142, and an active layer 144 of an optical bio-disc 110 are shown. Portions of reflective layer 142 are removed (or openings were created when deposited) to produce viewing windows 200 through which light can be directed at the locations of capture zones 140 where the antibodies are to be affixed. FIG. 24A shows five such capture zones 140, the first thereof being designated as capture zone 141. The active layer 144 is preferably polystyrene, which is spin-coated over the reflective layer 142 to form a smooth surface with a thickness of about 40 to 300 microns. Streptavidin 182 is then deposited over each capture zone 140 and 141, and the disc 110 is incubated at room temperature in a humidity chamber for approximately 30 minutes (FIG. 24B). The disc 110 is washed to remove unbound streptavidin 182, and then spin-dried to completely remove moisture from the surface of the disc 110 (FIG. 24C). A reference mark or calibration dot 202 is deposited over the first capture zone 141 and biotinylated secondary antibodies 186 are deposited over each successive capture zone 140 (FIGS. 24D and 24E). The disc 110 is then incubated at room temperature in a humidity chamber for preferably 30 minutes (FIG. 24E). Unbound secondary antibodies 186 are removed with a PBS wash, and the disc 110 is spin-dried to remove surface moisture (FIG. 24F). Primary antibodies 188 (FIG. 18F) then are deposited over each capture zone 140 (FIG. 24G). The disc 110 is then incubated at room temperature in a humidity chamber for approximately 30 minutes (FIG. 24H). Unbound primary antibodies 188 are removed with a PBS wash, and the disc 110 is spin-dried to remove surface moisture (FIG. 24I). A respective adhesive member 118 is attached to the active layer 144. The cap portion 116 (FIG. 2) may be similarly formed from polycarbonate and is preferably coated with the reflective surface 146 on the

bottom as best shown in FIG. 4. The cap portion 116 is integrally attached to adhesive member 118 thereby forming flow channels 130 within the disc (FIG. 24J). A blocking agent 204, such as StabilGuard®, is injected into each flow channel 130 to quickly and effectively block any remaining nonspecific binding sites of the active layer 144 (FIG. 24K). The disc 110 is incubated at room temperature in a humidity chamber for 30 minutes, and any remaining solution is completely removed via vacuum (FIG. 24L).

[0281] A second embodiment of the second implementation of the invention involves construction of a reflective disc 110 without the use of a cross-linking system to immobilize the capture agents within the flow channels 130 of the bio-disc 110. In this embodiment, a reference mark or calibration dot 202 is deposited over the first window 141 and non-biotinylated secondary antibodies 186 (FIG. 18D) are deposited directly onto the active layer 144 (FIG. 24A) over each successive capture zone 140. The disc 110 is then incubated at room temperature in a humidity chamber for 30 minutes (FIG. 24E). Unbound secondary antibodies 186 are removed with a PBS wash, and the disc 110 is spun-dried to remove surface moisture (FIG. 24F). Primary antibodies 188 (FIG. 18F) then are deposited over each capture zone 140 (FIG. 24G). The disc 110 is then incubated at room temperature in a humidity chamber for 30 minutes (FIG. 24H). Unbound primary antibodies 188 are removed with a PBS wash, and the disc 110 is spin-dried to remove surface moisture (FIG. 24I). An adhesive member 118 is attached to the active layer 144. As with the prior embodiments, the cap portion 116 of this embodiment may be formed from polycarbonate and is preferably coated with a reflective surface 146 on the bottom as best shown in FIG. 4. The cap portion 116 is integrally attached to adhesive member 118 thereby forming flow channels 130 within the disc (FIG. 24J). A blocking agent 204, such as StabilGuard®, is injected into each flow channel 130 to quickly and effectively block any remaining nonspecific binding sites of the active layer 144 (FIG. 24K). The disc is incubated at room temperature in a humidity chamber for preferably 30 minutes in this embodiment, and any remaining solution is completely removed via vacuum (FIG. 24L). FIG. 25 shows a cross-sectional view of the completed reflective bio-disc 110 without use of a cross-linking system.

[0282] A third embodiment of the second implementation of the invention involves construction of a transmissive disc 110 utilizing a cross-linking system to immobilize the capture agents within the flow channels 130 of the bio-disc 110. In this embodiment, the substrate 120, semi-reflective layer 143 without viewing windows 200 (FIG. 24A), and active layer 144 are presented as shown in FIG. 5. Deposition of streptavidin 182, biotinylated secondary antibodies 186, primary antibodies 188, reference dot 202, and blocking agent 204 are as described above, and as shown in FIGS. 24B-24K. The adhesive member 118 is attached to the active layer 144 in a similar manner. The optically transparent cap portion 116 (FIG. 5) may be formed from polycarbonate. The cap portion 116 is integrally attached to adhesive member 118 thereby forming flow channels 130 within the disc 110 (FIG. 24J). FIG. 26 shows a cross-sectional view of the completed transmissive bio-disc 110 utilizing a cross-linking system. It should be understood that a fourth embodiment of this second implementation may be constructed by those skilled in the art. The fourth embodiment involves construction of a transmissive disc 110 without the

use of a cross-linking system to immobilize the capture agents within the flow channels of the bio-disc 110 (FIGS. 21 and 22).

[0283] Implementation III

[0284] FIGS. 27A-D are pictorial representations of analyte capture according to a third implementation of the present invention. FIGS. 27A and 27B show capture of CD4⁺ T-cells 190 and CD8⁺ T-cells 194 by primary antibodies 188 (FIG. 18F). The primary antibodies 188 are bound to secondary antibodies 186 (FIG. 18D), which are bound to a strand of DCHO. The DCHO is immobilized on the active layer 144 of the bio-disc 110 (FIGS. 4 and 9). FIGS. 27C and 27D show another embodiment of the same implementation of the invention without the secondary antibodies 186. In this embodiment, the primary antibodies 188 are bound directly to the DCHO, which is immobilized on the active layer 144 of the bio-disc 110.

[0285] FIG. 28A is a pictorial flow diagram showing preparation of antibody-DCHO complexes while FIGS. 28B-28J are multiple views showing construction of an embodiment of the third implementation of the invention. The first embodiment of this third implementation involves construction of a reflective disc utilizing the cross-linking agent DCHO to immobilize the capture agents within the flow channels 130 of the bio-disc 110. FIG. 28A shows preparation of the DCHO-antibody complex 199. Equal concentrations of DCHO 198 and secondary antibodies 186 are mixed and allowed to combine, thereby forming the DCHO-antibody (secondary) complex 199. Referring to FIG. 28B, a light-transparent substrate 120, a reflective layer 142, and an active layer 144 of an optical bio-disc 110 are shown. Portions of reflective layer 142 are removed (or opening created when deposited) to produce viewing windows 200 through which light can be directed at the locations of capture zones 140 where the antibodies are to be affixed. FIG. 28B shows five such capture zones 140, the first thereof indicated as capture zone 141. The active layer 144 is preferably polystyrene, which is spin-coated over the reflective layer 142 to form a smooth surface with a thickness of about 40 to 300 microns. DCHO-antibody complex (secondary) 199 is then deposited over each capture zone 140, and the disc 110 is incubated at room temperature in a humidity chamber for 30 minutes in this specific embodiment (FIG. 28C). The disc 110 is washed to remove unbound DCHO-antibody complex (secondary) 199, and then spin-dried to completely remove moisture from the surface of the disc 110 (FIG. 28D). A reference dot 202 is deposited over the first capture zone 141 and primary antibodies 188 are deposited over each successive capture zone 140 (FIG. 28E). The disc 110 is then incubated at room temperature in a humidity chamber for 30 minutes (FIG. 28F). Unbound primary antibodies 188 are removed with a PBS wash, and the disc 110 is spin-dried to remove surface moisture (FIG. 28G). The adhesive member or channel layer 118 is attached to the active layer 144. As with the above embodiments, the cap portion 116 generally shown in FIG. 2 may be formed from polycarbonate and is preferably coated with a reflective surface 146 on the bottom as best shown in FIG. 4. In the present embodiment, the cap portion 116 is integrally attached to adhesive member 118 thereby forming flow channels 130 within the disc as illustrated in FIG. 28H. A blocking agent 204, such as StabilGuard®, is injected into each flow channel 130 to quickly and effec-

tively block any remaining nonspecific binding sites of the active layer 144. This step is shown in FIG. 28I. The disc 110 is incubated at room temperature in a humidity chamber for approximately 30 minutes in this embodiment, and any remaining solution is completely removed via vacuum as represented in FIG. 28J.

[0286] According to the manufacturing aspects of this invention, FIGS. 28A-28J also show a method of making an optical assay disc for performing a cluster designation count. This method of making an optical assay disc includes the steps of providing a cross-linker in a tube, adding a capture agent to the tube, allowing the cross-linker and the capture agent to combine (forming a complex), providing a substrate, coating the substrate with an active layer, depositing the complex onto the active layer, and attaching a cap portion to the active layer using an adhesive member. In this embodiment, the cross-linker is aldehyde-activated dextran. The capture agents are for binding with cell surface antigens. In an alternate embodiment, the capture agents are for binding with primary capture agents having a selective affinity for cell surface antigens. In a preferred embodiment, the cell surface antigens are independently selected from the CD family of antigens. In a more preferred embodiment, the cell surface antigens are independently selected from the group consisting of CD3, CD4, CD8, and CD45.

[0287] A second embodiment of the third implementation of the invention involves construction of a reflective disc 110 without the use of a secondary antibody to immobilize the primary capture agents within the flow channels 130 of the bio-disc 110. In this embodiment, equal concentrations of DCHO 198 and primary antibodies 188 are mixed and allowed to combine, thereby forming the DCHO-antibody (primary) complex 199 represented in FIG. 28A. A reference or calibration mark 202 is deposited over the first window 141 and the DCHO-antibody (primary) complex 199 are deposited onto the active layer 144 (FIG. 28C) over each successive capture zone 140. The disc 110 is then incubated at room temperature in a humidity chamber for preferably 30 minutes and unbound DCHO-antibody (primary) complex 199 is removed with a PBS wash. The disc 110 is spin-dried to remove surface moisture as illustrated in FIG. 28D. The adhesive member or channel layer 118 is similarly attached to the active layer 144. The cap portion 116 may be formed from polycarbonate and is preferably coated with a reflective surface 146 on the bottom as best shown in FIG. 4. The cap portion 116 is integrally attached to adhesive member 118 thereby forming flow channels 130 within the disc as shown in FIG. 28H. A blocking agent 204, such as StabilGuard®, is injected into each flow channel 130 to quickly and effectively block any remaining nonspecific binding sites of the active layer 144 (FIG. 28I). The disc is incubated at room temperature in a humidity chamber for approximately 30 minutes, and any remaining solution is completely removed via vacuum as illustrated in FIG. 28J. FIG. 29 shows a cross-sectional view of the completed reflective bio-disc 110 without use of secondary antibodies.

[0288] A third embodiment of the third implementation of the invention involves construction of a transmissive disc 110 utilizing the cross-linker DCHO to immobilize the capture agents within the flow channels 130 of the bio-disc 110. In this embodiment, the substrate 120, semi-reflective layer 143 without viewing windows 200 (FIG. 28B), and active layer 144 are presented as shown in FIG. 5. Depo-

sition of DCHO-antibody (secondary) complex 199, primary antibodies 188, reference mark or dot 202, and blocking agent 204 are as described above, and as shown in FIGS. 28C-28I. The adhesive member or channel layer 118 is attached to the active layer 144. An optically transparent cap portion 116 (FIG. 5) may be formed from polycarbonate. The cap portion 116 is integrally attached to adhesive member 118 thereby forming flow channels 130 within the disc 110. FIG. 30 shows a cross-sectional view of the completed transmissive bio-disc 110 utilizing a DCHO-antibody (secondary) complex 199 and primary antibodies 188. It should be understood that a fourth embodiment of the third implementation may be constructed by those skilled in the art. The fourth embodiment involves construction of a transmissive disc 110 without the use secondary antibodies to immobilize the primary capture agents within the flow channels of the bio-disc 110 (FIGS. 29 and 30). Other embodiments may involve use of streptavidin 182 (FIG. 18A) and biotin 184 (FIG. 18B) to immobilize primary or secondary antibodies to the active layer 144 of the bio-disc 110. Immobilized antibodies (both primary and secondary) can thereafter be complexed with DCHO 198 for increased concentrations of capture agents within the flow channels 130 of the bio-disc 110.

[0289] Implementation IV

[0290] FIG. 31A is a top plan view of an optical bio-disc 110 showing four fluidic circuits 128 each having several capture zones 140 for a specific cell surface marker and a negative control zone. As shown, each fluidic circuit is dedicated to having capture zones 140 specifically directed to CD3, CD4, CD8, and CD45. Any other desired pattern or combination of cell surface antigens such as, for example, other CD markers or cell surface markers may be employed. In this particular implementation, for each individual fluidic circuit it is necessary to have only a single common capture agent in each of the capture zones 140. The disc shown in FIG. 31A is specifically suited for use with the cell capture chemistries and methods described below in FIGS. 31B-31E, 44A-44D, 45, and 46. The bio-disc 110 represented in FIG. 31A may be either a reflective or transmissive disc.

[0291] Turning now to FIGS. 31B-31E, there is shown pictorial representations of analyte capture in a fourth implementation of the present invention. FIGS. 31B and 31C show capture by secondary antibodies 186 of CD4⁺ cells 190 and CD8⁺ cells 194 that were previously combined with primary antibodies 188 (FIG. 18F) to form primary antibody-cell complexes. The primary antibodies 188 are bound to biotinylated secondary antibodies 186 (FIG. 18E) immobilized on the active layer 144 of the bio-disc 110 (FIGS. 4 and 9) by the cross-linking agent streptavidin 182. FIGS. 31D and 31E show another embodiment of the same implementation of the invention without a cross-linking system. In this embodiment, the secondary antibodies 186 are immobilized directly on the active layer 144 of the bio-disc 110.

[0292] FIGS. 32A-32I are cross-sectional views showing construction of an embodiment of the fourth implementation of the invention. The first embodiment involves construction of a reflective disc utilizing a cross-linking system to immobilize the capture agents within the flow channels of the bio-disc. Referring to FIG. 32A, a light-transparent substrate 120, a reflective layer 142, and an active layer 144 of

an optical bio-disc **110** are shown. Portions of reflective layer **142** are removed (or openings were created when deposited) to produce viewing windows **200** through which light can be directed at the locations of capture zones **140** where the antibodies are to be affixed. **FIG. 32A** shows five such capture zones **140**, the first thereof designated as capture zone **141**. The active layer **144** is preferably polystyrene, which is spin-coated over the reflective layer **142** to form a smooth surface with a thickness of about 40 to 300 microns. Streptavidin **182** is then deposited over each capture zone **140** and **141**, and the disc **110** is incubated at room temperature in a humidity chamber for approximately 30 minutes as represented in **FIG. 32B**. The disc **110** is washed to remove unbound streptavidin **182**, and then spin-dried to completely remove moisture from the surface of the disc **110** as illustrated in **FIG. 32C**. A reference mark or calibration dot **202** is deposited over the first capture zone **141** and biotinylated secondary antibodies **186** are deposited over each successive capture zone **140** as shown in **FIGS. 32D and 32E**. The disc **110** is then incubated at room temperature in a humidity chamber for approximately 30 minutes in this embodiment. This step is illustrated in **FIG. 32E**. Unbound secondary antibodies **186** are removed with a PBS wash, and the disc **110** is spin-dried to remove surface moisture. This step of the present method is represented in **FIG. 32F**. An adhesive member or channel layer **118** is similarly applied to the active layer **144**. A respective cap portion **116** is preferably formed from polycarbonate and coated with a reflective surface **146** on the bottom as best shown in **FIG. 4**. The cap portion **116** is integrally attached to adhesive member **118** thereby forming flow channels **130** within the disc as shown in **FIG. 32G**. A blocking agent **204**, such as StabilGuard®), is injected into each flow channel **130** to quickly and effectively block any remaining nonspecific binding sites of the active layer **144**. This step is shown in **FIG. 32H**. The disc **110** is incubated at room temperature in a humidity chamber for preferably 30 minutes, and any remaining solution is completely removed via vacuum as represented in **FIG. 32I**.

[0293] A second embodiment of the fourth implementation of the invention involves construction of a reflective disc **110** without the use of a cross-linking system to immobilize the capture agents within the flow channels **130** of the bio-disc **110**. In this embodiment, a reference mark or calibration dot **202** is deposited over the first window **141** and non-biotinylated secondary antibodies **186**, shown generally in **FIG. 18D**, are deposited directly onto the active layer **144** over each successive capture zone **140**. The disc **110** is then incubated at room temperature in a humidity chamber for 30 minutes. Unbound secondary antibodies **186** are removed with a PBS wash, and the disc **110** is spin-dried to remove surface moisture. An adhesive member **118** is attached to the active layer **144**. A cap portion **116** may be formed from polycarbonate and is preferably coated with a reflective surface **146** on the bottom as best shown in **FIG. 4**. The cap portion **116** is integrally attached to adhesive member **118** thereby forming flow channels **130** within the disc. A blocking agent **204**, such as StabilGuard®, is injected into each flow channel **130** to quickly and effectively block any remaining nonspecific binding sites of the active layer **144**. The disc is incubated at room temperature in a humidity chamber for approximately 30 minutes, and any remaining solution is completely removed via vacuum.

FIG. 33 shows a cross-sectional view of the completed reflective bio-disc **110** without use of a cross-linking system.

[0294] A third embodiment of the fourth implementation of the invention involves construction of a transmissive disc **110** utilizing a cross-linking system to immobilize the capture agents within the flow channels **130** of the bio-disc **110**. In this embodiment, the substrate **120**, semi-reflective layer **143** without viewing windows **200** (**FIG. 32A**), and active layer **144** are presented as shown in **FIG. 5**. Deposition of streptavidin **182**, biotinylated primary antibodies **188**, reference mark **202**, and blocking agent **204** are as described above, and as shown in **FIGS. 32B-32H**. An adhesive member **118** is attached to the active layer **144**. An optically transparent cap portion **116** (**FIG. 5**) may be formed from polycarbonate. The cap portion **116** is integrally attached to adhesive member **118** thereby forming flow channels **130** within the disc **110** (**FIG. 32G**). **FIG. 34** shows a cross-sectional view of the completed transmissive bio-disc **110** utilizing a cross-linking system. It should be understood that a fourth embodiment of the fourth implementation may be constructed by those skilled in the art. The fourth embodiment involves construction of a transmissive disc **110** without the use of a cross-linking system to immobilize the secondary capture agents within the flow channels of the bio-disc **110** (**FIGS. 33 and 34**).

[0295] Cluster Designation Count—Implementation I

[0296] **FIG. 35A** is a top plan view of an optical bio-disc **110** showing four fluidic circuits **128** each having several capture zones **140** for four different specific cell surface markers and a negative control zone. As shown, each fluidic circuit is dedicated to having four capture zones **140** each one specifically directed to CD4, CD8, CD3, and CD45. Any other desired pattern or combination of cell surface antigens such as, for example, other CD markers or cell surface markers may be employed. In this particular implementation, there is no restriction that for each individual fluidic circuit it is necessary to have only a single common capture agent in each of the capture zones **140**. On the contrary, in this implementation, it is desirable to have capture zones with different capture agents. These capture zones may be arranged, for example, in an array format having at least a 2-by-2 matrix. The disc shown in **FIG. 35A** is specifically suited for use with the cell capture chemistries and methods described below in **FIGS. 35B-35D, 36, 37, 38A-38C, 39, 40, 41A-41C, 42, and 43**. The bio-disc **110** represented in **FIG. 35A** may be either a reflective or transmissive disc.

[0297] Referring next to **FIGS. 35B-35D**, analysis of a purified and washed MNC sample (**FIG. 17A**) using a bio-disc of the first implementation of the invention is shown. The flow channels **130** of a bio-disc **110** are flooded with the MNC sample as represented in **FIG. 35B**. Capillary action, pressure applied with an external applicator, and/or centrifugal force (i.e., the force on a body in curvilinear motion directed away from the center or curvature or axis of rotation) act upon the test sample to achieve contact with the primary antibodies **188**. As shown in **FIG. 35C**, the primary antibodies come into contact with and then capture any CD4⁺ cells **190** and CD8⁺ cells **194** present within the test sample. The optical drive motor **162** (**FIG. 10**) spins the disc, which clears the capture zones **140** (**FIG. 201**) of unbound T-cells. The incident beam **152** of an optical disc drive **112** (**FIG. 1**) interacts with the captured CD4⁺ cells

190 and CD8⁺ cells 194, FIG. 35D, and the return beam 154 is reflected to the detector 157 (FIG. 10) for processing and analysis.

[0298] FIG. 36 shows analysis of the same test sample from FIGS. 35B-35D, using the second embodiment of the first implementation of the invention. The flow channels 130 of a bio-disc 110 are flooded with the MNC sample (FIG. 35B). Capillary action, pressure applied with an external applicator, and/or centrifugal force (i.e., the force on a body in curvilinear motion directed away from the center or curvature or axis of rotation) act upon the test sample to achieve contact with the primary antibodies 188. The primary antibodies come into contact with, and capture any CD4⁺ T-cells 190 and CD8⁺ T-cells 194 present within the test sample (FIG. 35C). The optical drive motor 162 (FIG. 10) spins the disc, which clears the capture zones 140 (FIG. 20I) of unbound T-cells. The incident beam 152 of an optical disc drive 112 (FIG. 1) interacts with the captured CD4⁺ T-cells 190 and CD8⁺ T-cells 194 (FIG. 35D) and the return beam 154 is reflected to the detector 157 (FIG. 10) for processing and analysis.

[0299] FIG. 37 shows analysis of the same test sample from FIGS. 35B-35D, using the third embodiment of the first implementation of the invention. The flow channels 130 of a bio-disc 110 are flooded with the MNC sample (FIG. 35B). Capillary action, pressure applied with an external applicator, and/or centrifugal force (i.e., the force on a body in curvilinear motion directed away from the center or curvature or axis of rotation) act upon the test sample to achieve contact with the primary antibodies 188. The primary antibodies come into contact with and then capture any CD4⁺ T-cells 190 and CD8⁺ T-cells 194 present within the test sample (FIG. 35C). The optical drive motor 162 (FIG. 10) spins the disc, which clears the capture zones 140 (FIG. 20I) of unbound T-cells. The incident beam 152 of an optical disc drive 112 (FIG. 1) interacts with the captured CD4⁺ T-cells 190 and CD8⁺ T-cells 194 (FIG. 35D) and the transmitted beam 156 is passed to the detector 157 (FIG. 10) for processing and analysis.

[0300] Cluster Designation Count—Implementation II

[0301] With reference now to FIGS. 38A-38C, analysis of a purified and washed MNC sample (FIG. 17A) using a bio-disc of the second implementation of the invention is shown. The flow channels 130 of a bio-disc 110 are flooded with the MNC sample as shown in FIG. 38A. Capillary action, pressure applied with an external applicator, and/or centrifugal force (i.e., the force on a body in curvilinear motion directed away from the center or curvature or axis of rotation) act upon the test sample to achieve contact with the primary antibodies 188. The primary antibodies come into contact with, and capture any CD4⁺ T-cells 190 and CD8⁺ T-cells 194 present within the test sample. This step is illustrated in FIG. 38B. The optical drive motor 162 (FIG. 10) spins the disc, which clears the capture zones 140 (FIG. 24L) of unbound T-cells. The incident beam 152 of an optical disc drive 112 (FIG. 1) interacts with the captured CD4⁺ T-cells 190 and CD8⁺ T-cells 194, FIG. 38C, and the return beam 154 is reflected to the detector 157 (FIG. 10) for processing and analysis.

[0302] FIG. 39 shows analysis of the same test sample from FIGS. 38A-38C, using the second embodiment of the second implementation of the invention. The flow channels

130 of a bio-disc 110 are flooded with the MNC sample (FIG. 38A). Capillary action, pressure applied with an external applicator, and/or centrifugal force (i.e., the force on a body in curvilinear motion directed away from the center or curvature or axis of rotation) act upon the test sample to achieve contact with the primary antibodies 188. The primary antibodies come into contact with, and capture any CD4⁺ T-cells 190 and CD8⁺ T-cells 194 present within the test sample (FIG. 38B). The optical drive motor 162 (FIG. 10) spins the disc, which clears the capture zones 140 (FIG. 24L) of unbound T-cells. The incident beam 152 of an optical disc drive 112 (FIG. 1) interacts with the captured CD4⁺ T-cells 190 and CD8⁺ T-cells 194 and (FIG. 38C) the return beam 154 is reflected to the detector 157 (FIG. 10) for processing and analysis.

[0303] FIG. 40 shows analysis of the same test sample from FIGS. 38A-38C, using the third embodiment of the second implementation of the invention. The flow channels 130 of a bio-disc 110 are flooded with the MNC sample (FIG. 38A). Capillary action, pressure applied with an external applicator, and/or centrifugal force (i.e., the force on a body in curvilinear motion directed away from the center or curvature or axis of rotation) act upon the test sample to achieve contact with the primary antibodies 188. The primary antibodies come into contact with, and capture any CD4⁺ T-cells 190 and CD8⁺ T-cells 194 present within the test sample (FIG. 38B). The optical drive motor 162 (FIG. 10) spins the disc, which clears the capture zones 140 (FIG. 24L) of unbound T-cells. The incident beam 152 of an optical disc drive 112 (FIG. 1) interacts with the captured CD4⁺ T-cells 190 and CD8⁺ T-cells 194 (FIG. 38C) and the transmitted beam 156 is passed to the detector 157 (FIG. 10) for processing and analysis.

[0304] Cluster Designation Count—Implementation III

[0305] Turning next to FIGS. 41A-41C, analysis of a purified and washed MNC sample (FIG. 17A) using a bio-disc of the third implementation of the invention is shown. The flow channels 130 of a bio-disc 110 are flooded with the MNC sample as illustrated in FIG. 41A. Capillary action, pressure applied with an external applicator, and/or centrifugal force (i.e., the force on a body in curvilinear motion directed away from the center or curvature or axis of rotation) act upon the test sample to achieve contact with the primary antibodies 188. The primary antibodies come into contact with and then capture any CD4⁺ T-cells 190 and CD8⁺ T-cells 194 present within the test sample. This step of the present method is shown FIG. 41B. The optical drive motor 162 (FIG. 10) spins the disc, which clears the capture zones 140 (FIG. 28J) of unbound T-cells. The incident beam 152 of an optical disc drive 112 (FIG. 1) interacts with the captured CD4⁺ T-cells 190 and CD8⁺ T-cells 194, FIG. 41C, and the return beam 154 is reflected to the detector 157 (FIG. 10) for processing and analysis.

[0306] FIG. 42 shows analysis of the same test sample from FIGS. 41A-41C, using the second embodiment of the third implementation of the invention. The flow channels 130 of a bio-disc 110 are flooded with the MNC sample (FIG. 41A). Capillary action, pressure applied with an external applicator, and/or centrifugal force (i.e., the force on a body in curvilinear motion directed away from the center or curvature or axis of rotation) act upon the test sample to achieve contact with the primary antibodies 188.

The primary antibodies come into contact with, and capture any CD4⁺ T-cells 190 and CD8⁺ T-cells 194 present within the test sample, (FIG. 41B). The optical drive motor 162 (FIG. 10) spins the disc, which clears the capture zones 140 (FIG. 28J) of unbound T-cells. The incident beam 152 of an optical disc drive 112 (FIG. 1) interacts with the captured CD4⁺ T-cells 190 and CD8⁺ T-cells 194 (FIG. 41C) and the return beam 154 is reflected to the detector 157 (FIG. 10) for processing and analysis.

[0307] FIG. 43 shows analysis of the same test sample from FIGS. 41A-41C, using the third embodiment of the third implementation of the invention. The flow channels 130 of a bio-disc 110 are flooded with the MNC sample (FIG. 41A). Capillary action, pressure applied with an external applicator, and/or centrifugal force (i.e., the force on a body in curvilinear motion directed away from the center or curvature or axis of rotation) act upon the test sample to achieve contact with the primary antibodies 188. The primary antibodies come into contact with, and capture any CD4⁺ T-cells 190 and CD8⁺ T-cells 194 present within the test sample (FIG. 41B). The optical drive motor 162 (FIG. 10) spins the disc, which clears the capture zones 140 (FIG. 28J) of unbound T-cells. The incident beam 152 of an optical disc drive 112 (FIG. 1) interacts with the captured CD4⁺ T-cells 190 and CD8⁺ T-cells 194 (FIG. 41C) and the transmitted beam 156 is passed to the detector 157 (FIG. 10) for processing and analysis.

[0308] Cluster Designation Count—Implementation IV

[0309] Referring now to FIGS. 44A-44D, analysis of a purified and washed MNC sample (FIG. 17A) using a bio-disc of the fourth implementation of the invention is shown. FIG. 44A is a pictorial flow diagram showing preparation of the primary antibody-T-cell complex. The MNC suspension containing both CD4⁺ T-cells 190 and CD8⁺ T-cells 194 is mixed with primary antibodies 188, and allowed to bind, thereby forming the primary antibody-T-cell complexes. As would be apparent to one of skill in the art, other cells with different surface markers may also be tagged using the current implementation. As shown in FIG. 44B, the flow channels 130 of a bio-disc 110 are flooded with the primary antibody-T-cell complexes. Capillary action, pressure applied with an external applicator, and/or centrifugal force (i.e., the force on a body in curvilinear motion directed away from the center or curvature or axis of rotation) act upon the test sample to achieve contact with the secondary antibodies 188 immobilized on the active layer 144 of the bio-disc 110. The secondary antibodies 186, having an affinity for the primary antibody-T-cell complexes capture the complexes as shown in FIG. 44C. The optical drive motor 162 (FIG. 10) spins the disc, which clears the capture zones 140 (FIG. 32I) of unbound complexes. The incident beam 152 of the optical disc drive 112 (FIG. 1) interacts with the captured complexes, FIG. 44D, and the return beam 154 is reflected to the detector 157 (FIG. 10) for processing and analysis.

[0310] FIGS. 44A-44D also show another principal aspect of the methods of this invention. In conjunction with FIG. 17A, there is provided another method of performing a cluster designation count. This method includes the steps of (1) providing a blood sample in a tube containing a separation gradient, (2) rotating the tube at a time and speed sufficient to separate the blood sample into layers, (3)

resuspending a MNC layer containing T-cells to form a MNC suspension, (4) adding a primary antibody to the MNC suspension to form a primary antibody-T-cell complex, (5) providing a sample of the primary antibody-T-cell complex on a disc surface that includes at least one capture zone containing at least one capture agent, (6) loading the disc into an optical reader, (7) directing an incident beam of electromagnetic radiation to the capture zone, (8) detecting a beam of electromagnetic radiation formed after interacting with the disc at the capture zone, (9) converting the detected beam into an output signal, and (10) analyzing the output signal to extract information relating to the number of cells captured at the capture zone. In one embodiment of this method, the optical disc is constructed with a reflective layer such that light directed to the capture zone and not striking a cell is reflected. In another embodiment of this method, the optical disc is constructed such that light directed to the capture zone and not striking a cell is transmitted through the optical disc.

[0311] FIG. 45 shows analysis of the same test sample from FIGS. 44A-44D, using the second embodiment of the fourth implementation of the invention. The flow channels 130 of a bio-disc 110 are flooded with the primary antibody-T-cell complexes (FIG. 44B). Capillary action, pressure applied with an external applicator, and/or centrifugal force (i.e., the force on a body in curvilinear motion directed away from the center or curvature or axis of rotation) act upon the test sample to achieve contact with the secondary antibodies 188 immobilized on the active layer 144 of the bio-disc 110. The secondary antibodies 186, having an affinity for the primary antibody-T-cell complexes capture the complexes (FIG. 44C). The optical drive motor 162 (FIG. 10) spins the disc, which clears the capture zones 140 (FIG. 32I) of unbound complexes. The incident beam 152 of the optical disc drive 112 (FIG. 1) interacts with the captured complexes (FIG. 44D) and the return beam 154 is reflected to the detector 157 (FIG. 10) for processing and analysis.

[0312] FIG. 46 shows analysis of the same test sample from FIGS. 44A-44D, using the third embodiment of the fourth implementation of the invention. The flow channels 130 of a bio-disc 110 are flooded with the primary antibody-T-cell complexes (FIG. 44B). Capillary action, pressure applied with an external applicator, and/or centrifugal force (i.e., the force on a body in curvilinear motion directed away from the center or curvature or axis of rotation) act upon the test sample to achieve contact with the secondary antibodies 188 immobilized on the active layer 144 of the bio-disc 110. The secondary antibodies 186, having an affinity for the primary antibody-T-cell complexes capture the complexes (FIG. 44C). The optical drive motor 162 (FIG. 10) spins the disc, which clears the capture zones 140 (FIG. 32I) of unbound complexes. The incident beam 152 of an optical disc drive 112 (FIG. 1) interacts with the captured complexes (FIG. 44D) and the transmitted beam 156 is passed to the detector 157 (FIG. 10) for processing and analysis.

[0313] Those skilled in the art will appreciate in light of these teachings that one or more embodiments of one or more implementations of the methods of the invention may be combined without departing from the scope and spirit of this invention.

[0314] Cell Detection and Related Software

[0315] Referring now to FIG. 47, there is shown the optical bio-disc 110 including a fluid circuit 128 for holding

a sample. **FIG. 47** also shows the fluidic circuit **47** enlarged to illustrate different capture or target zones **140** and the target zone **141** including the reference mark or calibration dot **202**. In this specific embodiment, five capture zones **140** are employed, each respectively implemented to capture CD4+ cells, CD8+ cells, CD3+ cells, CD45+ cells, and myoglobin as the negative control.

[0316] **FIG. 48A** an image obtained from a different embodiment including six capture zones **140** each implemented, respectively, to capture CD45+ cells, CD15+ cells, CD4+ cells CD8+ cells, a second capture zone for CD15+ cells, and a second capture zone for CD45+ cells. In this embodiment, the two CD45 and CD15 capture zones may be used as a validation control or check to verify that the capture efficiency and expected count results are in agreement. **FIG. 48A** also shows a series of cell surface antigens with enlarged views for CD4, CD8, and a control. As indicated here, the image is of a number of cells shown against a background field. **FIG. 48B** represents a close up view of the control, CD4, and CD8 capture zones from an actual microscope image compared to a bio-disc derived image according to the present invention. **FIG. 49** illustrates another comparison in greater detail of an actual microscope image and a corresponding bio-disc image according to the present invention. As represented in **FIGS. 48B and 49**, the inventors have found that the bio-disc images are of equal quality and resolution compared to those obtainable from a microscope. These images thus demonstrate that individual cells can be made visible against a background using the apparatus and methods of the present invention. Methods for detecting investigational features are described in more detail in commonly assigned U.S. Provisional Application Serial Nos. 60/270,095 and 60/292,108 each entitled "Signal Processing Apparatus and Methods for Obtaining Signal Signatures of Investigational Features Detected on a Surface of an Optical Disc Assembly" respectively filed on Feb. 2, 2001 and May 18, 2001, and in commonly assigned U.S. patent application Ser. No. 10/043,688 entitled "Optical Disc Analysis System Including Related Methods For Biological and Medical Imaging" as filed on Jan. 10, 2002, all of which are incorporated herein by reference.

[0317] These cells can be detected through one of a variety of different methods including, for example, using edge detection hardware or software to detect and count sufficiently large changes in the level of transmitted or reflected light and thus count the transitions and hence the cells. Another method, described in more detail below, uses image or pattern recognition software to identify cells against the background. Image recognition can distinguish WBCs from RBCs, and also distinguish neutrophils, monocytes, basophils, eosinophils, granulocytes, and lymphocytes.

[0318] An optical disc with tracks on the order of 1.6 microns apart can be used to image cells or aggregates on the disc. For example, a white blood cell would typically have a diameter of at least 5 and as many as 12 tracks, and therefore an image of that white blood cell can be obtained.

[0319] To obtain such an image, a transmissive disc of the type shown in **FIGS. 2, 3 and 4** may be used (although a reflective disc would be operable), and a disc drive system of the type shown in **FIG. 10** including trigger sensor **160** and top detector **158**. Trigger detector **158** detects a trigger mark **126** in a transmissive disc and provides a signal to a

computer that data is to be collected and/or processed when that mark is detected. As the light source passes across the tracks in the viewing window, an image is obtained for the received transmitted light. The top detector in this case can be a single detector, or an array of multiple detector elements oriented in the radial and/or circumferential direction. Such detectors and detection methods are described, for example, in commonly assigned U.S. Provisional Application Serial No. 60/247,465 entitled "Optical Disc Drive For Bio-Optical Disc" filed Nov. 9, 2000; U.S. Provisional Application Serial No. 60/293,093 entitled "Disc Drive Assembly For Optical Bio-Discs" filed May 22, 2001; and U.S. patent application Ser. No. 10/008,156 entitled "Disc Drive System and Methods for Use with Bio-discs" filed Nov. 9, 2001, each of which is incorporated herein by reference in its entirety.

[0320] After images such as those in **FIGS. 48A, 48B and 49** are obtained, the image data can be processed further with image recognition software designed to identify desired features. It is further desirable that the image recognition software not only have the ability to distinguish cells from background, but also one type of cell from another.

[0321] With reference now to **FIG. 50**, there is shown an image derived from investigational data that includes both red blood cells and white blood cells. As indicated in the enlarged views, these white and red cells have clearly distinct characteristics and thus can be detected against the background and can also be distinguished from each other with image recognition. In addition, it is also possible to distinguish types of white blood cells from each other, including lymphocytes, monocytes, neutrophils, eosinophils, granulocytes, and basophils.

[0322] **FIG. 51** shows a sample field with a number of cells with a plus sign indicating each object that is identified as a cell. After the number of cells have been detected for every zone or any number of desired zones, resulting cell count data can be displayed in a single screen that provides an easy to view representation such as that shown in **FIG. 52**. As depicted in **FIG. 52**, the specific cell counts are provided along with a bar graph to demonstrate relative numbers of cells. In the case of a CD4/CD8 analysis, the system can also produce a CD4/CD8 ratio as well as any other desired mathematical calculation or comparison. **FIG. 53** provides a different view of the process showing cells in an image field being converted to a CD4 count, a CD8 count, and a ratio, with the output indicating that the ratio is in a normal range.

[0323] Aspects of the present invention relating to cell detection and associated processing methods are also disclosed in U.S. Provisional Application Serial No. 60/316,273 entitled "Capture Layer Assemblies and Optical Bio-Discs for Immunophenotyping" filed Aug. 31, 2001; U.S. Provisional Application Serial No. 60/318,026 entitled "Methods for Imaging Blood cells, Blood-Borne Parasites and Pathogens, and Other Biological Matter Including Related Optical Bio-Discs and Drive Assemblies" filed Sep. 7, 2001; U.S. Provisional Application Serial No. 60/322,527 entitled "Optical Analysis Discs Including Microfluidic Circuits for Performing Cell Counts" filed Sep. 14, 2001; U.S. Provisional Application Serial No. 60/322,040 entitled "Optical Analysis Discs Including Fluidic Circuits for Optical Imaging and Quantitative Evaluation of Blood Cells Including Lymphocytes" filed Sep. 11, 2001; U.S. Provi-

sional Application Serial No. 60/322,863 entitled "Methods for Differential Cell Counts Including Leukocytes and Use of Optical Bio-Disc for Performing Same" filed Sep. 12, 2001; and U.S. Provisional Application Serial No. 60/322,793 entitled "Methods for Reducing Non-Specific Binding of Cells on Optical Bio-Discs Utilizing Charged Matter Including Heparin, Plasma, or Poly-Lysine" filed Sep. 17, 2001, all of which are herein incorporated by reference.

[0324] Differential Cell Count Methods and Related Software

[0325] A number of methods and related algorithms for white blood cell counting using optical disc data are herein discussed in further detail. These methods and related algorithms are not limited to counting white blood cells, but may be readily applied to conducting counts of any type of cellular matter including, but not limited to, red blood cells, white blood cells, beads, and any other objects, both biological and non-biological, that produce similar optical signatures that can be detected by an optical reader.

[0326] For the purposes of illustration, the following description of the methods and algorithms related to the present invention as described with reference to FIGS. 54-58, are directed to white blood cell counting. With some modifications, these methods and algorithms can be applied to counting other types of cells, or objects similar in size to white blood cells. The data evaluation aspects of the cell counting methods and algorithms are described generally herein to provide related background for the methods and apparatus of the present invention. Methods and algorithms for capturing and processing investigational data from the optical bio-disc has general broad applicability and has been disclosed in further detail in commonly assigned U.S. Provisional Application No. 60/291,233 entitled "Variable Sampling Control For Rendering Pixelation of Analysis Results In Optical Bio-Disc Assembly And Apparatus Relating Thereto" filed May 16, 2001 which is herein incorporated by reference and the above incorporated U.S. Provisional Application No. 60/404,921 entitled "Methods For Differential Cell Counts Including Related Apparatus And Software For Performing Same".

[0327] In the following discussion, the basic scheme of the methods and algorithms with a brief explanation is presented. As illustrated in FIG. 10, information concerning attributes of the biological test sample is retrieved from the optical bio-disc 110 in the form of a beam of electromagnetic radiation that has been modified or modulated by interaction with the test sample. In the case of the reflective optical bio-disc discussed in conjunction with FIGS. 2, 3, 4, 11, 13, and 15, the return beam 154 carries the information about the biological sample. As discussed above, such information about the biological sample is contained in the return beam essentially only when the incident beam is within the flow channel 130 or target zones 140 and thus in contact with the sample. In the reflective embodiment of the bio-disc 110, the return beam 154 may also carry information encoded in or on the reflective layer 142 or otherwise encoded in the wobble grooves 170 illustrated in FIGS. 13 and 14. As would be apparent to one of skill in the art, pre-recorded information is contained in the return beam 154 of the reflective disc with target zones, only when the corresponding incident beam is in contact with the reflective layer 142. Such information is not contained in the return beam 154

when the incident beam 152 is in an area where the information bearing reflective layer 142 has been removed or is otherwise absent. In the case of the transmissive optical bio-disc discussed in conjunction with FIGS. 5, 6, 8, 9, 12, 14, and 16, the transmitted beam 156 carries the information about the biological sample.

[0328] With continuing reference to FIG. 10, the information about the biological test sample, whether it is obtained from the return beam 154 of the reflective disc or the transmitted beam 156 of the transmissive disc, is directed to a processor 166 for signal processing. This processing involves transformation of the analog signal detected by the bottom detector 157 (reflective disc) or the top detector 158 (transmissive disc) to a discrete digital form.

[0329] Referring next to FIG. 54, the signal transformation involves sampling the analog signal 210 at fixed time intervals 212, and encoding the corresponding instantaneous analog amplitude 214 of the signal as a discrete binary integer 216. Sampling is started at some start time 218 and stopped at some end time 220. The two common values associated with any analog-to-digital conversion process are sampling frequency and bit depth. The sampling frequency, also called the sampling rate, is the number of samples taken per unit time. A higher sampling frequency yields a smaller time interval 212 between consecutive samples, which results in a higher fidelity of the digital signal 222 compared to the original analog signal 210. Bit depth is the number of bits used in each sample point to encode the sampled amplitude 214 of the analog signal 210. The greater the bit depth, the better the binary integer 216 will approximate the original analog amplitude 214. In the present embodiment, the sampling rate is 8 MHz with a bit depth of 12 bits per sample, allowing an integer sample range of 0 to 4095 (0 to $(2^n - 1)$, where n is the bit depth. This combination may change to accommodate the particular accuracy necessary in other embodiments. By way of example and not limitation, it may be desirable to increase sampling frequency in embodiments involving methods for counting beads, which are generally smaller than cells. The sampled data is then sent to processor 166 for analog-to-digital transformation.

[0330] During the analog-to-digital transformation, each consecutive sample point 224 along the laser path is stored consecutively on disc or in memory as a one-dimensional array 226. Each consecutive track contributes an independent one-dimensional array, which yields a two-dimensional array 228 (FIG. 57A) that is analogous to an image.

[0331] FIG. 55 is a perspective view of an optical bio-disc 110 of the present invention with an enlarged detailed perspective view of the section indicated showing a captured white blood cell 230 positioned relative to the tracks 232 of the optical bio-disc. As shown, the interaction of incident beam 152 with white blood cell 230 yields a signal-containing beam, either in the form of a return beam 154 of the reflective disc or a transmitted beam 156 of the transmissive disc, which is detected by either of detectors 157 or 158.

[0332] FIG. 56A is another graphical representation of the white blood cell 230 positioned relative to the tracks 232 of the optical bio-disc 110 shown in FIG. 55. As shown in FIGS. 55 and 56A, the white blood cell 230 covers approximately four tracks A, B, C, and D. FIG. 56B shows a series of signature traces derived from the white blood cell 210 of FIGS. 55 and 56A. As indicated in FIG. 56B, the detection

system provides four analogue signals A, B, C, and D corresponding to tracks A, B, C, and D. As further shown in FIG. 56B, each of the analogue signals A, B, C, and D carries specific information about the white blood cell 230. Thus as illustrated, a scan over a white blood cell 230 yields distinct perturbations of the incident beam that can be detected and processed. The analog signature traces (signals) 210 are then directed to processor 166 for transformation to an analogous digital signal 222 as shown in FIGS. 57A and 57C as discussed in further detail below.

[0333] FIG. 57 is a graphical representation illustrating the relationship between FIGS. 57A, 57B, 57C, and 57D. FIGS. 57A, 57B, 57C, and 57D are pictorial graphical representations of transformation of the signature traces from FIG. 56B into digital signals 222 that are stored as one-dimensional arrays 226 and combined into a two-dimensional array 228 for data input 244.

[0334] With particular reference now to FIG. 57A, there is shown sampled analog signals 210 from tracks A and B of the optical bio-disc shown in FIGS. 55 and 56A. Processor 166 then encodes the corresponding instantaneous analog amplitude 214 of the analog signal 210 as a discrete binary integer 216 (see FIG. 54). The resulting series of data points is the digital signal 222 that is analogous to the sampled analog signal 210.

[0335] Referring next to FIG. 57B, digital signal 222 from tracks A and B (FIG. 57A) is stored as an independent one-dimensional memory array 226. Each consecutive track contributes a corresponding one-dimensional array, which when combined with the previous one-dimensional arrays, yields a two-dimensional array 228 that is analogous to an image. The digital data is then stored in memory or on disc as a two-dimensional array 228 of sample points 224 (FIG. 54) that represent the relative intensity of the return beam 154 or transmitted beam 156 (FIG. 55) at a particular point in the sample area. The two-dimensional array is then stored in memory or on disc in the form of a raw file or image file 240 as represented in FIG. 57B. The data stored in the image file 240 is then retrieved 242 to memory and used as data input 244 to analyzer 168 shown in FIG. 10.

[0336] FIG. 57C shows sampled analog signals 210 from tracks C and D of the optical bio-disc shown in FIGS. 55 and 56A. Processor 166 then encodes the corresponding instantaneous analog amplitude 214 of the analog signal 210 as a discrete binary integer 216 (FIG. 54). The resulting series of data points is the digital signal 222 that is analogous to the sampled analog signal 210.

[0337] Referring now to FIG. 57D, digital signal 222 from tracks C and D is stored as an independent one-dimensional memory array 226. Each consecutive track contributes a corresponding one-dimensional array, which when combined with the previous one-dimensional arrays, yields a two-dimensional array 228 that is analogous to an image. As above, the digital data is then stored in memory or on disc as a two-dimensional array 228 of sample points 224 (FIG. 54) that represent the relative intensity of the return beam 154 or transmitted beam 156 (FIG. 55) at a particular point in the sample area. The two-dimensional array is then stored in memory or on disc in the form of a raw file or image file 240 as shown in FIG. 57B. As indicated above, the data stored in the image file 240 is then retrieved 242 to memory and used as data input 244 to analyzer 168 FIG. 10.

[0338] The computational and processing algorithms of the present invention are stored in analyzer 168 (FIG. 10) and applied to the input data 244 to produce useful output results 262 (FIG. 58) that may be displayed on the display monitor 114 (FIG. 10). With reference now to FIG. 58 there is shown a logic flow chart of the principal steps for data evaluation according to the processing methods and computational algorithms of the present invention. A first principal step of the present processing method involves receipt of the input data 244. As described above, data evaluation starts with an array of integers in the range of 0 to 4096.

[0339] The next principle step 246 is selecting an area of the disc for counting. Once this area is defined, an objective then becomes making an actual count of all white blood cells contained in the defined area. The implementation of step 246 depends on the configuration of the disc and user's options. By way of example and not limitation, embodiments of the invention using discs with windows such as the target zones 140 shown in FIGS. 2 and 5, the software recognizes the windows and crops a section thereof for analysis and counting. In one preferred embodiment, such as that illustrated in FIG. 2, the target zones or windows have the shape of 1x2 mm rectangles with a semicircular section on each end thereof. In this embodiment, the software crops a standard rectangle of 1x2 mm area inside a respective window. In an aspect of this embodiment, the reader may take several consecutive sample values to compare the number of cells in several different windows.

[0340] In embodiments of the invention using a transmissive disc without windows, as shown in FIGS. 5, 6, 8, and 9, step 246 may be performed in one of two different manners. The position of the standard rectangle is chosen either by positioning its center relative to a point with fixed coordinates, or by finding reference mark 202 (see for example FIGS. 24L, 25, and 26), which is a spot of dark dye. In the case where a reference mark 202 is employed, a dye with a desired contrast is deposited in a specific position 141 (FIG. 20E for example) on the disc with respect to two clusters of cells. The optical disc reader is then directed to skip to the center of one of the clusters of cells and the standard rectangle is then centered around the selected cluster.

[0341] As for the user options mentioned above in regard to step 246, the user may specify a desired sample area shape for cell counting, such as a rectangular area, by direct interaction with mouse selection or otherwise. In the present embodiment of the software, this involves using the mouse to click and drag a rectangle over the desired portion of the optical bio-disc-derived image that is displayed on a monitor 114. Regardless of the evaluation area selection method, a respective rectangular area is evaluated for counting in the next step 248.

[0342] The third principal step in FIG. 58 is step 248, which is directed to background illumination uniformization. This process corrects possible background uniformity fluctuations caused in some hardware configurations. Background illumination uniformization offsets the intensity level of each sample point such that the overall background, or the portion of the image that is not cells, approaches a plane with an arbitrary background value $V_{\text{background}}$. While $V_{\text{background}}$ may be decided in many ways, such as taking the average value over the standard rectangular sample area, in

the present embodiment, the value is set to 2000. The value V at each point P of the selected rectangular sample area is replaced with the number $(V_{\text{background}} + (V - \text{average value over the neighborhood of } P))$ and truncated, if necessary, to fit the actual possible range of values, which is 0 to 4095 in a preferred embodiment of the present invention. The dimensions of the neighborhood are chosen to be sufficiently larger than the size of a cell and sufficiently smaller than the size of the standard rectangle.

[0343] The next step in the flow chart of FIG. 58 is a normalization step 250. In conducting normalization step 250, a linear transform is performed with the data in the standard rectangular sample area so that the average becomes 2000 with a standard deviation of 600. If necessary, the values are truncated to fit the range 0 to 4096. This step 250, as well as the background illumination uniformization step 248, makes the software less sensitive to hardware modifications and tuning. By way of example and not limitation, the signal gain in the detection circuitry, such as top detector 158 (FIG. 55), may change without significantly affecting the resultant cell counts.

[0344] As shown in FIG. 58, a filtering step 252 is next performed. For each point P in the standard rectangle, the number of points in the neighborhood of P , with dimensions smaller than indicated in step 248, with values sufficiently distinct from $V_{\text{background}}$ is calculated. The points calculated should approximate the size of a cell in the image. If this number is large enough, the value at P remains as it was; otherwise it is assigned to $V_{\text{background}}$. This filtering operation is performed to remove noise, and in the optimal case only cells remain in the image while the background is uniformly equal $V_{\text{background}}$.

[0345] An optional step 254 directed to removing bad components may be performed as indicated in FIG. 58. Defects such as scratches, bubbles, dirt, and other similar irregularities may pass through filtering step 252. These defects may cause cell counting errors either directly or by affecting the overall distribution in the images histogram. Typically, these defects are sufficiently larger in size than cells and can be removed in step 254 as follows. First a binary image with the same dimensions as the selected region is formed. A in the binary image is defined as white, if the value at the corresponding point of the original image is equal to $V_{\text{background}}$ and black otherwise. Next, connected components of black points are extracted. Then subsequent erosion and expansion are applied to regularize the view of components. And finally, components that are larger than a defined threshold are removed. In one embodiment of this optional step, the component is removed from the original image by assigning the corresponding sample points in the original image with the value $V_{\text{background}}$. The threshold that determines which components constitute countable objects and which are to be removed is a user-defined value. This threshold may also vary depending on the investigational feature being counted i.e. white blood cells, red blood cells, or other biological matter. After optional step 254, steps 248, 250, and 252 are preferably repeated.

[0346] The next principal processing step shown in FIG. 58 is step 256, which is directed to counting cells by bright centers. The counting step 256 consists of several substeps. The first of these substeps includes performing a convolution. In this convolution substep, an auxiliary array referred

to as a convolved picture is formed. The value of the convolved picture at point P is the result of integration of a picture after filtering in the circular neighborhood of P . More precisely, for one specific embodiment, the function that is integrated, is the function that equals $v - 2000$ when v is greater than 2000 and 0 when v is less than or equal to 2000. The next substep performed in counting step 256 is finding the local maxima of the convolved picture in the neighborhood of a radius about the size of a cell. Next, duplicate local maxima with the same value in a closed neighborhood of each other are avoided. In the last substep in counting step 256, the remaining local maxima are declared to mark cells.

[0347] In some hardware configurations, some cells may appear without bright centers. In these instances, only a dark rim is visible and the following two optional steps 258 and 260 are useful.

[0348] Step 258 is directed to removing found cells from the picture. In step 258, the circular region around the center of each found cell is filled with the value 2000 so that the cells with both bright centers and dark rims would not be found twice.

[0349] Step 260 is directed to counting additional cells by dark rims. Two transforms are made with the image after step 258. In the first substep of this routine, substep (a), the value v at each point is replaced with $(2000 - v)$ and if the result is negative it is replaced with zero. In substep (b), the resulting picture is then convolved with a ring of inner radius $R1$ and outer radius $R2$. $R1$ and $R2$ are, respectively, the minimal and the maximal expected radius of a cell, the ring being shifted, subsequently, in substep (d) to the left, right, up and down. In substep (c), the results of four shifts are summed. After this transform, the image of a dark rim cell looks like a four petal flower. Finally in substep (d), maxima of the function obtained in substep (c) are found in a manner to that employed in counting step 256. They are declared to mark cells omitted in step 256.

[0350] After counting step 256, or after counting step 260 when optionally employed, the last principal step illustrated in FIG. 58 is a results output step 262. The number of cells found in the standard rectangle is displayed on the monitor 114 shown in FIGS. 1 and 5, and each cell identified is marked with a red cross on the displayed optical bio-disc-derived 0 image.

[0351] Referring to FIG. 59 there is depicted, a pictorial representation showing an analysis chamber having different capture zones for analysis of white blood cells. The buffy coat or white blood cell sample is isolated from whole blood by various methods including lysis, positive or negative selection, and gradient centrifugation or any combination of these methods. Further details relating to sample preparation is described below in Examples 1, 2, and 3 and also disclosed in, for example, commonly assigned, co-pending U.S. Provisional Application Serial No. 60/382,319 entitled "Methods For Isolation Of T-Lymphocytes For Use In Differential Cell Counting And Use Of Optical Bio-Disc For Performing Same" filed May 22, 2002. This application is herein incorporated by reference in its entirety. Once the MNCs or buffy coat is isolated, the MNCs are then introduced into the analysis chamber where cells of interest bind to their respective capture agents and the analysis is carried out following the procedure outlined below in Example 7, for example.

[0352] Referring now to **FIGS. 60A and 60B**, there are illustrated pictorial representations showing analysis chambers having different capture zones for analysis of red and white blood cells. **FIG. 60A** shows a red blood cell ABO analysis and a buffy coat CD Marker assay having capture zones for CD makers 2, 19, 44, 4, and 8. **FIG. 60B** shows another red blood cell analysis using IgM capture antibodies with specific affinity to A, B, O, and Rh surface markers. There is also shown, in **FIG. 60B** a white blood cell analysis using whole blood as the sample. In this assay, white blood cells (T-cells, B-cells, Eosinophils, Basophils, Neutrophils, Monocytes, and Lymphocytes) are captured in specific capture zones and a secondary gate is used to identify and quantify the number of cells captured. The secondary gate used to identify the cells of interest are groups of beads; each group having a unique physical characteristic and signal agents, specific to a type of cell, attached thereto. The physical characteristics may include size, shape, texture, color, fluorescence, and phosphorescence. For example, Group A beads may be 1 μm yellow-green (YG) fluorescent beads having anti-T-cell signal antigens attached thereto. The Group A cells would then specifically bind to the captured T-cells thereby tagging the T-cells with 1 μm YG fluorescent beads. Another group of beads, Group B, 2 μm red fluorescent beads, having attached thereto antibodies against monocytes may be used to tag the monocytes, for example. The secondary gate allows for a more accurate assessment of the number of cells captured since it will tag only the cells of interest and not non-specifically bound cells within a particular capture zone. Further details relating to using beads or microspheres to tag cells of interest is disclosed in, for example, commonly assigned, co-pending U.S. Provisional Application Serial No. 60/382,944 entitled "Methods and Apparatus for Use in Detection and Quantitation of Cell Populations and Use of Optical Bio-Disc for Performing Same" filed May 24, 2002. This application is herein incorporated by reference in its entirety.

[0353] Referring next to **FIG. 61**, there is shown a graph showing the number of CD4+ cells, from a blood sample, captured as a function of cell concentration within the capture zones in a CD marker assay using the optical bio-disc. The graph shows the increase in cell capture relative to the increase in the number of cells in the sample. The cells were captured using CD4 antibodies from two sources (antibody A from DAKO and antibody B from Serotech). The graph further shows a similar trend and capture efficiencies using both capture antibodies. Further details regarding the experimental procedures followed to perform this experiment is discussed in Example 7 below.

[0354] Referring now to **FIGS. 62A and 62B**, there are shown scatter plots of data of CD4/CD8 ratios from blood samples analyzed using the optical bio-disc of the present invention and FACS analysis. The data used to generate these graphs are shown below in Table 8. Details relating to the experimental procedures followed to collect the data shown is discussed below in Example 8.

[0355] Experimental Details

[0356] While this invention has been described in detail with reference to the drawing figures, certain examples and further illustrations of the invention are presented below.

EXAMPLE 1

[0357] **FIG. 17A** illustrates a pictorial flow chart showing the preparation of a sample, use of a bio-disc, and the provision of results which are shown in greater detail in **FIGS. 52 and 53**. The details of the following example such as the individual time duration of process steps, rotation rates, and other details are more particular than those described above with reference to **FIGS. 17A, 52, and 53**. The basic steps of the present example are, nonetheless, similar to those described above.

[0358] A. Disc Manufacturing Including Substrate Preparation and Chemistry Deposition

[0359] In this example, a reflective disc or transmissive disc substrate **120** (**FIGS. 2 and 5**, respectively) is cleaned using an air gun to remove any dust particles. The disc is rinsed twice with iso-propanol, using a spin coater. A 2% polystyrene is spin coated on the disc to give a very thick coating throughout.

[0360] The chemistry is then deposited. One embodiment includes a three step deposition protocol that incubates: streptavidin, incubated for 30 minutes; biotinylated first antibody incubated for 60 minutes; and a second capture antibody incubated for 30 minutes. The first antibody can be raised in a first species (e.g., sheep) against a type of immunoglobulin (e.g., IgG, IgE, IgM) of a second species (e.g., mouse). The second capture antibody is raised in the second species against a specific cell surface antigen (e.g., CD4, CD8). The steps are done at room temperature in a humidity chamber using washing and drying steps between depositions.

[0361] A 1 μl ratio of 1 mg/ml streptavidin in phosphate buffered saline is layered over each window and incubated for 30 minutes. Excess streptavidin is rinsed off using distilled water and the disc is dried. Equal volumes of biotinylated anti-mouse IgG (125 $\mu\text{g}/\text{ml}$ in PBS) and activated dextran aldehyde (200 $\mu\text{g}/\text{ml}$) are combined. Dextran aldehyde (DCHO)-biotinylated anti-mouse IgG is layered over streptavidin in each capture window and incubated for 60 minutes or overnight in refrigerator. Excess reagent is rinsed and the disc is spun dry.

[0362] As shown in **FIG. 47**, there can be a number of radially oriented viewing windows with different tests, such as CD4 (window 2), CD8 (window 3), CD3 (window 4), and CD45 (window 5), and negative control (window 6), using mouse IgG antibodies against these human cell surface antigens. This prepared substrate is incubated for 30 minutes or overnight in the refrigerator.

[0363] The pattern of chemistry deposition is provided below in Table 5.

TABLE 5

Window	1-2	3-4	5-6	7-8
1 st Layer	Streptavidin	Streptavidin	Streptavidin	Streptavidin
Secondary Antibodies	B-anti Mouse IgG + DCHO	B-anti Mouse IgG + DCHO	B-anti Mouse IgG + DCHO	B-anti Mouse IgG + DCHO
Primary Antibodies	Mouse Anti-human CD8	Mouse Anti-human CD3	Mouse Anti-human CD45	Mouse Anti-human CD45

TABLE 5-continued

Window	1-2	3-4	5-6	7-8
	human CD4			

[0364] B. Disc Assembly

[0365] The disc is assembled using an adhesive layer that may, for example, be 25, 50, or 100 microns thick (channel layer **118** in **FIGS. 2 and 5**), with a stamped out portion, such as a U-shape or "e-rad" channel, to create a fluidic channel, and a clear cap **116** (**FIG. 5**, for use with a transmissive disc with a top detector) or a cap **116** with a reflective layer **142** located over the capture zones (**FIG. 2**, for use with a reflective disc with a bottom detector).

[0366] In one embodiment, the disc is a forward Wobble Set FDL21:13707 or FDL21:1270 CD-R disc coated with 300 nm of gold as the encoded information layer. On a reflective disc, viewing windows of size 2x1 mm oval are etched out of the reflective layer by known lithography techniques. In some designs of transmissive disc, no separate viewing windows are etched, and the entire disc is available for use. In this particular example, the channel layer is formed from Fraylock adhesive DBL 201 Rev C 3M94661. The cover is a clear disc with 48 sample inlets each with a diameter of 0.040 inches located equidistantly at radius 26 mm. The data disc is scanned and read with the software at speed 4x and sample rate 2.67 MHz using CD4/CD8 counting software.

[0367] C. Disc Leak Check

[0368] Because blood is being analyzed, the disc can be leak checked first to make sure none of the chambers leak during spinning of the disc with the sample in situ. Each channel is filled with a blocking agent, such as StabilGuard and PBS-Tween. The block is for at least 1 hour. The disc is spun at 5000 rpm for 5 minutes to leak proof and check disc stability. After checking for leaks, the disc is placed in a vacuum chamber for 24 hours. After vacuuming for 24 hours, discs are placed in a vacuum pouch and stored in refrigerator until use.

[0369] D. Sample Collection, Preparation, and Application to Disc

[0370] The following section is directed to sample processing steps which are generally shown in **FIG. 17A**. Mononuclear cells (MNC) are purified by a density gradient centrifugation method, e.g., using a Becton Dickinson CPT Vacutainer. Blood (4-8 ml) is collected directly into a 4 or 8 ml EDTA containing CPT Vacutainer. The tubes are centrifuged at 1500 to 1800xg in a biohazard centrifuge with horizontal rotor and swing out buckets for 25 minutes at room temperature. The blood is preferably used within two hours of collection. After centrifugation, plasma overlying the mononuclear cell fraction is removed, leaving about 2 mm of plasma above an MNC layer. MNC are collected and washed with PBS. Cells are pelleted by centrifuge at 300xg for 10 minutes at room temperature. The supernatant is removed and the pellet containing the MNC is resuspended in PBS by tapping the tube gently. One more washes are done at 300xg for 10 minutes each at room temperature to

remove platelets. The final pellet is resuspended to a cell count of 10,000 cells/pl. An 18 μ l volume of the MNC is introduced to one or more the analysis chamber or channel, incubated for 15 minutes at room temperature with the disc stationary. The channels are sealed. The disc is then spun at 3000 rpm for 3 to 4 minutes using a disc drive. The disc is preferably scanned and read with the software at speed 4x and sample rate 2.67 MHz.

[0371] If a blood sample cannot be processed immediately, mononuclear cells after the first centrifugation can be resuspended in plasma by gently inverting the CPT tube several times and stored for up to 24 hours at room temperature. Within 24 hours, the cells in the plasma can be collected and washed as described above.

[0372] E. CD4/CD8 Assay Format

[0373] The assay in this example is a generic homogeneous solid phase cell capture assay for the rapid determination of absolute number of CD4+ and CD8+ T-lymphocyte populations and ratio of CD4+/CD8+ lymphocytes in blood samples. The test, which is run within a small chamber incorporated into a CD-ROM, determines the number of CD4+, CD8+, CD2+, CD3+ and CD45+ cells captured by the specific antibodies on the capture zones in 7 μ l of mononuclear cells (MNC) isolated from whole blood. The test is based upon the principle of localized cell capture on specific locations on the disc. Several specific cell capture zones are created on the disc by localized application of capture chemistries based upon monoclonal or polyclonal antibodies to particular blood cell surface antigens. Upon flooding the chamber with the MNC blood (30,000 cells/pl), cells expressing antigens CD4, CD8, CD2, CD3 and CD45 are captured in the capture zones on the disc. Also incorporated within the bar code are defined negative control areas.

[0374] F. On-Disc Analysis

[0375] MNC cells, prepared in step D above (18 μ l in PBS), are injected into the disc chamber, and inlet and outlet ports of the chamber are sealed. The disc is incubated for 15 minutes at room temperature, and then scanned using a 780 nm laser in an optical drive with a top detector to image the capture field as described above.

[0376] Software is encoded on the disc to instruct the drive to automatically perform the following acts: (a) centrifuge the disc to spin off excess unbound cells in one or more stages, (b) image specific capture windows, and (c) process data including counting the specifically-captured cells in each capture zone and deriving the ratio of CD4/CD8 (or which ever ratio is programmed to be determined).

[0377] During the processing step, the software reads across each capture zone image and marks cells as it encounters them. For example, following estimation of number of CD4+ and CD8+ cells, the software calculates the ratio of CD4+/CD8+ cells and displays both the absolute numbers of cells in CD4+, CD8+, CD3+ and CD45+ capture zones per microliter of whole blood and also the CD4+/CD8+ ratio. The entire process takes about 12 minutes from inserting the disc into the optical drive to obtaining the numbers and ratios.

[0378] G. Reagents Used

[0379] Streptavidin (Sigma, cat. # S-4762): Add de-ionized water to make a 5 mg/ml solution, aliquot and store at -30° C. To use, add Tris buffer for a final concentration of 1 mg/ml.

[0380] Positive control: CD45 (Sigma, Lot # 038H4892, cat # C7556). Store at $2-8^{\circ}$ C.

[0381] Secondary capture antibody: Biotinylated anti-mouse IgG (raised in sheep, Vector laboratories, lot # L0602, Catalog # BA-9200) Stock solution 1.5 mg/ml made in distilled water. Working b-IgG solution 125 μ g/ml in 0.1M PBS. Store at $2-8^{\circ}$ C. May be kept at -30° C. for long term storage.

[0382] Aldehyde activated Dextran (Pierce, lot # 97111761, cat # 1856167). Stock solution stock solution 5 mg/ml in PBS, store at $2-8^{\circ}$ C.

[0383] Primary capture antibody: CD4 (DAKO, cat # M0716), CD8 (DAKO, cat # M0707), CD2 (DAKO, cat # M720), CD45 (DAKO, cat # M0701), CD14 (DAKO, cat # M825), and CD3 (DAKO, cat # M7193). Store at $2-8^{\circ}$ C.

[0384] Negative control: Mouse IgG1 (DAKO, cat # X0931). Store at $2-8^{\circ}$ C.

[0385] Phosphate Buffered Saline (PBS), pH 7.4 (Life Technologies/GIBCO BRL, cat. # 10010-023) or equivalent. Store at room temperature

[0386] Isopropyl alcohol, 90-100%

[0387] H. RBC Lysing Protocol**[0388]** Ammonium Chloride Lysing Buffer

[0389] A 1xstock of ammonium chloride lysing buffer should be stored at 2 to 8° C. Comprised of 0.155M NH_4Cl , 10 mM KHCO_3 , and 0.1 mM disodium EDTA; pH 7.3 to 7.4. Store at $2-8^{\circ}$ C. Bring to room temperature prior to use.

[0390] Procedure

[0391] 1. For every 100 μ l of blood add 2 ml of lysing buffer. (It is preferable to do this procedure in a biohazard hood.)

[0392] 2. Vortex and incubate for 15 minutes at room temperature.

[0393] 3. Centrifuge the blood at $500\times g$ for 5 minutes at room temperature, using the centrifuge in the biohazard hood.

[0394] 4. Remove supernatant and wash cells with 2% FCS or FBS in PBS. Centrifuge cells.

[0395] 5. Calculate the total amount of WBCs and make the final concentration of WBCs 10,000 cells/pi for sample injection.

EXAMPLE 2

[0396] Mononuclear Cells Separation Procedure

[0397] Use Becton and Dickinson Vacutainer CPT (BD catalog # 362760 for 4 ml, # 362761 for 8 ml) cell preparation tubes with sodium citrate. Do procedure in biohazard hood following all biohazard precautions. Steps:

[0398] 1. Collect blood directly into a 4 or 8 ml EDTA containing CPT Vacutainer.

[0399] If the blood sample is already in an anticoagulant, pour off the EDTA in the Vacutainer first and then pour 6-8 mls of blood sample into the CPT tube.

[0400] 2. Centrifuge the tube at 1500 to $1800\times g$ in a biohazard centrifuge with horizontal rotor and swing out buckets for 25 minutes at room temperature. For best results, the blood should be centrifuged within two hours of collection. However, blood older than 2 hours may be centrifuged with a decrease in MNC number and increase in RBC contamination.

[0401] 3. After centrifugation, remove the plasma leaving about 2 mm of plasma above the MNC layer. Collect and transfer the whitish mononuclear layer into a 15 ml conical centrifuge tube.

[0402] 4. Add 10-15 mls with PBS to MNC layer, gently mix the cells by inverting the centrifuge tube several times.

[0403] 5. Wash cells by centrifuge at $200\times g$ for 10 minutes at room temperature in biohazard centrifuge.

[0404] 6. Remove supernatant. Resuspend cells by tapping the tube gently.

[0405] 7. Wash one more time in 10 ml of PBS. Centrifuge at $200-300\times g$ for 10 minutes at room temperature to remove platelets.

[0406] 8. Remove supernatant and resuspend pellet in 50 μ l PBS.

[0407] 9. Estimate cell counts in the sample. Run CBC or dilute 2 μ l of cells to 18 μ l of trypan blue, gently mix and count cells with a hemocytometer.

[0408] Make up the sample to a final cell count of 10,000 cells/ μ l for analysis.

[0409] 10. If the cells cannot be processed immediately, resuspend mononuclear cells after the first centrifugation (step 2 above) in the separated plasma by gently inverting the CPT tube several times and store for up to 24 hours at room temperature. Within 24 hours, collect the cells in the plasma and continue with the washes as described above.

[0410] Total cell counts per μ l=number of cells in 25 small squares \times (times) 100.

EXAMPLE 3

Isolation of MNCS From Whole Blood Using Histopaque-1077

[0411] 1 ml of Histopaque-1077 was placed in a 15 ml centrifuge tube and 1 ml of whole blood is gently layered over that. Then centrifuged at $400\times g$ for 30 min at room temperature. The mixture was aspirated carefully with a pasture pipette and the opaque interface transferred to centrifuged tube. Then 10 ml of PBS was added to the centrifuge tube. The solution was then centrifuged at $250\times g$ for 10 min. The supernatant was decanted and the cell pellet was resuspended in 10.0 ml PBS and spun at $250\times g$ for 10 min. The cells were then washed one more time by resuspending the pellet in 10 ml PBS and spinning at $250\times g$. The final cell pellet was resuspended in 0.5 ml PBS.

EXAMPLE 4

Disc Preparation and Chemistry Deposition

[0412] A. Disc Manufacturing Including Substrate Preparation and Chemistry Deposition

[0413] In this example, a transmissive disc substrate was cleaned with an air gun to remove dust. The disc was then mounted in the spin coater and rinsed twice with a steady stream of iso-propanol. Next, a polystyrene solution with 2% polystyrene dissolved in 310 mls of toluene and 65 mls of iso-propanol was evenly coated onto the disc.

[0414] For the secondary antibody deposition, a fresh solution of activated dextran aldehyde (200 ug/ml in PBS) was combined with an equal volume of the Vector IgG (125 ug/ml in PBS). Using manual pin deposition, approximately 1 ul of the IgG+DCHO complex was deposited in each capture zone on the disc. The disc was incubated in a humidity chamber for 60 minutes. Excess antibody was rinsed off with D. I. water and the disc was spun dry.

[0415] For the primary antibody, DAKO CD4 was diluted to 50 ug/ml in PBS, DAKO CD8 was diluted to 25 ug/ml in PBS, and DAKO CD45 was diluted to 145 ug/ml in PBS. Using the manual pin applicator, deposited approximately 1 ul of each primary antibody on top of the absorbed secondary antibodies. Incubated in the humidity chamber for 30 minutes. Rinsed off the excess antibody with PBS and spun dry the disc.

[0416] B. Disc Assembly

[0417] The cover disc used was clear with a Fraylock adhesive channel layer attached thereto. Stamped into the adhesive was 4 U-shaped channels that created the fluidic circuits. The cover was placed onto the transmissive disc substrate so that the fluid channels were over the antibody zones. Next, to secure the discs together, they were passed through a disc press 8 times.

[0418] C. Disc Leak Check, Blocking

[0419] Each fluid channel was filled with StableGuard and incubated for 1 hour. During the incubation, the disc was spun in the spin coater for 5 minutes at 5000 rpm. After the spin, the disc channels were checked for leaks. Next, the StableGuard was aspirated out of the channels, and the disc was placed under vacuum in a vacuum chamber overnight. The next morning, the disc was placed in a vacuum pouch and stored at 4° C.

EXAMPLE 5

Lymphoma Cell Lines

[0420] A. Cell Line Description

[0421] The cell line is a human T-cell lymphoma with the designation SUP-TI. The ATCC cat# is CRL-1942. This line was derived from malignant cells collected from the malignant pleural effusion of an 8 year old child with T-LL. The cells express multiple T lineage markers. The antigen expression is CD1a+, CD3+, CD4+, CD5+, and CD8+. The cultures are maintained between 2×10^5 and 2×10^6 cells/ml by two weekly additions and replacements of RPMI media with 10%FBS. The cultures are suspensions that are incubated horizontally at 37° C. in a 5% CO₂ air atmosphere.

[0422] B. SUP-TI Preparation

[0423] Estimate the cell concentration using a hemocytometer. Concentrate the cells to the desired concentration by centrifuging and removing the excess supernatant.

EXAMPLE 6

Disc Check using Lymphoma Cell Lines

[0424] A. Testing Antibodies With SUP-TI Cells

[0425] A disc was prepared according to Example 4 using a 25 um adhesive/channel layer. SUP-TI cell concentration was determined using a hemocytometer. The cells were concentrated to 30,000 cells/ul. They were injected into 2 channels in the analysis disc. After 15 minutes, the disc was centrifuged at 3000 rpm for 5 minutes. Light micrographs were taken of the cells captured on the capture zones and the cells were counted. The results from this experiment are shown below in Table 6.

TABLE 6

Number of SUP-TI Cells Captured on the capture zones			
Channel #	CD4	CD8	CD45
1	3160	3200	3400
2	3264	3144	3008

EXAMPLE 7

CD Marker Test of a Blood Sample

[0426] A. Preparing a Clinical Blood Sample

[0427] 12 ml of blood (sample # 176) was drawn at 10:00am into an EDTA tube. At 12:00 pm, 3 ml of blood was layered over 3 mls of Histopaque 1077 in 4x15 ml tubes. The tubes were centrifuged for 30 minutes at 400xg, at room temperature. After centrifugation, the upper plasma, within 0.5 cm of the opaque MNC layer, was aspirated off. The remaining opaque MNC layers was transferred the to a clean 15 ml tube and added 12 ml of PBS. Repeated this for the remaining 3 tubes.

[0428] Washing

[0429] The cell suspensions were then centrifuged at 250xg for 10 minutes. The supernatants were then aspirated off. And the cells resuspended in 14 ml of PBS. The suspensions were centrifuged at 250xg for 10 minutes again. Aspirated off the supernatants and resuspended each cell pellet with 175 ul of PBS. Then all 4 MNC suspensions were combined and the cell concentration determined by running a CBC with a Cell Dyne 1600 cell counter. The final volume was 650 uls with a concentration of 23.5K cells/ul. We then performed 6 serial dilutions to end up with concentrations ranging from 1000 cells/ul to 100,000 cells/ul, shown below in Table 7.

[0430] B. Comparing CD4 antibodies from DAKO and Serotec

[0431] Two discs (#338 & 339) with 6 channels each were made according to Example 4 using a 100 um adhesive layer. Another primary CD4 antibody from Serotec (50 ug/ml, cat# LN1298) was also used in this experiment.

[0432] We injected each channel of disc 338 with one serial dilution of sample 176. After 15 minutes, the disc was centrifuged at 3000 rpm for 5 minutes. Light micrographs were taken of the cells captured on the chemistry zones and the cells were counted using a cell counting software (Metamorph). Alternatively, the number of cells captured may be evaluated using an optical disc reader and accompanying software. This procedure was for disc 339. The counts of cells captured on the DAKO CD4 chemistry zones were compared to the cells captured on the Serotec CD4 chemistry zones. The data collected from this experiment is shown in Table 7 and in FIG. 61.

filed Sep. 20, 2001; U.S. Provisional Application Serial No. 60/324,336 entitled "Methods for Reducing Bubbles in Fluidic Chambers Using Polyvinyl Alcohol and Related Techniques for Achieving Same in Optical Bio-Discs" filed Sep. 24, 2001; U.S. Provisional Application Serial No. 60/326,800 entitled "Sealing Methods for Containment of Hazardous Biological Materials within Optical Analysis Disc Assemblies" filed Oct. 3, 2001; U.S. Provisional Application Serial No. 60/328,246 entitled "Methods for Calculating Qualitative and Quantitative Ratios of Helper/Inducer-Suppressor/Cytotoxic T-Lymphocytes Using Optical Bio-Disc Platform" filed Oct. 10, 2001; U.S. Provisional

TABLE 7

Cells Captured using DAKO and Serotec CD4 Capture Antibodies						
Sample 176 Cell Concentration	CD4 Dako Disc 338	CD4 Dako Disc 339	CD4 Dako Averages	CD4 Serotek Disc 338	CD4 Serotek Disc 339	CD4 Serotek Averages
1,000 cells/ul	76	59	68	71	49	60
5,000 cells/ul	282	211	247	287	273	280
10,000 cells/ul	559	526	543	530	525	528
25,000 cells/ul	1168	1205	1187	1015	1040	1028
50,000 cells/ul	2459	2595	2527	1726	1981	1854
100,000 cells/ul	3686	3835	3761	3513	3372	3443

EXAMPLE 8

Comparing Disc CD4/CD8 Ratios to FACS
CD4/CD8 Ratios

[0433] Clinical samples (Nos. 154,156,157,159 & 175) were prepared similar to example 7, using only 3 mls of blood each, with the final sample concentrations 10,000 cells/ul.

[0434] Discs (Nos. 272,275,276,267 & 327) were prepared similar to example 4, using 100 um adhesive channels.

[0435] Each sample was injected into each corresponding disc as shown below in Table 8. After 15 minutes, the disc was centrifuged at 3000 rpm for 5 minutes. Light micrographs were then taken of the cells captured on the chemistry zones and the cells were counted. Each clinical sample also had a FACS analysis performed. The results from this experiment is shown below in Table 8 and in FIGS. 62A and 62B.

TABLE 8

CD4/CD8 Ratios of Blood using the Optical Bio-Disc and FACS			
Disc #	Sample #	Disc CD4/CD8	FACS CD4/CD8
272	154	0.32	0.48
275	156	0.99	0.87
276	157	2.68	2.46
267	159	1.48	1.65
327	175	1.18	1.32

[0436] Concluding Statements

[0437] Aspects of the present invention relating to the apparatus, methods, and processes disclosed herein are also presented in U.S. Provisional Application Serial No. 60/323,682 entitled "Methods for Reducing Non-Specific Binding of Cells on Optical Bio-Discs Utilizing Blocking Agents"

Application Serial No. 60/386,072 entitled "Quantitative and Qualitative Methods for Characterizing Cancerous Blood Cells Including Leukemic Blood Samples Using Optical Bio-Disc Platform" filed Oct. 19, 2001; U.S. Provisional Application Serial No. 60/386,073 entitled "Methods for Quantitative and Qualitative Characterization of Cancerous Blood Cells Including Lymphoma Blood Samples Using Optical Bio-Disc Platform" filed Oct. 19, 2001; U.S. Provisional Application Serial No. 60/386,071 entitled "Methods for Specific Cell Capture by Off-Site Incubation of Primary Antibodies with Sample and Subsequent Capture by Surface-Bound Secondary Antibodies and Optical Bio-Disc Including Same" filed Oct. 26, 2001; U.S. Provisional Application Serial No. 60/344,977 entitled "Quantitative and Qualitative Methods for Cell Isolation and Typing Including Immunophenotyping" filed Nov. 7, 2001; and U.S. Provisional Application Serial No. 60/349,975 entitled "Methods for Reducing Non-Specific Binding of Cells on Optical Bio-Discs Utilizing Charged Matter Including Heparin, Plasma, or Poly-Lysine" filed Nov. 9, 2001, all of which are herein incorporated by reference.

[0438] While this invention has been described in detail with reference to certain preferred embodiments and technical examples, it should be appreciated that the present invention is not limited to those precise embodiments or examples. Rather, in view of the present disclosure which describes the current best mode for practicing the invention, many modifications and variations would present themselves to those of skill in the art without departing from the scope and spirit of this invention. The scope of the invention is, therefore, indicated by the following claims rather than by the foregoing description. All changes, modifications, and variations coming within the meaning and range of equivalency of the claims are to be considered within their scope.

What is claimed is:

1. A system for detecting blood cell analytes in biological samples, said system comprising:

- an optical disc having a substrate;
- a cap parallel to said substrate;
- a chamber between said substrate and cap, said chamber including capture zones;
- a capture layer associated with said substrate at the capture zones, such that a first capture zone has first cell capture agents and a second capture zone has a second cell capture agents;
- a light source for directing light to the disc at the capture zones;
- a detector for detecting light reflected from or transmitted through the disc at the capture zones and providing a signal; and
- a processor for using the signal to count items in the sample bound to the capture agents.

2. A method for detecting blood cell analytes in biological samples employing an optical disc and disc drive, said method comprising the steps of:

- providing a sample of cells on a disc surface in a chamber in of the disc, the chamber including at least one capture zone with a capture agent;
- loading the disc into an optical reader;
- rotating the optical disc;
- directing an incident beam of electromagnetic radiation toward the capture zone;
- detecting a beam of electromagnetic radiation formed after interacting with the disc at the capture zone;
- converting the detected beam into an output signal; and
- analyzing the output signal to extract therefrom information relating to the number of cells captured at the capture zone.

3. The method according to claim 2 wherein the chamber with the disc surface supporting the sample is internal to the disc and is bounded on opposite sides by a substrate and cap.

4. The method according to claim 2 wherein the optical disc is constructed with a reflective layer such that light directed to the capture zone and not striking a cell is reflected.

5. The method according to claim 2 wherein the optical disc is constructed such that light directed to the capture zone and not striking a cell is transmitted through the optical disc, the disc being between the light source and the detector.

6. The method according to claim 2 wherein the disc surface is coated with a first group of cell capture agents.

7. The method according to claim 6 wherein the cell capture agents define a discrete capture zone.

8. The method according to claim 7 wherein a second group of cell capture agents define a second discrete capture zones in a predetermined pattern.

9. The method according to claim 8 wherein the first and second capture zones are in one chamber.

10. The method according to claim 6 wherein the cell capture agents are for binding with cell surface antigens.

11. The method according to claim 10 wherein the cell surface antigen is selected from the CD family of antigens.

12. A method of performing a cluster designation count with an optical disc and disc drive, said method comprising the steps of:

- providing a blood sample in a first tube having a separation gradient;
- rotating the first tube at a time and speed sufficient to separate the blood sample into layers;
- resuspending an MNC layer containing T-cells to thereby form an MNC suspension;
- providing a sample of the MNC suspension on an optical disc surface including at least one capture zone with at least one capture agent;
- loading the optical disc into an optical reader;
- rotating the optical disc;
- directing an incident beam of electromagnetic radiation toward said at least one capture zone;
- detecting a beam of electromagnetic radiation formed after interacting with the disc at the capture zone;
- converting the detected beam into an output signal; and
- analyzing the output signal to extract therefrom information relating to the number of cells captured at the capture zone.

13. The method according to claim 12 wherein the separation gradient is provided by a matrix that forms a density gradient upon centrifugation.

14. The method according to claim 12 wherein the first tube further contains an anticoagulant.

15. The method according to claim 14 wherein the anticoagulant is EDTA.

16. The method according to claim 14 wherein the anticoagulant is acid citrate dextran.

17. The method according to claim 14 wherein the anticoagulant is heparin.

18. The method according to claim 12 wherein the surface is internal to the disc and is bounded on opposite sides by a substrate and a cap.

19. The method according to claim 12 wherein the optical disc is constructed with a reflective layer such that light directed to the capture zone and not striking a cell is reflected.

20. The method according to claim 12 wherein the optical disc is constructed such that light directed to the capture zone and not striking a cell is transmitted through the optical disc, the disc being between the light source and the detector.

21. The method according to claim 12 wherein the disc surface is coated with a first group of capture agents.

22. The method according to claim 21 wherein the capture agents are antibodies.

23. The method according to claim 22 wherein the antibodies are selected from the group including monoclonal, polyclonal, and recombinantly created antibodies.

24. The method according to claim 21 wherein the capture agents are immobilized on the disc surface by a cross-linking system.

25. The method according to claim 20 wherein the capture agents are immobilized directly on the disc surface.

26. The method according to claim 20 wherein the capture agents define one or more discrete capture zones.

27. The method according to claim 26 wherein the one or more capture zones are located within one or more chambers within the optical disc.

28. The method according to claim 20 wherein the capture agents have a selective affinity for cell surface antigens.

29. The method according to claim 28 wherein the cell surface antigens are selected from the CD family of antigens.

30. The method according to claim 29 wherein the cell surface antigens are selected from the group consisting of CD3, CD4, CD8, and CD45.

31. The method according to claim 12 wherein the blood sample comprises normal blood cells, leukemic blood cells, or lymphomic blood cells.

* * * * *

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摘要(译)

在光学生物盘上进行临床诊断测定，并用盘驱动器读取。公开了使用光学生物盘检测和定量生物样品中的特定血细胞分析物的方法和装置。用于确定生物样品中特定类型的血细胞的量的方法包括将抗体结合到椎间盘上的捕获区，向捕获区提供样品，去除未结合在捕获区中的样品的部分，并计算绑定的细胞。还描述了用于利用光盘和盘驱动器执行簇指定计数的方法和装置，以及用于制造用于执行这种簇指定计数的光学测定盘的方法。

