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(54) **METHOD FOR DETERMINING THE STATUS OF AN INDIVIDUAL**

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(57) **ABSTRACT**

Methods of determining status of an individual based on the use of biological specimen and analysis of reference population of cells are described.

METHOD FOR DETERMINING THE STATUS OF AN INDIVIDUAL

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 61/055,362, filed May 22, 2008, which application is incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] Despite great gains in knowledge over the past several decades in the fields of genetics and cellular and molecular biology, this expansion of knowledge has not translated into commensurate advances in the diagnosis or prognosis of disease, or the ability to predict or assess response to therapy. New methods for diagnosis and prognosis that harness the advances in the biologic sciences are needed.

SUMMARY OF THE INVENTION

[0003] The present invention relates to determining the status of an individual. More specifically, the present invention is a method for treating a population of reference cells with a biological specimen derived from an individual.

[0004] One embodiment of the present invention is a method for determining the status of an individual, comprising subjecting a reference population of cells to a biological specimen obtained from the individual; determining the activation states of a plurality of activatable elements in said reference population of cells; and determining the status of the individual based on the activation state of the reference population of cells.

[0005] Another embodiment of the invention is a method for determining the status of an individual, comprising, obtaining a biological specimen from an individual; applying the biological specimen, or a fraction thereof, to a reference population of cells; assessing activatable elements in the reference population of cells; and comparing activatable elements of the reference cell line that has been contacted with the biological specimen with activatable elements of the reference population of cells that has not been contacted with the biological specimen to determine the status of the individual.

[0006] Another embodiment of the invention is a method for determining the status of an individual comprising: obtaining one or more elements of a cellular environmental from the individual; applying said element or elements to a reference population of cells; determining the activation state of an intracellular activatable element in the reference population of cells; classifying one or more cells of the reference population of cells into one or more classes based on the activation state; and determining the status of the individual by linking the one or more classes to a clinical outcome.

[0007] Another method of the present invention is a method for determining the status of an individual, comprising, obtaining blood from the individual; fractionating the blood into sera; applying the sera to a reference population of cells; assessing the activatable elements in the reference population of cells; comparing the activatable elements of the reference population of cells to that of the reference population of cells that has not been contacted with the sera.

[0008] In some embodiments, the biological specimen or cellular environment is used as a modulator. Alternatively, one or more modulators can be derived from the biological specimen or cellular environment.

[0009] In the above methods, the biological specimen, or cellular environment can comprise: sera, whole blood, ascites, plasma, cell extract, cerebrospinal fluid, saliva, urine, whole cells, lavage or rinse of cavities. The methods may be useful for therapeutic choice, disease diagnosis or prognosis. The reference population of cells may be a homogeneous cell line, a defined mixture of homogeneous cell lines, a homogeneous cell population, a mixture of cells, or a library of cells. The reference population of cells can be obtained from the individual whose status is being determined or from a different individual. In some embodiments, the reference population of cells is obtained from a mammal that is a different mammal than the individual whose status is being determined. Additionally, the modulator may be fractionated into serum components, which comprise cytokines, hormones, and chemokines; Igs; or cellular components, which comprise white blood cells, dendritic cells, platelets, and red blood cells. Additionally, the modulator may be the liquid or cellular environment that surrounds or previously surrounded cells from the individual. Also, the individual may have cancer, sepsis, inflammatory, infectious, immunologic, or an autoimmune disease. Additionally, the activation state of the reference population of cells that has not been contacted with the sera, or biological specimen, or cellular environment, may be stored in a database and the comparisons between the activation state of the reference population of cells that has, and has not been contacted with the sera, or biological specimen, or cellular environment, may be performed on a computer. In one embodiment, the determination or assessment of the activation state of the cells is by flow cytometry.

INCORPORATION BY REFERENCE

[0010] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

DETAILED DESCRIPTION OF THE INVENTION

[0011] The present invention incorporates information disclosed in other applications and texts. The following patent and other publications are hereby incorporated by reference in their entireties: Haskell et al, Cancer Treatment, 5th Ed., W.B. Saunders and Co., 2001; Weinberg, The Biology of Cancer, Garland Science, 2007; Alberts et al., The Cell, 4th Ed., Garland Science, 2002; Vogelstein and Kinzler, The Genetic Basis of Human Cancer, 2d Ed., McGraw Hill, 2002; Michael, Biochemical Pathways, John Wiley and Sons, 1999; Immunobiology, Janeway et al. 7th Ed., Garland, and Leroith and Bondy, Growth Factors and Cytokines in Health and Disease, A Multi Volume Treatise, Volumes 1A and 1B, Growth Factors, 1996. Patent applications that are also incorporated by reference include U.S. Ser. Nos. 10/193,462; 11/655,785; 11/655,789; 10/346,620; 11/655,821; 10/898,734; 11/338,957; 61/048,886; 61/048,920 and 61/048,657. Some commercial reagents, protocols, software and instruments that are useful in some embodiments of the present invention are available at the Becton Dickinson Website http://www.bdbiosciences.com/features/products/, and the Beckman Coulter website, http://www.beckmancoulter.com/Default.asp?bhfv=7. Relevant articles include High-content single-cell drug screening with phosphospecific flow cytometry, Krutzik et al., Nature Chemical Biology, 23 Dec. 2007;

Irish et al., F1t3 Y591 duplication and Bc1-2 over expression are detected in acute myeloid leukemia cells with high levels of phosphorylated wild-type p53, Neoplasia, 2007, and Irish et al., Single cell profiling of potentiated phospho-protein networks in cancer cells, Cell, Vol. 118, 1-20 Jul. 23, 2004; Schulz, K. R., et al., Single-cell phospho-protein analysis by flow cytometry, Curr Protoc Immunol, 2007, 78:8 8.17.1-20; Krutzik, P. O., et al., Coordinate analysis of murine immune cell surface markers and intracellular phosphoproteins by flow cytometry, J Immunol. Aug. 15, 2005; 175(4):2357-65; Krutzik, P. O., et al., Characterization of the murine immunological signaling network with phosphospecific flow cytometry, J Immunol. Aug. 15, 2005;175(4):2366-73; Shulz et al., Current Protocols in Immunology 2007, 78:8.17.1-20; and Krutzik, P. O. and Nolan, G. P., Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events, Cytometry A. 2003 October;55 (2):61-70. Experimental and process protocols and other helpful information can be found at http:/proteomices.stanford.edu.

Introduction

[0012] A cell of any lineage or type co-exists in an environment which may be liquid and/or solid and consist of proteins, carbohydrates, lipids, nutrients, cytokines, growth factors and/or other cells or tissues. Any of these environmental factors may have an effect on the viability, growth and/or differentiation of a given cell. For example, in response to a given environmental cue a cell might receive a message or signal to grow, stop growing, differentiate into a different cell type, or to secrete new or different cytokines or growth factors. These new or different cytokines or factors that are secreted then add to the environmental milieu and may have effects on the initial cell or other cells or tissues at a distance that come into contact with the factors. This is especially important when the cell of interest is a cancerous cell. The effect of a biological specimen, (e.g. serum from an individual having or suspected to have a condition) on reference cells can be used to determine the status of an individual (e.g. diagnose a condition). The effect of the biological specimen can be measured, for example, on cells from the individual whose status is being determined, cells from a different individual, cells from a different mammal or cell lines.

[0013] One aspect of this invention provides a method for determining the status of an individual comprising; obtaining a biological specimen by removing an element or elements of the physiological environment, be it in the form of sera, plasma, ascites, cerebrospinal fluid, saliva, urine lipid, carbohydrate, protein, or in the form of a cell, or a plurality of cells or tissue, from the individual and the application of said element(s) to a reference population of cells, such as an established cell line(s), finding the activation state of an intracellular activatable element in this reference population of cells; classifying the cells of this reference population of cells into one or more classes based on the activation state; and determining the status of the individual by linking the signaling and classification data of the reference population of cells to clinical outcomes data. These outcomes may be related to an individual's state of health, the presence or absence of a pathologic or pre-pathologic condition, response to a therapeutic, prognosis, and/or likelihood of relapse or progression of a pre-pathologic or pathologic condition.

[0014] In some embodiments, this invention is directed to methods and compositions for diagnosis, prognosis and for methods of treatment. In some embodiments, the status of the reference population of cells is used, e.g., in diagnosis or prognosis of a condition, patient selection for therapy, to monitor treatment, modify therapeutic regimens, and to further optimize the selection of therapeutic agents. Hence, therapeutic regimens can be individualized and tailored according to the data obtained prior to, and at different times over the course of treatment, thereby providing a regimen that is individually appropriate.

[0015] In some embodiments, the present invention is directed to methods for classifying a biological specimen or sample derived from an individual having or suspected of having a condition, e.g., a neoplastic, autoimmune or a hernatopoietic condition. The invention allows for identification of prognostically and therapeutically relevant subgroups of conditions and prediction of the clinical course of an individual. The methods of the invention provide tools useful in the treatment of an individual afflicted with a condition, including but not limited to methods for assigning a risk group, methods of predicting a refractory or resistant response to drugs, an increased risk of relapse, methods of predicting an increased risk of developing secondary complications, methods of choosing a therapy for an individual, methods of predicting response to a therapy for an individual, methods of determining the efficacy of a therapy in an individual, methods for determining the dosing regimen and methods of determining the prognosis for an individual. The present invention provides methods that can serve as a prognostic indicator to predict the course of a condition, e.g. whether the course of a neoplastic, immunologic or a hematopoietic condition in an individual will be aggressive or indolent, thereby aiding the clinician in managing the patient and evaluating the modality of treatment to be used.

[0016] In some embodiments, the invention is directed to methods for determining the activation level of one or more activatable elements in a reference population of cells upon treatment with a biological specimen derived from the patient, such as a sample from the cellular environment, or a modulator derived from the patient and another modulator, either derived from the patient or from an external source that may be better characterized. Examples of well characterized external modulators include cytokines, chemokines, hormones and pharmaceutical agents. The activation of an activatable element in the cell upon treatment with one or more modulators can reveal operative pathways in a condition that can then be used, e.g., as an indicator to predict course of the condition, identify risk group, predict an increased risk of developing secondary complications, choose a therapy for an individual, predict response to a therapy for an individual, determine the efficacy of a therapy in an individual, and determine the prognosis for an individual. For example, the activation of an activatable element in a reference cell in response to a biological specimen can reveal one or more factors present in the biological specimen that might contribute to the pathology of the condition. In addition, the activation of an activatable element in a reference cell obtained from the individual whose status is being determined in response to a biological specimen from said individual can reveal operative pathways that can be used to make a determination regarding the status of the individual.

[0017] In some embodiments, the invention is directed to methods for determining the status of an individual by using a biological specimen or a sample of the cellular environment of an individual, and classifying its effect on a reference

population of cells by contacting the cells with the specimen or sample, determining the presence or absence of an increase in activation level of an activatable element in the cell relative to non treated cells in the reference population, and classifying the cell based on the presence/absence of the increase/decrease in the activation of the activatable element.

[0018] In some embodiments, the invention is directed to methods of determining a phenotypic profile of a reference population of cells by exposing the population of cells to a plurality of fractions of the biological specimen or cellular environment as modulators in separate cultures, either alone or in combination with other external modulators, deter-mining the presence or absence of an increase in activation level of an activatable element in the cell population from each of the separate culture and classifying the cell population based on the presence or absence of the increase in the activation of the activatable element from each of the separate culture.

[0019] In some embodiments, the invention is directed to methods of classifying a reference population of cells by contacting the cells with at least one modulator in addition to the biological specimen or cellular environment obtained from the individual, where the additional modulator is an inhibitor, such as H_2O_2, a member of the IMIDS family, such as Revlimid (sold by CelGene, Summit, N.J., see www.celgene.com or www.revlimid.com), in combination with other modulators such as PMA, thapsigargin, thrombopoietin, IGF-1, GM-CSF, G-CSF, crythropoetin, SCF, SDF, IFNα, WFNγ, BAFF, April, SDF 1a, CD40L, TNF-α, interleulcin, cytokine or growth factor, hormone, receptor ligand or co-factor and/or a combination thereof. Then, determining the presence or absence of an increase in activation level of an activatable element in the cell population, and classifying the cell population based on the presence or absence of the increase in the activation of the activatable element. Other modulators that may be used in combination with the present invention are shown below or are found in the following references U.S. Ser. Nos. 10/193,462; 11/655,785; 11/655,789; 10/346,620; 11/655,821; 10/898,734; 11/338,957; 61/048,886; 61/048,920 and 61/048,657.

[0020] The subject invention also provides kits for use in determining the physiological status of cells in a sample, the kit comprising one or more specific binding elements for signaling molecules, and may additionally comprise one or more therapeutic agents. The kit may further comprise a software package for data analysis of the physiological status, which may include reference profiles for comparison with the test profile.

Methods

[0021] In some embodiments, the invention provides methods, including methods to determine the physiological status of an individual, e.g., by determining the activation level of an activatable element within a reference cell or cell population, upon contact with one or more modulators inherent in a biological specimen or a sample of the cellular environment of the individual, potentially in combination with other, known modulators. In some embodiments, the invention provides methods, including methods to classify a cell according to the status of an activatable element in a cellular pathway. The information can be used in prognosis and diagnosis, including susceptibility to disease(s), status of a diseased state and response to changes, in the environment, such as the passage of time, treatment with drugs or other modalities. The physiological status of the cells may be classified according to the activation of cellular pathways of interest. The cells can also be classified as to their ability to respond to therapeutic agents and treatments.

[0022] The biological specimen, cellular environment or other modulator can be isolated from body samples, such as, but not limited to, smears, sputum, biopsies, secretions, cerebrospinal fluid, bile, sera, whole blood, ascites, plasma, cell extract, whole cells, lavage or rinse of cavities, lymph fluid, urine and feces, or tissue which has been removed from organs, such as breast, lung, intestine, skin, cervix, prostate, and stomach. The biological specimen can be a fraction of the above specimen or a derivative of the specimen. For example, a tissue sample can comprise a region of functionally related cells or adjacent cells. Such samples can comprise complex populations of cells, which can be assayed as a population, or separated into sub-populations. The cells can also be used to produce a cell culture extract which may be used in the present method. Such cellular and acellular samples can be separated by centrifugation, elutriation, density gradient separation, apheresis, affinity selection, panning, FACS, centrifugation with Hypaque, etc. By using antibodies specific for markers identified with particular cell types, a relatively homogeneous population of cells may be obtained. Alternatively, a heterogeneous cell population can be used. Cells can also be separated by using filters. For example, whole blood can be applied to filters that are engineered to contain pore sizes that select for the desired cell type or class. Rare pathogenic cells can be filtered out of diluted, whole blood following the lysis of red blood cells by using filters with pore sizes between 5 to 10 μm, as disclosed in U.S. patent application Ser. No. 09/790,673. Once a sample is obtained, it can be used directly, cryopreserved, or maintained in appropriate culture medium for short periods of time. Methods to isolate one or more cells for use according to the methods of this invention are performed according to standard techniques and protocols well-established in the art.

[0023] Cells obtained as a biologic specimen from the patient may serve a dual purpose as either the modulator, or, if another biologic specimen (modulator) is applied to them, they may serve as the reference cell population. Suitable cells for preparing the biological specimen as either a modulator or as the reference population of cells, include those cell types associated in a wide variety of disease conditions, even while in a non-diseased state. Accordingly, suitable eukaryotic cell types include, but are not limited to, tumor cells of all types (e.g. melanoma, myeloid leukemia, carcinomas of the lung, breast, ovaries, colon, kidney, prostate, pancreas and testes), cardiomyocytes, dendritic cells, endothelial cells, epithelial cells, lymphocytes (T-cell and B cell), mast cells, eosinophils, vascular intimal cells, macrophages, natural killer cells, erythrocytes, hepatocytes, leukocytes including mononuclear leukocytes, stem cells such as haemopoetic, neural, skin, lung, kidney, liver and myocyte stem cells, osteoclasts, chondrocytes and other connective tissue cells, keratinocytes, melanocytes, liver cells, kidney cells, and adipocytes. Suitable cells also include primary disease state cells, such as primary tumor cells.

[0024] In some embodiments, the reference population of cells can be obtained from a different individual than the individual whose status is being determined, e.g., a healthy individual. In other embodiments, the reference population of cells can be obtained from a different mammal than the individual whose status is being determined. For example, the

individual whose status is being determined can be a human and the reference population of cells can be obtained from mice.

[0025] In some embodiments, the reference population of cells can also include known research cells including but limited to Jurkat T-cells, NIH3T3 cells, CHO, COS, U937, TF-1 etc. See the ATCC cell line catalog hereby expressly incorporated by reference. In some embodiments, the reference population of cells is cultured in a media suitable for revealing the activation level of an activatable element (e.g. RPMI, DMEM) in the presence, or absence, of serum such as fetal bovine serum, bovine serum, human serum, porcine serum, horse serum, or goat serum. When serum is present in the media it could be present at a level ranging from 0.0001% to 30%.

[0026] In some embodiments, the reference population of cells comprises a hematopoietic cell. Examples of hematopoietic cells include but are not limited to pluripotent hematopoietic stem cells, B-lymphocyte lineage progenitor or derived cells, T-tymphocyte lineage progenitor or derived cells, NK cell lineage progenitor or derived cells, granulocyte lineage progenitor or derived cells, monocyte lineage progenitor or derived cells, megakaryocyte lineage progenitor or derived cells and erythroid lineage progenitor or derived cells. In some embodiments, the cells used in the present invention as either the biological specimen or the reference population of cells are taken from a patient.

[0027] The term "patient" or "individual" as used herein includes humans as well as other mammals. The methods generally involve determining the status of an activatable element. The methods also involve determining the status of a plurality of activatable elements.

[0028] In some embodiments, the invention provides a method of classifying a cell by determining the presence or absence of an increase or decrease in activation level of an activatable element in the cell upon treatment with the biological specimen, cell environment potentially in combination with one or more additional modulators, and classifying the cell based on the presence or absence of the increase or decrease in the activation of the activatable element. In some embodiments of the invention, the activation level of the activatable element is determined by contacting the cell with a binding element that is specific for an activation state of the activatable element. In some embodiments, a cell is classified according to the activation level of a plurality of activatable elements after the cell have been subjected to a modulator. In some embodiments of the invention, the activation levels of a plurality of activatable elements are determined by contacting a cell with a plurality of binding element, where each binding element is specific for an activation state of an activatable element.

[0029] The classification of a cell according to the status of an activatable element can comprise classifying the cell as a cell that is correlated with a clinical outcome. In some embodiments, the clinical outcome is the prognosis and/or diagnosis of a condition. In some embodiments, the clinical outcome is the presence or absence of a neoplastic, immunologic or a hematopoietic condition. Neoplastic conditions may include solid tumors. The solid tumor may be any solid tumor amenable to sampling for direct or indirect analysis; solid tumors include but are not limited to head and neck cancer including brain, thyroid cancer, breast cancer, lung cancer, mesothelioma, germ cell tumors, ovarian cancer, liver cancer, gastric carcinoma, colon cancer, prostate cancer, pancreatic cancer, melanoma, bladder cancer, renal cancer, prostate cancer, testicular cancer, cervical cancer, endometrial cancer, myosarcoma, Iciomyosarcoma and other soft tissue sarcomas, osteosarcoma, Ewing's sarcoma, retinoblastoma, rhabdomyosarcoma, Wilm's tumor, and neuroblastoma. Immunologic diseases and disorders include sepsis, allergic, disorders of immune function, and autoimmune diseases and conditions. Allergic diseases and disorders include but are not limited to allergic rhinitis, allergic conjunctivitis, allergic asthmna, atopic eczema, atopic dermatitis, and food allergy. Immunodeficiencies include but are not limited to severe combined immunodeficiency (SCID), hypereosiniphic syndrome, chronic granulomatous disease, leukocyte adhesion deficiency I and II, hyper IgE syndrome, Chediak Higashi, neutrophilias, neutropenias, aplasias, Agammaglobulinemia, hyper-IgM syndromes, DiGeorge/Velocardial-facial syndromes and Interferon gamma-TH1 pathway defects. Autoimmune and immune dysregulation disorders include but are not limited to rheumatoid arthritis, diabetes, systemic lupus erythematosus, Graves' disease, Graves ophthalmopathy, Crohn's disease, multiple sclerosis, psoriasis, systemic sclerosis, goiter and struma lymphomatosa (Hashimoto's thyroiditis, lymphadenoid goiter), alopecia aerata, autoimmune myocarditis, lichen sclerosis, autoimmune uveitis, Addison's disease, atrophic gastritis, myasthenia gravis, idiopathic thrombocytopenic purpura, hemolytic anemia, primary biliary cirrhosis, Wegener's granulomatosis, polyarteritis nodosa, and inflammatory bowel disease, allograft rejection and tissue destructive from allergic reactions to infectious microorganisms or to environmental antigens. Hematopoietic conditions include but are not limited to Non-Hodgkin Lymphoma, Hodgkin or other lymphomas, acute or chronic leukemias, polycythemias, thrombocythemias, multiple myeloma or plasma cell disorders, e.g., amyloidosis and Waldenstrom's macroglobulinemia, myelodysplastic disorders, mycloproliferative disorders, myelofibroses, or atypical immune lymphoproliferations. In some embodiments, the neoplastic or hematopoietic condition is non-B lineage derived, such as Acute myeloid leukemia (AML), Chronic Myeloid Leukemia (CML), non-B cell Acute lymphocytic leukemia (ALL), non-B cell lymphomas, myelodysplastic disorders, myeloproliferative disorders, myelofibroses, polycythemias, thrombocythemias, or non-B atypical immune lymphoproliferations, Chronic Lymphocytic Leukemia (CLL), B lymphocyte lineage leukemia, B lymphocyte lineage lymphoma, Multiple Myeloma, or plasma cell disorders, e.g., amyloidosis or Waldenstrom's macroglobulinemia. In some embodiments, the clinical outcome is the presence or absence of a neoplastic or a hematopoietic condition, such as those mentioned above. In some embodiments, the clinical outcome is the staging or grading of a neoplastic or hematopoietic condition. Examples of staging include, but are not limited to, aggressive, indolent, benign, refractory, Roman Numeral staging, TNM Staging, Rai staging, Binet staging, WHO classification, FAB classification, IPSS score, WPSS score, limited stage, extensive stage, staging according to cellular markers such as ZAP70, IgVH mutational status, Flt 3, androgen or estrogen receptor positivity, occult, including information that may inform on time to progression, progression free survival, overall survival, or event-free survival.

[0030] In some embodiments, methods and compositions are provided for the classification of a cell according to the activation level of an activatable element, e.g., in a cellular pathway wherein the classification comprises classifying a

cell as a cell that is correlated to a patient response to a treatment. In some embodiments, the patient response is selected from the group consisting of complete response, remission, partial response, nodular partial response, no response, progressive disease, stable disease and adverse reaction, or as determined by RECIST or other response criteria.

[0031] The classification of a cell according to the status of an activatable element can comprise selecting a method of treatment. Example of methods of treatments include, but are not limited to chemotherapy, biological therapy, radiation therapy, bone marrow transplantation, Peripheral stem cell transplantation, umbilical cord blood transplantation, autologous stem cell transplantation, allogeneic stem cell transplantation, syngeneic stem cell transplantation, surgery, induction therapy, maintenance therapy, watchful waiting, and holistic/alternative therapy.

[0032] The modulator can be a biological specimen or a sample of the cellular environment of an individual. Other modulators may be added to the biological specimen or sample of the cellular environment. For example, these additional modulators can be an inhibitor or a compound capable of impacting cellular signaling networks. The modulators inherent in the biological specimen or cell environment may not have been chemically or biologically characterized after removal from an individual and prior to contact with the reference population of cells.

[0033] Known modulators may be used in addition to the uncharacterized modulators. Examples of known modulators include but are not limited to growth factors, cytokines, chemokines, adhesion molecule modulators, drugs, hormones, small molecules, polynucleotides, oligonucleotides, miRNAs, siRNAs, antibodies, natural compounds, lactones, chemotherapeutic agents, immune modulators, carbohydrates, proteases, ions, reactive oxygen species, radiation, physical parameters such as heat, cold, UV radiation, peptides, and protein fragments, either alone or in the context of cells, cells themselves, viruses, and biological and non-biological complexes (e.g. beads, plates, viral envelopes, antigen presentation molecules such as major histocompatibility complex). Specific examples include H_2O_2, PMA, Revlimid, TNF-α, G-CSF, GM-CSF, FLT3L, IGF-1, SCF, erythropoetin, thrombopoetin, interferons, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-27 BAFF, April, SDF1a, CD40L, Imiquimod, poly-CpG, IL-7, IL-6, IL-10, IL-27, IL-4. See also U.S. Ser. Nos. 10/193,462; 11/655,785; 11/655,789; 10/346,620; 11/655,821; 10/898,734; 11/338,957; 61/048,886; 61/048,920 and 61/048,657 for additional modulators.

[0034] In some embodiments, the modulator is an activator. In some embodiments the modulator is an inhibitor. In some embodiments, the invention provides methods for classifying a reference cell by contacting the cell with an inhibitor derived from the patient, determining the presence or absence of an increase in activation level of an activatable element in the cell, and classifying the cell based on the presence or absence of the increase in the activation of the activatable element. In some embodiments, a cell is classified according to the activation level of a plurality of activatable elements after the cell have been subjected to an inhibitor. In some embodiments, the inhibitor is an inhibitor of a cellular factor or a plurality of factors that participates in a signaling cascade in the cell. In some embodiments, the inhibitor is added in addition to the biologic specimen modulator as a second modulator. In some cases this inhibitor is a phosphatase inhibitor. Examples of phosphatase inhibitors include, but are not limited to H_2O_2, members of the IMIDS family, such as Revlimid, siRNA, miRNA, Cantharidin, (−)-p-Bromotetramisole, Microcystin LR, Sodium Orthovanadate, Sodium Pervanadate, Vanadyl sulfate, Sodium oxodiperoxo(1,10-phenanthroline)vanadate, bis(maltolato)oxovanadium(IV), Sodium Molybdate, Sodium Perm olybdate, Sodium Tartrate, Imidazole, Sodium Fluoride, β-Glycerophosphate, Sodium Pyrophosphate Decahydrate, Calyculin A, Discodermia calyx, bpV(phen), mpV(pic), DMHV, Cypermethrin, Dephostatin, Okadaic Acid, NIPP-1, N-(9,10-Dioxo-9,10-dihydro-phenanthren-2-yl)-2,2-dimethyl-propionamide, α-Bromo-4-hydroxyacetophenone, 4-Hydroxyphenacyl Br, α-Bromo-4-methoxyacetophenone, 4-Methoxyphenacyl Br, α-Bromo-4-(carboxymethoxy)acetophenone, 4-(Carboxymethoxy)phenacyl Br, and bis(4-Trifluoromethylsulfonamidophenyl)-1,4-diisopropylbenzene, phenyarsine oxide, Pyrrolidine Dithiocarbarnate, and Aluminium fluoride.

[0035] In some embodiments, the invention provides methods for correlating and/or classifying an activation state of a reference population of cells with a clinical outcome in an individual, wherein the presence of the alteration is indicative of a clinical outcome. In some embodiments, the activation levels of a plurality of activatable elements are determined by contacting the cell with a plurality of binding elements, where each binding element is specific for an activation state of an activatable element. The clinical outcome can be any clinical outcome described herein.

[0036] In some embodiments, patterns and profiles of activatable elements that are cellular components of a cellular signaling pathway are detected using the methods described herein. For example, patterns and profiles of one or more phosphorylated polypeptide are detected using methods known in art including those described herein.

[0037] In some embodiments, a reference population of cells is used in assigning a risk group, predicting an increased risk of relapse, predicting an increased risk of developing secondary complications, choosing a therapy for an individual, predicting response to a therapy for an individual, determining the efficacy of a therapy in an individual, and/or determining the prognosis for an individual. The reference population of cells can be a homogeneous cell line, a defined mixture of homogeneous cell lines, a homogeneous cell population, a mixture of cells, or a library of cells. The reference population of cells can be obtained from the individual whose status is being determined or from a different individual. In some embodiments, the reference population of cells is obtained from a manual different that the individual whose status is being determined. In some embodiments the reference population of cells is a cell line.

Conditions

[0038] The methods of the invention are applicable to any condition in an individual involving, indicated by, and/or arising from, in whole or in part, altered physiological status in a cell. The term "physiological status" includes mechanical, physical, and biochemical functions in a cell. In some embodiments, the physiological status of a cell is determined by measuring characteristics of cellular components of a cellular pathway. Cellular pathways are well known in the art. In some embodiments the cellular pathway is a signaling pathway. Signaling pathways are also well known in the art (see, e.g., Hunter T., Cell 100(1): 113-27 (2000); Cell Signaling

Technology, Inc., 2002 Catalogue, Pathway Diagrams pgs. 232-253; Weinberg, Chapter 6, The biology of Cancer, 2007; and Blume-Jensen and Hunter, Nature, vol 411, 17 May 2001, p 355-365). A condition involving or characterized by altered physiological status may be readily identified, for example, by determining the state in a cell of one or more activatable elements, as taught herein.

[0039] In certain embodiments of the invention, the condition is a neoplastic, immunologic or hematopoietic condition. In some embodiments, the neoplastic, immunologic or hematopoietic condition is selected from the group consisting of solid tumors such as head and neck cancer including brain, thyroid cancer, breast cancer, lung cancer, mesothelioma, germ cell tumors, ovarian cancer, liver cancer, gastric carcinoma, colon cancer, prostate cancer, pancreatic cancer, melanoma, bladder cancer, renal cancer, prostate cancer, testicular cancer, cervical cancer, endometrial cancer, myosarcoma, leiomyosarcoma and other soft tissue sarcomas, osteosarcoma, Ewing's sarcoma, retinoblastoma, rhabdomyosarcoma, Wilm's tumor, and neuroblastoma, sepsis, allergic diseases and disorders that include but are not limited to allergic rhinitis, allergic conjunctivitis, allergic asthma, atopic eczema, atopic dermatitis, and food allergy, immunodeficiencies including but not limited to severe combined immunodeficiency (SCID), hypereosiniphic syndrome, chronic granulomatous disease, leukocyte adhesion deficiency I and II, hyper IgE syndrome, Chediak Higashi, neutrophilias, neutropenias, aplasias, agammaglobulinemia, hyper-IgM syndromes, DiGeorge/Velocardial-facial syndromes and Interferon gamma-TH1 pathway defects, autoimmune and immune dysregulation disorders that include but are not limited to rheumatoid arthritis, diabetes, systemic lupus erythematosus, Graves' disease, Graves ophthalmopathy, Crohn's disease, multiple sclerosis, psoriasis, systemic sclerosis, goiter and struma lymphomatosa (Hashimoto's thyroiditis, lymphadenoid goiter), alopecia aerata, autoimmune myocarditis, lichen sclerosis, autoimmune uveitis, Addison's disease, atrophic gastritis, myasthenia gravis, idiopathic thrombocytopenic purpura, hemolytic anemia, primary biliary cirrhosis, Wegener's granulomatosis, polyarteritis nodosa, and inflammatory bowel disease, allograft rejection and tissue destructive from allergic reactions to infectious microorganisms or to environmental antigens, and hematopoietic conditions that include but are not limited to Non-Hodgkin Lymphoma, Hodgkin or other lymphomas, acute or chronic leukemias, polycythemias, thrombocythemias, multiple myeloma or plasma cell disorders, e.g., amyloidosis and Waldenstrom's macroglobulinemia, myelodysplastic disorders, myeloproliferative disorders, myelofibroses, or atypical immune lymphoproliferations. In some embodiments, the neoplastic or hematopoietic condition is non-B lineage derived, such as Acute myeloid leukemia (AML), Chronic Myeloid Leukemia (CML), non-B cell Acute lymphocytic leukemia (ALL), non-B cell lymphomas, myelodysplastic disorders, myeloproliferative disorders, myelofibroses, polycythemias, thrombocythemias, or non-B atypical immune lymphoproliferations, Chronic Lymphocytic Leukemia (CLL), B lymphocyte lineage leukemia, B lymphocyte lineage lymphoma, Multiple Myeloma, or plasma cell disorders, e.g., amyloidosis or Waldenstrom's macroglobulinemia.

[0040] In some embodiments, the neoplastic or hematopoietic condition is non-B lineage derived. Examples of non- B lineage derived neoplastic or hematopoictic condition include, but are not limited to, Acute myeloid leukemia (AML), Chronic Myeloid Leukemia (CML), non-B cell Acute lymphocytic leukemia (ALL), non-B cell lymphomas, myclodysplastic disorders, myeloproliferative disorders, myelofibroses, polycythemias, thrombocythemias, and non-B atypical immune lymphoproliferations.

[0041] In some embodiments, the neoplastic or hematopoietic condition is a B-Cell or B cell lineage derived disorder. Examples of B-Cell or B cell lineage derived neoplastic or hematopoietic condition include but are not limited to Chronic Lymphocytic Leukemia (CLL), B lymphocyte lineage leukemia, B lymphocyte lineage lymphoma, Multiple Mycloma, and plasma cell disorders, including amyloidosis and Waldenstrom's macroglobulinemia.

[0042] Other conditions within the scope of the present invention include, but are not limited to, cancers such as gliomas, lung cancer, colon cancer and prostate cancer. Specific signaling pathway alterations have been described for many cancers, including loss of PTEN and resulting activation of Akt signaling in prostate cancer (Whang Y E. Proc Natl Acad Sci USA Apr. 28, 1998;95(9):5246-50), increased IGF-1 expression in prostate cancer (Schaefer et al., Science Oct. 9, 1998, 282: 199a), EGFR overexpression and resulting ERK activation in glioma cancer (Thomas C Y. Int J Cancer Mar. 10, 2003;104(1):19-27), expression of HER2 in breast cancers (Menard et al. Oncogene. Sep. 29 2003, 22(42):6570-8), and APC mutation and activated Wnt signaling in colon cancer (Bienz M. Curr Opin Genet Dev Oct. 9, 1999(5):595-603).

[0043] Diseases other than cancer involving altered physiological status are also encompassed by the present invention. For example, it has been shown that diabetes involves underlying signaling changes, namely resistance to insulin and failure to activate downstream signaling through IRS (Burks D J, White M F. Diabetes 2001 February;50 Suppl 1:S140-5). Similarly, cardiovascular disease has been shown to involve hypertrophy of the cardiac cells involving multiple pathways such as the PKC family (Malhotra A. Mol Cell Biochem 2001 September;225 (1-):97-107). Inflammatory diseases, such as rheumatoid arthritis, are known to involve the chemokine receptors and disrupted downstream signaling (D'Ambrosio D. J hmmunol Methods 2003 February;273 (1-2):3-13). The invention is not limited to diseases presently known to involve altered cellular function, but includes diseases subsequently shown to involve physiological alterations or anomalies.

Activatable elements

[0044] The methods and compositions of the invention may be employed to examine and profile the status of any activatable element in a cellular pathway, or collections of such activatable elements. Single or multiple distinct pathways may be profiled (sequentially or simultaneously), or subsets of activatable elements within a single pathway or across multiple pathways may be examined (again, sequentially or simultaneously).

[0045] The activation state of an individual activatable element is either in the on or off state. As an illustrative example, and without intending to be limited to any theory, an individual phosphorylatable site on a protein will either be phosphorylated and then be in the "on" state or it will not be phosphorylated and hence, it will be in the "off" state. See Blume-Jensen and Hunter, Nature, vol 411, 17 May 2001, p 355-365. The terms "on" and "off," when applied to an activatable element that is a part of a cellular constituent, are used here to describe the state of the activatable element (e.g., phosphorylated is "on" and non-phosphorylated is "off"), and

not the overall state of the cellular constituent of which it is a part. Typically, a cell possesses a plurality of a particular protein or other constituent with a particular activatable element and this plurality of proteins or constituents usually has some proteins or constituents whose individual activatable element is in the on state and other proteins or constituents whose individual activatable element is in the off state. Since the activation state of each activatable element is measured through the use of a binding element that recognizes a specific activation state, only those activatable elements in the specific activation state recognized by the binding element, representing some fraction of the total number of activatable elements, will be bound by the binding element to generate a measurable signal. The measurable signal corresponding to the summation of individual activatable elements of a particular type that are activated in a single cell is the "activation level" for that activatable element in that cell.

[0046] Activation levels for a particular activatable element may vary among individual cells so that when a plurality of cells is analyzed, the activation levels follow a distribution. The distribution may be a normal distribution, also known as a Gaussian distribution, or it may be of another type. Different populations of cells may have different distributions of activation levels that can then serve to distinguish between the populations.

[0047] In some embodiments, the basis for classifying cells may use the distribution of activation levels for one or more specific activatable elements which will differ among different phenotypes. A certain activation level, or more typically a range of activation levels for one or more activatable elements seen in a cell or a population of cells, is indicative that that cell or population of cells belongs to a distinctive phenotype. Other measurements, such as cellular levels (e.g., expression levels) of biomolecules that may not contain activatable elements, may also be used to classify cells in addition to activation levels of activatable elements; it will be appreciated that these levels also will follow a distribution, similar to activatable elements. Thus, the activation level or levels of one or more activatable elements, optionally in conjunction with levels of one or more levels of biomolecules that may not contain activatable elements, of cell or a population of cells may be used to classify a cell or a population of cells into a class. Once the activation level of intracellular activatable elements of individual single cells is known they can be placed into one or more classes, e.g., a class that corresponds to a phenotype. A class encompasses a class of cells wherein every cell has the same or substantially the same known activation level, or range of activation levels, of one or more intracellular activatable elements. For example, if the activation levels of five intracellular activatable elements are analyzed, predefined classes that encompass one or more of the intracellular activatable elements can be constructed based on the activation level, or ranges of the activation levels, of each of these five elements. It is understood that activation levels can exist as a distribution and that an activation level of a particular element used to classify a cell may be a particular point on the distribution but more typically may be a portion of the distribution.

[0048] In some embodiments, the basis for classifying cells may use the position of a cell in a contour or density plot. The contour or density plot represents the number of cells that share a characteristic such as the activation level of activatable proteins in response to a modulator. For example, when referring to activation levels of activatable elements in response to one or more modulators, normal individuals and patients with a condition might show populations with increased activation levels in response to the one or more modulators. However, the number of cells that have a specific activation level (e.g. specific amount of an activatable element) might be different between normal individuals and patients with a condition. Thus, a cell can be classified according to its location within a given region in the contour or density plot.

[0049] In addition to activation levels of intracellular activatable elements, expression levels of intracellular or extracellular biomolecules, e.g., proteins, may be used alone or in combination with activation states of activatable elements to classify cells. Further, additional cellular elements, e.g., biomolecules or molecular complexes such as RNA, DNA, carbohydrates, metabolites, and the like, may be used in conjunction with activatable states, expression levels or any combination of activatable states and expression levels in the classification of cells encompassed here.

[0050] In some embodiments, other characteristics that affect the status of a cellular constituent may also be used to classify a cell. Examples include the translocation of biomolecules or changes in their turnover rates and the formation and disassociation of complexes of biomolecule. Such complexes can include multi-protein complexes, multi-lipid complexes, homo- or hetero-dimers or oligomers, and combinations thereof. Other characteristics include proteolytic cleavage, e.g. from exposure of a cell to an extracellular protease or from the intracellular proteolytic cleavage of a biomolecule.

[0051] Additional elements may also be used to classify a cell, such as the expression level of extracellular or intracellular markers, nuclear antigens, enzymatic activity, protein expression and localization, cell cycle analysis, chromosomal analysis, cell volume, and morphological characteristics like granularity and size of nucleus or other distinguishing characteristics. For example, myeloid lineage cells can be further subdivided based on the expression of cell surface markers such as CD14, C15, or CD33, CD34 and CD45.

[0052] Alternatively, predefined classes of cells can be aggregated based upon shared characteristics that may include inclusion in one or more additional predefined class or the presence of extracellular or intracellular markers, similar gene expression profile, nuclear antigens, enzymatic activity, protein expression and localization, cell cycle analysis, chromosomal analysis, cell volume, and morphological characteristics like granularity and size of nucleus or other distinguishing characteristics.

[0053] In some embodiments, the physiological status of one or more cells is determined by examining and profiling the activation level of one or more activatable elements in a cellular pathway. In some embodiments, a cell is classified according to the activation level of a plurality of activatable elements. In some embodiments, a hematopoietic cell is classified according to the activation levels of a plurality of activatable elements. In some embodiments, the activation levels of one or more activatable elements of a hematopoietic cell are correlated with a condition. In some embodiments, the activation levels of one or more activatable elements of a hematopoietic cell are correlated with a neoplastic, autoimmune or hematopoietic condition as described herein. Examples of hematopoietic cells include but are not limited to pluripotent hematopoietic stem cells, B-lymphocyte lineage progenitor or derived cells, T- lymphocyte lineage progenitor or derived cells, NK cell lineage progenitor or derived cells,

myeloid lineage progenitor or derived cells, granulocyte lineage progenitor or derived cells, monocyte lineage progenitor or derived cells, megakaryocyte lineage progenitor or derived cells and erythroid lineage progenitor or derived cells. In some embodiments, the hematopoietic cell is a myeloid lineage progenitor or derived cell as described herein.

[0054] In some embodiments, the activation level of one or more activatable elements in single cells in the sample is determined. Cellular constituents that may include activatable elements include without limitation proteins, carbohydrates, lipids, nucleic acids and metabolites. The activatable element may be a portion of the cellular constituent, for example, an amino acid residue in a protein that may undergo phosphorylation, or it may be the cellular constituent itself, for example, a protein that is activated by translocation, change in conformation (due to, e.g., change in pH or ion concentration), by proteolytic cleavage, and the like. Upon activation, a change occurs to the activatable element, such as covalent modification of the activatable element (e.g., binding of a molecule or group to the activatable element, such as phosphorylation) or a conformational change. Such changes generally contribute to changes in particular biological, biochemical, or physical properties of the cellular constituent that contains the activatable element. The state of the cellular constituent that contains the activatable element is determined to some degree, though not necessarily completely, by the state of a particular activatable element of the cellular constituent. For example, a protein may have multiple activatable elements, and the particular activation states of these elements may overall determine the activation state of the protein; the state of a single activatable element is not necessarily determinative. Additional factors, such as the binding of other proteins, pH, ion concentration, interaction with other cellular constituents, and the like, can also affect the state of the cellular constituent.

[0055] In some embodiments, the activation levels of a plurality of intracellular activatable elements in single cells are determined. In some embodiments, at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 intracellular activatable elements are determined.

[0056] Activation states of activatable elements may result from chemical additions or modifications of biomolecules and include biochemical processes such as glycosylation, phosphorylation, acetylation, methylation, biotinylation, glutamylation, glycylation, hydroxylation, isomerization, prenylation, myristoylation, lipoylation, phosphopantetheinylation, sulfation, ISGylation, nitrosylation, palmitoylation, SUMOylation, ubiquitination, neddylation, citrullination, amidation, and disulfide bond formation, disulfide bond reduction. Other possible chemical additions or modifications of biomolecules include the formation of protein carbonyls, direct modifications of protein side chains, such as o-tyrosine, chloro-, nitrotyrosine, and dityrosine, and protein adducts derived from reactions with carbohydrate and lipid derivatives. Other modifications may be non-covalent, such as binding of a ligand or binding of an allosteric modulator.

[0057] In some embodiments, the activatable element is a protein. Examples of proteins that may include activatable elements include, but are not limited to kinases, phosphatases, lipid signaling molecules, adaptor/scaffold proteins, cytokines, cytokine regulators, ubiquitination enzymes, adhesion molecules, cytoskeletal/contractile proteins, heterotrimeric G proteins, small molecular weight GTPases, guanine nucleotide exchange factors, GTPase activating proteins, caspases, proteins involved in apoptosis, cell cycle regulators, molecular chaperones, metabolic enzymes, vesicular transport proteins, hydroxylases, isomerases, deacetylases, methylases, demethylases, tumor suppressor genes, proteases, ion channels, molecular transporters, transcription factors/DNA binding factors, regulators of transcription, and regulators of translation. Examples of activatable elements, activation states and methods of determining the activation level of activatable elements are described in US Publication Number 20060073474 entitled "Methods and compositions for detecting the activation state of multiple proteins in single cells" and US Publication Number 20050112700 entitled "Methods and compositions for risk stratification" the content of which are incorporate here by reference. See also U.S. Ser. Nos. 61/048,886, 61/048,920 and Shulz et al, Current Protocols in Immunology 2007, 7:8.17.1-20.

[0058] In some embodiments, the protein that may be activated is selected from the group consisting of HER receptors, PDGF receptors, FLT3 receptor, Kit receptor, FGF receptors, Eph receptors, Trk receptors, IGF receptors, Insulin receptor, Met receptor, Ret, VEGF receptors, erythropoetin receptor, thromobopoetin receptor, CD114, CD116, TIE1, TIE2, FAK, Jak1, Jak2, Jak3, Tyk2, Src, Lyn, Fyn, Lck, Fgr, Yes, Csk, Abl, Btk, ZAP70, Syk, IRAKs, cRaf, ARaf, BRAF, Mos, Lim kinase, ILK, Tp1, ALK, TGFβ, receptors, BMP receptors, MEKKs, ASK, MLKs, DLK, PAKs, Mek 1, Mek 2, MKK3/6, MKK4/7, ASK1, Cot, NIK, Bub, Myt 1, Weel, Casein kinases, PDK1, SGK1, SGK2, SGK3, Akt1, Akt2, Akt3, p90Rsks, p70S6Kinase,Prks, PKCs, PKAs, ROCK 1, ROCK 2, Auroras, CaMKs, MNKs, AMPKs, MELK, MARKs, Chk1, Chk2, LKB-1, MAPKAPKs, Pim1, Pim2, Pim3, IKKs, Cdks, Jnks, Erks, IKKs, GSK3α, GSK3β, Cdks, CLKs, PKR, PI3-Kinase class 1, class 2, class 3, mTor, SAPK/JNK1,2,3, p38s, PKR, DNA-PK, ATM, ATR, Receptor protein tyrosine phosphatases (RPTPs), LAR phosphatase, CD45, Non receptor tyrosine phosphatases (NPRTPs), SHPs, MAP kinase phosphatases (MKPs), Dual Specificity phosphatases (DUSPs), CDC25 phosphatases, Low molecular weight tyrosine phosphatase, Eyes absent (EYA) tyrosine phosphatases, Slingshot phosphatases (SSH), serine phosphatases, PP2A, PP2B, PP2C, PP1, PP5, inositol phosphatases, PTEN, SHIPs, myotubularins, phosphoinositide kinases, phopsholipases, prostaglandin synthases, 5-lipoxygenase, sphingosine kinases, sphingomyelinases, adaptor/scaffold proteins, She, Grb2, BLNK, LAT, B cell adaptor for P13-kinase (BCAP), SLAP, Dok, KSR, MyD88, Crk, CrkL, GAD, Nck, Grb2 associated binder (GAB), Fas associated death domain (FADD), TRADD, TRAF2, RIP, T-Cell leukemia family, IL-2, IL-4, IL-8, IL-6, interferon γ, interferon α, suppressors of cytokine signaling (SOCs), Cbl, SCF ubiquitination ligase complex, APC/C, adhesion molecules, integrins, Immunoglobulin-like adhesion molecules, selectins, cadherins, catenins, focal adhesion kinase, p130CAS, fodrin, actin, paxillin, myosin, myosin binding proteins, tubulin, eg5/KSP, CENPs, β-adrenergic receptors, muscarinic receptors, adenylyl cyclase receptors, small molecular weight GTPases, H-Ras, K-Ras, N-Ras, Ran, Rac, Rho, Cdc42, Arfs, RABs, RHEB, Vav, Tiam, Sos, Dbl, PRK, TSC1,2, Ras-GAP, Arf-GAPs, Rho-GAPs, caspases, Caspase 2, Caspase 3, Caspase 6, Caspase 7, Caspase 8, Caspase 9, Bc1-2, Mc1-1, Bc1-XL, Bc1-w, Bc1-B, Al, Bax, Bak, Bok, Bik, Bad, Bid, Bim, Bmf, Hrk, Noxa, Puma, IAPs, XIAP, Smac, Cdk4, Cdk 6, Cdk 2, Cdkl, Cdk 7, Cyclin D, Cyclin E, Cyclin A, Cyclin B, Rb, p16,

p14Arf, p27KIP, p21CIP, molecular chaperones, Hsp90s, Hsp70, Hsp27, metabolic enzymes, Acetyl-CoAa Carboxylase, ATP citrate lyase, nitric oxide synthase, caveolins, endosomal sorting complex required for transport (ESCRT) proteins, vesicular protein sorting (Vsps), hydroxylases, prolyl-hydroxylases PHD-1, 2 and 3, asparagine hydroxylase FLH transferases, Pinl prolyl isomerase, topoisomerases, deacetylases, Histone deacetylases, sirtuins, histone acetylases, CBP/P300 family, MYST family, ATF2, DNA methyl transferases, Histone H3K4 demethylases, H3K27, JHDM2A, UTX, VHL, WT-1, p53, Hdm, PTEN, ubiquitin proteases, urokinase-type plasminogen activator (uPA) and uPA receptor (UPAR) system, cathepsins, metalloproteinases, esterases, hydrolases, separase, potassium channels, sodium channels, multi-drug resistance proteins, P-Gycoprotein, nucleoside transporters, Ets, Elk, SMADs, Rel-A (p65-NFKB), CREB, NFAT, ATF-2, AFT, Myc, Fos, Spl, Egr-1, T-bet, β-catenin, HIFs, FOXOs, E2Fs, SRFs, TCFs, Egr-1, -catenin, FOXO STAT1, STAT 3, STAT 4, STAT 5, STAT 6, p53, WT-1, HMGA, pS6, 4EPB-1, eIF4E-binding protein, RNA polymerase, initiation factors, elongation factors.

[0059] In some embodiments of the invention, the methods described herein are employed to determine the activation level of an activatable element, e.g., in a cellular pathway. Methods and compositions are provided for the classification of a cell according to the activation level of an activatable element in a cellular pathway. The cell can be a hematopoietic cell and examples are shown above.

[0060] In some embodiments, the classification of a cell according to activation level of an activatable element, e.g., in a cellular pathway comprises classifying the cell as a cell that is correlated with a clinical outcome. Examples of clinical outcomes, staging, as well as patient responses are also shown above.

[0061] In some embodiments, methods and compositions are provided for the classification of a cell according to the activation level of an activatable element, e.g., in a cellular pathway wherein the classification comprises classifying the cell as a cell that is correlated with minimal residual disease or emerging resistance.

[0062] A. Signaling Pathways

[0063] In some embodiments, the methods of the invention are employed to determine the status of an activatable element in a signaling pathway. In some embodiments, a reference cell is classified, as described herein, according to the activation level of one or more activatable elements in one or more signaling pathways. Signaling pathways and their members have been extensively described. See (Hunter T. Cell Jan. 7, 2000;100(1): 13-27; Weinberg, 2007; and Blume-Jensen and Hunter, Nature, vol 411, 17 May 2001, p 355-365 cited above). Exemplary signaling pathways include the following pathways and their members: the JAK-STAT pathway including JAKs, STATs 2,3 4 and 5, the FLT3L signaling pathway, the MAP kinase pathway including Ras, Raf, MEK, ERK and elk; the P13K/Akt pathway including PI-3-kinase, PDK1, Akt and Bad; the NF-KB pathway including IKKs, IkB and NF-κB and the Wnt pathway including frizzled receptors, beta-catenin, APC and other co-factors and TCF (see Cell Signaling Technology, Inc. 2002 Catalog pages 231-279 and Hunter T., supra.). In some embodiments of the invention, the correlated activatable elements being assayed (or the signaling proteins being examined) are members of the MAP kinase, Akt, NFκB, WNT, STAT and/or PKC signaling pathways.

[0064] In some embodiments, the methods of the invention are employed to determine the status of a signaling protein in a signaling pathway known in the art including those described herein. Exemplary types of signaling proteins within the scope of the present invention include, but are not limited to, kinases, kinase substrates (i.e. phosphorylated substrates), phosphatases, phosphatase substrates, binding proteins (such as 14-3-3), receptor ligands and receptors (cell surface receptor tyrosine kinases and nuclear receptors)). Kinases and protein binding domains, for example, have been well described (see, e.g., Cell Signaling Technology, Inc., 2002 Catalogue "The Human Protein Kinases" and "Protein Interaction Domains" pgs. 254-279).

[0065] Exemplary signaling proteins include, but are not limited to, kinases, HER receptors, PDGF receptors, Kit receptor, FGF receptors, Eph receptors, Trk receptors, IGF receptors, Insulin receptor, Met receptor, Ret, VEGF receptors, TIE1, TIE2, FAK, Jak1, Jak2, Jak3, Tyk2, Src, Lyn, Fyn, Lck, Fgr, Yes, Csk, Abl, Btk, ZAP70, Syk, IRAKs, cRaf, ARaf, BRAF, Mos, Lim kinase, ILK, Tpl, ALK, TGFβ receptors, BMP receptors, MEKKs, ASK, MLKs, DLK, PAKs, Mek 1, Mek 2, MKK3/6, MKK4/7, ASK1,Cot, NIK, Bub, Myt 1, Weel, Casein kinases, PDK1, SGK1, SGK2, SGK3, Akt1, Akt2, Akt3, p90Rsks, p70S6Kinase,Prks, PKCs, PKAs, ROCK 1, ROCK 2, Auroras, CaMKs, MNKs, AMPKs, MELK, MARKs, Chk1, Chk2, LKB-1, MAPKAPKs, Pim1, Pim2, Pim3, IKKs, Cdks, Jnks, Erks, IKKs, GSK3α, GSK3β, Cdks, CLKs, PKR, PI3-Kinase class 1, class 2, class 3, mTor, SAPK/JNK1,2,3, p38s, PKR, DNA-PK, ATM, ATR, phosphatases, Receptor protein tyrosine phosphatases (RPTPs), LAR phosphatase, CD45, Non receptor tyrosine phosphatases (NPRTPs), SHPs, MAP kinase phosphatases MKPs), Dual Specificity phosphatases (DUSPs), CDC25 phosphatases, low molecular weight tyrosine phosphatase, Eyes absent (EYA) tyrosine phosphatases, Slingshot phosphatases (SSH), serine phosphatases, PP2A, PP2B, PP2C, PP1, PP5, inositol phosphatases, PTEN, SHIPs, myotubularins, lipid signaling, phosphoinositide kinases, phopsholipases, prostaglandin synthases, 5-lipoxygenase, sphingosine kinases, sphingomyelinases, adaptor/scaffold proteins, Shc, Grb2, BLNK, LAT, B cell adaptor for PI3-kinase (BCAP), SLAP, Dok, KSR, MyD88, Crk, CrkL, GAD, Nek, Grb2 associated binder (GAB), Fas associated death domain (FADD), TRADD, TRAF2, RIP, T-Cell leukemia family, cytokines, IL-2, IL-4, IL-8, IL-6, interferon γ, interferon α, cytokine regulators, suppressors of cytolcine signaling (SOCs), ubiquitination enzymes, Cbl, SCF ubiquitination ligase complex, APC/C, adhesion molecules, integrins, Immunoglobulin-like adhesion molecules, selectins, cadherins, catenins, focal adhesion kinase, p130CAS, cytoskeletal/contractile proteins, fodrin, actin, paxillin, myosin, myosin binding proteins, tubulin, eg5/KSP, CENPs, heterotrimeric G proteins, β-adrenergic receptors, muscarinic receptors, adenylyl cyclase receptors, small molecular weight GTPases, H-Ras, K-Ras, N-Ras, Ran, Rac, Rho, Cdc42, Arfs, RABs, RHEB, guanine nucleotide exchange factors, Vav, Tiam, Sos, Dbl, PRK, TSC1,2, GTPase activating proteins, Ras-GAP, Arf-GAPs, Rho-GAPs, caspases, Caspase 2, Caspase 3, Caspase 6, Caspase 7, Caspase 8, Caspase 9, proteins involved in apoptosis, Bc1-2, Mc1-1, Bc1-XL, Bc1-w, Bc1*x*-B, Al, Bax, Bak, Bok, Bik, Bad, Bid, Bim, Bmf, Hrk, Noxa, Puma, IAPs, XIAP, Smac, cell cycle regulators, Cdk4, Cdk 6, Cdk 2, Cdk1, Cdk 7, Cyclin D, Cyclin E, Cyclin A, Cyclin B, Rb, p16, p14Arf,

p27KIP, p21CIP, molecular chaperones, Hsp90s, Hsp70, Hsp27, metabolic enzymes, Acetyl-CoAa Carboxylase, ATP citrate lyase, nitric oxide synthase, vesicular transport proteins, caveolins, endosomal sorting complex required for transport (ESCRT) proteins, vesicular protein sorting (Vsps), hydroxylases, prolyl-hydroxylases PHD-1, 2 and 3, asparagine hydroxylase FIH transferases, isomerases, Pin1 prolyl isomerase, topoisomerases, deacetylases, Histone deacetylases, sirtuins, acetylases, histone acetylases, CBP/P300 family, MYST family, ATF2, methylases, DNA methyl transferases, demethylases, Histone H3K4 demethylases, H3K27, JHDM2A, UTX, tumor suppressor genes, VHL, WT-1, p53, Hdm, PTEN, proteases, ubiquitin proteases, urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR) system, cathepsins, metalloproteinases, esterases, hydrolases, separase, ion channels, potassium channels, sodium channels, molecular transporters, multi-drug resistance proteins, P-Gycoprotein, nucleoside transporters, transcription factors/ DNA binding proteins, Ets, Elk, SMADs, Rel-A (p65-NFKB), CREB, NFAT, ATF-2, AFT, Myc, Fos, Spl, Egr-1, T-bet,β-catenin, HIFs, FOXOs, E2Fs, SRFs, TCFs, Egr-1, β-catenin, FOXO STAT1, STAT 3, STAT 4, STAT 5, STAT 6, p53, WT-1, HMGA, regulators of translation, pS6, 4EPB-1, eIF4E-binding protein, regulators of transcription, RNA polymerase, initiation factors, and elongation factors.

[0066] In some embodiments the protein is selected from the group consisting of P13-Kinase (p85, p110a, p110b, p110d), Jak1, Jak2, SOCs, Rac, Rho, Cdc42, Ras-GAP, Vav, Tiam, Sos, Dbl, Nck, Gab, PRK, SHP1, and SHP2, SHIP1, SHIP2, sSHIP, PTEN, Shc, Grb2, PDK1, SGK, Akt1, Akt2, Akt3, TSC1,2, Rheb, mTor, 4EBP-1, p70S6Kinase, S6, LKB-1, AMPK, PFK, Acetyl-CoAa Carboxylase, DokS, Rafs, Mos, Tp12, MEK1/2, MLK3, TAK, DLK, MKK3/6, MEKK1,4, MLK3, ASK1, MKK4/7, SAPK/JNK1,2,3, p38s, Erk1/2, Syk, Btk, BLNK, LAT, ZAP70, Lck, Cbl, SLP-76, PLCyi, PLCy2, STAT1, STAT 3, STAT 4, STAT 5, STAT 6, FAK, p130CAS, PAKs, LIMK1/2, Hsp90, Hsp70, Hsp27, SMADs, Rel-A (p65-NFKB), CREB, Histone H2B, HATs, HDACs, PKR, Rb, Cyclin D, Cyclin E, Cyclin A, Cyclin B, P16, p14Arf, p27KIP, p21CIP, Cdk4, Cdk6, Cdk7, Cdk1, Cdk2, Cdk9, Cdc25,A/B/C, Abl, E2F, FADD, TRADD, TRAF2, RIP, Myd88, BAD, Bc1-2, Mc1-1, Bc1-XL, Caspase 2, Caspase 3, Caspase 6, Caspase 7, Caspase 8, Caspase 9, IAPs, Smack Fodrin, Actin, Src, Lyn, Fyn, Lck, NIK, IκB, p65(Re1A), IKKα, PKA, PKCα, PKCβ, PKCθ, PKCδ, CAMK, Elk, AFT, Myc, Egr-1, NFAT, ATF-2, Mdm2, p53, DNA-PK, Chk1, Chk2, ATM, ATR, βcatenin, CrkL, GSK3α, GSK3β, and FOXO.

[0067] In some embodiments of the invention, the methods described herein are employed to determine the status of an activatable element in a signaling pathway. See U.S. Ser. Nos. 61/048,886 and 61/048,920 which are incorporated. Methods and compositions are provided for the classification of a cell according to the status of an activatable element in a signaling pathway. The cell can be a hematopoietic cell. Examples of hematopoietic cells are shown above.

[0068] In some embodiments, the classification of a cell according to the status of an activatable element in a signaling pathway comprises classifying the cell as a cell that is correlated with a clinical outcome. Examples of clinical outcome, staging, patient responses and classifications are shown above.

Binding Element

[0069] In some embodiments of the invention, the activation level of an activatable element is determined. One embodiment makes this determination by contacting a reference cell with a binding element that is specific for an activation state of the activatable element. The term "Binding element" includes any molecule, e.g., peptide, nucleic acid, small organic molecule which is capable of detecting an activation state of an activatable element over another activation state of the activatable element. Binding elements and labels for binding elements are shown in U.S. Ser. Nos. /048, 886; 61/048,920 and 61/048,657.

[0070] In some embodiments, the binding element is a peptide, polypeptide, oligopeptide or a protein. The peptide, polypeptide, oligopeptide or protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures. Thus "amino acid", or "peptide residue", as used herein include both naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline and noreleucine are considered amino acids for the purposes of the invention. The side chains may be in either the (R) or the (S) configuration. In some embodiments, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard in vivo degradation. Proteins including non-naturally occurring amino acids may be synthesized or in some cases, made recombinantly; see van Hest et al., FEBS Lett 428:(1-2) 68-70 May 22, 1998 and Tang et al., Abstr. Pap Am. Chem. S218: U138 Part 2 Aug. 22, 1999, both of which are expressly incorporated by reference herein.

[0071] Methods of the present invention may be used to detect any particular activatable element in a sample that is antigenically detectable and antigenically distinguishable from other activatable element which is present in the sample. For example, the activation state-specific antibodies of the present invention can be used in the present methods to identify distinct signaling cascades of a subset or subpopulation of complex cell populations; and the ordering of protein activation (e.g., kIinase activation) in potential signaling hierarchies. Hence, in some embodiments the expression and phosphorylation of one or more polypeptides are detected and quantified using methods of the present invention. In some embodiments, the expression and phosphorylation of one or more polypeptides that are cellular components of a cellular pathway are detected and quantified using methods of the present invention. As used herein, the term "activation state-specific antibody" or "activation state antibody" or grammatical equivalents thereof, refer to an antibody that specifically binds to a corresponding and specific antigen. Preferably, the corresponding and specific antigen is a specific form of an activatable element. Also preferably, the binding of the activation state-specific antibody is indicative of a specific activation state of a specific activatable element.

[0072] In some embodiments, the binding element is an antibody. In some embodiment, the binding element is an activation state-specific antibody.

[0073] The term "antibody" includes full length antibodies and antibody fragments, and may refer to a natural antibody from any organism, an engineered antibody, or an antibody generated recombinantly for experimental, therapeutic, or other purposes as further defined below. Examples of antibody fragments, as are known in the art, such as Fab, Fab', F(ab')2, Fv, scFv, or other antigen-binding subsequences of antibodies, either produced by the modification of whole antibodies or those synthesized de novo using recombinant DNA technologies. The term "antibody" comprises mono-

clonal and polyclonal antibodies. Antibodies can be antagonists, agonists, neutralizing, inhibitory, or stimulatory. They can be humanized, aglycosylated, bound to solid supports, and posses other variations. See U.S. Ser. Nos. 61/048,886; 61/048,920 and 61/048,657 for more information about antibodies as binding elements.

[0074] As pointed out above, activation state specific antibodies can be used to detect kinase activity, however additional means for determining kinase activation are provided by the present invention. For example, substrates that are specifically recognized by protein kinases and phosphorylated thereby are known. Antibodies that specifically bind to such phosphorylated substrates but do not bind to such non-phosphorylated substrates (phospho-substrate antibodies) may be used to determine the presence of activated kinase in a sample.

[0075] The antigenicity of an activated isoform of an activatable element is distinguishable from the antigenicity of non-activated isoform of an activatable element or from the antigenicity of an isoform of a different activation state. In some embodiments, an activated isoform of an element possesses an epitope that is absent in a non-activated isoform of an element, or vice versa. In some embodiments, this difference is due to covalent addition of moieties to an element, such as phosphate moieties, or due to a structural change in an element, as through protein cleavage, or due to an otherwise induced conformational change in an element which causes the element to present the same sequence in an antigenically distinguishable way. In some embodiments, such a conformational change causes an activated isoform of an element to present at least one epitope that is not present in a non-activated isoform, or to not present at least one epitope that is presented by a non-activated isoform of the element. In some embodiments, the epitopes for the distinguishing antibodies are centered around the active site of the element, although as is known in the art, conformational changes in one area of an element may cause alterations in different areas of the element as well.

[0076] Many antibodies, many of which are commercially available (for example, see Cell Signaling Technology, www.cellsignal.com or Becton Dickinson, www.bd.com) have been produced which specifically bind to the phosphorylated isoform of a protein but do not specifically bind to a non-phosphorylated isoform of a protein. Many such antibodies have been produced for the study of signal transducing proteins which are reversibly phosphorylated. Particularly, many such antibodies have been produced which specifically bind to phosphorylated, activated isoforms of protein. Examples of proteins that can be analyzed with the methods described herein include, but are not limited to, kinases, HER receptors, PDGF receptors, FLT3 receptor, Kit receptor, FGF receptors, Eph receptors, Trk receptors, IGF receptors, Insulin receptor, Met receptor, Ret, VEGF receptors, TIE1, TIE2, erythropoetin receptor, thromobopoetin receptor, CD114, CD116, FAK, Jak1, Jak2, Jak3, Tyk2, Src, Lyn, Fyn, Lck, Fgr, Yes, Csk, Abl, Btk, ZAP70, Syk IRAKs, cRaf, ARaf, BRAF, Mos, Lim kinase, ILK, Tp1, ALK, TGFβ receptors, BMP receptors, MEKKs, ASK, MLKs, DLK, PAKs, Mek 1, Mek 2, MKK3/6, MKK4/7, ASK1,Cot, NIK, Bub, Myt 1, Weel, Casein kinases, PDK1, SCK1, SGK2, SGK3, Akt1, Akt2, Akt3, p90Rsks, p70S6Kinase,Prks, PKCs, PKAs, ROCK 1, ROCK 2, Auroras, CaMKs, MNKs, AMPKs, MELK, MARKs, Chk1, Chk2, LKB-1, MAPKAPKs, Pim1, Pim2, Pim3, IKKs, Cdks, Jnks, Erks, IKKs, USK3α; GSK3β, Cdks, CLKs, PKR, PI3-Kinase class 1, class 2, class 3, mTor, SAPK/JNK1,2,3, p38s, PKR, DNA-PK, ATM, ATR, phosphatases, Receptor protein tyrosine phosphatases (RPTPs), LAR phospbatase, CD45, Non receptor tyrosine phosphatases (NPRTPs), SHPs, MAP kinase phosphatases (MKPs), Dual Specificity phosphatases (DUSPs), CDC25 phosphatases, Low molecular weight tyrosine phosphatase, Eyes absent (EYA) tyrosine phosphatases, Slingshot phosphatases (SSH), serine phosphatases, PP2A, PP2B, PP2C, PP1, PPS, inositol phosphatases, PTEN, SHIPs, myotubularins, lipid signaling, phosphoinositide kinases, phopsholipases, prostaglandin synthases, 5-lipoxygenase, sphingosine kinases, sphingomyelinases, adaptor/scaffold proteins, Shc, Grb2, BLNK, LAT, B cell adaptor for PI3-kinase (BCAP), SLAP, Dok, KSR, MyD88, Crk, CrkL, GAD, Nck, Grb2 associated binder (GAB), Fas associated death domain (FADD), TRADD, TRAF2, RIP, T-Cell leukemia family, cytokines, IL-2, IL-4, IL-8, IL-6, interferon γ, interferon α, cytokine regulators, suppressors of cytokine signaling (SOCs), ubiquitination enzymes, Cbl, SCF ubiquitination ligase complex, APC/C, adhesion molecules, integrins, Immunoglobulin-like adhesion molecules, selectins, cadherins, catenins, focal adhesion kinase, p130CAS, cytoskeletallcontractile proteins, fodrin, actin, paxillin, myosin, myosin binding proteins, tubulin, eg5/KSP, CENPs, heterotrimeric G proteins, β-adrenergic receptors, muscarinic receptors, adenylyl cyclase receptors, small molecular weight GTPases, H-Ras, K-Ras, N-Ras, Ran, Rac, Rho, Cdc42, Arfs, RABs, RHEB, guanine nucleotide exchange factors, Vav, Tiam, Sos, Dbl, PRK, TSC1,2, GTPase activating proteins, Ras-GAP, Arf-GAPs, Rho-GAPs, caspases, Caspase 2, Caspase 3, Caspase 6, Caspase 7, Caspase 8, Caspase 9, proteins involved in apoptosis, Bc1-2, Mc1-1, Bc1-XL, Bc1-w, Bc1-B, Al, Bax, Bak, Bok, Bik, Bad, Bid, Bim, Bmf, Hrk, Noxa, Puma, IAPs, XIAP, Smac, cell cycle regulators, Cdk4, Cdk 6, Cdk 2, Cdk1, Cdk 7, Cyclin D, Cyclin E, Cyclin A, Cyclin B, Rb, p16, p14Arf, p27KIP, p21CIP, molecular chaperones, Hsp90s, Hsp70, Hsp27, metabolic enzymes, Acetyl-CoAa Carboxylase, ATP citrate lyase, nitric oxide synthase, vesicular transport proteins, caveolins, endosomal sorting complex required for transport (ESCRT) proteins, vesicular protein sorting (Vsps), hydroxylases, prolyl-hydroxylases PHD-1, 2 and 3, asparagine hydroxylase FIH transferases, isomerases, Pin1 prolyl isomerase, topoisomerases, deacetylases, Histone deacetylases, sirtuins, acetylases, histone acetylases, CBP/P300 family, MYST family, ATF2, methylases, DNA methyl transferases, demethylases, Histone H3K4 demethylases, H3K27, JHDM2A, UTX, tumor suppressor genes, VHL, WT-1, p53, Hdm, PTEN, proteases, ubiquitin proteases, urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR) system, cathepsins, metalloproteinases, esterases, hydrolases, separase, ion channels, potassium channels, sodium channels, molecular transporters, multiAug resistance proteins, P-Gycoprotein, nucleoside transporters, transcription factors/DNA binding proteins, Ets, Elk, SMADs, Rel-A (p65-NFKB), CREB, NFAT, ATF-2, AFT, Myc, Fos, Spl, Egr-1, T-bet, β-catenin, HIFs, FOXOs, E2Fs, SRFs, TCFs, Egr-1, β-FOXO STAT1, STAT 3, STAT 4, STAT 5, STAT 6, p53, WT-1, HMGA, regulators of translation, pS6, 4EPB-1, eIF4E-binding protein, regulators of transcription, RNA polymerase, initiation factors, elongation factors. In some embodiments, the protein is S6.

[0077] In some embodiments, an epitope-recognizing fragment of an activation state antibody rather than the whole

antibody is used. In some embodiments, the epitope-recognizing fragment is immobilized. In some embodiments, the antibody light chain that recognizes an epitope is used. A recombinant nucleic acid encoding a light chain gene product that recognizes an epitope may be used to produce such an antibody fragment by recombinant means well known in the art.

[0078] In alternative embodiments of the instant invention, aromatic amino acids of protein binding elements may be replaced with other molecules. See U.S. Ser. Nos. 61/048,886; 61/048,920 and 61/048,657.

[0079] In some embodiments, the activation state-specific binding element is a peptide comprising a recognition structure that binds to a target structure on an activatable protein. A variety of recognition structures are well known in the art and can be made using methods known in the art, including by phage display libraries (see e.g., Gururaja et al. Chem. Biol. (2000) 7:515-27; Houimel et al., Eur. J. Immunol. (2001) 31:353545; Cochran et al. J. Am. Chem. Soc. (2001) 123:625-32; Houimel et al. Int. J. Cancer (2001) 92:748-55, each incorporated herein by reference). Further, fluorophores can be attached to such antibodies for use in the methods of the present invention.

[0080] A variety of recognitions structures are known in the art (e.g., Cochran et al., J. Am. Chem. Soc. (2001) 123:625-32; Boer et al., Blood (2002) 100:467-73, each expressly incorporated herein by reference)) and can be produced using methods known in the art (see e.g., Boer et al., Blood (2002) 100:467-73; Gualillo et al., Mol. Cell Endocrinol. (2002) 190:83-9, each expressly incorporated herein by reference)), including for example combinatorial chemistry methods for producing recognition structures such as polymers with affinity for a target structure on an activatable protein (see e.g., Barn et al., J. Comb. Chem. (2001) 3:534-41; Ju et al., Biotechnol. (1999) 64:232-9, each expressly incorporated herein by reference). In another embodiment, the activation state-specific antibody is a protein that only binds to an isoform of a specific activatable protein that is phosphorylated and does not bind to the isoform of this activatable protein when it is not phosphorylated or nonphosphorylated. In another embodiment the activation state-specific antibody is a protein that only binds to an isoform of an activatable protein that is intracellular and not extracellular, or vice versa. In a some embodiment, the recognition structure is an anti-laminin single-chain antibody fragment (scFv) (see e.g., Sanz et al., Gene Therapy (2002) 9:1049-53; Tse et al., J. Mol. Biol. (2002) 317:85-94, each expressly incorporated herein by reference).

[0081] In some embodiments the binding element is a nucleic acid. The term "nucleic acid" include nucleic acid analogs, for example, phosphoramide (Beaucage et al., Tetrahedron 49(10):1925 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al., Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem. Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica Scripta 26:141 91986)), phosphorothioate (Mag et al., Nucleic Acids Res. 19:1437 (1991); and U.S. Pat. No. 5,644,048), phosphorodithioate (Briu et al., J. Am. Chem. Soc. 111:2321 (1989), O-methylphopboroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc. 114:1895 (1992); Meier et al., Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson et al., Nature 380:207 (1996), all of which are incorporated by reference). Other analog nucleic acids include those with positive backbones (Denpcy et al., Proc. Natl. Acad. Sci. USA 92:6097 (1995); non-ionic backbones (U.S. Pat. Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi et al., Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); Letsinger et al., Nucleoside & Nucleotide 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y. S. Sanghui and P. Dan Cook, Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Pat. Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series **580**, "Carbohydrate Modifications in Antisense Research", Ed. Y. S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp 169-176). Several nucleic acid analogs are described in Rawls, C & E News Jun. 2, 1997 page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments.

[0082] In some embodiment the binding element is a small organic compound. Binding elements can be synthesized from a series of substrates that can be chemically modified. "Chemically modified" herein includes traditional chemical reactions as well as enzymatic reactions. These substrates generally include, but are not limited to, alkyl groups (including alkanes, alkenes, alkynes and heteroalkyl), aryl groups (including arenes and heteroaryl), alcohols, ethers, amines, aldehydes, ketones, acids, esters, amides, cyclic compounds, heterocyclic compounds (including purines, pyrimidines, benzodiazepins, beta-lactams, tetracylines, cephalosporins, and carbohydrates), steroids (including estrogens, androgens, cortisone, ecodysone, etc.), alkaloids (including ergots, vinca, curare, pyrollizdine, and mitomycines), organometallic compounds, hetero-atom bearing compounds, amino acids, and nucleosides. Chemical (including enzymatic) reactions may be done on the moieties to form new substrates or binding elements that can then be used in the present invention.

[0083] In some embodiments the binding element is a carbohydrate. As used herein the term carbohydrate is meant to include any compound with the general formula $(CH_2O)_n$. Examples of carbohydrates are di-, tri- and oligosaccharides, as well polysaccharides such as glycogen, cellulose, and starches.

[0084] In some embodiments the binding element is a lipid. As used herein the term lipid herein is meant to include any water insoluble organic molecule that is soluble in nonpolar organic solvents. Examples of lipids are steroids, such as cholesterol, and phospholipids such as sphingomeylin.

[0085] Examples of activatable elements, activation states and methods of determining the activation level of activatable elements are described in US publication number 20060073474 entitled "Methods and compositions for detecting the activation state of multiple proteins in single cells" and US publication number 20050112700 entitled

"Methods and compositions for risk stratification" the content of which are incorporate here by reference.

[0086] A. Labels

[0087] The methods and compositions of the instant invention provide binding elements comprising a label or tag. By label is meant a molecule that can be directly (i.e., a primary label) or indirectly (i.e., a secondary label) detected; for example a label can be visualized and/or measured or otherwise identified so that its presence or absence can be known. Binding elements and labels for binding elements are shown in U.S. Ser. Nos. /048,886; 61/048,920 and 61/048,657.

[0088] A compound can be directly or indirectly conjugated to a label which provides a detectable signal, e.g. radioisotopes, fluorescers, enzymes, antibodies, particles such as magnetic particles, chemiluminescers, molecules that can be detected by mass spec, or specific binding molecules, etc. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. Examples of labels include, but are not limited to, optical fluorescent and chromogenic dyes including labels, label enzymes and radioisotopes. In some embodiments of the invention, these labels may be conjugated to the binding elements.

[0089] In some embodiments, one or more binding elements are uniquely labeled. Using the example of two activation state specific antibodies, by "uniquely labeled" is meant that a first activation state antibody recognizing a first activated element comprises a first label, and second activation state antibody recognizing a second activated element comprises a second label, wherein the first and second labels are detectable and distinguishable, making the first antibody and the second antibody uniquely labeled.

[0090] In general, labels fall into four classes: a) isotopic labels, which may be radioactive or heavy isotopes; b) magnetic, electrical, thermal labels; c) colored, optical labels including luminescent, phosphorous and fluorescent dyes or moieties; and d) binding partners. Labels can also include enzymes (horseradish peroxidase, etc.) and magnetic particles. In some embodiments, the detection label is a primary label. A primary label is one that can be directly detected, such as a fluorophore.

[0091] Labels include optical labels such as fluorescent dyes or moieties. Fluorophores can be either "small molecule" fluors, or proteinaceous fluors (e.g. green fluorescent proteins and all variants thereof).

[0092] In some embodiments, activation state-specific antibodies are labeled with quantum dots as disclosed by Chattopadhyay, P. K. et al. Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry. Nat. Med. 12, 972-977 (2006). Quantum dot labels are commercially available through Invitrogen, http://probes.invitrogen.com/products/qdot/.

[0093] Quantum dot labeled antibodies can be used alone or they can be employed in conjunction with organic fluorochrome-conjugated antibodies to increase the total number of labels available. As the number of labeled antibodies increase so does the ability for subtyping known cell populations. Additionally, activation state-specific antibodies can be labeled using chelated or caged lanthanides as disclosed by Erkcki, J. et al. Lanthanide chelates as new fluorochrome labels for cytochemistry. J. Histochemistry Cytochemistry, 36:1449-1451, 1988, and U.S. Pat. No. 7,018850, entitled Salicylamide-Lanthanide Complexes for Use as Luminescent Markers. Other methods of detecting fluorescence may also be used, e.g., Quantum dot methods (see, e.g., Goldman et al., J. Am. Chem. Soc. (2002) 124:6378-82; Pathak et al. J. Am. Chem. Soc. (2001) 123:41034; and Remade et al., Proc. Natl. Sci. USA (2000) 18:553-8, each expressly incorporated herein by reference) as well as confocal microscopy.

[0094] In some embodiments, the activatable elements are labeled with tags suitable for Inductively Coupled Plasma Mass Spectrometer (ICP-MS) as disclosed in Tanner et al. Spectrochimica Acta Part B: Atomic Spectroscopy, 2007 March;62(3):188-195.

[0095] Alternatively, detection systems based on FRET, discussed in detail below, may be used. FRET finds use in the instant invention, for example, in detecting activation states that involve clustering or multimerization wherein the proximity of two FRET labels is altered due to activation. In some embodiments, at least two fluorescent labels are used which are members of a fluorescence resonance energy transfer (FRET) pair.

[0096] The methods and composition of the present invention may also make use of label enzymes. By label enzyme is meant an enzyme that may be reacted in the presence of a label enzyme substrate that produces a detectable product. Suitable label enzymes for use in the present invention include but are not limited to, horseradish peroxidase, alkaline phosphatase and glucose oxidase. Methods for the use of such substrates are well known in the art. The presence of the label enzyme is generally revealed through the enzyme's catalysis of a reaction with a label enzyme substrate, producing an identifiable product. Such products may be opaque, such as the reaction of horseradish peroxidase with tetramethyl benzedine, and may have a variety of colors. Other label enzyme substrates, such as Luminol (available from Pierce Chemical Co.), have been developed that produce fluorescent reaction products. Methods for identifying label enzymes with label enzyme substrates are well known in the art and many commercial kits are available. Examples and methods for the use of various label enzymes are described in Savage et al., Previews 247:6-9 (1998), Young, J. Virol. Methods 24:227-236 (1989), which are each hereby incorporated by reference in their entirety.

[0097] By radioisotope is meant any radioactive molecule. Suitable radioisotopes for use in the invention include, but are not limited to ^{14}C, ^{3}H, ^{32}P, ^{33}P, ^{35}S, ^{125}I and ^{131}I. The use of radioisotopes as labels is well known in the art.

[0098] As mentioned, labels may be indirectly detected, that is, the tag is a partner of a binding pair. By "partner of a binding pair" is meant one of a first and a second moiety, wherein the first and the second moiety have a specific binding affinity for each other. Suitable binding pairs for use in the invention include, but are not limited to, antigens/antibodies (for example, digoxigenin/anti-digoxigenin, dinitrophenyl (DNP)/anti-DNP, dansyl-X-anti-dansyl, Fluorescein/anti-fluorescein, lucifer yellow/anti-lucifer yellow, and rhodamine anti-rhodamine), biotin/avidin (or biotin/streptavidin) and calmodulin binding protein (CBP)/calmodulin. Other suitable binding pairs include polypeptides such as the FLAG-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide Martin et al., Science, 255: 192-194 (192)9; tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)] and the antibodies each thereto. As will be appreciated by those in the art, binding pair partners may be used in applications other than for labeling, as is described herein.

[0099] As will be appreciated by those in the art, a partner of one binding pair may also be a partner of another binding pair. For example, an antigen (first moiety) may bind to a first antibody (second moiety) that may, in turn, be an antigen for a second antibody (third moiety). It will be further appreciated that such a circumstance allows indirect binding of a first moiety and a third moiety via an intermediary second moiety that is a binding pair partner to each.

[0100] As will be appreciated by those in the arts a partner of a binding pair may comprise a label, as described above. It will further be appreciated that this allows for a tag to be indirectly labeled upon the binding of a binding partner comprising a label. Attaching a label to a tag that is a partner of a binding pair, as just described, is referred to herein as "indirect labeling".

[0101] By "surface substrate binding molecule" or "attachment tag" and grammatical equivalents thereof is meant a molecule have binding affinity for a specific surface substrate, which substrate is generally a member of a binding pair applied, incorporated or otherwise attached to a surface. Suitable surface substrate binding molecules and their surface substrates include, but are not limited to poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags and Nickel substrate; the Glutathione-S Transferase tag and its antibody substrate (available from Pierce Chemical); the flu HA tag polypeptide and its antibody 12CA5 substrate [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the SF9, 3C7, 6E10, G4, B7 and 9E10 antibody substrates thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody substrate [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. In general, surface binding substrate molecules useful in the present invention include, but are not limited to, polyhistidine structures (His-tags) that bind nickel substrates, antigens that bind to surface substrates comprising antibody, haptens that bind to avidin substrate (e.g., biotin) and CBP that binds to surface substrate comprising calmodulin.

Alternative Activation State Indicators

[0102] An alternative activation state indicator useful with the instant invention is one that allows for the detection of activation by indicating the result of such activation. For example, phosphorylation of a substrate can be used to detect the activation of the kinase responsible for phosphorylating that substrate. Similarly, cleavage of a substrate can be used as an indicator of the activation of a protease responsible for such cleavage. Methods are well known in the art that allow coupling of such indications to detectable signals, such as the labels and tags described above in connection with binding elements. For example, cleavage of a substrate can result in the removal of a quenching moiety and thus allowing for a detectable signal being produced from a previously quenched label.

Modulators

[0103] In some embodiments, the methods and composition utilize a modulator. A modulator can be a biological specimen or sample of a cellular or physiological environment from an individual, which may be a heterogeneous sample without complete chemical or biological characterization. Collection of the modulator specimen may occur directly from the individual, or be obtained indirectly. An illustrative example would be to remove a cellular sample from the individual, and then culture that sample to obtain modulators. Other modulators may be used in addition to the biological specimen or sample of a cellular environment which may be a more characterized agent. These other modulators may be contacted with the cells in addition to the biological specimen or sample of a cellular environment. A modulator can be an activator, an inhibitor or a compound capable of impacting a cellular pathway. Modulators can be uncharacterized or characterized as known compounds.

[0104] Modulation can be performed in a variety of environments. In some embodiments, cells are exposed to a modulator immediately after collection of the modulator. In some embodiments where there is a mixed population of cells, purification of cells may or may not be performed after modulation. In some embodiments, whole blood is collected to which a modulator is added. In some embodiments, cells are modulated after processing for single cells or purified fractions of single cells. As an illustrative example, whole blood can be collected and processed for an enriched fraction of lymphocytes that is then exposed to a modulator. Modulation can include exposing cells to more than one modulator.

[0105] In some embodiments, a reference population of cells is cultured with the biological specimen or cellular environment modulator in a suitable media. In some embodiments, the media is a growth media. In some embodiments, the growth media is a complex media that may include serum. In some embodiments, the growth media comprises serum. In some embodiments, the serum is selected from the group consisting of fetal bovine serum, bovine serum, human serum, porcine serum, horse serum, and goat serum. In some embodiments, the serum level ranges from **0.0001**% to **30** %. In some embodiments, the growth media is a chemically defined minimal media and is without serum. In some embodiments, cells are cultured in a differentiating media.

[0106] Modulators that may be added in addition to a biological specimen or sample of a cellular environment from an individual include chemical and biological entities. Modulators can act extracellularly or intracellularly. Chemical and biological modulators include growth factors, cytokines, neurotransmitters, adhesion molecules, hormones, small molecules, inorganic compounds, polynucleotides, antibodies, natural compounds, lectins, lactones, chemotherapeutic agents, biological response modifiers, carbohydrate, proteases and free radicals. Modulators include complex and undefined biologic compositions that may comprise cellular or botanical extracts, cellular or glandular secretions, physiologic fluids such as serum, amniotic fluid, whole urine, ascites, plasma, cell extract, whole cells, lavage or rinse of cavities. Modulators that may be added in addition to a biological specimen or sample of a cellular environment from an individual include physical and environmental stimuli, as well as chemical and biological as listed above. These modulators also can act extracellularly or intracellularly. Physical and environmental modulators include electromagnetic, ultraviolet, infrared or particulate radiation, redox potential and pH, the presence or absences of nutrients, changes in temperature, changes in oxygen partial pressure, changes in ion concentrations and the application of oxidative stress. Modulators can be endogenous or exogenous and may produce different effects depending on the concentration and duration of exposure to the single cells or whether they are used in combination or sequentially with other modulators. Modulators can act directly on the activatable elements or

indirectly through the interaction with one or more intermediary biomolecule. Indirect modulation includes alterations of gene expression wherein the expressed gene product is the activatable element or is a modulator of the activatable element.

[0107] In some embodiments the modulator that may be added in addition to a biological specimen or sample of a cellular environment from an individual is known and is selected from the group consisting of growth factor, cytokine, adhesion molecule modulator, drugs, hormone, small molecule, polynucleotide, antibodies, natural compounds, lactones, chemotherapeutic agents, immune modulator, carbohydrate, proteases, ions, reactive oxygen species, peptides, and protein fragments, either alone or in the context of cells, cells themselves, viruses, and biological and non-biological complexes (e.g. beads, plates, viral envelopes, antigen presentation molecules such as major histocompatibility complex). In some embodiments, the modulator that may be added in addition to a biological specimen or sample of a cellular environment from an individual is a physical stimuli such as heat, cold, UV radiation, and radiation. In some embodiments, the modulator is an activator. In some embodiments the modulator is an inhibitor. In some embodiments, reference cells are exposed to one or more modulators. In some embodiments, reference cells are exposed to multiple modulators. In some embodiments, reference cells are exposed to at least two modulators.

Detection

[0108] In practicing the methods of this invention, the detection of the status of the one or more activatable elements can be carried out by a person, such as a technician in the laboratory. Alternatively, the detection of the status of the one or more activatable elements can be carried out using automated systems. In either case, the detection of the status of the one or more activatable elements for use according to the methods of this invention is performed according to standard techniques and protocols well-established in the art,

[0109] One or more activatable elements can be detected and/or quantified by any method that detect and/or quantitates the presence of the activatable element of interest. Such methods may include radioimmunoassay (WRA) or enzyme linked immunoabsorbance assay (ELISA), immunohistochemistry, immunofluorescent histochemistry with or without confocal microscopy, reversed phase assays, homogeneous enzyme immunoassays, and related non-enzymatic techniques, Western blots, whole cell staining, immunoelectronmicroscopy, nucleic acid amplification, gene array, protein array, mass spectrometry, patch clamp, 2-dimensional gel electrophoresis, differential display gel electrophoresis, microsphere-based multiplex protein assays, label-free cellular assays and flow cytometry, etc. U.S. Pat. No. 4,568,649 describes ligand detection systems, which employ scintillation counting. These techniques are particularly useful for modified protein parameters. Cell readouts for proteins and other cell determinants can be obtained using fluorescent or otherwise tagged reporter molecules. Flow cytometry methods are useful for measuring intracellular parameters. See U.S. patent Ser. No. 10/898,734 and Shulz et al., Current Protocols in Immunology, 2007, 78:8.17.1-20 which are incorporated by reference in their entireties.

[0110] In some embodiments, the present invention provides methods for determining an activatable element's activation profile for a single cell. The methods may comprise analyzing cells by flow cytometry on the basis of the activation level of at least two activatable elements. Binding elements (e.g. activation state-specific antibodies) are used to analyze cells on the basis of activatable element activation level, and can be detected as described below. Alternatively, non-binding elements systems as described above can be used in any system described herein.

[0111] When using fluorescent labeled components in the methods and compositions of the present invention, it will recognize that different types of fluorescent monitoring systems, e.g., Cytometric measurement device systems, can be used to practice the invention. In some embodiments, flow cytometric systems are used or systems dedicated to high throughput screening, e.g. 96 well or greater microtiter plates. Methods of performing assays on fluorescent materials are well known in the art and are described in, e.g., Lakowicz, J. R., Principles of Fluorescence Spectroscopy, New York: Plenum Press (1983); Herman, B., Resonance energy transfer microscopy, in: Fluorescence Microscopy of Living Cells in Culture, Part B, Methods in Cell Biology, vol. 30, ed. Taylor, D. L. & Wang, Y. -L., San Diego: Academic Press (1989), pp. 219-243; Turro, N. J., Modern Molecular Photochemistry, Menlo Park: Benjamin/Cummings Publishing Col, Inc. (1978), pp. 296-361.

[0112] Fluorescence in a sample can be measured using a fluorimeter. In general, excitation radiation, from an excitation source having a first wavelength, passes through excitation optics. The excitation optics cause the excitation radiation to excite the sample. In response, fluorescent proteins in the sample emit radiation that has a wavelength that is different from the excitation wavelength. Collection optics then collect the emission from the sample. The device can include a temperature controller to maintain the sample at a specific temperature while it is being scanned. According to one embodiment, a multi-axis translation stage moves a microtiter plate holding a plurality of samples in order to position different wells to be exposed. The multi-axis translation stage, temperature controller, auto-focusing feature, and electronics associated with imaging and data collection can be managed by an appropriately programmed digital computer. The computer also can transform the data collected during the assay into another format for presentation. In general, known robotic systems and components can be used.

[0113] Other methods of detecting fluorescence may also be used, e.g., Quantum dot methods (see, e.g., Goldman et al., J. Am. Chem. Soc. (2002) 124:6378-82; Pathak et al. J. Am. Chem. Soc. (2001) 123:41034; and Remade et al., Proc. Natl. Sci. USA (2000) 18:553-8, each expressly incorporated herein by reference) as well as confocal microscopy. In general, flow cytometry involves the passage of individual cells through the path of a laser beam. The scattering the beam and excitation of any fluorescent molecules attached to, or found within, the cell is detected by photomultiplier tubes to create a readable output, e.g. size, granularity, or fluorescent intensity.

[0114] The detecting, sorting, or isolating step of the methods of the present invention can entail fluorescence-activated cell sorting (FACS) techniques, where FACS is used to select cells from the population containing a particular surface marker, or the selection step can entail the use of magnetically responsive particles as retrievable supports for target cell capture and/or background removal. A variety of FACS systems are known in the art and can be used in the methods of the invention (see e.g., WO99/54494, filed Apr. 16, 1999;

U.S. Ser. No. 20010006787, filed Jul. 5, 2001, each expressly incorporated herein by reference).

[0115] In some embodiments, a FACS cell sorter (e.g. a FACSVantage™ Cell Sorter, Becton Dickinson Immunocytometry Systems, San Jose, Calif.) is used to sort and collect cells that may used as a modulator or as a population of reference cells. In some embodiments, the modulator or reference cells are first contacted with fluorescent-labeled binding elements (e.g. antibodies) directed against specific elements. In such an embodiment, the amount of bound binding element on each cell can be measured by passing droplets containing the cells through the cell sorter. By imparting an electromagnetic charge to droplets containing the positive cells, the cells can be separated from other cells. The positively selected cells can then be harvested in sterile collection vessels. These cell-sorting procedures are described in detail, for example, in the FACSVantage™. Training Manual, with particular reference to sections 3-11 to 3-28 and 10-1 to 10-17, which is hereby incorporated by reference in its entirety.

[0116] In another embodiment, positive cells can be sorted using magnetic separation of cells based on the presence of an isoform of an activatable element. In such separation techniques, cells to be positively selected are first contacted with specific binding element (e.g., an antibody or reagent that binds an isoform of an activatable element). The cells are then contacted with retrievable particles (e.g., magnetically responsive particles) that are coupled with a reagent that binds the specific element. The cell-binding element-particle complex can then be physically separated from non-positive or non-labeled cells, for example, using a magnetic field. When using magnetically responsive particles, the positive or labeled cells can be retained in a container using a magnetic filed while the negative cells are removed. These and similar separation procedures are described, for example, in the Baxter Immunotherapy Isolex training manual which is hereby incorporated in its entirety.

[0117] In some embodiments, methods for the determination of a receptor element activation state profile for a single cell are provided. The methods comprise providing a population of cells and analyze the population of cells by flow cytometry. Preferably, cells are analyzed on the basis of the activation level of at least two activatable elements. In some embodiments, a multiplicity of activatable element activation-state antibodies is used to simultaneously determine the activation level of a multiplicity of elements.

[0118] In some embodiment, cell analysis by flow cytometry on the basis of the activation level of at least two elements is combined with a determination of other flow cytometry readable outputs, such as the presence of surface markers, granularity and cell size to provide a correlation between the activation level of a multiplicity of elements and other cell qualities measurable by flow cytometry for single cells,

[0119] As will be appreciated, the present invention also provides for the ordering of element clustering events in signal transduction. Particularly, the present invention allows the artisan to construct an element clustering and activation hierarchy based on the correlation of levels of clustering and activation of a multiplicity of elements within single cells. Ordering can be accomplished by comparing the activation level of a cell or cell population with a control at a single time point, or by comparing cells at multiple time points to observe subpopulations arising out of the others.

[0120] As will be appreciated, these methods provide for the identification of distinct signaling cascades for both artificial and stimulatory conditions in cell populations, such a peripheral blood mononuclear cells, or naive and memory lymphocytes.

[0121] When necessary, cells are dispersed into a single cell suspension, e.g. by enzymatic digestion with a suitable protease, e.g. collagenase, dispase, etc.; and the like, An appropriate solution is used for dispersion or suspension. Such solution will generally be a balanced salt solution, e.g. normal saline, PBS, Hanks balanced salt solution, etc., conveniently supplemented with fetal calf serum or other naturally occurring factors, in conjunction with an acceptable buffer at low concentration, generally from **5-25** mM. Convenient buffers include HEPES 1 phosphate buffers, lactate buffers, etc. The cells may be fixed, e.g. with 3% paraformaldehyde, and are usually permeabilized, e.g. with ice cold methanol; HEPES-buffered PBS containing 0.1% saponin, 3% BSA; covering for 2 min in acetone at −200C; and the like as known in the art and according to the methods described herein.

[0122] In some embodiments, one or more cells are contained in a well of a 96 well plate or other commercially available multiwell plate. In an alternate embodiment, the reaction mixture or cells are in a cytometric measurement device. Other multiwell plates useful in the present invention include, but are not limited to 384 well plates and 1536 well plates. Still other vessels for containing the reaction mixture or cells and useful in the present invention will be apparent to the skilled artisan.

[0123] The addition of the components of the assay for detecting the activation level or activity of an activatable element, or modulation of such activation level or activity, may be sequential or in a predetermined order or grouping under conditions appropriate for the activity that is assayed for. Such conditions are described here and known in the art. Moreover, further guidance is provided below (see, e.g., in the Examples).

[0124] In some embodiments, the activation level of an activatable element is measured using Inductively Coupled Plasma Mass Spectrometer (ICP-MS). A binding element that has been labeled with a specific element binds to the activativatable. When the cell is introduced into the ICP, it is atomized and ionized. The elemental composition of the cell, including the labeled binding element that is bound to the activatable element, is measured. The presence and intensity of the signals corresponding to the labels on the binding element indicates the level of the activatable element on that cell (Tanner et al. Spectrochimica Acta Part B: Atomic Spectroscopy, 2007 March, 62(3):188-195.).

[0125] As will be appreciated by one of skill in the art, the instant methods and compositions find use in a variety of other assay formats in addition to flow cytometry analysis. For example, a chip analogous to a DNA chip can be used in the methods of the present invention. Arrayers and methods for spotting nucleic acids on a chip in a prefigured array are known. In addition, protein chips and methods for synthesis are known. These methods and materials may be adapted for the purpose of affixing activation state binding elements to a chip in a prefigured array. In some embodiments, such a chip comprises a multiplicity of element activation state binding elements, and is used to determine an element activation state profile for elements present on the surface of a cell. See U.S. Pat. No. 5,744,934.

[0126] In some embodiments confocal microscopy can be used to detect activation profiles for individual cells. Confocal microscopy relies on the serial collection of light from spatially filtered individual specimen points, which is then electronically processed to render a magnified image of the specimen. The signal processing involved confocal microscopy has the additional capability of detecting labeled binding elements within single cells, accordingly in this embodiment the cells can be labeled with one or more binding elements. In some embodiments the binding elements used in connection with confocal microscopy are antibodies conjugated to fluorescent labels, however other binding elements, such as other proteins or nucleic acids are also possible.

[0127] In some embodiments, the methods and compositions of the instant invention can be used in conjunction with an "In-Cell Western Assay." In such an assay, cells are initially grown in standard tissue culture flasks using standard tissue culture techniques. Once grown to optimum confluency, the growth media is removed and cells are washed and trypsinized. The cells can then be counted and volumes sufficient to transfer the appropriate number of cells are aliquoted into microwell plates (e.g., Nunc™ 96 Microwell™ plates). The individual wells are then grown to optimum confluency in complete media whereupon the media is replaced with serum-free media. At this point controls are untouched, but experimental wells are incubated with a modulator, e.g. EGF. After incubation with the modulator cells are fixed and stained with labeled antibodies to the activation elements being investigated. Once the cells are labeled, the plates can be scanned using an imager such as the Odyssey Imager (LiCor, Lincoln Nebr.) using techniques described in the Odyssey Operator's Manual v1.2., which is hereby incorporated in its entirety. Data obtained by scanning of the multiwell plate can be analyzed and activation profiles determined as described below.

[0128] In some embodiments, the detecting is by high pressure liquid chromatography (HPLC), for example, reverse phase BPLC, and in a further aspect, the detecting is by mass spectrometry.

[0129] These instruments can fit in a sterile laminar flow or fume hood, or arc enclosed, self-contained systems, for cell culture growth and transformation in multi-well plates or tubes and for hazardous operations. The living cells may be grown under controlled growth conditions, with controls for temperature, humidity, and gas for time series of the live cell assays. Automated transformation of cells and automated colony pickers may facilitate rapid screening of desired cells.

[0130] Flow cytometry or capillary electrophoresis formats can be used for individual capture of magnetic and other beads, particles, cells, and organisms.

[0131] Flexible hardware and software allow instrument adaptability for multiple applications. The software program modules allow creation, modification, and running of methods. The system diagnostic modules allow instrument alignment, correct connections, and motor operations. Customized tools, labware, and liquid, particle, cell and organism transfer patterns allow different applications to be performed. Databases allow method and parameter storage. Robotic and computer interfaces allow communication between instruments.

[0132] In some embodiments, the methods of the invention include the use of liquid handling components. The liquid handling systems can include robotic systems comprising any number of components. In addition, any or all of the steps outlined herein may be automated; thus, for example, the systems may be completely or partially automated.

[0133] As will be appreciated by those in the art, there are a wide variety of components which can be used, including, but not limited to, one or more robotic arms; plate handlers for the positioning of microplates; automated lid or cap handlers to remove and replace lids for wells on non-cross contamination plates; tip assemblies for sample distribution with disposable tips; washable tip assemblies for sample distribution; 96 well loading blocks; cooled reagent racks; microtiter plate pipette positions (optionally cooled); stacking towers for plates and tips; and computer systems. See U.S. Ser. No. 61/048,657 which is incorporated by reference in its entirety.

[0134] Fully robotic or microfluidic systems include automated liquid-, particle-, cell- and organism-handling including high throughput pipetting to perform all steps of screening applications. This includes liquid, particle, cell, and organism manipulations such as aspiration, dispensing, mixing, diluting, washing, accurate volumetric transfers; retrieving, and discarding of pipet tips; and repetitive pipetting of identical volumes for multiple deliveries from a single sample aspiration. These manipulations are cross-contamination-free liquid, particle, cell, and organism transfers. This instrument performs automated replication of microplate samples to filters, membranes, and/or daughter plates, high-density transfers, full-plate serial dilutions, and high capacity operation.

[0135] In some embodiments, chemically derivatized particles, plates, cartridges, tubes, magnetic particles, or other solid phase matrix with specificity to the assay components are used. The binding surfaces of microplates, tubes or any solid phase matrices include non-polar surfaces, highly polar surfaces, modified dextran coating to promote covalent binding, antibody coating, affinity media to bind fusion proteins or peptides, surface-fixed proteins such as recombinant protein A or G, nuelcotide resins or coatings, and other affinity matrix are useful in this invention.

[0136] In some embodiments, platforms for multi-well plates, multi-tubes, holders, cartridges, minitubes, deep-well plates, microfuge tubes, cryovials, square well plates, filters, chips, optic fibers, beads, and other solid-phase matrices or platform with various volumes are accommodated on an upgradable modular platform for additional capacity. This modular platform includes a variable speed orbital shaker, and multi-position work decks for source samples, sample and reagent dilution, assay plates, sample and reagent reservoirs, pipette tips, and an active wash station. In some embodiments, the methods of the invention include the use of a plate reader. See U.S. Ser. No. 61/048,657.

[0137] In some embodiments, thermocycler and thermoregulating systems are used for stabilizing the temperature of heat exchangers such as controlled blocks or platforms to provide accurate temperature control of incubating samples from 0° C. to 100° C.

[0138] In some embodiments, interchangeable pipet heads (single or multi-channel) with single or multiple magnetic probes, affinity probes, or pipetters robotically manipulate the liquid, particles, cells, and organisms. Multi-well or multi-tube magnetic separators or platforms manipulate liquid, particles, cells, and organisms in single or multiple sample formats.

[0139] In some embodiments, the instrumentation will include a detector, which can be a wide variety of different detectors, depending on the labels and assay. In some embodi-

ments, useful detectors include a microscope(s) with multiple channels of fluorescence; plate readers to provide fluorescent, ultraviolet and visible spectrophotometric detection with single and dual wavelength endpoint and kinetics capability, fluorescence resonance energy transfer (FRET), luminescence, quenching, two-photon excitation, and intensity redistribution; CCD cameras to capture and transform data and images into quantifiable formats; and a computer workstation.

[0140] In some embodiments, the robotic apparatus includes a central processing unit which communicates with a memory and a set of input/output devices (e.g., keyboard, mouse, monitor, printer, etc.) through a bus. Again, as outlined below, this may be in addition to or in place of the CPU for the multiplexing devices of the invention. The general interaction between a central processing unit, a memory, input/output devices, and a bus is known in the art. Thus, a variety of different procedures, depending on the experiments to be run, are stored in the CPU memory. See U.S. Ser. No. 61/048,657 which is incorporated by reference in its entirety.

[0141] These robotic fluid handling systems can utilize any number of different reagents, including buffers, reagents, samples, washes, assay components such as label probes, etc.

[0142] Any of the steps above can be performed by a computer program product that comprises a computer executable logic that is recorded on a computer readable medium. For example, the computer program can execute some or all of the following functions: (i) exposing reference population of cells to one or more modulators, (ii) exposing reference population of cells to one or more binding elements, (iii) detecting the activation levels of one or more activatable elements, and (iv) classifying one or more cells into one or more classes based on the activation level.

[0143] The computer executable logic can work in any computer that may be any of a variety of types of general-purpose computers such as a personal computer, network server, workstation, or other computer platform now or later developed. In some embodiments, a computer program product is described comprising a computer usable medium having the computer executable logic (computer software program, including program code) stored therein. The computer executable logic can be executed by a processor, causing the processor to perform functions described herein. In other embodiments, some functions are implemented primarily in hardware using, for example, a hardware state machine. Implementation of the hardware state machine so as to perform the functions described herein will be apparent to those skilled in the relevant arts.

[0144] The program can provide a method of determining the status of an individual by accessing data that reflects the activation level of one or more activatable elements in the reference population of cells.

Analysis

[0145] Advances in flow cytometry have enabled the individual cell enumeration of up to thirteen simultaneous parameters and are moving towards the study of genomic and proteomic data subsets. See Krutzik et al and Irish et al. above as well as Irish, Jour. Immunol., 2006, 177: 1581-1589. Likewise, advances in other techniques (e.g. microarrays) allow for the identification of multiple activatable elements. As the number of parameters, epitopes, and samples have increased, the complexity of experiments and the challenges of data analysis have grown rapidly. An additional layer of data complexity has been added by the development of stimulation panels which enable the study of activatable elements under a growing set of experimental conditions. Methods for the analysis of multiple parameters are well known in the art.

[0146] In some embodiments where flow cytometry is used, flow cytometry experiments are arrayed and the results are approximated as fold changes using a heat map to facilitate evaluation. Generally speaking, arrayed flow cytometry experiments simplify multidimensional flow cytometry data based on experimental design and observed differences between flow cytometry samples. One common way of comparing changes in a set of flow cytometry samples is to overlay histograms of one parameter on the same plot. Arrayed flow cytometry experiments ideally contain a reference sample against which experimental samples are compared. This reference sample is placed in the first position of the array, and subsequent experimental samples follow the control in the sequence. Reference samples can include normal and/or cells associated with a condition (e.g. tumor cells).

[0147] See the references cited in U.S. Ser. Nos. 61/048,886 and 61/048,920 for analysis techniques of flow cytometry data. See also the references cited above.

[0148] Examples of analysis for activatable elements are described in US publication number 20060073474 entitled "Methods and compositions for detecting the activation state of multiple proteins in single cells" and US publication number 20050112700 entitled "Methods and compositions for risk stratification" the content of which are incorporate here by reference. See also U.S. Ser. Nos. 61/048,886 and 61/048,920 for more examples of know modulators.

Kits

[0149] In some embodiments the invention provides kits. Kits provided by the invention may comprise one or more of the state-specific binding element described herein, such as phospho-specific antibodies. In some embodiments, the kit comprises one or more of the phospho-specific antibodies specific for the proteins selected from the group consisting of PI3-Kinase p85, p1 10a, p1 10b, p1 10d), Jak1, Jak2, SOCs, Rac, Rho, Cdc42, Ras-GAP, Vav, Tiam, Sos, Dbl, Nck, Gab, PRK, SHP1, and SHP2, SHIP1, SHIP2, sSHIP, PTEN, She, Grb2, PDK1, SGK, Akt1, Akt2, Akt3, TSC1,2, Rheb, mTor, 4EBP-1, p70S6Kinase, S6, LKB-1, AMPK, PFK, Acetyl-CoAa Carboxylase, DokS, Rafs, Mos, Tp12, MEK1/2, MLK3, TAK, DLK, MKK3/6, MEKK1,4, MLK3, ASK1, MKK4/7, SAPK/JNK1,2,3, p38s, Erk1/2, Syk, Btk, BLNK, LAT, ZAP70, Lck, Cbl, SLP-76, PLCγ_1, PLCγ2, STAT1, STAT 3, STAT 4, STAT 5, STAT 6, FAK, p130CAS, PAKs, LIMK1/2, Hsp90, Hsp70, Hsp27, SMADs, Rel-A (p65-NFKB), CREB, Histone H2B, HATs, HDACs, PKR, Rb, Cyclin D, Cyclin E, Cyclin A, Cyclin B, P16, p14Arf, p27KIP, p21CIP, Cdk4, Cdk6, Cdk7, Cdk1, Cdk2, Cdk9, Cdc25,A/B/C, Abl, E2F, FADD, TRADD, TRAF2, RIP, Myd88, BAD, Bc1-2, MG1-1, Bc1-XL, Caspase 2, Caspase 3, Caspase 6, Caspase 7, Caspase 8, Caspase 9, lAPs, Smac, Fodrin, Actin, Src, Lyn, Fyn, Lck, NIK, IκB, p65(RelA), IKKα, PKA, PKCα, PKCβ, PKCθ, PKCδ, CAMKC, Elk, AFT, Myc, Egr-1, NFAT, ATF-2, Mdm2, p53, DNA-PK, Chk1, Chk2, ATM, ATR, βcatenin, CrkL, GSK3α, GSK3β, and FOXO. In some embodiments, the kit comprises one or more of the phospho-specific antibodies specific for the proteins selected from the group consisting of Erk, Syk, Zap70, Lck, Btk, BLNK, Cbl, PLCγ2, Akt, RelA, p38, S6. In some embodiments, the kit comprises one or more of the phospho-

specific antibodies specific for the proteins selected from the group consisting of Akt1, Akt2, Akt3, SAPK/JNK1,2,3, p38s, Erk1/2, Syk, ZAP70, Btk, BLNK, Lck, PLCγ, PLCiγ2, STAT1, STAT 3, STAT 4, STAT 5, STAT 6, CREB, Lyn, p-S6, Cbl, NF-κB, GSK3β, CARMA/Bc110 and Tc1-1.

[0150] Kits provided by the invention may comprise one or more of the modulators described herein. In some embodiments, the kit comprises one or more modulators selected from the group consisting of, H_2O_2, a member of the IMIDS family such as Revlimid, TNF-α, PMA, thapsigargin, G-CSF, GM-CSF, FLT3L, IGF-1, SCF, erythropoetin, thrombopoetin, interferons, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-27 BAFF, April, SDFla, CD40L, and a combination thereof.

[0151] Such kits enable the detection of activatable elements by sensitive cellular assay methods, such as IHC and flow cytometry, which are suitable for the clinical detection, prognosis, and screening of cells and tissue from patients, such as leukemia patients, having a disease involving altered pathway signaling.

[0152] Such kits may also comprise tools and reagents to isolate a biological specimen from an individual. The kits of the invention may also comprise tools and reagent to isolate one or more components (e.g. cytokines) from the biological specimen.

[0153] Such kits may additionally comprise one or more therapeutic agents. The kit may further comprise a software package for data analysis of the physiological status, which may include reference profiles for comparison with the test profile.

[0154] Such kits may also include information, such as scientific literature references, package insert materials, clinical trial results, and/or summaries of these and the like, which indicate or establish the activities and/or advantages of the composition, and/or which describe dosing, administration, side effects, drug interactions, or other information useful to the health care provider. Such information may be based on the results of various studies, for example, studies using experimental animals involving in vivo models and studies based on human clinical trials. Kits described herein can be provided, marketed and/or promoted to health providers, including physicians, nurses, pharmacists, formulary officials, and the like. Kits may also, in some embodiments, be marketed directly to the consumer.

[0155] The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are expressly incorporated by reference in their entirety.

EXAMPLES

Example 1

[0156] The present illustrative example represents how to treat and analyze cells in one embodiment of the present invention. There are several steps in the process, such as the step where a modulator such as serum from an individual is added, the staining step and the flow cytometry step. The stimulation step of the phospho-flow procedure can start with vials of cryopreserved cells and end with cells fixed and perrneabilized in methanol. Then the cells can be incubated with an antibody directed to a particular protein of interest and then analyzed using a flow cytometer.

[0157] The materials used in this example include thawing medium which comprises PBS-CMF+10% FBS +2 mM EDTA; 70 um Cell Strainer (BD); anti-CD45 antibody conjugated to Alexa 700 (Invitrogen) used at 1 ul per sample; propidium iodide (PI) solution (Sigma 10 ml, 1 mg/ml) used at 1 ug/ml; RPMI+1% FBS medium; media A comprising RPMI+1% FBS+1×Penn/Strep; Live/Dead Reagent, Amine Aqua (Invitrogen); 2 ml, 96-Deep Well, U-bottom polypropylene plates (Nunc); 300 ul 96-Channel Extended-Length D.A.R.T. tips for Hydra (Matrix); Phosphate Buffered Saline (PBS) (NediaTech); 16% Paraformaldehyde (Electron Microscopy Sciences); 100% Methanol (EMD) stored at −20C; Transtar 96 dispensing apparatus (Costar); Transtar 96 Disposable Cartridges (Costar, Polystyrene, Sterile); Transtar reservoir (Costar); and foil plate sealers.

[0158] a. Serum isolation

[0159] Venous blood samples can be collected from each patient into 10 ml vacutainer tubes with SST gel and clot activator (Ref-368510 Becton Dickinson Systems UK). The tubes are kept in vertical position for at least 1 hour at room temperature until the clot is formed. The tubes are centrifuged 3,000 rpm for 10 min at room temperature. The serum (supernatant) is then transferred to a new sterile tube and centrifuges again at 2,500 rpm for 10 min at room temperature in order to pellet potentially remaining cells on isolated serum. The serum can be stored at −20° C. until use or follow with exposure of cells to the serum.

[0160] b. Isolation of PMBC to Use as Reference Cells:

[0161] 1. Venous blood samples from each patient or healthy donors are collected into **3** or 10 ml vacutainer tubes with K3/EDTA (lavender tube) (3 ml—Ref. #367652#—or 10 ml—Ref. #368457#—Becton Dickinson Systems UK). Tubes are centrifuged at 2,500 rpm for 15 min at room temperature and the supematant is discarded. The blood samples are transferred into a 20 ml sterile tube and add 2-3 volumes of erythrocyte lysis buffer (155 mM NH4Cl [8.3 g/I], Hepes 10 mM [10 ml 1M/l], pH: 7.0). The samples are incubated at room temperature for 30 min on a rocking platform. The samples are centrifuged at 3,000 rpm for 10 min at room temperature. The cells are resuspended in media. Cells can be stored for later use or can be used immediately.

[0162] c. Thawing Cell and Live/Dead Staining:

[0163] Cryopreserved cells are thawed in a 37° C. water bath and gently resuspended in the vial and transferred to the 15 mL conical tube. The 15 mL tube is centrifuged at 930 RPM (200×g) for 8 minutes at room temperature. The supernatant is aspirated and the pellet is gently resuspended in 1 mL media A. The cell suspension is filtered through a 70 um cell strainer into a new 15 mL tube. The cell strainer is rinsed with 1 mL media A and another 12 ml of media A into the 15 mL tube. The cells are mixed into an even suspension. A 20 μL aliquot is immediately removed into a 96-well plate containing **180** /μL PBS+4% FBS+CD45 Alexa 700+PI to determine cell count and viability post spin. After the determination, the 15 mL tubes are centrifuged at 930 RPM (200×g) for 8 minutes at room temperature. The supematant is aspirated and the cell pellet is gently resuspended in 4 mL PBS+4 μL Amine Aqua and incubated for 15 min in a 37° C. incubator. 10 mL RPMI+1% FBS is added to the cell suspension and the tube is inverted to mix the cells. The 15 mL tubes are centrifuged at 930 RPM (200×g) for 8 minutes at room temperature. The cells are resuspended in Media A at the desired cell concen-

tration (1.25×10^6/mL). For samples with low numbers of cells (<18.5×10^6), the cells are resuspended in up to 15 mL media. For samples with high numbers of cells (>18.5×10^6), the volume is raised to 10 mL with media A and the desired volume is transferred to a new 15 mL tube, and the cell concentration is adjusted to 1.25×10^6 cells/ml. 1.6 mL of the above cell suspension (concentration at 1.25×10^6 cells/ml) is transferred into wells of a multi-well plate. From this plate, 80 ul is dispensed into each well of a subsequent plate. The plates are covered with a lid (Nunc) and placed in a 37° C. incubator for 2 hours to rest.

[0164] d. Addition to a Modulator to the Cells

[0165] Serum from step (a) or one or more components isolated from the serum can be used as a modulator. Additional modulators can also be used. A concentration for each modulator that is five folds more (5×) than the final concentration is prepared using Media A as diluent. 5× stimuli are arrayed into wells of a standard 96 well v-bottom plate that correspond to the wells on the plate with cells to be stimulated.

[0166] Preparation of fixative: Stock vial contains 16% paraformaldehyde which is diluted with PBS to a concentration that is 1.5×. The stock vial is placed in a 37° C. water bath.

[0167] Adding the modulator: The cell plate(s) are taken out of the incubator and placed in a 37° C. water bath next to the pipette apparatus. The cell plate is taken from the water bath and gently swirled to resuspend any settled cells. With pipettor, the stimulant is dispensed into the cell plate and vortexed at "7" for 5 seconds. The deep well plate is put back into the water bath.

[0168] Adding Fixative: 200 μl of the fixative solution (final concentration at 1.6%) is dispensed into wells and then mixed on the titer plate shaker on high for 5 seconds. The plate is covered with foil sealer and incubated in a 37° C. water bath for 10 minutes. The plate is spun for 6 minutes at 2000 rpm at room temperature. The cells are aspirated using a 96 well plate aspirator (VP Scientific). The plate is vortexed to resuspend cell pellets in the residual volume. The pellet is ensured to be dispersed before the Methanol step (see cell permeabilization) or clumping will occur.

[0169] Cell Permeabilization: Permeability agent, for example methanol, is added slowly and while the plate is vortexing. To do this, the cell plate is placed on titer plate shaker and made sure it is secure. The plate is set to shake using the highest setting. A pipetter is used to add 0.6 mls of 100% methanol to the plate wells. The plate(s) are put on ice until this step has been completed for all plates. Plates are covered with a foil seal using the plate roller to achieve a tight fit. At this stage the plates may be stored at −80° C.

[0170] e. Staining Protocol

[0171] Reagents for staining include FACS/Stain Buffer-PBS+0.1% Bovine serum albumen (BSA)+0.05% Sodium Azide; Diluted Bead Mix-1 mL FACS buffer+1 drop anti-mouse Ig Beads+1 drop negative control beads. The general protocol for staining cells is as follows, although numerous variations on the protocol may be used for staining cells:

[0172] Cells are thawed if cryopreserved. Cells are pelleted at 2000 rpm 5 minutes. Supernatant is aspirated with vacuum aspirator. Plate is vortexed on a "plate vortex" for 5-10 seconds. Cells are washed with 1 mL FACS buffer. Repeat the spin, aspirate and vortex steps as above. 50 μL of FACS/stain buffer with the desired, previously optimized, antibody cocktail is added to two rows of cells at a time and agitate the plate. The plate is covered and incubated in a shaker for 30 minutes at room temperature (RT). During this incubation, the compensation plate is prepared. For the compensation plate, in a standard 96 well V-bottom plate, 20 μL of "diluted bead mix" is added per well. Each well gets 5 μL of 1 fluorophor conjugated control IgG (examples: Alexa488, PE, Pac Blue, Aqua, Alexa647, Alexa700). For the Aqua well, add 200 uL of Aqua± cells, Incubate the plate for 10 minutes at RT. Wash by adding 200 μL FACS/stain buffer, centrifuge at 2000 rpm for 5 minutes, and remove supernatant. Repeat the washing step and resuspend the cells/beads in 200 μL FACS/stain buffer and transfer to a U-bottom 96 well plate. After 30 min, 1 mL FACS/stain buffer is added and the plate is incubated on a plate shaker for 5 minutes at room temperature. Centrifuge, aspirate and vortex cells as described above. 1 mL FACS/stain buffer is added to the plate and the plate is covered and incubated on a plate shaker for 5 minutes at room temperature. Repeat the above two steps and resuspend the cells in 75 μl FACS/stain buffer. The cells are analyzed using a flow cytometer, such as a LSR-II (Becton Disckinson). All wells are selected and Loader Settings are described below: Flow Rate: 2 uL/sec; Sample Volume: 40 uL; Mix volume: 40 uL; Mixing Speed: 250 uL/sec; # Mixes: 5; Wash Volume: 800 uL; STANDARD MODE. When a plate has completed, a Batch analysis is performed to ensure no clogging.

[0173] d. Gating Protocol

[0174] Data acquired from the flow cytometer are analyzed with Flovo software (Treestar, Inc). The Flow cytometry data is first gated on single cells (to exclude doublets) using Forward Scatter Characteristics Area and Height (FSC-A, FSC-H). Single cells are gated on live cells by excluding dead cells that stain positive with an amine reactive viability dye (Aqua-Invitrogen). Live, single cells are then gated for subpopulations using antibodies that recognize surface markers for different populations. For example when analyzing a sample from a patient having or suspected of having AML, markers such as: CD45++, CD33− for lymphocytes, CD45++, CD33++for monocytes+granulocytes and CD45+, CD33+ for leukemic blasts, can be used. Signaling, determined by the antibodies that interact with intracellular signaling molecules, in these subpopulation gates is analyzed.

[0175] The data can then be analyzed using various metrics, such as basal level of a protein or the basal level of phosphorylation in the absence of a stimulant, total phosphorylated protein, or fold change (by comparing the change in phosphorylation in the absence of a stimulant to the level of phosphorylation seen after treatment with a stimulant), on each of the cell populations that are defined by the gates in one or more dimensions. These metrics are then organized in a database tagged by: the Donor ID, plate identification (ID), well ID, gated population, stain, and modulator. These metrics tabulated from the database are then combined with the clinical data to identify nodes that are correlated with a pre-specified clinical variable (for example; response or non response to therapy) of interest.

[0176] A diagnsose can be made based on the results from the data analysis.

Example 2

[0177] Scenarios of how this invention might be used to advance the diagnosis or prognosis of disease, or the ability to predict or assess response to therapy are outlined in the following two paragraphs.

[0178] An individual presents to their primary medical doctor with lymphadenopathy, fever, and shortness of breath. Radiologic examination reveals a large anterior mediastinal mass. The patient is diagnosed with a T cell lymphoma. Using an embodiment of the present invention, the peripheral blood of the patient might be removed and sera collected. Sera could then be fractionated and applied to a reference cell line and activatable elements assessed. The classification of this reference population from prior experience might reveal that this patient has a particular sub-type of T cell lymphoma that has an excellent prognosis. This invention might also inform the physician that the patient should be treated with a particular drug.

[0179] An individual presents to her medical oncologist with recurrent ascites after therapy for ovarian cancer. Using this invention the ascites could be tapped, cellular debris spun out, and the fluid fraction applied to a reference cell line. Activatable elements could be assessed and a classification made based on prior experience that could identify the class of therapeutic that the patient should receive as therapy for this recurrent ovarian cancer.

[0180] An individual presents to their primary medical doctor with fatigue and bone pain. Initial evaluation reveals an elevated calcium level and anemia. The patient is found to have Bence-Jones proteins in the urine and is subsequently diagnosed with multiple myeloma. Using an embodiment of the present invention, this patient's urine sample could be applied to a reference cell line and activatable elements assessed. The classification of this reference population from prior experience might reveal that this patient has a particular sub-type of multiple myeloma that should be treated with a particular class of therapeutics.

[0181] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

What is claimed is:

1. A method for determining the status of an individual, comprising

subjecting a reference population of cells to a biological specimen obtained from an individual;

determining activation states of a plurality of activatable elements in said reference population of cells; and

determining the status of the individual based on the activation states of said plurality of activatable elements of the reference population of cells.

2. A method for determining the status of an individual, comprising,

obtaining a biological specimen from an individual;

applying the biological specimen, a derivative or fraction thereof, to a reference population of cells;

assessing the activation state of a plurality of activatable elements in the reference population of cells;

comparing activatable elements of the reference cell line that has been contacted with the biological specimen with activatable elements of the reference population of cells that has not been contacted with the biological specimen to determine the status of the individual.

3. A method for determining the status of an individual comprising:

obtaining one or more elements of a cellular environmental from the individual;

applying said element or elements to a reference population of cells;

determining the activation state of an intracellular activatable element in the reference population of cells;

classifying one or more cells of the reference population of cells into one or more classes based on the activation state; and

determining the status of the individual by linking the one or more classes to a clinical outcome.

4. A method for determining the status of an individual, comprising,

obtaining blood from the individual;

fractionating the blood into sera;

applying the sera to a reference population of cells;

assessing the activation state of a plurality of activatable elements in the reference population of cells;

comparing the activatable elements of the reference population of cells to that of the reference population of cells that has not been contacted with the sera.

5. A method in accordance with claim **1** wherein the biological specimen can contain a cellular environment which can comprise: sera, whole blood, ascites, plasma, cell extract, whole cells, lavage or rinse of cavities.

6. A method in accordance with claims **1** to **4** wherein the method is useful for therapeutic choice, disease diagnosis or prognosis.

7. A method in accordance with claims **1** to **4** wherein the reference population of cells is a homogeneous cell line, a defined mixture of homogeneous cell lines, a homogeneous cell population, a mixture of cells, or a library of cells.

8. A method in accordance with claims **1** to **3** wherein the biological specimen or cellular environment may be fractionated.

9. A method in accordance with claims **1** to **3** wherein the biological specimen or cellular environment may be fractionated into serum components or cellular components, wherein the serum components are selected from the group consisting of cytoldnes, hormones, chemoltines, an Igs, and wherein the cellular components are selected from a group consisting of white blood cells, dendritic cells, platelets, and red cells.

10. A method in accordance with claims **1** to **3** wherein the biological specimen or cellular environment modulator is the liquid environment that surrounds or surrounded cells from the individual.

11. A method in accordance with claims **1** to **4** wherein the individual has cancer, inflammatory, infectious, or an immunologic disease.

12. A method in accordance with claims **1** to **4** wherein the activation state of a reference population of cells that has not been contacted with the sera, or biological specimen, or cellular environment, is stored in a database.

13. A method in accordance with claims **2** or **4** wherein the comparisons between the activation state of the reference population of cells that has, and has not been contacted with the sera, or biological specimen, or cellular environment, is performed on a computer.

14. A method in accordance with claims **1** to **4** wherein the determination or assessment of the activation state of the cells is by flow cytometry.

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专利名称(译)	确定个人状态的方法		
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申请(专利权)人(译)	NODALITY INC.		
当前申请(专利权)人(译)	NODALITY INC.		
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摘要(译)

描述了基于使用生物样本确定个体状态的方法和参考细胞群的分析。