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Clinical Flow Cytometry, a Hypothesis-Driven Discipline of Modern Cytomics

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Recently, two major books have been published that summarize the historical aspects and recent achievements of practical flow cytometry (1,2). Both emphasize the role played by this newly developed technical discipline in the development of scientific (1) and diagnostic platforms during late 20th-century medicine (2). Indeed, the gray box called a flow cytometer is the result of a multidisciplinary collaboration between engineers, biophysicists, biochemists, histopathologists, molecular cytologists, hematologists, immunologists, and quality controllers, with a more recent contribution from physicians specializing in the human immunodeficiency virus (HIV), oncologists, and epidemiologists (Table 1).

The foresight by the "fathers" has been astonishing (1). In his book, Howard Shapiro reminds us that flow cytometry, used to investigate cells in a flow system instead of on a static microscope, was put into practice by Coulter when he used impedance to count red cells. Rapid cell spectroscopy was introduced by Kamentsky to leukocyte differential counting with computer-assisted displays. Then optical cell counters, fluorescence dies, lasers, and photomultiplier tubes were added by Fulwyler and Jones. Immunologic concepts and reagents were introduced by the Herzenbergs and were fully primed to harvest the gems of the incipient monoclonal antibody revolution. The first commercial cytometer with precision engineering of the flow cells was launched by Goehde. These initial steps were duly followed by the release of a series of cytometers that showed increasing sensitivity, computerization, and practicality in research and routine laboratory work. This is illustrated by science historians Keating and Cambrosio in their book that blends the sociological aspects of medical technological developments with witness accounts (2).

Flow cytometry currently is a colorful, practical discipline that has become available to medicine at the appropriate time for various pressing clinical applications, thus rewarding the scientists who had the vision to foresee these needs. These contributions established cytometry, including flow technology, image analysis, and advanced microscopy, among the leading trends of modern biomedicine and health care.

After the launch of the Human Genome Project (3), two areas, genomics and proteomics, have been majestically promoted on both sides of the Atlantic and in the East (4). Genomics includes the identification of genes and gene regulatory processes, and proteomics investigates the abundance of proteins simultaneously with the changes associated with alterations of the functional state of the cell. Such a "pseudo-functional" approach aims to extend the study of quantitative changes during differentiation, proliferation, and signaling of different cell types (5). Clearly, there is an enormous, newly generated influx of information here, but it is not certain that a mere analysis of genes and protein structure, even in its extended format that includes the interaction of various biomolecules, will provide all of the necessary information to understand function and regulation at the level of living cells and organisms. Hence, the concept of cytomics has been introduced recently (6,7), for two reasons. First, cytomics is the cell-oriented analysis of molecules and their functions in cellular systems and/or organs, referred to as *cytomes*. Second, cytomics is the new, absolutely essential, interface between biosciences and clinical medicine. Importantly, advanced cytometry, including the traits defined in Table 1, represent the driving engine of cytomics. A new paradigm is that cytomics is the combined, interrelated force for cytometry, proteomics, and genomics (6,7).

There are, however, significant differences between these three modern trends. First, it is in the realm of cytometry, with the major contribution of flow systems, where the appropriate functional analysis of cells, the display and regulation of functional molecules, and the differentiation pathways are successfully carried out. Animal models and human diseases provide targets for these studies. Second, it is the cytometric field where the diagnostic tests, for malignancies (cf. 8,9) and the cellular analysis of infectious diseases (10), currently harvest the richest crop of practical results, whereas in the fields of genomics and proteomics only the seeds are sown for the practically useful multiarray systems. Third, these two fields at their current developmental state, are essentially observational sciences that primarily document the observed heterogeneity. As a marked contrast, the research by flow cytometry has been, from the beginning, primarily

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Table 1
*Scientific and Technical Contributions to Flow Cytometry**

Optical filter technology
Compact engineering and robust design with stable flow characteristics
Increase in the speed and storage capacity of computers
Florescent dyes, including new Alexa dyes, for constructing molecular probes
Wide variety of monochromatic light sources including laser technology
Precision syringes for volumetric measurements (e.g., on Cytoron)
Monoclonal antibodies to identify clusters of differentiation (CD) antigens
Techniques to recognize antigens on the surface and inside the cells
Separation techniques for viable cells by flow sorting and magnetic beads
Fixatives to preserve cellular antigens and provide stabilized biological standards
Clinical needs for using flow cytometry as a method of choice in diagnosis
Clinical protocols agreed and propagated by international committees
Quality control organizations for external assessment
Internet and mobile telephone services for education and services at large

*Reviewed by Shapiro (1) and Keating and Cambrosio (2).

hypothesis driven and backed up by sophisticated experimental designs and clearly defined clinical needs. In this review, some of the basic hypotheses that have contributed to the introduction of clinical applications are summarized to document the hypothesis-driven nature of flow cytometry research.

PROCEDURE

Six hypotheses are described that have been conceived with the flow cytometric technology in mind to answer biological and/or clinical diagnostic questions. In each case, a short account of the background is followed by the relevant hypothesis. Then the implications of the results are summarized, with additional observations that substantiate the results observed and explain the significance of the findings.

SIX HYPOTHESES

Preparation of Anti-ALL Antiserum: Relevance to Normal Precursors and Leukemia

A fluorescence-activated cell sorter (FACS) from Becton-Dickinson, the first to arrive to Europe in 1974, was operated by David Capellaro at the ICRF Tumour Immunology Unit, London (Fig. 1). This sorter was used for leukemia and stem cell research by Greaves and colleagues. Around that time it was already known that the membrane markers for murine and human T and B cells were also present on these cells' malignant counterparts (11,12). As the common form of acute lymphoid leukemia (ALL) remained negative (non-T, non-B ALL), Greaves and Brown made a rabbit anti–non-T, non-B ALL antiserum (13); in this laboratory other antisera to T cells, myeloid cells, and class II antigens also were used for immunodiagnosis (Fig. 2).

Hypothesis 1A: A non-T, non-B ALL antiserum will react with (a small number of) normal precursor cells, the "target cells," from which this particular form of malignancy derives (14).

The anti-ALL antiserum was shown to react with an antigen of 100 kD (CALLA; later proven to be CD10) (2,16), strongly expressed on most common ALLs (Fig. 2-1), and weakly expressed on some T-ALL (Fig. 2-7), but undetectable on acute myeloid leukemias and chronic lymphoid leukemia. As predicted, this was the first anti-precursor cell reagent; the reactivity is shown in Table 2. Once this cell type, also referred to as the pro-B cell, and

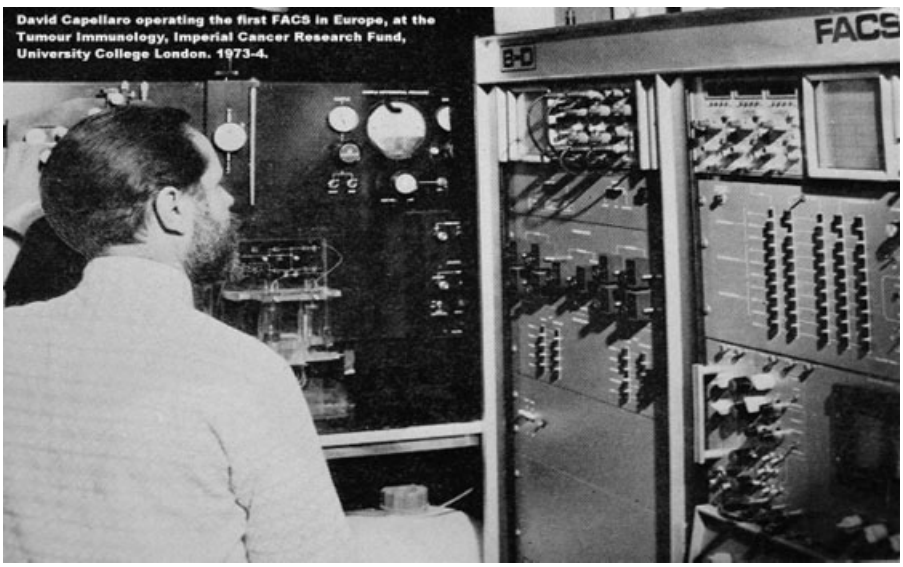

FIG. 1. The fluorescence activated cell sorter (FACS) at University College London, 1973 to 1974, operated by David Capellaro.

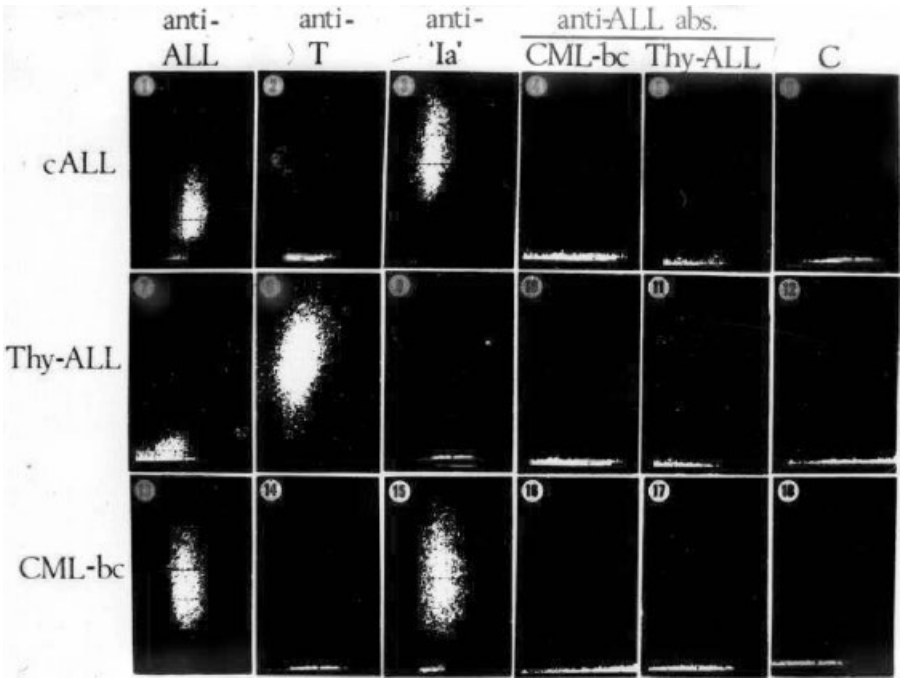

FIG. 2. The reactivity of heterologous anti-ALL, anti-T, and anti–class II antisera with the common form of ALL (cALL), thymic ALL (Thy-ALL), and a "lymphoid" blast crisis of chronic myeloid leukemia (CML-bc). The results of the absorption of the antiserum made against ALL with Thy-ALL and CML-bc show that the antigen recognized by this diagnostic reagent is shared between them (14). This was the common ALL antigen, with CD10 of 100 k (15,16).

its close relative, the cytoplasmic immunoglobulin (Ig) positive pre-B cells were identified, these cell types were characterized extensively by flow cytometry, cell sorting, biochemistry, and gene rearrangements (2,14) (also see below).

Hypothesis 1B: The same early lymphoid precursor also can be involved in malignancies that may develop as a result of different pathologic mechanism(s), e.g., in Philadelphia chromosome positive (Ph′) chronic myeloid leukemia (CML) of pluripotential hemopoietic stem cell origin (13,17,18).

Anti-ALL serum reacted with the "lymphoid" blasts in CML (Fig. 2-13) (14,17). When the anti–non-T, non-B ALL antiserum was cross-absorbed with Ph′ lymphoid blasts (Fig. 2-4) or with T-ALL blasts (Fig. 2-5), the reactivity to common ALL was removed and vice versa (Figs. 2-10 and 2-17). Thus, the antiserum detected the same precursor cell-related moieties (the CD10 antigen) (15,16) in the different diseases. In Ph′-positive disease that the lymphoid blasts were frequently seen admixed with other cell types such as maturing myeloid cells and myeloblasts (17), cell sorting with FACS showed that only the lymphoid blasts had the CALLA-positive phenotype (Fig. 3) (18,19). Similar cell sorting studies (20) have shown that class II antigens are expressed more widely on lymphoid and myeloid blasts but not on differentiating forms (Fig. 4). These investigations have established the immunophenotyping platform for leukemias (2).

Enriching Hybridoma Clones by Sorting

The FACS-1 had already been in operation in Stanford when Len and Lea Herzenberg visited Milstein's laboratory in Cambridge, United Kingdom. Here the method of monoclonal antibody production was discovered while attempting to answer a hypothesis, that one B cell produces a single antibody (21). The hybridoma technique includes the fusion of B cells and immortal plasma cells,

Table 2
*Anti-ALL Antiserum Detects Normal Precursor Cells**

Tissue	Source	No. of cases	%Anti-ALL reactive cells
Normal hemopoietic tissue	Fetal	15	1–20
	Children	6	
	Adults	44	<0.1
Regenerating hemopoietic tissue	After chemotherapy	7	5–50
	After X-R + bone marrow transplant	4	
Nonmalignant proliferative disorders	Neonatal "leukemoid" reaction	4	5–35

*FromGreaves and Janossy (14) with permission. ALL, acute lymphoid leukemia; X-R,??.

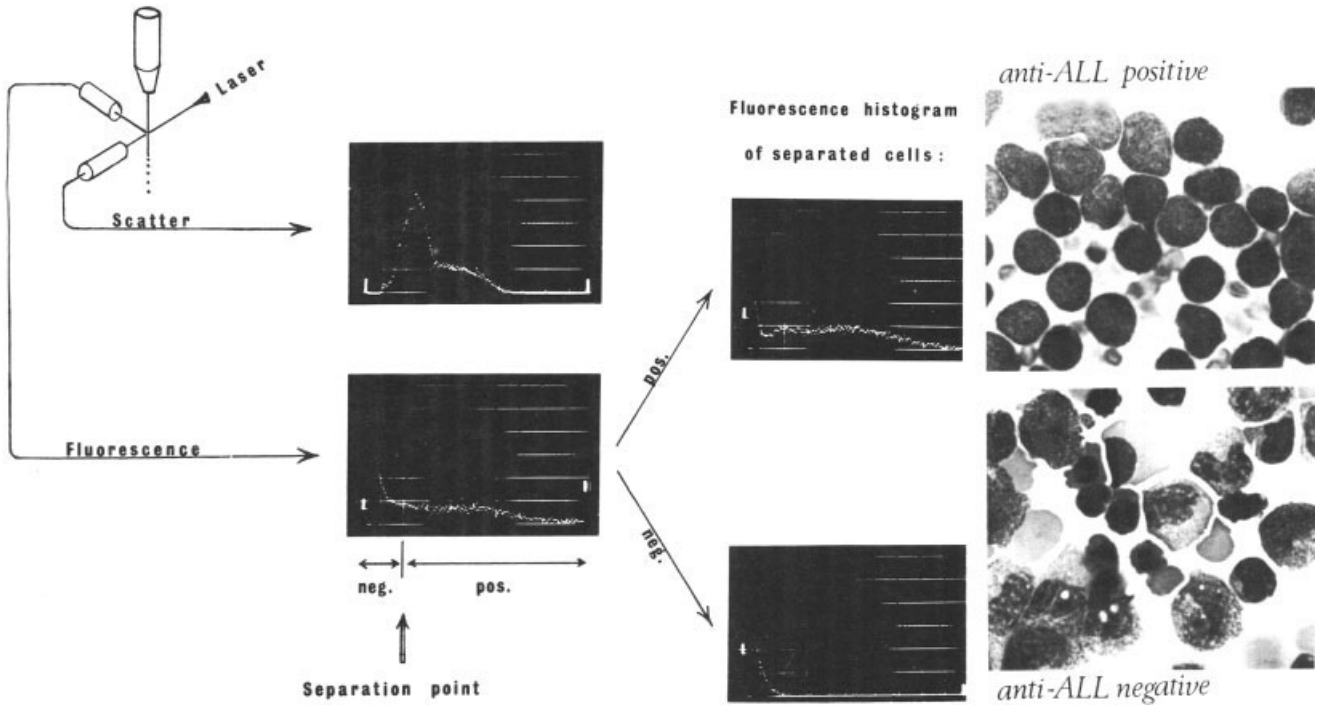

FIG. 3. Heterogeneity of cell phenotypes in CML blast crisis: analysis using the FACS. Cells are labeled with anti-cALL serum and separated according to fluorescence as indicated in the lower oscilloscope screen picture. Cells of lymphoid morphology sort into the positive fraction and the nonlymphoid cells in to the negative (18,19).

and it is cumbersome to screen for the viable hybrids that produce the correct antibodies.

Hypothesis 2: It is suggested that viable monoclonal hybrids carry surface Ig with antibody specificity and that, after having been labeled with antigen-coated fluorescing microspheres, the progenitors can be sorted into specific antibody secreting fractions to facilitate the selection of "wanted" clones (22).

In this study, viable and dead cells could be distinguished by flow analysis. Rare viable hybrids with antigen binding were recognized and enriched by sorting. The antigen-coated fluorospheres adhered to the hybrid cells that expressed antigen-specific receptors, providing a signal bright enough to do the sorting. At the time, only fluorescent microspheres provided a signal bright enough for this purpose. Subsequently, fluorochromes of higher intensity proteins also could be labeled with fluoro-

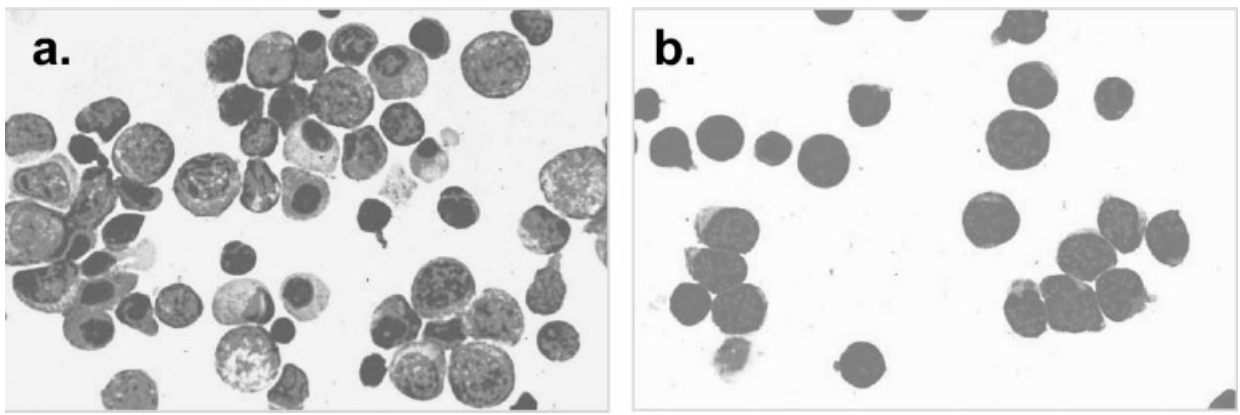

FIG. 4. Heterogeneity of cell types in CML blast crisis. A mixed myeloid and lymphoid blast crisis was stained with an anti–class II antiserum and sorted as in Figure 3. **a:** The negative fraction contains differentiating granulocytic and erythroid cells. **b:** In the class II-positive fraction, lymphoid and myeloblasts are seen (20).

Table 3
*Secretion and Microsphere-Binding Properties of FACS-Sorted Clones Derived From an Anti-Ig-la-Producing Clone**

No. of microspheres bound by sorted progenitor cell			Properties of clones derived from sorted progenitors	
52–70 (bright)	3–5 (dull)	0 (negative)	Secretion of anti-Ig-Ia	Binding of Ig-Ia microspheres
10	10	11	+	+
0	0	12	—	—
14	14	0	+	Not done

*From Parks et al. (22) with permission. FACS, fluorescence-activated cell sorting; Ig, immunoglobulin.

chromes and used for positively selecting hybridomas of known specificity (23). In culture, the enriched progenitors developed into clones that secreted specific antibodies against the antigens (mouse Ig-1a; Table 3). The beauty of this experiment is that it combined basic science with a practical purpose: to prepare better reagents and to use these in flow cytometry.

Characterization of Differentiation Lineages Using Monoclonal Antibodies for Multiparameter Analysis

The successive Leucocyte Typing Workshops marshaled monoclonal antibodies into CD groups (16,24), and multiparameter analysis became feasible by the tandem use of monoclonal antibodies labeled with different fluorochromes (25). Mike Loken and Leon Terstappen, in collaboration with Curt Civin, had an organized approach to characterizing the expression of these CD groups. The description of erythroid (26) and the B-lymphoid lineage (Fig. 5) (27,28) was soon followed by the characterization of the neutrophil lineage (29), and monocytic differentiation from $CD34^+$ cells in the bone marrow and cord blood was described by Knapp et al. (30,31).

Hypothesis 3: Flow cytometry, with its multiparameter analytical approach, is ideally suited to indicate the steps of differentiation along a given cell lineage in situ (e.g., in the bone marrow) (27,28) and to prove this scheme by following the development of lineage precursors in vitro (30–32).

The example of B-lymphoid development from early precursors, through CALLA (CD10)–positive pro-B cells to $Sigma^+$ B lymphocytes (Fig. 5) is a model for the highly controlled sequential acquisition of cell surface antigens during B-lymphocyte development. The most immature cells identifiable in the bone marrow express CD34 and HLA-DR. The earliest recognizable B-lineage cells ($CD19^+$, bright $CD10^+$) also express CD34 and terminal deoxynucleotidyl transferase in the nucleus and are proliferating. The progression of cells from stage II to stage III is marked by the acquisition of CD20, HLA-DQ, and SmIgM. A caveat is that $SmIgD^+$ B cells (last stage) may not be an integral or sequential part of this linear progression but perhaps represent cells that seed back to bone marrow from the periphery (33). The cultivation of $CD34^+$ precursor cells in vitro has confirmed these B-cell and myeloid differentiation schemes (30,31).

Aberrant Leukemia-Associated Combinations for Detecting Minimal Residual Disease (MRD) by Multiparameter Flow Cytometry

Morphology of leukemic cells shows severe asynchrony, e.g., between cytoplasmic and nuclear maturation antigen expressions. As a consequence, although the individual antigens tested are not specific for leukemias (see

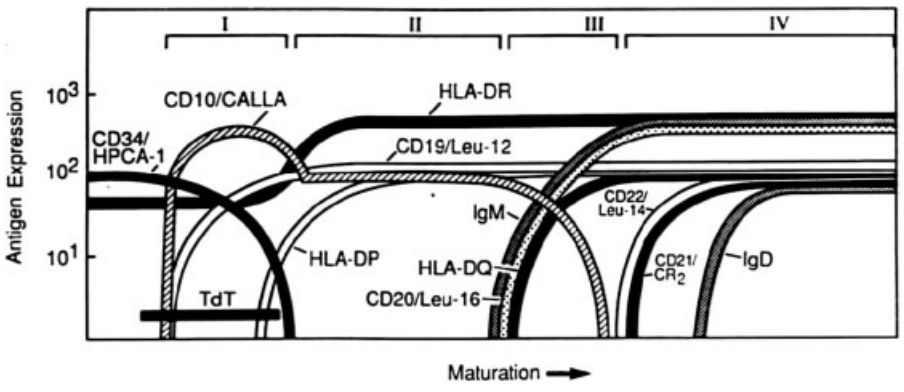

FIG. 5. Stages of B-lymphoid differentiation in the normal bone marrow, investigated with multiparameter analysis using combined staining with monoclonal antibodies labeled with different fluorochromes. The reagents used were CD34, CD10, anti–terminal deoxynucleotidyl transferase, anti–class II (HLA-DR, -DP, and -DQ), CD19, CD20, CD21, and IgM (for membrane labeling; SmIgM). B-lymphoid precursors at phase II contain cytoplasmic IgM (not shown); see Greaves and Janossy (14). From Loken et al. (27,28).

above), the asynchrony of marker display is leukemia associated (34). Such an analysis, by definition, includes multiparameter analysis that is optimally performed on a flow cytometer (35–37).

Hypothesis 4: Differentiating normal cells express their markers in an orderly fashion when studied with three-color (35) or four-color (36,37) antibody combinations, whereas leukemia cells frequently show aberrant dysregulation that is their characteristic feature. In patients retested at the end of remission induction in full morphologic remission with sensitive techniques to identify MRD, the presence of aberrant cells may have a profound prognostic significance (38).

The regularity of marker expression in normal bone marrow for CD10/CD20/CD19 (Fig. 6a) and CD34/CD38/CD19 combinations (Fig. 6b) is depicted. The three populations of normal precursors cell types such as the early ($CD34^+$, $CD10^+$) precursors, intermediate forms and the newly emerging ($CD10^-$, $CD20^+$) B cells are shown as dots (with colours in ref. 35). In the white unallocated areas, aberrant leukemic phenotypes reside, representing 59.7% and 53.9% of ALL cases; the frequency of ALL cases that exhibit the individual aberrant combinations is also shown (35).

These tests for MRD are regarded to be useful if they can identify fewer then one leukemic cells per 10^4 normal cells. The three- and four-color combinations used for T- and B-lineage ALL and for acute myeloid leukemia at the St. Jude Children's Hospital, Memphis, Tennessee, USA and at the University Hospital, Salamanca, Spain are published in detail (37). In clinical practice, the children are studied at the end of remission induction by conventional morphologic methods. If no abnormality is seen, the bone marrow is retested by flow cytometry for MRD because this technique is more sensitive to detect residual leukemia. The prognostic significance of these diagnostic results has been proven in a large cohort by observing the relapses that developed during subsequent years. The patients were grouped into four categories: (a) non-detectable MRD, (b) MRD detectable but less than 0.1%, (c) MRD between 0.1% and 1%, and (d) MRD greater than 1%. Five years later, the relapse rates were 10%, 23%, 43%, and 72%, indicating the clinical use of the assay (36,38). These tests for MRD are complementary to molecular techniques. Both of these serve as surrogate endpoints in the clinical testing of the new therapeutic approaches.

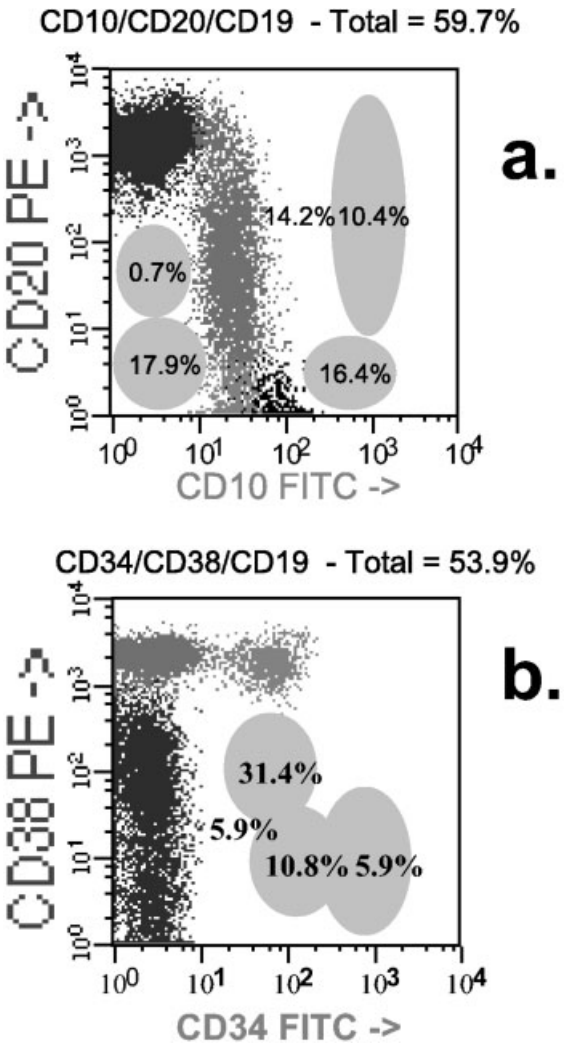

FIG. 6. Identification of leukemic blast cells of the B-lymphoid lineage by irregular aberrant marker expression. Normal B-cell precursors provide a regular differentiation pathway that can be investigated by different combinations such as CD10/CD20/CD19 (**a**) or CD34/CD38/CD19 (**b**). Leukemic cells frequently fall outside these normal boundaries and occupy different positions in the "white area." In a and b, 53% to 59% of ALL cases can be regarded as aberrant, but other combinations exist, and most cases of ALL can be followed with one or another combination staining. The percentage values are the proportions of ALL cases that are positioned as aberrant. For example, 10.4% of cases show much higher CD10 display on ALL blasts than on normal $CD20^+$ B-cell forms (35).

Standards for Quantitative Flow Cytometry

The studies described above already gave the impression that the marker expression on normal leucocytes was regular and that the flow cytometers provided reliable quantitative results. These premises can be studied further by methods that quantitate antibody-binding capacity (ABC) expressed as molecules per cell (39). Of these, the indirect immunofluorescence tests, such as the Quantitative Indirect Immuno-Fluorescence (QIFI) test (40), is the most reliable because the first antibody used to bind to the membrane antigens at saturating concentrations remains unconjugated and therefore unmodified; the fluorochrome-labeled second antibody, used at saturating level, is then a universal reagent used in every assay. Thus, the results observed are comparable and allow the construction of "league tables" for the expression of different membrane antigens on different cell types (39,40). For example, the CD45 antigens on lymphocytes are expressed at four times higher levels (at 200×10^3 molecules/cell) than CD4 antigens on "helper-type" T cells (50×10^3 molecules/cell) and 10 times higher than CD19 antigens on B lymphocytes (20×10^3 molecules/cell; Fig. 7a).

Hypothesis 5: When tested by the QIFI test, some markers will prove to be regularly maintained at their character-

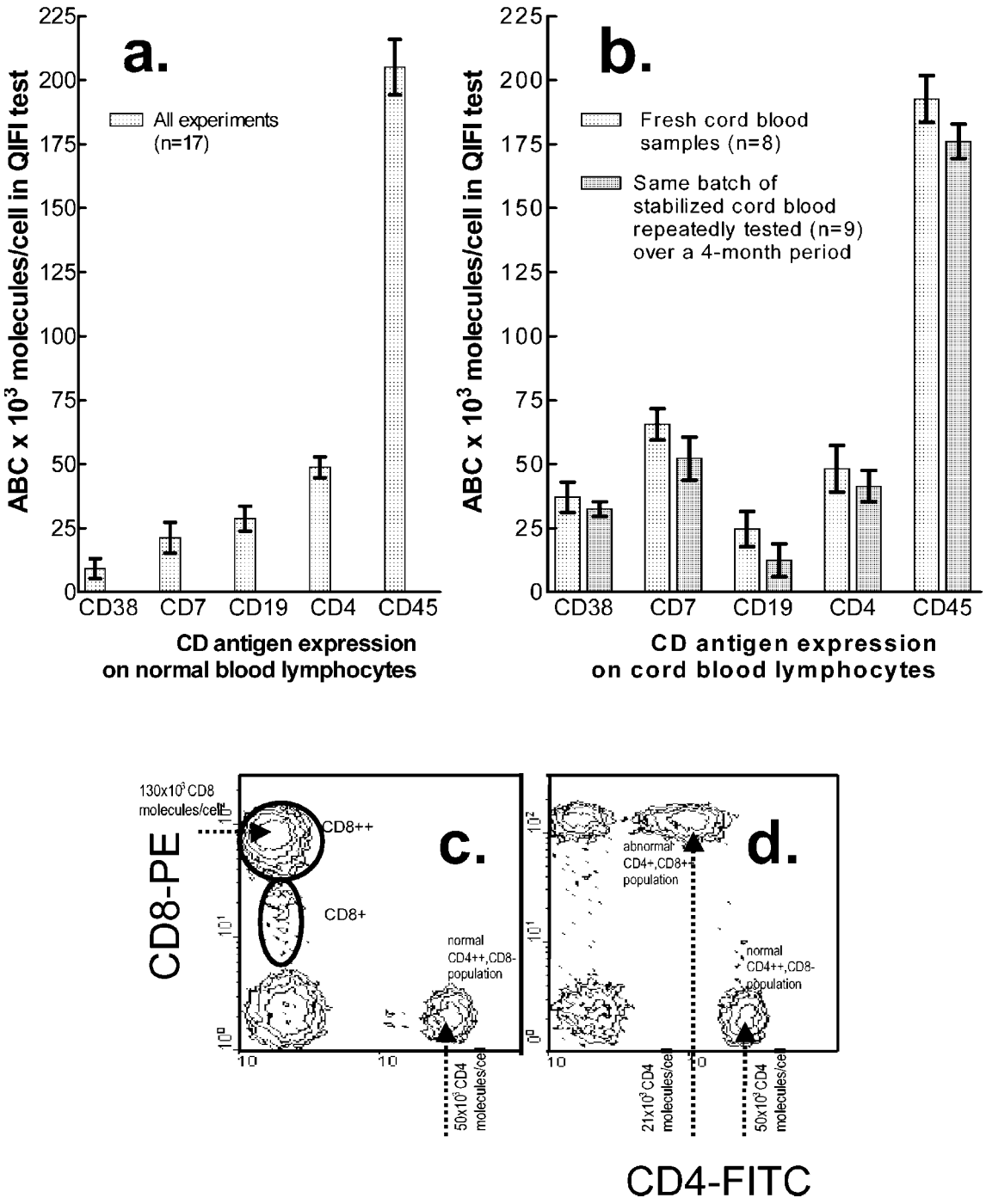

FIG. 7. Expressions of CD38, CD7, CD19, CD4, and CD45 antigens on lymphocytes from different donors (**a**) and the preservation of these antigens on stabilized cell preparations prepared from fresh cord blood cells (**b**). The ABC was determined by using the indirect quantitative immunofluorescence method referred to as the QIFI test (40). The expressions of CD4 and CD45 molecules is particularly regular and robust, so these moieties can be used as controls for quantitative flow cytometry (41). Once the ABC values are known, these help to interpret the staining of normal lymphocytes (**c**) and aberrant populations (**d**) in a quantitative manner.

istic known levels of expression in different people, to be used as acceptable biological controls; further, these molecules might be stabilized by fixation to provide cellular standards of long shelf-life (41).

The ABC values for the expression of various CD antigens on lympho-, mono-, and granulocytes have been documented using the QIFI assay (40). The hierarchy of expression is known and the individual personal varia-

tions for CD45 and CD4 antigen expression are less than 10% (Fig. 7a). These antigens, also tested on cord blood cells for CD45, CD4, and CD38, are maintained at a stable high level on blood preparations stabilized for long-term use (Fig. 7b). These preparations of long shelf-life (42) serve as standards in the stabilized cellular immunofluorescence assay (41). With the help of these standards during flow cytometry, the values of mean fluorescence intensity can be replaced by values of ABC (molecules per cell).

In normal blood, $CD4^+$ T cells uniformly express 50×10^3 ABCs/cell (Fig. 7c). $CD8^+$ cells are heterogeneous: "proper" $CD3^+$,$CD8^{++}$ T cells exhibit 130×10^3 molecules, whereas the $CD3^-$,$CD8^+$ natural killer cells display 10 to 100×10^3 CD8 ABCs/cell (Fig. 7c). This picture is complicated by the existence of minor (<5%) populations that can be $CD3^+/CD4^-/CD8^-$ or $CD4^+/CD8^+$ (43,44). Only in occasional cases can aberrant T cells be seen in numbers higher than 10%. In such a special case (Fig. 7d), the cells are $CD8^+$ T cells with extra low-level (20×10^3) CD4 display (monoclonal chronic T chronic lymphoid leukemia?). These simple quantitative methods, based on normal blood standards and on stabilized blood preparations, have a wide application in precisely characterizing the aberrant display of various antigens in leukemia and lymphoma (45).

Quantitative Flow Cytometry, the Method of Choice for Affordable CD4 T-Cell Enumeration

The progressive depletion of $CD4^+$ T-lymphocytes is the central event in the pathogenesis of HIV infection. The level of these cells in the peripheral blood is therefore the single most important parameter for monitoring disease associated with HIV infection. Ever since the first case report on acquired immunodeficiency syndrome (AIDS) (46), flow cytometry has remained the gold standard for CD4 T-cell enumeration in HIV disease. Recently, however, this virtually unassailable position has been placed into jeopardy. The introduction of generic drugs in resource-poor countries has decreased the costs for antiretroviral therapy, but the cost of CD4 tests has been perceived to remain as high as $20 to $30. For these reasons, non-flow CD4 T-cell enumeration methods have been offered to replace the gold standards (47).

Two relevant issues need to be discussed here. First, physicians in industrialized countries may request a full panel of comprehensive lymphocyte subset analysis (including T-subset, B, and natural killer counts), whereas those in clinics with a large HIV burden might require only CD4 T-cell counts. Second, the superiority of primary immunologic gating recently has been established when compared with the previously preferred strategies based on morphologic scatter gating (48,49). Optimally, a single histogram of antibody reactivity (fluorescence intensity; y axis) versus side light scatter (x axis) should be used when $CD4^+$ lymphocytes are defined by auto-gating (Fig. 8a,b). The rationale for this shift to a more robust gating strategy is that markers such as CD4 and CD45 are maintained relatively well in aging samples at the time when scatter features deteriorate quickly (49,50).

Hypothesis 6A: Clinical flow cytometry tests for CD4 T-cell enumeration can be simplified and performed by using a single CD4 antibody; with the current improved gating protocol, these assays will be more robust then the previous more expensive methods (43).

The primary CD4 gating strategy described in Figure 8a,b using a single CD4 antibody was used on blood samples from more than 600 individuals, including HIV^+ patients; the results were compared with the absolute CD4 counts obtained on a single-platform flow cytometer, the Cytoron-absolute, which uses nine reagents (Fig. 8c). The correlation was excellent, and Bland-Altman statistics showed a mean difference of -2 cells/μl. Thus, CD4 T-cell enumeration, a simple method, can be performed economically in resource-poor conditions, particularly if simpler cytometers are also designed for this purpose (10,52). In children, CD4 and CD45 antibodies are required (50). Indeed, in these areas, the price of the $CD4^+$ T test has decreased to less than $8 (10).

Hypothesis 6B: The main advantage of CD4 counting by flow is that this quantitative method effortlessly discriminates between $CD4^{++}$ lymphocytes and $CD4^+$ monocytes; many non-flow methods are unable to achieve this without additional efforts for removing monocytes and remain dangerous to use when $CD4^+$ T-cell lymphopenia is associated with infections, such as tuberculosis and parasitic diseases.

Normally, CD4 staining on the flow cytometer discriminates between $CD4^{++}$ T cells of smaller size displaying 50×10^3 molecules/cell versus $CD4^+$ monocytes of larger size displaying 12×10^3 molecules/cell irrespective of the fluorochromes used (Fig. 8a,b). The enzyme-linked immunosorbent assay (ELISA) method such as TRAx, however, measure total CD4 antigen in blood after cells have been destroyed (52). Under normal conditions, when most CD4 antigens originate from $CD4^+$ lymphocytes, the monocyte- and eosinophil-derived CD4 antigens (53) are incorporated into the background during the construction of the ELISA calibration curve. Nevertheless, in patients with AIDS and tuberculosis, this balance changes, and with TRAx ELISA $CD4^+$ T cells (that might be referred to as "fantom T cells") are detectable that do not seem to exist when tested by flow cytometry (Fig. 8d). This is disturbing because, based on TRAx in Africa, AIDS-associated diseases started to appear at CD4 counts well above the 200/μl value, but these might have been skewed by monocyte-derived CD4 molecules (54). It is therefore incorrect to suggest elevating the levels of "AIDS-defining conditions" from the currently accepted CD4 200/μl value when these suggestions are based merely on the findings of TRAx-like assays. Instead, a suitably simplified and inexpensive flow-based CD4 counting method should remain the gold standard for monitoring patients during antiretroviral therapy.

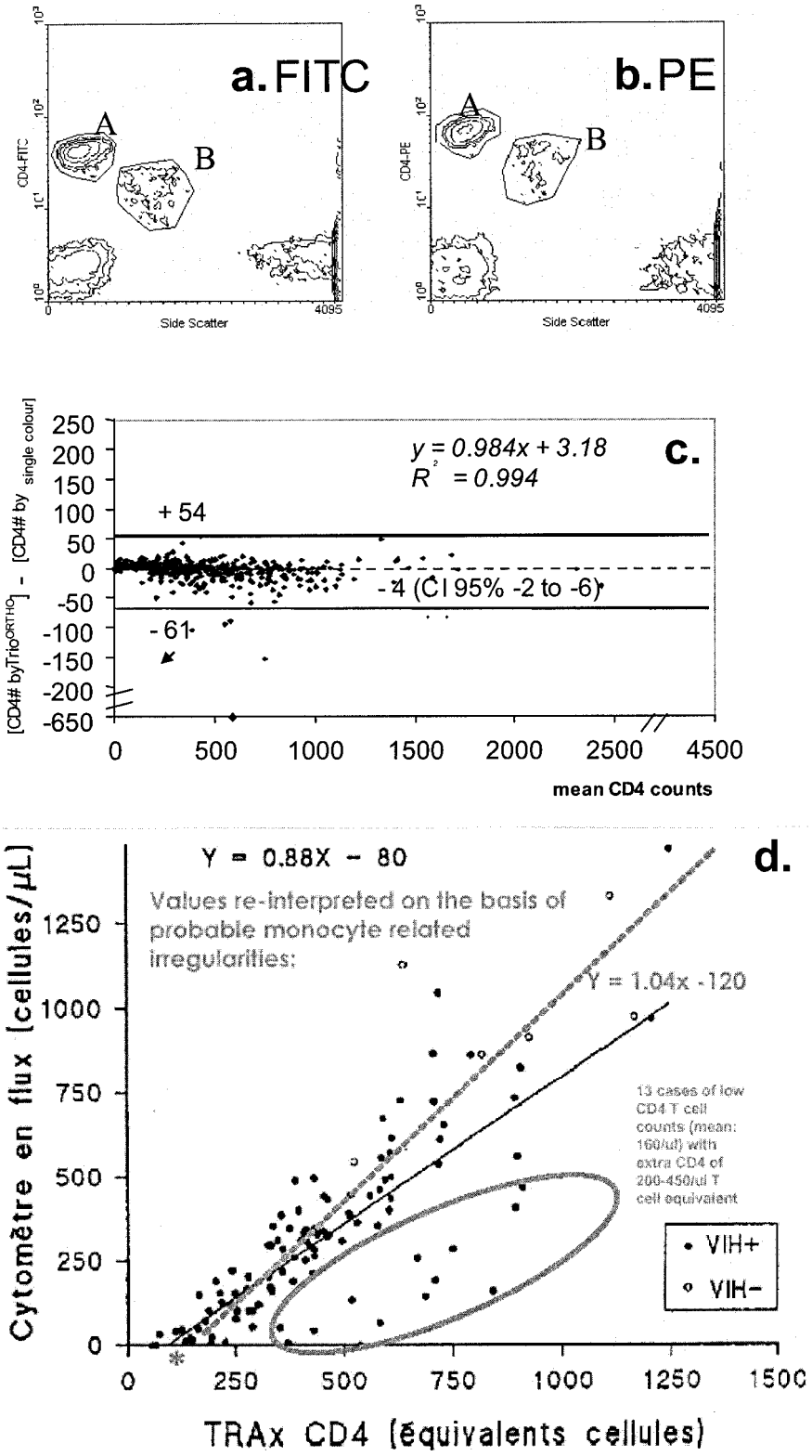

FIG. 8. It is possible to count $CD4^+$ T lymphocytes using a single CD4 antibody labeled with a choice of fluorochromes such as fluorescein isothiocyanate (FITC; **a**) or phycoerythrin (PE; **b**). Primary immunologic gating using CD4 intensity on the y axis and side scatter on the x axis is recommended; the small $CD4^{++}$ lymphocytes are reliably discriminated from the larger $CD4^+$ monocytes. **c:** The tests using a single CD4 antibody provide virtually identical CD4 counts without bias when compared with other protocols that use more reagents (43). **d:** Other assays, such as the TRAx method (based on CD4 ELISA after lysing the cells), are not as reliable as flow cytometry because it is as documented by comparing the TRAx CD4 values (x axis) with the flow cytometric CD4 counts (y axis). The 12 CD4 lymphopenic cases show misleadingly high TRAx CD4 values. This extra CD4 might derive from activated monocytes and/or eosinophils in patients with infectious disease such as tuberculosis (54).

DISCUSSION

Despite the extraordinary accumulation of new information on genes and proteins, the disciplines of genomics and proteomics alone are unlikely to be in an optimal constellation to unravel the functional complexities of cells, cellular systems, and organs. It is therefore an inev-

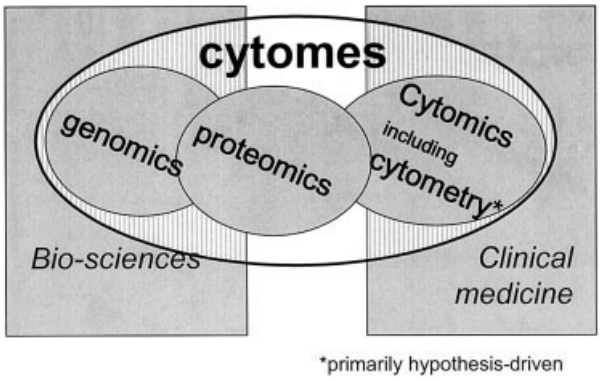

FIG. 9. The concept of cytomics includes the three main areas of genomics, proteomics, and cytometry to elucidate cellular and organ functions and to link biosciences with their medical applications. Cytometry is a distinctly hypothesis-driven discipline, as illustrated in this summary.

itable development that the discipline of cytomics, with its technical driving force represented by flow cytometry, image analysis, and modern microscopy, has been enlisted recently to contribute to a new phase of scientific development (6,7). Cytomics is an approach oriented toward cells and systems using the available information in a functional context. The freshness of this approach is testified by the fact that the investigations, using flow cytometry as the technology, have always been, from their early stages of development to the present day, hypothesis oriented (Fig. 9).

In this review, the examples have been selected primarily to document two aspects of the hypotheses listed: (a) their intention to bring the academic science closer to diagnostic medicine to provide support for the idea that cytomics constitutes a new interface between the biosciences and clinical medicine; and (b) to provide practical benefits in addition to immunologic scientific understanding.

In reality, the field covered by flow cytometry is far richer than suggested above. The volume of science that is clarified by the continued activities of Leucocyte Typing Workshops by identifying cellular antigens, through the monoclonal antibody technology and their CD classification (55), rivals the achievements of the proteomics discipline and is more interesting to immunologists due to its essentially functional approach. The ease of how the monoclonal antibody revolution thrives on flow cytometry (and vice versa) is well demonstrated above. It is difficult to see how these two arms of science (i.e., CD numerology and proteomics) can avoid collaborating fully.

Similarly, in this review, the modern breakthroughs of flow cytometry to develop 13 channels of analysis by employing multiple lasers, optical paths, and arrays of antibodies conjugated with new sets of fluorochromes (56) have not been detailed. However, these investigations are poised to sort out the minute details of immunoregulatory disorders in infections such as HIV (57) and in animal and human genetic disorders such as the defects of the newly discovered regulatory $CD4^+$ T cells (58,59).

Moreover, the emerging new multitasking platforms show the promise of combining the diagnostic multiplexing bead-array systems (60) with flow cytometric analysis of cell populations, including $CD4^+$ T-cell enumeration (61). This is the latest "one-stop shopping" concept (63), which is more serious than its flippant name suggests. This last development is a notable example to demonstrate two remarkable facts: (a) the current highly efficient multiarray systems, with contributions from cytomics, will soon directly influence clinical diagnosis in a novel way (61); and (b) flow cytometry is an irrepressible part of the current technical developments, although sometimes arriving in the disguise of multiarray technology.

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