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(54) **METHOD FOR THE MULTIDIMENSIONAL DETECTION OF ABERRANT PHENOTYPES IN NEOPLASTIC CELLS TO BE USED TO MONITOR MINIMAL DISEASE LEVELS USING FLOW CYTOMETRY**

VERFAHREN ZUM MEHRDIMENSIONALEN NACHWEIS ANOMALER PHÄNOTYPEN IN NEOPLASTISCHEN ZELLEN ZUR VERWENDUNG BEI DER ÜBERWACHUNG MINIMALER KRANKHEITSNIVEAUS MITTELS DURCHFLUSSZYTOMETRIE

DETECTION MULTIDIMENSIONNELLE DE PHENOTYPES ABERRANTS DANS DES CELLULES NEOPLASIQUES POUR SURVEILLER DES NIVEAUX MINIMUM DE MALADIES A L'AIDE DE LA CYTOMETRIE DE FLUX

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## Description

### CROSS-REFERENCE TO RELATED APPLICATIONS

**[0001]** This application claims the benefit of Provisional Application Serial No. 60/451,738 filed Mar. 4, 2003.

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

**[0002]** This invention relates to the field of flow cytometry and more particularly to the sequential detection of aberrant patterns of protein expression on neoplastic cells and the identification of minimal numbers of neoplastic cells present among a major population of normal cells from blood, bone marrow, spinal fluid and lymph node samples. The invention enables: 1) the unequivocal identification of the aberrant phenotypes carried by neoplastic cells that allow their sensitive and specific identification, 2) the estimation of their utility for the identification of minimal numbers of neoplastic cells displaying an identically aberrant phenotype in another sample from the same individual obtained simultaneously or subsequently, and 3) the calculation of the distribution of neoplastic cells in blood, bone marrow, spinal fluid and lymph node samples both in terms of the percentage of cells and the number of cells per microliter of sample, by means of multiparameter flow cytometry analysis of the cells present in a sample stained with multiple combinations of monoclonal antibodies mixed with a quantified microbead (microparticle) suspension.

#### 2. Background of the Invention

**[0003]** In U.S. Pat. No. 5,047,321, Loken and Terstappen described the multiparameter analysis of cellular components in a body fluid. The body fluids described included blood and bone marrow. Using a combination of two nucleic acid dyes, a fluorescently labeled monoclonal antibody and two light scatter parameters, Loken and Terstappen were able to discriminate among various cellular components of blood and bone marrow, count the number of cells within each component and provide a differential analysis of each of them. Through the combined staining with the LDS-751 (Exciton) DNA-dye, the thiazol orange (TO, Molecular Probes, Inc) RNA-dye and a fluorescently labeled anti-CD45 monoclonal antibody, together with the measurement of forward and sideward light scatter on whole blood and bone marrow aspirates, these authors were able to identify and differentiate between, nucleated red cells, erythrocytes, reticulocytes, platelets, lymphocytes, monocytes, neutrophil granulocytes, basophilic granulocytes, eosinophilic granulocytes and precursors of all nucleated cells. However they could not show the ability of this approach to specifically differentiate between normal and neoplastic cells, coexisting in the same sample.

**[0004]** In U.S. Pat. No. 6,287,791, Terstappen and Chen describe a further refinement of the U.S. Pat. No. 5,047,321, but they did not show any better characterization of the different leukocyte populations.

5 **[0005]** In U.S. Pat. No. 5,137,809, Loken and Sha describe the multiparameter analysis of cellular components in bone marrow. The authors describe the use, in a first step, of a combination of monoclonal antibodies each labeled with a different fluorochrome, to stain all  
10 leukocytes and of further combinations to stain selected populations of leukocytes, in a second step.

**[0006]** All the methods described above were able to identify several populations of normal leukocytes present in blood and bone marrow samples and were only identifying selected subpopulations as identified by the specific combination of monoclonal antibodies and nucleic acid dyes used; nevertheless, they were not able to provide an approach for the specific and reproducible identification of neoplastic cells admixed naturally or artificially with normal cells in a sample. Also these approaches allow enumeration of the subpopulations of normal leukocytes identified in terms of percentage of total leukocytes. Moreover, by using these methods it is not possible to easily link and directly compare the information on the amount of light scatter and fluorescence measured for cells contained in a first sample to that of cells containing in a second different sample, especially if they derive from different tissues from the same individual, from different individuals or if they have been  
20 measured under different conditions.

**[0007]** In U.S. Pat. No. 5,627,037, Ward et al propose a one step technique for the calculation of the number of one or more cell populations contained in a given volume of a blood sample. This approach employs a mixture of reagents containing a mixture of one or more cell markers, a fluorescent quantified microparticle and a fixative. The technique described by Ward et al allows the calculation of the absolute counts of leukocytes, such as CD4+T-cells, but does not provide any specific indication of the exact procedures to be applied for the enumeration of individual subpopulations of blood leukocytes.

**[0008]** In the last decade many different reports have been published which show that neoplastic cells from a great majority of patients suffering from hematological malignancies display aberrant patterns of antigen expression as detected through the use of several triple and quadruple combinations of monoclonal antibodies analyzed by flow cytometry (Reviewed in Vidriales et al. Best Clin Res Pract, 2003; 16:599-612). These abnormal patterns of antigen expression are never detected in normal cells and they include one or more of the following subtypes: 1) cross-lineage antigen expression, 2) asynchronous antigen expression, 3) antigen over- and under-expression, 4) abnormally high or low light scatter properties, and 5) ectopic phenotypes. Based on these abnormalities several disease-type specific panels of three- and four-color combinations of monoclonal antibody reagents have been proposed for the systematic

identification of leukemic cells expressing aberrant phenotypes, in virtually every patient with precursor B-acute lymphoblastic leukemia (BCP-ALL; Lucio et al, Leukemia, 2001; 15: 1185-1192), T-ALL (Porwit-MacDonald, Leukemia, 2000; 14: 816-825), acute myeloblastic leukemia (AML; San Miguel et al, Blood, 2001; 98: 1746-1751), B-cell chronic lymphocytic leukemia (Rawstrom et al, Blood 2001; 98: 29-35) and other B-cell chronic lymphoproliferative disorders (Sanchez et al, Leukemia, 2002; 16: 1460-1469), among other diseases.

**[0009]** In all the approaches described so far for the identification of aberrant phenotypes expressed by neoplastic cells, data interpretation is carried out by an experienced person, with extensive knowledge on the patterns of protein expression on normal cells. Through this approach it has been shown that neoplastic cells from patients studied at first diagnosis and at relapse frequently show more than one aberrant phenotype, especially if relatively large panels of combinations of three or four monoclonal antibodies are used for their identification. Based on all the aberrant phenotypes detected at diagnosis, new 3- or 4-color combinations of monoclonal antibodies for the specific investigation of these neoplastic cell-specific phenotypes are designed and tested for their further use for the investigation of minimal infiltration by neoplastic cells in other samples obtained concurrently (e.g. for staging purposes) or subsequently (e.g. for monitoring the disease and the therapy). However, the need for data interpretation by an expert person with a high amount of knowledge and experience on the patterns of protein expression differentially observed in normal versus neoplastic cells, makes the identification of aberrant phenotypes subjective and difficult to reproduce. Moreover, many of these aberrant phenotypes are only present in a subset of all leukemic cells present in a given sample and they may change in the same patient, and even in another sample from the same tissue, with time. This further makes the identification of aberrant phenotypes, apart from being subjective, uncertain, with potentially occurring false negative and positive results. In addition, current knowledge about the phenotypes of normal cells from blood, bone marrow, spinal fluid and lymph nodes occurring at frequencies of less than 10.sup.-4 is very limited; this impacts negatively in the sensitivity of these approaches for detecting minimal numbers of neoplastic cells among a majority of normal blood and bone marrow cells which under the best technical and biological conditions, is currently of between 10.sup.-3 (detection of one neoplastic cells among 1000 normal cells) and 10.sup.-6 (one neoplastic cell in one million normal cells) depending on the exact lineage, type and maturation stage of the neoplastic cells, the aberrant phenotype used for the identification of the neoplastic cells, and the type of specimen studied.

#### DESCRIPTION OF THE INVENTION

**[0010]** The present invention relates to a new method

to improve the sensitivity, specificity and reproducibility of the detection of aberrant phenotypes expressed by neoplastic cells, wherein aberrant phenotypes refer to patterns of protein expression on neoplastic cells which differ from the pattern of normal or reactive non-neoplastic cells, present in bone marrow, peripheral blood, spinal fluid and lymph nodes.

**[0011]** The invention comprises the steps of: 1) separately stain one or more normal/reactive samples and a population of reference microparticles, and one neoplastic sample and a population of reference microparticles, with identical or at least partially overlapping multiple combinations of at least four monoclonal antibodies, each monoclonal antibody in each combination being conjugated to a different fluorochrome and each combination of monoclonal antibodies having in common at least three fluorochrome conjugated monoclonal antibodies, said monoclonal antibodies being capable of recognizing a different antigen which is expressed at different quantities on the various populations of leucocytes and on the populations of neoplastic cells in a sample, 2) sequentially measure at least two light scatter and at least four distinct fluorescence emissions associated to large numbers of cells stained with each of the combinations of monoclonal antibodies from the normal/reactive samples and the neoplastic sample, and the said populations of reference microparticles, using flow cytometry, 3) store two independent list mode data files each containing the information obtained as described in step (2) about the specific light scatter and fluorescence characteristics of each individual cell and reference microparticle analyzed, one containing information corresponding to the cells and reference microparticle from the normal/reactive samples and the other containing information corresponding to cells from the tumor sample and reference microparticle, 4) create new data files by mixing, at known proportions, list mode data on cellular events and reference microparticle events from the data file containing the light scatter and fluorescence information obtained as described in step (2) about the cells and reference microparticles present in the neoplastic sample into the data file containing information obtained as described in step (2) about the cells and reference microparticles present in the normal samples, wherein information from two different data files is merged after adjusting a relative position of populations of cellular events measured according to reference microparticles, 5) define in a multidimensional space generated by the flow cytometric measurements of light scatter and fluorescence emissions, those areas occupied by events corresponding to normal cells and those areas corresponding to empty spaces in normal/reactive samples and that may be occupied by tumor cells in neoplastic samples, 6) sequentially identify in the new merged data files as described in step (4) those events corresponding to neoplastic cells and those events corresponding to normal cells in a multidimensional space generated by the flow cytometric measurements of light scatter and fluores-

cence emissions, 7) establish the most relevant phenotypic aberrations displayed by the neoplastic cells as compared to their normal counterpart, that allow their unequivocal and clear sensitive and specific identification of those events corresponding to the neoplastic cells contained in the merged data file. This procedure simulates dilutional experiments of neoplastic cells in a normal sample through the use of computational procedures and could be used by mixing the information either directly from two or more distinct data files or after adjusting the relative position of the populations of cellular events measured according to pre-established standards.

**[0012]** As briefly described above, the first step of the method of this invention comprises staining of one or more normal/reactive samples and a population of reference microparticles, and of one neoplastic sample and a population of reference microparticles with multiple combinations of at least four monoclonal antibodies, each monoclonal antibody in each combination being conjugated to a different fluorochrome and each combination having in common a subgroup of at least three fluorochrome conjugated monoclonal antibodies, said monoclonal antibodies being capable of recognizing a different antigen which is expressed at different quantities on the various populations of leucocytes and on the populations of neoplastic cells in a sample.

**[0013]** The normal and neoplastic samples are of one of the following types: blood, bone marrow, spinal fluid and lymph nodes.

**[0014]** The neoplastic samples may contain hematopoietic tumor cells or neoplastic cells from any non-hematopoietic origin such as breast cancer, renal carcinomas, prostate tumors, lung cancer, bladder carcinomas and gastric tumors, among others.

**[0015]** The neoplastic samples are obtained at first diagnosis, relapse and at any time period after diagnosis; they may contain high numbers of neoplastic cells or minimal disease. They might have been stained directly after obtained and after being cultured in vitro.

**[0016]** The panel of combinations of monoclonal antibodies used to stain the neoplastic sample is identical to the panel of monoclonal antibody combinations used for the staining of the normal/reactive samples; alternatively the former panel can be shorter than the latter, but a part of the former panel is fully contained in the latter panel of combinations of monoclonal antibodies.

**[0017]** For each pair of panels used to stain normal/reactive and neoplastic samples, the clone of each monoclonal antibody reagent used in each individual combination of monoclonal antibodies, and the fluorochrome to which it is conjugated, are identical in the two panels of monoclonal antibody combinations.

**[0018]** The number of fluorescence components in each of the multiple combinations of monoclonal antibodies comprises at least four different fluorochromes, each linked to a different monoclonal antibody, whose fluorescence emission is distinguishable from that of the other fluorochrome-conjugated monoclonal antibodies. Fluorescence labels that can be used in the practice of this invention include fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin, alexa fluor 488, alexa 647, pacific blue, alexa fluor 405, cyanin 5 (Cy5), cyanin5.5 (Cy5.5) and conjugates thereof coupled to PE, to APC or to PerCP (e.g. PE/Cy5, PE/Cy5.5, PE/Cy7, APC/Cy7 and PerCP/Cy5.5). Each monoclonal antibody is capable of recognizing a different antigen, which is expressed in different quantities on the various populations of leukocytes and on the populations of neoplastic cells in a sample. The exact monoclonal antibodies which are common to all combinations of monoclonal antibodies used to stain a pair of normal/reactive and neoplastic samples may vary depending on the type, the lineage and the maturation stage of the tumor cells of interest, contained in the neoplastic sample.

**[0019]** Once selected the panel of multicolor combinations of monoclonal antibodies to be used for staining purposes, the cells in a known volume of sample are mixed together with a lysing solution, a fluorescent microparticle at known quantities and the mixture of monoclonal antibody reagents. For each of the multiple combinations of monoclonal antibodies used to stain a sample, a different aliquot containing a replicate of the sample mixed with a lysing solution, a fluorescent microparticle and the specific combination of monoclonal antibodies is used. After the nucleated cells are stained and the non-nucleated red cells are lysed, the sample can be washed and measured in a flow cytometer wherein the cells are passed one by one through one or more sensing regions, or it can be directly analyzed in a flow cytometer without mediating any washing step. Alternatively, the sample can be mixed together with only the monoclonal antibodies and a population of fluorescent microparticles and, once stained, measured directly in the flow cytometer.

**[0020]** The population of reference microparticles may be uniform or composed of multiple populations of microbeads of differing size, differing fluorescence amounts, density, shape, volume, adhesion characteristics and other physico-chemical properties. A population of microparticles may be composed of fluorescent particles, of microparticles whose surface is covered with anti-immunoglobulin antibodies or a mixture of both. In order to be able to calculate the absolute number of a population of cells present in a microliter of sample, a quantified population of microparticles in known numbers per volume of sample is added.

**[0021]** In each of the sensing regions of the flow cytometer, the cells and the microparticles are individually exposed to a source of light at a one or more wavelengths; for each stained cell in a sample of cells taken from blood, bone marrow, spinal fluid and lymph node and for each microparticle, at least two measures of light scatter are taken and at least four measures of fluorescence are taken. The information recorded for all light scatter and fluorescence measures of each individual cell and of each individual microparticle analyzed, is stored in a data

storage and analysis device, such as a computer. Information for each sample aliquot analyzed containing microbeads and cells stained with a combination of monoclonal antibodies, can be stored in a separate data file; however, in a preferred embodiment of this invention, information on the microbeads and the cells stained with all combinations of monoclonal antibodies used to stain a sample is stored in a single file through sequential acquisition of information on each separate aliquot of the sample/microparticle mixture stained with a different combination of monoclonal antibodies; specific identification of those events corresponding to each aliquot stained with a different combination of monoclonal antibodies that have been sequentially measured may be attained through the use of a parameter such as "time of acquisition". In any case, data on the normal/reactive samples and on the neoplastic sample are stored separately in different data files. In U.S. Pat. No. 4,284,412 and in U.S. Pat. No. 4,727,020, the configuration and the use of a typical flow cytometer instrument equipped with a data storage computer system and with a single and a double light source are described, respectively. In these systems, data is usually stored in a list mode FCS (flow cytometry standard) format. In this format, information for each individual event is listed for all parameters measured in a sequential way according to the sequence at which each cell and microparticle was measured in the flow cytometer.

**[0022]** If information from a single sample is contained in independent data files each of the data files containing information on the light scatter and fluorescence characteristics of those events corresponding to a mixture of microparticles and cells stained with a specific combination of monoclonal antibodies—information from all data files corresponding to the multiple combinations of monoclonal antibodies with which the sample has been stained, is merged into a single data file. In every data file resulting from merging the data contained in two or more different data files, the information corresponding to each individual data file merged is ordered in a separate list before or after the list mode data containing information on the events of the other data files with which it has been merged.

**[0023]** A data file containing information about cells from normal/reactive samples that have been measured in a flow cytometer may be composed of information obtained after merging multiple data files corresponding to an identical number of sample aliquots each stained with a different combination of monoclonal antibodies, all of which correspond either to one single normal/reactive sample or to multiple normal/reactive samples. A data file resulting from merging the information collected for two or more normal/reactive samples, each stained separately with an identical panel of multiple combinations of monoclonal antibodies, is composed of normal/reactive samples from randomly selected individuals, from individuals from a well-defined age interval or from any other group of individuals defined according to their age,

gender, and underlying non-neoplastic conditions, among other characteristics.

**[0024]** For the identification of the aberrant phenotypes expressed by neoplastic cells which have been stained with a specific panel of multiple combinations of monoclonal antibodies, new data files are generated by mixing at well known ratios data corresponding to cellular events and reference microparticle events from the data file containing the light scatter and fluorescence information obtained as described in step (2) about the cells and reference microparticles present in the neoplastic sample into the data file containing information on the cells and reference microparticles present in the normal/reactive samples, wherein information from two different data files is merged after adjusting a relative position of populations of cellular events measured according to reference microparticles. In the merged data file containing information on cellular events from both the neoplastic sample and the normal/reactive samples, events corresponding to each of the two data files are ordered one after the other in a list mode data format and they may be labeled differently in any multidimensional space generated by the flow cytometric measurements of light scatter and fluorescence emissions, using software tools (e.g. dots painted in different colors).

**[0025]** If data files containing information on the light scatter and fluorescence characteristics of cells from multiple different samples are merged, and they have been acquired under different conditions (e.g. distinct instrument settings), in different flow cytometry instruments or at different times (e.g. in different days, months or years), the relative position of each cellular event in the multidimensional space formed by all parameters measured (light scatter and fluorescence emissions), is adjusted automatically by software operations based on the relative changes observed in the position of the populations of internal reference microparticles measured simultaneously with the cells, for each of the samples. After adjusted, the normalization process is controlled because the corrected position of those events corresponding to the microparticles and to identical populations of normal/reactive cells contained in the multiple samples merged, overlaps.

**[0026]** Different data files containing information about cellular events measured in one or more normal samples and one neoplastic sample may be generated. For that purpose all the information corresponding to the whole cellularity measured in the neoplastic sample or different parts of it corresponding to the whole information measured in variable numbers of randomly selected events representative of the neoplastic cells present in this file are added to the information of the data file obtained after measuring normal/reactive samples. This procedure simulates dilutional experiments of neoplastic cells in a normal sample through the use of computational procedures. Accordingly, serial dilutions of data about individual events of for instance 1/1, 1/10, 1/100, 1/1000, 1/10000, 1/100000, 1/1000000, and 1/10000000

(events/events) can be created by merging data from a file corresponding to normal/reactive samples with data from a file corresponding to a neoplastic sample.

**[0027]** Once the data files from normal/reactive samples and from a neoplastic sample have been merged into a single data file, the neoplastic cells present in this new merged data file are identified using software tools, as those populations of at least 15 events falling into the spaces that remain empty in the initial file containing only information on the cells of the normal/reactive samples. By quantifying the amount of light scatter and fluorescence measured for each individual population of neoplastic cells as compared to those populations of normal cells stained with identical panels, statistical information on the most discriminant aberrant phenotypes displayed by the neoplastic cells can be derived. Each aberrant phenotype may be composed of a combination of two or more light scatter and fluorescence measures and it allows the unequivocal, sensitive and specific identification of those events corresponding to the neoplastic cells present in the merged data file as being different from all populations of events corresponding to normal/reactive cells contained in the same merged data file.

**[0028]** The procedure of this invention could also be used to detect abnormal patterns of antigen expression to monitor cell activation and a cell function.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

**[0029]** The invention will be illustrated by two examples which do not limit its areas of application as follows

##### EXAMPLE 1.

###### 1. Samples

**[0030]** Five mL of peripheral blood (PB) was obtained by venipuncture from 10 healthy volunteers and placed in VACUTAINER.TM. (Becton Dickinson, New Jersey, N.J.) tube containing EDTA as anticoagulant. In addition 5 mL of a PB sample from a patient diagnosed with B-cell chronic lymphocytic leukemia (B-CLL) with an absolute lymphocytosis of  $5 \times 10^9/L$  were also obtained.

###### 2. Sample Preparation

**[0031]** After gentle mixing the sample, 200  $\mu$ L of each PB sample containing between  $10 \times 10^6$  and  $2 \times 10^6$  nucleated cells was placed in six different replicate tubes and washed for 5 minutes at 540 g with 2 mL/tube of phosphate buffered saline. Then, to each tube from each PB sample one of the following five-color combinations of monoclonal antibodies was added, each monoclonal antibody conjugated with a different fluorochrome (FITC/PE/PE-Texas red/PerCP-Cy5.5/APC) being added at saturating amounts in a vol-

ume of 5  $\mu$ L: 1) CD22/CD23/CD19/CD45/CD5, 2) CD43/CD79b/CD19/CD45/CDS, 3) anti-Lambda/antika-ppa/CD19/CD45/CDS, 4) anti-IgM/CD27/CD19/CD45/CDS, 5) CD1c/CD10/CD19/CD45/CDS, 6) CD103/CD25/CD19/CD45/CD5 and, 7) CD20/Zap70/CD19/CD45/CD5. After gently mixing the samples were incubated for 15 minutes at room temperature in the darkness. After this incubation, 200  $\mu$ L of a microbead suspension (PerfectCOUNT, Cytognos, Salamanca, Spain), containing  $2 \times 10^3$  microbeads was added to each replicate tube.

###### 3. Data Acquisition

**[0032]** After gentle mixing the light scatter and fluorescence of the stained cells were measured in a FACSAria flow cytometer (Becton/Dickinson Biosciences BDB-, San Jos, Calif.) equipped with two emission laser lights tuned at 488 and 635 nm using the FACS DIVA (BDB) software. For each sample analyzed a single data file was collected which contained information on the forward (FSC) and sideward light scatter (SSC), and the fluorescence emissions of FITC, PE, PE-texas red, PE-Cy5.5 and APC for each cell and microbead analysed. In addition information corresponding to the time (of acquisition parameter) was also recorded. For each sample acquisition was performed for the different tubes in the sequence defined in the above section about "sample preparation" using CD45-associated red fluorescence as threshold. For each combination, information on 500.000 CD45+ cells was acquired. Acquisition was stopped between two consecutive tubes for 10 seconds.

###### 4.- Data Manipulation and Data Analysis

**[0033]** Prior to data analysis, list mode data contained in all the 10 files corresponding to the 10 normal PB samples was merged in an ordered way from sample 1 to sample 10 in a single data file. For that purpose data corresponding to the measures of FSC, SSC, FITC, PE, PE-Texas red, PE-Cy5.5 and APC fluorescence emission for each microbead and cellular event was introduced into a data base after being transformed into channel values (scaled from 0 to 2048) using the alphaFACS assistant 1.1a software (University of Stanford, Stanford, Calif.). An algorithm was then built based on the data from the normal sample using the Lymphogram B software (Version: beta-test, Cytognos, Salamanca, Spain) to identify through clustering analysis, all different populations of normal cells identified with the common parameters measured for all sample aliquots (FSC, SSC, CD19, CD45, CD5) as well as the microparticles measured in each sample aliquot. Statistical data of each cell population for each measured parameter was then used to estimate the multi-dimensional probability distribution function, e.g. by using Parzen windows (Duda R. O. et al., Pattern Classification, John Wiley & Sons 2001). One

or more prototypes (codebook vectors) are set to be the representatives of each population of cells and microbeads present in the data file collected for normal samples.

**[0034]** In parallel, a similar analysis was performed with the B-CLL datafile. The estimated distribution of the PerfectCount microbead particles obtained in the B-CLL data file was then compared to the position obtained in the Normal datafile. In the B-CLL the microbeads gave an identical distribution but in a distinct position in the space  $R_{sup.7}$ , shifts of different magnitude being observed in each dimension. Based on the shifts observed a correction function was applied and the position of all events in the B-CLL datafile was adjusted according to this function. After adjusted the new values for the events corresponding to the distribution of the populations of normal residual CD5+/CD45+/CD19 T-cells and of normal residual CD45+/SSChigh neutrophils coexisting with the neoplastic B-CLL cells were calculated in the B-CLL datafile and compared with those obtained for the same normal populations present in the Normal datafile. The distribution of both populations of events was identical in both data files ( $p > 0.05$ ). Then, the multi-dimensional statistical data of all cellular and microbead populations, including the neoplastic B-cell population was used to build up one or more prototypes and to estimate probability distribution function of the population of neoplastic B-CLL cells, in addition to those corresponding to the normal residual populations of cellular events present in the same data file.

**[0035]** After this step, a copy of the data file containing information on the 10 normal PB samples (NORMAL data file) was merged at different proportions with data corresponding to events from the B-CLL data file after adjusting the values for the position of each event from this datafile as described above. The following dilution factors of events from the datafile corresponding to the adjusted B-CLL datafile into the NORMAL data file were done: 99/1, 1/1, 1/10, 1/100, 1/1000, 1/10000, 1/100000, 1/1000000, 10000000 (NORMAL/B-CLL data files. Then, clustering analysis was performed for the merged NORMAL/B-CLL data files as described above. In all NORMAL/B-CLL data files, a population of CD5+/CD19+/CD45 high/CD20 low/CD11c-/CD22-/CD23+/CD43+/CD79b low/IgM low/CD27+/CD103-/CD25 low/kappa low/lambda-/ZAP70-/CD10- neoplastic B-cells, which was absent in the NORMAL data file, was identified. From the other populations, the most closely related one was represented by normal CD5+/CD19+/CD45 high B-cells. This latter population could be clearly distinguished from the former based on the combination of CD20, CD22, CD79b, CD43, CD5 and CD25 (markers that would define the aberrant phenotype of the neoplastic B-CLL cells).

**[0036]** Accordingly, while only a few embodiments of the present invention have been shown and described, it is obvious that many changes and modifications may be made thereunto without departing from the scope of

the invention.

## Claims

1. A method for the identification of aberrant phenotypes, wherein aberrant phenotypes refer to patterns of protein expression on neoplastic cells which differ from the pattern of normal or reactive non-neoplastic cells, wherein the method comprises the steps of: a) separately staining one or more normal/reactive samples and a population of reference microparticles, and one neoplastic sample and a population of reference microparticles with overlapping multiple combinations of at least four monoclonal antibodies, each monoclonal antibody in each combination being conjugated to a different fluorochrome and each combination having in common at least three fluorochrome conjugated monoclonal antibodies, said monoclonal antibodies being capable of recognizing a different antigen which is expressed at different quantities on the various populations of leucocytes and on the populations of neoplastic cells in a sample; b) sequentially measuring at least two light scatter and at least four distinct fluorescence emissions associated to large numbers of cells stained with each of the combinations of monoclonal antibodies from the normal/reactive samples and the neoplastic sample, and the said populations of reference microparticles, using flow cytometry; c) storing two independent list mode data files, one containing the information obtained as described in step (b) about the specific light scatter and fluorescence characteristics of each cell and reference microparticle analyzed from the normal/reactive samples and the other containing the information obtained as described in step (b) about the specific light scatter and fluorescence characteristics of each cell and reference microparticle analyzed from the neoplastic sample; d) creating new data files by merging, at known proportions the cellular events and reference microparticle events from the data file containing the light scatter and fluorescence information obtained as described in step (b) about the cells and reference microparticles present in the neoplastic sample into the data file containing the information obtained as described in step (b) about the cells and reference microparticles present in the normal/reactive samples; wherein information from two different data files is merged after adjusting a relative position of populations of cellular events measured according to reference microparticles; e) defining in a multidimensional space generated by the flow cytometric measurements of light scatter and fluorescence emissions, those areas occupied by events corresponding to normal cells and those areas corresponding to empty spaces in normal/reactive samples and that may be occupied by tumor cells in neoplastic sam-

- ples; f) sequentially identifying in the data files merged as described in step d), those events corresponding to neoplastic cells as different from the events corresponding to normal cells coexisting in a multidimensional space generated by the flow cytometric measurements of light scatter and fluorescence emissions; and g) establishing the most relevant phenotypic aberrations displayed by the neoplastic cells as compared to their normal counterpart, that allow their unequivocal, sensitive and specific identification in the merged data file.
2. The method of claim 1, wherein the samples comprise peripheral blood.
  3. The method of claim 1, wherein the samples comprise bone marrow.
  4. The method of claim 1, wherein the samples comprise spinal fluid.
  5. The method of claim 1, wherein the samples comprise lymph node.
  6. A method according to any of the previous claims, wherein more than one randomly selected normal sample is stained.
  7. A method according to any of the previous claims, wherein more than one normal sample is stained, all normal samples being selected from a well defined age group of individuals, or from any other group of individuals defined according to their gender and underlying non-neoplastic conditions.
  8. A method according to any of the claims 1 to 5, wherein the neoplastic samples contain hematopoietic tumor cells of one or more different types.
  9. A method according to any of the claims 1 to 5 and 8, wherein the neoplastic samples contain non-hematopoietic tumor cells of one or more different types.
  10. A method according to any of the claims 1 to 5 and 8 to 9, wherein the neoplastic samples contain both hematopoietic neoplastic cells and non-hematopoietic tumor cells.
  11. A method according to any of the claims 1 to 5 and 8 to 10, wherein the neoplastic samples are obtained at first diagnosis, relapse and at any time period after diagnosis.
  12. A method according to any the claims 1 to 5 and 8 to 11, wherein the neoplastic samples may contain high or minimal numbers of neoplastic cells.
  13. A method according to any of the previous claims, wherein the samples are stained directly after being obtained.
  14. A method according to any of the claims 1 to 12, wherein the samples are stained after being cultured in vitro.
  15. A method according to any of the previous claims, wherein the panels of multiple combinations of monoclonal antibodies used to stain neoplastic samples and normal/reactive samples are identical.
  16. A method according to any of the previous claims, wherein the panel of multiple combinations of monoclonal antibodies used to stain neoplastic samples are shorter than the panel of combinations of monoclonal antibodies used to stain normal/reactive samples, but the former panel is fully contained in the latter one.
  17. A method according to any of the previous claims, wherein for each pair of panels of combinations of monoclonal antibodies, an exact clone of each monoclonal antibody used in each individual combination of monoclonal antibodies, and a fluorochrome to which it is conjugated, are identical in the two panels of combinations of monoclonal antibodies.
  18. A method according to any of the previous claims, wherein a combination of compatible fluorochromes is selected from fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin chlorophyll protein (PerCP), allophycocyanin, alexa fluor 488, alexa 647, pacific blue, alexa fluor 405, cyanin 5 (Cy5, cyanin5.5(Cy5.5) and conjugates thereof coupled to PE, to APC or to PerCP (PE/Cy5, PE/Cy5.5, PE/Cy7, APC/Cy7 and PerCP/Cy5.5) or any additional compatible fluorochrome or fluorochrome-conjugates.
  19. A method according to any of the previous claims, wherein information on the light scatter and the fluorescence measures of those events stained with each combination of monoclonal antibodies conjugated to fluorochromes contained in a panel used to stain different replicate aliquots of the same sample are initially stored in separate data files.
  20. A method according to any of the claims 1 to 18, wherein information on the light scatter and the fluorescence measures of those events stained with all different combinations of monoclonal antibodies conjugated to fluorochromes contained in a panel used to stain different replicate aliquots of the same sample are stored in a single data file.
  21. A method according to any of the claims 1 to 19, wherein information from two distinct data files is

merged directly without any correction.

22. A method according to claim 1, wherein the population of reference microparticles is uniform.
23. A method according to any of the claims 1 and 22, wherein a population of reference microparticles is composed of multiple populations of microbeads of differing size, density, volume, shape, amount of fluorescence, adhesion characteristics or other physico-chemical properties.
24. A method according to any of the claims 1 and 22 to 23, wherein a population of microparticles is composed of fluorescent particles.
25. A method according to any of the claims 1 and 22 to 23, wherein a population of microparticles is composed of microparticles whose surface is covered with anti-immunoglobulin antibodies.
26. A method according to any of the claims 1 and 22 to 23, wherein a population of microparticles is composed of a mixture of fluorescent microparticles whose surface is covered with anti-immunoglobulin antibodies.
27. A method according to any of the claims 1 and 22 to 26, wherein the microparticles are added in known numbers.
28. A method according to any of the previous claims, wherein serial dilutions of events from a data file corresponding to a neoplastic sample stained with a panel of multiple monoclonal antibody combinations into a data file containing information on the light scatter and fluorescence measures of cells contained in one or more normal/reactive samples stained with an identical panel of monoclonal antibodies, are made in step (d) of the procedure described in claim 1, to evaluate the sensitivity at which a small number of neoplastic cells could be detected once diluted in normal/reactive cells at predefined known proportions.
29. A method according to any of the claims 1 to 28, wherein abnormal patterns of antigen expression related to cell activation and a cell function are detected.

#### Patentansprüche

1. Verfahren zur Identifizierung aberranter Phänotypen, wobei es sich bei den aberranten Phänotypen um Proteinexpressionsmuster auf neoplastischen Zellen handelt, die sich vom Muster der normalen oder reaktiven nicht neoplastischen Zellen unter-

scheiden, wobei das Verfahren folgende Schritte umfasst: a) getrenntes Färben einer oder mehrerer normaler/ reaktiver Proben und einer Population von Referenzmikropartikeln sowie einer neoplastischen Probe und einer Population von Referenzmikropartikeln mit einer Vielzahl von überlappenden Kombinationen aus mindestens vier monoklonalen Antikörpern, wobei jeder monoklonale Antikörper in jeder Kombination mit einem unterschiedlichen Fluorochrom konjugiert ist und jede Kombination mindestens drei Fluorochrom-konjugierte monoklonale Antikörper gemein hat, wobei besagte monoklonale Antikörper in der Lage sind, ein unterschiedliches Antigen zu erkennen, das in unterschiedlichen Mengen auf den verschiedenen Leukozyten-Populationen und auf den Populationen der neoplastischen Zellen in einer Probe exprimiert wird; b) sequenzielles Messen mittels Durchflusszytometrie von mindestens zwei Lichtstreuungen und mindestens vier verschiedenen Fluoreszenz-Emissionen, die mit einer großen Anzahl an mit jeder der Kombinationen aus monoklonalen Antikörpern gefärbten Zellen aus den normalen/ reaktiven Proben und der neoplastischen Probe sowie den besagten Populationen von Referenzmikropartikeln einhergehen; c) Speichern von zwei unabhängigen Dateien im Listenmodus, wobei die eine die wie in Schritt (b) beschrieben erhaltenen Informationen über die spezifischen Lichtstreuungs- und Fluoreszenzeigenschaften jeder analysierten Zelle und jedes analysierten Referenzmikropartikels aus den normalen/ reaktiven Proben enthält und die andere die wie in Schritt (b) beschrieben erhaltenen Informationen über die spezifischen Lichtstreuungs- und Fluoreszenzeigenschaften jeder analysierten Zelle und jedes analysierten Referenzmikropartikels aus der neoplastischen Probe enthält; d) Erstellen neuer Dateien durch Zusammenfügen, in bekannten Anteilen, der zellulären Ereignisse und der Ereignisse der Referenzmikropartikel aus der Datei, die die wie in Schritt (b) beschrieben erhaltenen Lichtstreuungs- und Fluoreszenzinformationen über die in der neoplastischen Probe vorhandenen Zellen und Referenzmikropartikel enthält, in die Datei, die die wie in Schritt (b) beschrieben erhaltenen Informationen über die in den normalen/ reaktiven Proben vorhandenen Zellen und Referenzmikropartikel enthält; wobei die Informationen aus zwei verschiedenen Dateien nach dem Einstellen einer relativen Position der Populationen der gemäß den Referenzmikropartikeln gemessenen zellulären Ereignisse zusammengefügt werden; Definieren in einem multidimensionalen, durch die durchflusszytometrischen Messungen der Lichtstreuung und Fluoreszenz-Emissionen erzeugten Raum derjenigen Bereiche, die durch die den normalen Zellen entsprechenden Ereignisse besetzt sind, und derjenigen Bereiche, die leeren Räumen in normalen/ reaktiven Proben entsprechen und in neoplastischen Proben durch Tu-

- morzellen besetzt sein können; f) sequenzielles Identifizieren in den wie in Schritt d) beschrieben zusammengeführten Dateien derjenigen den neoplastischen Zellen entsprechenden Ereignisse, die von den normalen Zellen entsprechenden Ereignissen verschieden sind, die in einem multidimensionalen Raum koexistieren, der durch die durchflusszytometrischen Messungen der Lichtstreuung und der Fluoreszenz-Emissionen erzeugt wird; und g) Feststellen der relevantesten phänotypischen Aberrationen, die die neoplastischen Zellen im Vergleich zu ihren normalen Gegenstücken zeigen und die deren eindeutige, empfindliche und spezifische Identifizierung in der zusammengeführten Datei ermöglichen.
2. Verfahren nach Anspruch 1, wobei die Proben peripheres Blut umfassen.
  3. Verfahren nach Anspruch 1, wobei die Proben Knochenmark umfassen.
  4. Verfahren nach Anspruch 1, wobei die Proben Rückenmarksflüssigkeit umfassen.
  5. Verfahren nach Anspruch 1, wobei die Proben Lymphknoten umfassen.
  6. Verfahren nach einem der vorangehenden Ansprüche, wobei mehr als eine normale, zufällig ausgewählte Probe gefärbt wird.
  7. Verfahren nach einem der vorangehenden Ansprüche, wobei mehr als eine normale Probe gefärbt wird und alle normalen Proben aus einer Gruppe von Individuen einer definierten Altersstufe oder aus einer anderen Gruppe von Individuen, die gemäß ihrem Geschlecht und ihren nicht neoplastischen Grunderkrankungen definiert ist, ausgewählt werden.
  8. Verfahren nach einem der Ansprüche 1 bis 5, wobei die neoplastischen Proben eine oder mehrere verschiedene Arten von hämatopoetischen Tumorzellen enthalten.
  9. Verfahren nach einem der Ansprüche 1 bis 5 und 8, wobei die neoplastischen Proben eine oder mehrere verschiedene Arten von nicht hämatopoetischen Tumorzellen enthalten.
  10. Verfahren nach einem der Ansprüche 1 bis 5 und 8 bis 9, wobei die neoplastischen Proben sowohl hämatopoetische neoplastische Zellen als auch nicht hämatopoetische Tumorzellen enthalten.
  11. Verfahren nach einem der Ansprüche 1 bis 5 und 8 bis 10, wobei die neoplastischen Proben bei der ersten Diagnose, bei einem Rückfall und innerhalb eines Zeitraums nach der Diagnose entnommen werden.
  12. Verfahren nach einem der Ansprüche 1 bis 5 und 8 bis 11, wobei die neoplastischen Proben eine hohe oder eine minimale Anzahl neoplastischer Zellen enthalten können.
  13. Verfahren nach einem der vorangehenden Ansprüche, wobei die Proben direkt nach deren Entnahme gefärbt werden.
  14. Verfahren nach einem der Ansprüche 1 bis 12, wobei die Proben gefärbt werden, nachdem sie *in vitro* gezüchtet wurden.
  15. Verfahren nach einem der vorangehenden Ansprüche, wobei die Gruppen zahlreicher Kombinationen aus monoklonalen Antikörpern, die zur Färbung der neoplastischen Proben und der normalen/reaktiven Proben eingesetzt werden, identisch sind.
  16. Verfahren nach einem der vorangehenden Ansprüche, wobei die Gruppe der zahlreichen Kombinationen aus monoklonalen Antikörpern, die zur Färbung der neoplastischen Proben eingesetzt werden, kleiner ist als die Gruppe der Kombinationen aus monoklonalen Antikörpern, die zur Färbung der normalen/reaktiven Proben eingesetzt werden, und wobei aber die erstgenannte Gruppe in der letzteren vollständig enthalten ist.
  17. Verfahren nach einem der vorangehenden Ansprüche, wobei für jedes Gruppenpaar der Kombinationen aus monoklonalen Antikörpern ein exakter Klon von jedem monoklonalen Antikörper, der in jeder einzelnen Kombination aus monoklonalen Antikörpern verwendet wird, und ein Fluorochrom, mit dem er konjugiert ist, in den beiden Gruppen von Kombinationen aus monoklonalen Antikörpern identisch sind.
  18. Verfahren nach einem der vorangehenden Ansprüche, wobei eine Kombination aus kompatiblen Fluorochromen aus Fluoreszein-Isothiocyanat (FITC), Phycoerythrin (PE), Peridinin-Chlorophyll-Protein (PerCP), Allophycocyanin, Alexa Fluor 488, Alexa 647, Pazifikblau, Alexa Fluor 405, Cyanin 5 (Cy5), Cyanin 5.5 (Cy5.5) und Konjugaten davon ausgewählt wird, die an PE, an APC oder an PerCP (PE/Cy5, PE/Cy5.5, PE/Cy7, APC/Cy7 und PerCP/Cy5.5) oder an ein zusätzliches kompatibles Fluorochrom oder an Fluorochrom-Konjugate gekoppelt sind.
  19. Verfahren nach einem der vorangehenden Ansprüche, wobei die Informationen über die Lichtstreuungs- und Fluoreszenzmessungen derjenigen Ereignisse, die mit jeder einzelnen Kombination aus

- Fluorochrom-konjugierten monoklonalen Antikörpern gefärbt wurden, die in einer Gruppe enthalten sind, die zur Färbung verschiedener wiederholter Aliquote derselben Probe verwendet wurde, anfänglich in getrennten Dateien gespeichert werden. 5
20. Verfahren nach einem der Ansprüche 1 bis 18, wobei die Informationen über die Lichtstreuungs- und Fluoreszenzmessungen derjenigen Ereignisse, die mit all den unterschiedlichen Kombinationen aus Fluorochrom-konjugierten monoklonalen Antikörpern gefärbt wurden, die in einer Gruppe enthalten sind, die zur Färbung verschiedener wiederholter Aliquote derselben Probe verwendet wurde, in einer einzigen Datei gespeichert werden. 10
21. Verfahren nach einem der Ansprüche 1 bis 19, wobei die Informationen aus den beiden unterschiedlichen Dateien direkt und ohne Korrektur zusammengefügt werden. 20
22. Verfahren nach Anspruch 1, wobei die Population von Referenzmikropartikeln einheitlich ist. 25
23. Verfahren nach einem der Ansprüche 1 und 22, wobei eine Population von Referenzmikropartikeln aus zahlreichen Populationen von Mikroperlen unterschiedlicher Größe, Dichte, Volumen, Form, Fluoreszenzmenge, Haftungseigenschaften oder anderen physikalischen und chemischen Eigenschaften besteht. 30
24. Verfahren nach einem der Ansprüche 1 und 22 bis 23, wobei eine Population von Mikropartikeln aus fluoreszierenden Partikeln besteht. 35
25. Verfahren nach einem der Ansprüche 1 und 22 bis 23, wobei eine Population von Mikropartikeln aus Mikropartikeln besteht, deren Oberfläche mit Anti-Immunglobulin-Antikörpern bedeckt ist. 40
26. Verfahren nach einem der Ansprüche 1 und 22 bis 23, wobei eine Population von Mikropartikeln aus einer Mischung fluoreszierender Mikropartikel besteht, deren Oberfläche mit Anti-Immunglobulin-Antikörpern bedeckt ist. 45
27. Verfahren nach einem der Ansprüche 1 und 22 bis 26, wobei die Mikropartikel in einer bekannten Anzahl hinzugefügt werden. 50
28. Verfahren nach einem der vorangehenden Ansprüche, wobei in Schritt (d) des in Anspruch 1 beschriebenen Verfahrens Verdünnungsreihen der Ereignisse aus einer Datei, die einer mit einer Gruppe von zahlreichen Kombinationen aus monoklonalen Antikörpern gefärbten neoplastischen Probe entspricht, in einer Datei, die Informationen über die Lichtstreu-

ungs- und Fluoreszenzmessungen der Zellen enthält, die in einer oder mehreren mit einer identischen Gruppe monoklonaler Antikörper gefärbten normalen/ reaktiven Proben enthalten sind, angelegt werden, um die Empfindlichkeit zu beurteilen, mit der eine kleine Anzahl neoplastischer Zellen nachgewiesen werden könnte, nachdem sie in normalen/ reaktiven Zellen zu vorbestimmten bekannten Anteilen verdünnt wurden.

29. Verfahren nach einem der Ansprüche 1 bis 28, wobei anormale Expressionsmuster von Antigenen, die mit Zellaktivierung und einer Zellfunktion zusammenhängen, nachgewiesen werden. 15

### Revendications

1. Un procédé pour l'identification de phénotypes aberrants, dans lequel les phénotypes aberrants se réfèrent à des modèles d'expression de protéines sur des cellules néoplasiques qui diffèrent du modèle de cellules normales ou réactives non néoplasiques, dans lequel le procédé comprend les étapes consistant à : a) colorer séparément un ou plusieurs échantillons normaux/réactifs et une population de microparticules de référence, et un échantillon néoplasique et une population de microparticules de référence avec des combinaisons multiples se chevauchant d'au moins quatre anticorps monoclonaux, chaque anticorps monoclonal dans chaque combinaison étant conjugué à un fluorochrome différent et chaque combinaison ayant en commun au moins trois anticorps monoclonaux conjugués à un fluorochrome, lesdits anticorps monoclonaux étant capables de reconnaître un antigène différent qui est exprimé en différentes quantités sur les diverses populations de leucocytes et sur les populations de cellules néoplasiques dans un échantillon ; b) mesurer séquentiellement au moins deux diffusions de lumière et au moins quatre émissions de fluorescence distincte associées à de grands nombres de cellules colorées avec chacune des combinaisons d'anticorps monoclonaux provenant des échantillons normaux/réactifs et de l'échantillon néoplasique, et lesdites populations de microparticules de référence, en utilisant la cytométrie en flux ; c) sauvegarder deux fichiers de données en mode liste indépendants, l'un contenant les informations obtenues de la façon décrite dans l'étape b) sur les caractéristiques spécifiques de diffusion de lumière et de fluorescence de chaque cellule et microparticule de référence analysées des échantillons normaux/réactifs et l'autre contenant les informations obtenues de la façon décrite dans l'étape b) sur les caractéristiques spécifiques de diffusion de lumière et de fluorescence de chaque cellule et microparticule de référence analysées de l'échantillon néoplasique ; d) créer de nouveaux fi-

- chiers de données en fusionnant, dans des proportions connues les événements cellulaires et les événements de microparticules de référence provenant du fichier de données contenant les informations de diffusion de lumière et de fluorescence obtenues de la façon décrite dans l'étape b) sur les cellules et microparticules de référence présentes dans l'échantillon néoplasique dans le fichier de données contenant les informations obtenues de la façon décrite dans l'étape b) sur les cellules et microparticules de référence présentes dans les échantillons normaux/réactifs ; dans lequel les informations provenant de deux fichiers de données différents sont fusionnées après l'ajustement d'une position relative de populations d'événements cellulaires mesurée selon des microparticules de référence ; e) définir dans un espace multidimensionnel généré par des mesures cytométriques en flux de diffusion de lumière et d'émissions de fluorescence, les zones occupées par des événements correspondant à des cellules normales et les zones correspondant à des espaces vides dans des échantillons normaux/réactifs et qui peuvent être occupés par des cellules tumorales dans des échantillons néoplasiques ; f) identifier séquentiellement dans les fichiers de données fusionnés de la façon décrite dans l'étape d), les événements correspondant à des cellules néoplasiques en tant qu'ils sont différents des événements correspondant à des cellules normales coexistant dans un espace multidimensionnel généré par les mesures cytométriques en flux de diffusion de lumière et d'émissions de fluorescence ; et g) déterminer les aberrations phénotypiques les plus pertinentes présentées par les cellules néoplasiques par comparaison avec leur contrepartie normale, qui permettent leur identification non équivoque, sensible et spécifique dans le fichier de données fusionnées.
2. Le procédé de la revendication 1, dans lequel les échantillons comprennent du sang périphérique.
  3. Le procédé de la revendication 1, dans lequel les échantillons comprennent de la moelle osseuse.
  4. Le procédé de la revendication 1, dans lequel les échantillons comprennent du liquide céphalo-rachidien.
  5. Le procédé de la revendication 1, dans lequel les échantillons comprennent un ganglion lymphatique.
  6. Un procédé selon n'importe laquelle des revendications précédentes, dans lequel plus d'un échantillon normal sélectionné de manière aléatoire est coloré.
  7. Un procédé selon n'importe laquelle des revendications précédentes, dans lequel plus d'un échantillon normal est coloré, tous les échantillons normaux étant sélectionnés dans un groupe d'âge bien défini d'individus, ou dans tout autre groupe d'individus définis selon leur sexe et leurs états non néoplasiques latents.
  8. Un procédé selon n'importe laquelle des revendications 1 à 5 et 8, dans lequel les échantillons néoplasiques contiennent des cellules tumorales hématopoïétiques d'un ou de plusieurs types différents.
  9. Un procédé selon n'importe laquelle des revendications 1 à 5 et 8, dans lequel les échantillons néoplasiques contiennent des cellules tumorales non hématopoïétiques d'un ou de plusieurs types différents.
  10. Un procédé selon n'importe laquelle des revendications 1 à 5 et 8 à 9, dans lequel les échantillons néoplasiques contiennent à la fois des cellules néoplasiques hématopoïétiques et des cellules tumorales non hématopoïétiques.
  11. Un procédé selon n'importe laquelle des revendications 1 à 5 et 8 à 10, dans lequel les échantillons néoplasiques sont obtenus lors du premier diagnostic, de la récurrence et à n'importe quelle période après le diagnostic.
  12. Un procédé selon n'importe laquelle des revendications 1 à 5 et 8 à 11, dans lequel les échantillons néoplasiques peuvent contenir des nombres élevés ou minima de cellules néoplasiques.
  13. Un procédé selon n'importe laquelle des revendications précédentes, dans lequel les échantillons sont colorés directement après avoir été obtenus.
  14. Un procédé selon n'importe laquelle des revendications 1 à 12, dans lequel les échantillons sont colorés après avoir été cultivés in vitro.
  15. Un procédé selon n'importe laquelle des revendications précédentes, dans lequel les panels de combinaisons multiples d'anticorps monoclonaux utilisés pour colorer des échantillons néoplasiques et des échantillons normaux/réactifs sont identiques.
  16. Un procédé selon n'importe laquelle des revendications précédentes, dans lequel le panel de combinaisons multiples d'anticorps monoclonaux utilisés pour colorer des échantillons néoplasiques est plus court que le panel de combinaisons d'anticorps monoclonaux utilisés pour colorer des échantillons normaux/réactifs, mais le premier panel est entièrement contenu dans le dernier.
  17. Un procédé selon n'importe laquelle des revendica-

- tions précédentes, dans lequel pour chaque paire de panels de combinaisons d'anticorps monoclonaux, un clone exact de chaque anticorps monoclonal utilisé dans chaque combinaison individuelle d'anticorps monoclonaux, et un fluorochrome auquel il est conjugué, sont identiques dans les deux panels de combinaisons d'anticorps monoclonaux.
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19. Un procédé selon n'importe laquelle des revendications précédentes, dans lequel une combinaison de fluorochromes compatibles est sélectionnée parmi isothiocyanate de fluorescéine (FITC), phycoérythrine (PE), protéine de chlorophylle de péricline (PerCP), allophycocyanine, alexa fluor 488, alexa 647, pacific blue, alexa fluor 405, cyanine 5 (Cy5, cyanin5.5(Cy5.5) et leurs conjugués couplés à PE, à APC ou à PerCP (PE/Cy5, PE/Cy5.5, PE/Cy7, APC/Cy7 et PerCP/Cy5.5) ou tout fluorochrome ou conjugué de fluorochrome compatible supplémentaire.
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20. Un procédé selon n'importe laquelle des revendications 1 à 18, dans lequel des informations sur la diffusion de la lumière et les mesures de fluorescence des événements colorés avec chaque combinaison d'anticorps monoclonaux conjugués à des fluorochromes contenus dans un panel utilisé pour colorer différentes aliquotes répétées du même échantillon sont initialement sauvegardées dans des fichiers de données séparés.
- 15
21. Un procédé selon n'importe laquelle des revendications 1 à 19, dans lequel des informations provenant de deux fichiers de données distincts sont fusionnées directement sans aucune correction.
- 20
22. Un procédé selon la revendication 1, dans lequel la population des microparticules de référence est uniforme.
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23. Un procédé selon n'importe laquelle des revendications 1 et 22, dans lequel une population de microparticules de référence est composée de multiples populations de microbilles de taille, densité, volume, forme, quantité de fluorescence, caractéristiques d'adhésion ou autres propriétés physico-chimiques qui diffèrent.
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24. Un procédé selon n'importe laquelle des revendications 1 et 22 à 23, dans lequel une population de microparticules est composée de particules fluorescentes.
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25. Un procédé selon n'importe laquelle des revendications 1 et 22 à 23, dans lequel une population de microparticules est composée de microparticules dont la surface est recouverte d'anticorps anti-immunoglobulines.
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26. Un procédé selon n'importe laquelle des revendications 1 et 22 à 23, dans lequel une population de microparticules est composée d'un mélange de microparticules fluorescentes dont la surface est recouverte d'anticorps anti-immunoglobulines.
- 45
27. Un procédé selon n'importe laquelle des revendications 1 et 22 à 26, dans lequel les microparticules sont ajoutées en nombres connus.
- 50
28. Un procédé selon n'importe laquelle des revendications précédentes, dans lequel des dilutions en série d'événements provenant d'un fichier de données correspondant à un échantillon néoplasique coloré avec un panel de multiples combinaisons d'anticorps monoclonaux dans un fichier de données contenant des informations sur la diffusion de la lumière et les mesures de fluorescence de cellules contenues dans un ou plusieurs échantillons normaux/réactifs colorés avec un panel identique d'anticorps monoclonaux, sont réalisées dans l'étape d) de la procédure décrite dans la revendication 1, pour évaluer la sensibilité à laquelle un petit nombre de cellules néoplasiques pourraient être détectées une fois diluées dans des cellules normales/réactives dans des proportions prédéfinies connues.
- 55
29. Un procédé selon n'importe laquelle des revendications 1 à 28, dans lequel des modèles d'expression d'antigène anormaux en rapport avec l'activation de cellules et une fonction cellulaire sont détectés.

**REFERENCES CITED IN THE DESCRIPTION**

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专利名称(译)	用于通过流式细胞术监测最小疾病水平的肿瘤细胞中异常表型的多维检测方法		
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优先权	10/791994 2004-03-03 US		
其他公开文献	EP1724581A1		
外部链接	<a href="#">Espacenet</a>		

#### 摘要(译)

一种检测存在于骨髓，外周血，脊髓液和淋巴结中的肿瘤细胞表达的异常表型的新方法，包括以下步骤：1) 分别染色一个或多个正常/反应样品和一个具有相同或部分重叠的肿瘤样品，单个抗体的多种组合，2) 依次测量与来自正常/反应性样品和肿瘤样品的单克隆抗体染色的大量细胞相关的荧光发射，3) 存储两个独立的列表模式数据文件，每个文件包含关于分析每个细胞的特定光散射和荧光特征，4) 通过将包含有关肿瘤样品中存在的细胞的信息的数据文件中的列表模式数据混合到包含正常样品中存在的细胞的信息的数据文件中来创建新的数据文件。，5) 定义由与正常细胞相对应的事件占据的那些区域那些区域对应于正常/反应样本中的空白区域，可能被肿瘤样本中的肿瘤细胞占据，6) 依次识别对应于肿瘤细胞的那些事件和那些与多维空间中共享的正常细胞相对应的事件，7) 与正常对应物相比，确定肿瘤细胞显示的最相关的表型畸变。