*review*
*Haematologica 1995; 80:69-81*

# FLOW CYTOMETRY: ITS APPLICATIONS IN HEMATOLOGY

***Alberto Orfao*, Alejandro Ruiz-Arguelles°, Francis Lacombe#, Kenneth Ault@, Giuseppe Basso§, Marco Danova****

**Laboratory of Hematology, University Hospital of Salamanca, Spain; °Clinical Laboratories, Puebla, Mexico; #Laboratory of Hematology, Haut-Leveque Hospital, Bordeaux, France; @Maine Medical Center, Cytometry Research Institute, South Portland, ME, USA; §Department of Pediatrics, University of Padova, Italy; **Internal Medicine and Medical Oncology, University and IRCCS San Matteo, Pavia, Italy*

**ABSTRACT**

**The techniques of flow cytometry are becoming more and more important for the clinical hematology laboratory. No longer a novelty confined to a few specialized institutions as it was 10 years ago, flow cytometry has blossomed into a mature discipline. The methodology is well-known, the mechanical apparatus is readily available, and the role it plays in clinical hematology is increasingly appreciated. The burgeoning number of scientific articles devoted to this topic attests to the interest it has aroused as a tool for both medical research and patient care. In fact, more than a thousand such papers are now published each year and it would be impossible to deal with all the methodologies and applications of FCM currently utilized or under development.**

**Throughout this paper four relevant hematologic fields are briefly discussed, in which FCM appears to be of great help at present: the immunophenotyping of leukemias and lymphomas, the measurement of proliferative activity and DNA ploidy in hematological malignancies, the detection of drug resistant leukemic cells and the use of FCM in the study of platelets.**

*Key words: flow cytometry, immunophenotype, DNA content, cell proliferation, drug resistance, platelets*



Flow cytometry (FCM) is a rather recent and innovative technology by means of which different cell characteristics are simultaneously analyzed on a single cell basis.[1-3] This is achieved by means of hydrodynamic focusing of cells that pass aligned one by one in front of a set of light detectors; at the same time they are illuminated by the flow cytometer light source, which is usually a laser beam. All cell parameters measured in this way can be divided into two main groups: 1) those related to light scattering, which mainly reflects the size of the cell and its internal complexity, and 2) those associated with the presence of one or more fluorochromes inside the cell or attached to the cell surface membrane, either in a natural (autofluorescence) or artificial (i.e. using fluorochrome conjugated monoclonal antibodies) way.

FCM has developed rapidly in recent years, its applications expanding in both basic and clinical research laboratories. This has been mainly due to the fact that this technology provides objective, sensitive, rapid and accurate measurements of a relatively broad range of cell characteristics[1,3,4] that include, among others, DNA and/or RNA cell content, the detection and quantification of cell antigens, the analysis of multidrug cell resistance, membrane potential, mitochondria and chromosomes, the measurement of intracytoplasmic changes affecting either pH or ions such as free $Ca^{++}$. In addition, FCM allows sorting of cells and subcellular components such as the chromosomes.[2,3]

When applied to the clinical laboratory, this information largely completes that obtained with other diagnostic tools. Thus, the use of

*Correspondence: Alberto Orfao, M.D., Ph.D., Servicio General de Citometria, Laboratorio de Hematologia, Hospital Universitario de Salamanca, 37007 Salamanca, Spain. Tel. international +34.23.291375. Fax international +34.23.264743.*
*Acknowledgments: part of the present paper traces the participation of some of the authors in a dedicated Symposium held during the XXV Congress of the International Society of Hematology, April 17-22, 1994, Cancùn (Mexico).*
*Received August 5, 1994; accepted October 27, 1994.*

FCM has expanded quickly in very different areas of medicine, including immunology, pathology, oncology, genetics, clinical biochemistry, microbiology and internal medicine; hematology represents one of the most important emerging arenas for FCM.[1-3,5] For several reasons FCM is ideally suited to the study of leukocytes, platelets and red cells, and there is an increasing number of clinically relevant aspects of the pathophysiology and function of these cells that can be measured using FCM.[1-3,5]

The present work provides a survey of the uses of this technology in the areas of hematology where it finds its principal applications.

### *The immunophenotyping of leukemia/lymphoma*

In the past few years FCM has become the most widely used approach for the immunophenotyping of leukemia patients. For several years it has been used for the classification of both acute lymphoblastic leukemia (ALL)[2,3,6,7] and acute leukemias following a primary myeloproliferative[8] or myelodysplastic syndrome,[9] since leukemia immunophenotyping in these groups of disorders has been shown to have diagnostic value (Table 1). In addition, the impact of these classifications has already been well established both from a prognostic and a therapeutic point of view.

In particular, the role of immunophenotyping has been demonstrated to be critical for obtaining good results with chemotherapy in pediatric patients. Using immunological markers it is possible to initially identify an ALL as T lineage (positivity to cyCD3 and CD7) or B lineage (positivity to CD79 and CD19). A later classification includes three major subgroups for T ALL: pre T ($CD1a^-$ $SCD3^-$), T thymic ($CD1a^+$ $SCD3^-$), T mature ($SCD3^+$), and four major subgroups for ALL of B origin: C-ALL ($CD10^+$ Cylg–), pre-B (Cylg+ Smlg-), transitional pre B-B ($Cylg^+$ $SmIg^+$), B ($Cylg^-$ $SmIg^+$). The prognostic impact of these subclassifications and of other monoclonal antibodies (CD34, CD20, CD45) has been investigated in various clinical trials but their relevance has not been completely confirmed.[10,11] However, CD10 positivity could divide the ALL into two different groups.

Table 1. Immunophenotypic diagnosis of leukemias: impact of individual positive markers.

| *Diagnostic group* | Diagnostic value of immunological markers | | |
|---|---|---|---|
| | *Major* | *Intermediate* | *Minor* |
| *acute leukemias* | | | |
| AML | cMPO | CD33, CD13, CD65, CD117 | CD15, CD11b CD14, CD11c |
| B-ALL | m/cCD22 m/cCD79 m/clg | CD19, CD20 | TDT |
| T-ALL | m/cCD3 m/cTCR | CD2, CD5 $CD7^{++}$ | TDT, $CD7^+$ |
| *chronic leukemias* | | | |
| B-lineage | | | |
| CLL | | mIg±, CD5 MRFC, CD23 | |
| PLL | | Fmc7 | |
| HCL | | Fmc7, $CD25^{++}$ CD11c, HC2, Bly7 | |
| MM | CD38 | $CD56^{++}$, $cIgG^{++}$ | |
| T-lineage | | | |
| CLL | | CD8 | $CD2^{++}$ |
| PLL | | | CD7 |
| ATLL | | CD25, CD4 | $CD2^{++}$ |
| SS | | CD4 | |
| NK-lineage | | $CD56^+/CD16^+$ $CD3^-$ | CD11b, CD2 CD11c, CD7 |

*ALL: acute lymphoblastic leukemia; AML: acute myeloblastic leukemia; CLL: chronic lymphocytic leukemia; MM: multiple myeloma; MDS: myelodysplastic syndrome; PLL: prolymphocytic leukemia; HCL: hairy cell leukemia; ATLL: adult T-cell leukemia/lymphoma; SS: Sezary syndrome. See also Reference 106.*

In fact, a correlation exists between antigen positivity and better prognosis for both T and B ALL. CD10 positivity is an important risk factor in the overall population, but in infants it identifies subgroups of diseases in which different therapeutic treatments are needed.[10-13]

Children with SIg positivity (B ALL) have a very poor prognosis when conventional chemotherapy protocols are employed, but they could achieve a good response by following a specific therapeutic approach;[14-16] however, if Cylg is also present (transitional pre B-B), the prognosis is good even when conventional protocols are utilized.[17]

In some cases of ALL (from 5 to 20% in different studies) the lymphoblasts express *myeloid*-associated antigens. The clinical impact of these immunophenotypes is not completely

clear, due to numerous problems principally connected with the antigens considered strictly *myeloid*, with the various chemotherapy trials and with the method used to detect the antigens (microscope, FCM, direct or indirect fluorescence).

A poorer prognosis has been demonstrated for adults and infants with lymphoblasts positive for myeloid antigens, while in children this difference is not always evident.[14,18-23] Flow cytometry plays a fundamental role in distinguishing these forms in which lymphoid and myeloid antigens are simultaneously expressed on the blast cells from those in which residual *normal* myeloid cells are present in the leukemic bone marrow, and from those leukemias in which two different populations are present, one lymphoid and one myeloid.

The fluorescence microscope is not able to identify the different morphological findings correctly, while biparametric FCM histograms based on scatter properties versus FITC-fluorescence could be routinely used to identify different antigen positivity in cell populations with different morphological patterns; however, these particular forms can be unequivocally classified only by simultaneously using reagents conjugated with different fluorochromes in double or triple combinations.

Regarding acute myeloblastic leukemia (AML), the contribution of FCM immunophenotyping of blast cells is particularly relevant for the diagnosis of acute megakaryoblastic leukemias[24,25] and for the microgranular variant of acute promyelocytic leukemia cases,[24,26] where the final diagnosis might not be completely clear on the basis of morphology.

Another important goal of AML flow cytometric immunophenotyping has been the classification of these patients on the basis of both the forward scatter/side scatter distribution pattern of the bast cells and their reactivity to monoclonal antibodies directed against antigens associated with different myeloid cell lineages and their differentiation stages.[26] Moreover, the expression of a certain number of antigens such as CD34, CD11b and CD7 has been related to the prognosis of the disease.[24,27-31]

Furthermore, a large percentage of AML express *lymphoid antigens* and these phenomena are sometimes correlated to specific subtypes of myeloid leukemias.[22,23] This particular aspect reflects population expansions not usually present or hardly represented in *normal* bone marrow, and it could be applied in monitoring the efficacy of chemotherapy when a morphological follow-up is not easy in AML in the presence of recovering bone marrow.

The use of FCM to study chronic lymphoproliferative disorders has had a particularly important impact on the differential diagnosis between reactive lymphocytosis and monoclonal diseases because this methodology allows a rapid, sensitive, objective and reproducible diagnosis and classification of these hematologic disorders.[3,7,31] Accordingly, the use of the CD19/κ light chain/λ light chain triple staining combination is of great help in assessing the presence of monoclonal B-cells in peripheral blood, bone marrow, lymph node or spleen single-cell suspensions.[3] In a similar way, the application of FCM together with the use of different monoclonal antibody combinations has contributed to the classification of other chronic lymphoproliferative disorders;[32] this is particularly relevant to the diagnosis of cases involving large granular lymphocytes, since it allows a distinction to be made between patients displaying a T-cell origin ($CD3^+$/T-cell $receptor^+$) and those in whom NK-cell lineage is involved ($CD3^-$/T-cell $receptor^-$).[3]

Recently, the use of FCM and its multidimensional analytical capabilities to study cell subpopulations that only represent a small proportion of the whole sample has become a major challenge for this technology.[2,3] Therefore the use of FCM in this area has focused on the detection of minimal residual disease in acute leukemias achieving morphologic complete remission.[33,34] Preliminary reports[34,35] indicate that the application of FCM together with the use of a set of different monoclonal antibody combinations may be a suitable methodology for the detection of minimal residual disease in a high proportion of acute lymphoblastic leukemia patients. Accordingly, it is suggested that this methodology can achieve a level of sensitivity that would allow the detection of one

leukemic cell among 100,000 normal cells. The demonstration of either the persistence of cells displaying leukemic phenotypes during chemotherapy or their increase after achieving complete remission would significantly contribute to an earlier diagnosis of leukemia relapse.

The recent introduction of commercial fixatives able to permeabilize cellular membranes, thus preserving surface immunoreactivity and cellular morphological aspects (on the basis of *scatter* properties), is an important step forward in circumventing the limitations of FCM in identifying intracellular antigens. Preliminary results have indicated the possibility of simultaneously detecting surface, cytoplasmic and nuclear antigens, which has wide range of utilization in the study of minimal residual disease and oncogene expression.[36] In particular, the detection of nuclear TdT in ALL bone marrow in combination with surface antigen identification permitted analysis of a large number of cells with distinct physical properties in order to reveal a minimal blast cell component.

### *The measurement of proliferative activity and DNA ploidy in hematological malignancies*

One of the applications of FCM which has had a major impact on the study of leukemias and lymphomas is the measurement of nuclear DNA content.[1,3,5,37-39] This methodology usually provides two different types of information.[37]

On the one hand, the proliferative activity of a certain cell population is assessed by means of analyzing its distribution throughout the different cell cycle phases, in particular the S-phase. Accordingly, the proportion of cells that display intermediate DNA content between that of normal diploid resting cells (2n)(G0/G1 cell cycle phases) and that of those cells which have duplicated their DNA and are about to divide (4n)(G2 phase and mitosis) is assessed.

On the other hand, the presence or absence of clonal abnormalities in the DNA content of a certain cell population as compared with that of normal cells (DNA index) at the same cell cycle phase is analyzed. These kinds of abnormalities have been termed cytofluorimetric DNA aneuploidy (Table 2).

More recently, FCM analysis of DNA content has been used together with several other FCM techniques[40] to characterize and distinguish the two different mechanisms of cell death: apoptosis and necrosis.[41,42] The most common marker of apoptotic cells in FCM is the decreased stainability of their DNA with a variety of fluorochromes.[40] Experimental and clinical studies based on this approach have been conducted in hematological malignancies with promising results.[43-45]

*Proliferative activity*

Analysis of cell cycle kinetics in malignant hematologic disorders has demonstrated the existence of important proliferative heterogeneity.[46,47] In particular, B-CLL patients display quite a low percentage of peripheral blood S-phase cells, usually less than 1%, with respect to those detected in both ALL and AML.[3,6] Non-Hodgkin's lymphoma (NHL) patients show an intermediate proliferative index.[3,47] Proliferative activity is measured either as the percentage of S-phase cells or by reactivity with the Ki67 monoclonal antibody, and it is lower in low-grade NHL than in the high-grade lymphomas.[38,47] Furthermore, there is great variability regarding the results reported in the literature, even when a single diagnostic group of patients is considered.[47] This is probably related to the methodologic approaches used such as the type of fluorochrome, the sample preparation technique, the method of analysis, the site of the study, the use of a technique that allows

Table 2. Flow cytometric DNA ploidy status and percentage of cells in DNA synthetic phase in hematological malignancies.

| *Diagnostic group* | *% DNA FCM aneuploid cases* | *% S-phase cells* |
|---|---|---|
| ALL | 25-35 | 4.6±3.2 |
| AML | 4-20 | 7.3±5.9 |
| B-CLL | 0-5 | 0.2±0.5 |
| MM | 40-80 | 3.3±2.6 |
| MDS | 10-50 | — |

AML: acute myeloblastic leukemia; B- CLL: chronic lymphocytic leukemia B-cell type; MM: multiple myeloma; MDS: myelodysplastic syndromes). Personal unpublished data from Orfao A, et al.

discrimination between normal and leukemic cells.[47,48] As an example, the mean percentage of S-phase cells found in previously published studies on AML varies between 5% and 11%. In addition, at present it is known that the time of DNA synthesis (Ts) and the cycling time (Tc) may show wide variability from one case to another. Ts variations from 4 to 49 hours and Tc variations from 16 to 292 hours have been reported.[6]

In order to gain further insight into this variation, several authors have investigated the existence of a relationship between the proliferative rate and the morphologic characteristics of the leukemic cells in different hematologic malignancies. As a result it has been observed that while in ALL L2 morphology is related to a higher mean percentage of S-phase cells than in L1 cases,[3,6] the monocytic subtypes in AML patients are associated with higher numbers of peripheral blood S-phase cells.[3,24] In a similar way, lower proliferative activity has been noted in refractory anemia (RA) and RA with sideroblasts as compared to RA with excess of blasts and chronic myelomonocytic leukemia.[6,47] There have been many studies in which the prognostic value of these parameters, both in terms of response to treatment and of survival, has been analyzed.[3,6,47-51]

Although there are very often differences between the results obtained by individual authors, it can be stated as a general rule that high proliferative activity has been associated with a lower survival rate. B-NHL and B-CLL cases with a higher proliferative rate and higher peripheral blood S-phase counts show a poorer prognosis.[6,47,52] Identical results have been described for multiple myeloma (MM) where a high S phase has been associated with a less fovarable prognosis.[2,3,6,53-56] Finally, myelodysplastic syndromes (MDS) with a greater percentage of bone marrow S-phase cells have been reported to have poor clinical outcomes.[3,6]

However, these results should be taken with caution since, with the exception of a small number of reports that mainly refer to NHL, B-CLL and AML, the independent prognostic value of S phase cells has been neither assessed nor detected.[48] As a matter of fact, there is still considerable controversy surrounding the possible prognostic information that can be obtained from analyzing cell proliferation during acute leukemia, especially since many conflicting results have been reported. Given the clear-cut role cytokinetic factors play in defining the susceptibility of leukemic cells to chemotherapy drugs,[46,57-59] these discrepancies are unexpected. Several factors are responsible for the discrepancies that emerge in this area. First of all, only small groups of patients have been studied, and the induction regimens employed in them showed considerable variation. Secondly, often the only kinetic parameter these investigations scrutinized was the percentage of cells in S-phase. This places severe restrictions on the analysis since the S-phase offers only limited insight into cell proliferation. In order to analyze the cell cycle properly in acute leukemia, the temporal parameters connected with cell proliferation also need to be considered.[60-62] A third drawback to these studies was the fact that an accurate estimate of the percentage of cycling cells (i.e. of the leukemic growth fraction) as well as of the resting (G0) cell fraction is essential for obtaining a more realistic picture of cell proliferation, but in spite of the FCM methods proposed[63,64] it has generally been difficult to estimate these parameters *in vivo*.

A possible way of avoiding these shortcomings could be based on the following principles. The first is to adopt a standard induction regimen in which pure S-phase-specific drugs (vincristine and arabinosylcytosine) are given prior to anthracyclines in the induction treatment of a large and homogeneous series of adult patients with AML. Next, utilize multiparameter FCM techniques to study: DNA content,[57] *in vivo* incorporation of bromodeoxyuridine (BrdU) for the temporal kinetic parameters (Ts and tumor potential doubling time, Tpot),[65] and expression of the proliferating cell nuclear antigen (PCNA/cyclin) as a marker of the leukemic growth fraction.[66-68]

An example of this can be found in a recent experience involving 68 adult patients with untreated AML who entered a clinical study. All patients received a BrdU infusion prior to starting remission induction therapy in order to

allow study of bone marrow (BM) blast kinetics.[69] For induction remission each patient received 2 courses of the same regimen that included sequential administration of vincristine and arabinosylcytosine followed by anthracyclines.[70] In this series when the 2n-4n%, BrdU-LI, Ts and Tpot were considered, the proliferative activity of CR patients was always higher than that of the NR ones. When the percentage of PCNA-positive blasts was considered, AML proliferative activity was significantly higher in CR patients that in those who did not respond, and it was also greater in those cases that showed CR and survival durations longer than the median values for the total patient population, and greater than in those patients whose CR and/or survival lasted less than the overall median intervals.[70]

When multivariate analysis was performed, the WBC count emerged as the most important parameter, followed directly by the Tpot of the proliferating (PCNA-positive) blasts; every other variable exerted a relatively negligible influence in the model. A particular effort was made to render the data obtained from the *in vivo* BrdU/DNA FCM assay more significant by including a determination of the leukemic growth fraction identified by means of a cell cycle-related protein together with FCM.[71-73]

These results confirm the importance of FCM in these kinds of studies and support the hypothesis that high proliferative activity represents a favorable prognostic indicator in the treatment of AML patients with protocols designed by taking cytokinetics into consideration.

One of the most challenging tasks for the future in this sense will be to improve and individualize treatment modalities based upon the biological features of the disease. This hope could become reality since a new group of agents are available that have the potential to modulate cell growth and differentiation at different levels, e.g. hemopoietic growth factors. Flow cytometry has been used successfully to detail the cytokinetic effects of some of these factors on human bone marrow,[74,75] and clinical trials in AML have been initiated that employ colony-stimulating factor treatment to push leukemic cells into proliferating in order to increase their sensitivity to cytotoxic agents.[76-78]

*Flow cytometric DNA ploidy*

The incidence of DNA aneuploidy in hematologic malignancies is lower than that detected by cytogenetic methods.[3,47] This is basically due to the fact that even under the best technical conditions FCM does not allow detection of compensated karyotypic abnormalities in those cases where there is no DNA loss or gain.[37] Moreover, simple monosomies and trisomies are undetectable in almost all occasions when this technique is used. However, FCM has the advantage of allowing study of interphase nuclei without the need to induce cell division. As a general rule, the incidence of DNA aneuploidy is quite low in CLL (< 6%) and in Hodgkin's disease, and high in multiple myeloma (MM) (40-80%).[6,47] Acute *de novo* leukemias show an intermediate incidence that ranges from 5 to 20% in AML and from 25% to 35% in adult and juvenile ALL, respectively.[6,37] The presence of hyperploidy within aneuploid cases is much more common than that of hypoploidy. The incidence of aneuploid cases that have a second aneuploid cell population is normally less than 10%. In NHL, the incidence of DNA aneuploid cases is greater in high-grade lymphomas.[37] In ALL patients the presence of DNA aneuploidy has been associated with both an L2 morphology and a common ($CD10^+$) phenotype,[3,6] while in MM, using bivariate FCM, a correlation has been demonstrated between this parameter and Ha-ras oncogene expression.[79]

In pediatric ALL patients a diploid DNA content at diagnosis is considered an important feature for correctly assigning the risk factor. A DNA index > 1.16 (corresponding approximately to total of 53 chromosomes) is a favorable independent prognostic factor, above all if it is associated with an age from 4 to < 8 years and a common immunophenotype ALL. The disease-free survival of these children with current chemotherapy protocols is > 97% at 3 years.[80]

Although data on the prognostic value of this parameter are frequently contradictory, it appears to be evident that hypoploid MM, MDS and ALL cases have a poorer prognosis

while hyperploid ALL patients have a better outcome.[3,47] Regarding AML patients DNA aneuploidy has not been shown to have a prognostic impact, unlike the relationship observed between karyotypic abnormalities and the clinical outcome of these patients.[2,6] In NHL, the existence of DNA aneuploidy has been associated with a less favorable prognosis, although it does not appear to be an independent prognostic factor.[47]

### *The detection of drug-resistant leukemic cells using flow cytometry*

Cellular resistance to chemotherapy in leukemia patients is multifactorial. However, both the absence of initial response to chemotherapy and the progression after remission can probably be explained to a large extent by the functional role of a limited number of detoxifying mechanisms that are also present in normal cells. Some of these have already been characterized from the biochemical point of view and specific assays for measuring this resistance have been established. Preliminary reports suggest that based on an assessment of drug-resistance mechanisms in leukemia, chemotherapy could probably be either established or adapted on an individual basis.[81]

Among the analytical methodologies available for the study of drug resistance, FCM is particularly suitable because it permits a quantitative measurement on a single cell basis which, in turn, leads to a rather sensitive and objective assessment of cell heterogeneity in leukemia.[82-87] At present different procedures have been tested to measure drug resistance in human malignancies, most of which deal with the multi-drug resistance phenotype through assessment of either the p-glycoprotein (gp-170) efflux pump or cellular glutathione content.[88,89] An alternative method uses FCM and two fluorochromes. The first, doxorubicin, is a drug involved in these mechanisms; the second, rhodamin 123, is a reagent that utilizes this mechanism to go in and out of the cells. These molecules, when excited by an argon laser, show emission at 560-600 nm and 510-530 nm, respectively. Blasts cells are incubated at 37°C in the presence of one of these fluorochromes, and their uptake is evaluated at different times in the presence or absence of MDR inhibitors (cyclosporine, verapamil). The difference in the fluorescence cell intensity with and without inhibitors is the expression of gp-170 function. It is also possible to use double fluorescence to study the mechanism in a particular cell subset by means of monoclonal atibodies with appropriate conjugation (FITC for doxorubicin, phycoerythrin for Ro123). This functional approach could be very interesting for identifying MDR resistant leukemia cells that are negative if examined by specific monoclonal antibodies or gene expression.[90,91]

Recently, a different method has been proposed by which drug-resistance could be measured for S-phase specific drugs after quantifying its effect on the inhibition of DNA-synthesis.[92,93] Its application to the measurement of ara-C resistance in AML patients will be discussed in this paper. Ara-C is one of the most effective agents for the treatment of human acute leukemias; it is an S-phase-specific agent and its active metabolite ara-CTP is a substrate for DNA polymerase that is incorporated into the DNA of those cells actively synthesizing DNA.[92] As a consequence, ara-C incorporation into cells strongly correlates with cytotoxicity and results in the inhibition of DNA synthesis.[93-95] In addition, a correlation between the inhibition of DNA synthesis and response to treatment with ara-C has been reported.[94,95]

The use of BrdU/DNA staining with an anti-BrdU monoclonal antibody and propidium iodide (PI) allows simultaneous assessment of both cell cycle distribution and the rate of DNA synthesis of a certain cell population in a simple and reproducible way.[92] Changes in the rate of DNA synthesis are quickly and accurately evaluated at FCM from BrdU/DNA bivariate distributions since the amount of BrdU incorporated over a short period of time is proportional to the amount of DNA synthesized during that period.

In order to have a reliable evaluation of the degree of resistance of leukemic cells to ara-C in clinical situations, it is necessary to determine the proportion of S-phase cells resistant to ara-C, as well as the level of BrdU incorporation

into ara-C resistant cells. Recently, a computerized method for analyzing BrdU/DNA bivariate distributions of HL60 cell lines that are sensitive or resistant to various doses of ara-C has been described. According to this method the degree of resistance is automatically obtained and is expressed as an index of ara-C resistance (RI). This RI is defined as the product of RS by MS, where RS is the ratio between the percentage of S-phase cells in the presence and absence of a certain dosage of ara-C, and MS is the ratio between the mean fluorescence channel (BrdU) of S-phase cells incubated in the absence of ara-C. When applied to leukemia cell lines, this method has been shown to quantify the effect of ara-C on the inhibition of DNA-synthesis. A similar degree of correlation was found when this technique was used to assess resistance to chemotherapy in a group of 121 AML patients treated with a protocol that included daunorubicin (50-60 mg/m$^2$/day, 3 days) and ara-C (100 mg/m$^2$/day, continuous infusion, 7-10 days).[97] In order to evaluate RI properly in this study a minimum of 3% S-phase cells after 48 hours of cell culture was considered essential, and this requirement was met by 96 out of the 121 patients. Of these, 81 (84%) achieved complete remission (CR) and 15 (16%) did not. RI was calculated for each patient with two different doses of ara-C that encompassed the presumed plasma concentration of the drug (0.1 mg/mL and 1 mg/mL); RI(0.1) and RI(1) correspond to the doses of ara-C used.

Based on the results obtained after pulsing cultured cells with BrdU in the presence and absence of ara-C (0.1 and 1 mg/mL), patients were grouped into three categories. Group I included those cases displaying an RI(0.1) and an RI(1) below 8, while Group III was formed by patients with both an RI(0.1) and an RI(1) over 8. Group II AML cases showed an RI(0.1) greater than 8 and an RI(1) less than 8. Upon analyzing response to chemotherapy, it was observed that this classification was able to discriminate between ara-C sensitive and resistant cases since most of the patients in group I achieved CR (62/63), while all the cases included in Group III (9/9) were in failure. Group II showed an intermediate distribution, with 19 out of 24 patients achieving CR.[97] While in this study an RI threshold of 8 was the optimal level for discriminating between ara-C sensitive and resistant cases, this value needs to be confirmed in larger series of patients. The results of this study demonstrated that RI is independent of the shape of the BrdU labelling distribution, and that slightly resistant homogeneous blast cell populations as well as individual resistant cells among ara-C sensitive leukemic cells can be detected with this method.

It was concluded that when this approach is used to measure ara-C resistant AML cases it is essential to analyze both the percentage of S-phase cells after ara-C treatment and the extent of BrdU incorporation within these cells, in order to assess both the percentage of potentially resistant cells and their degree of resistance.[94,97]

### *The use of flow cytometry in studying platelets*

Flow cytometric analysis of human platelets represents a relatively new, emerging field of application of this technology in both clinical and research hematology.[2,3] For several reasons FCM is ideally suited for studying platelets, and at present there are several clinically relevant aspects of platelet pathophysiology and function that can be analyzed with FCM. The array of surface glycoproteins expressed on platelets has been extensively studied. Most of the major surface structures have been assigned functions and their structure has been determined at the molecular level. The availability of monoclonal antibodies directed against both functional and structural determinants on these molecules permits most of the flow cytometric techniques utilized in this area.[2,3] There are essentially three functional responses that platelets are capable of making when they are stimulated.[99] They can release the contents of their granules (release), they can bind to a surface (adhesion), and they can aggregate with other platelets (aggregation). Flow cytometry is an excellent tool for studying each of these functions.[2,99] Compared to other standard methods, FCM offers a number of advantages for the study of platelets. These include: 1) the need for relatively small amounts of blood, especially when the patient

is thrombocytopenic; 2) the ability to obtain information about the physiologic state of platelets with an absolute minimum of manipulation; 3) the ability to positively identify platelets and thus be certain that the measurements are not contaminated by other cell types or by *debris*. In addition, FCM presents the same major advantages for the study of platelets that it does for other applications, namely the ability to detect functional subpopulations, and the ability to obtain correlated measurements of several cellular properties simultaneously. These last two advantages, when applied to platelets, have immediately led to fascinating new observations that were never before possible with any other technique, and which had not been previously suspected.

At present there are at least four applications of FCM to the study of platelets that have potential clinical relevance.[2,3] These include the measurement of platelet-associated immunoglobulins, the analysis of platelet nucleic acid content, the diagnosis of primary thrombocytopathies and the assessment of both platelet activation and function. Of these, probably the most widely used is the measurement of platelet-associated immunoglobulins (PAIg), sometimes referred to as *antiplatelet antibodies*.[4] The goal of measuring platelet-associated Ig is simply stated. It is desirable to be able to quantitate the amount of Ig on platelets because there are a number of disorders in which the binding of antiplatelet antibodies can be reasonably suspected as the cause of the premature destruction of platelets. In essence the use of the flow cytometer involves much the same approach as other PAIg immunoassays. One must prepare platelets from blood, label them with a fluoresceinated anti-Ig antibody, and then measure the amount of fluorescence associated with the platelets. There are, however, several significant differences. First, when using the flow cytometer it is not necessary to purify the platelet preparation to nearly the extent required by other methods.[2,4] This is because the flow cytometer is capable of directly identifying platelets even when they are heavily contaminated with erythrocytes and leukocytes. Thus, because the cytometer can reliably identify platelets, PAIg determination is restricted to platelets and contaminating cells do not influence the measurement as they would in an immunoassay.[2-4] Second, the cytometer carries out the determination of PAIg on individual platelets. If such a measurement is made on 100,000 or even on 10,000 platelets the statistical accuracy of the average level of PAIg is extremely good. Thus it is not necessary to process very much blood in order to obtain a sufficient number of platelets for flow cytometer analysis. One mL of blood from a patient with a platelet count of $10\times10^9$/L contains $10^6$ platelets and should be enough. Third, the measurement is performed with platelets in suspension. Thus there is no concern that Ig which might have been trapped in the interstices between platelets will influence the measurement. Finally, the flow cytometric method is easily adapted to the determination of several different antigens on the same sample. Thus, for example, the measurement of platelet-associated IgG, IgM and C3 can be accomplished on the same sample with no changes in the basic technique.[2-4]

Using the dye known as thiazole orange, it is now possible to determine individual platelet nucleic acid content by flow cytometry.[3,100,101] When applied to platelets this method may represent a clinically useful technique based on the results of different groups.[100,101] It is postulated that the *reticulated platelets*[102] detected in this way represent recently released platelets which mature rapidly in circulation, and that their measurement is an estimate of the rate of thrombopoiesis in the same sense that an erythrocyte reticulocyte count is a measure of erythropoiesis. It is likely that the ability to measure the proportion of young platelets easily will prove to be clinically useful in the differential diagnosis of unexplained thrombocytopenia.

The development and availability of different monoclonal antibodies directed against platelet-associated glycoproteins that have already been characterized from a functional point of view has rapidly increased the use of FCM in the study of platelets.[103,104] Accordingly, the possibility of using this technology to identify the abnormal expression of gp IIb/IIIa and gpIb on the surface of platelets is now being utilized in many laboratories to support the diagnosis of Glanz-

mann's disease and Bernard-Soulier syndrome, respectively. Recently, several groups described immunological markers of platelet activation which, in combination with FCM, may prove to be of considerable clinical utility. In addition to their possible clinical applications, the areas in research for such markers are too numerous to describe here. There are a number of situations in which one might expect platelet activation markers to demonstrate clinical utility. These can be broadly classified as those areas involving *biocompatibility* issues, platelet storage and transfusion, those involving assessment of cardiovascular disease and assessment of platelet dysfunction. Data are currently available to show that: 1) platelets become activated during storage under blood bank conditions, and activated platelets do not remain in the patient's circulation; 2) platelets become activated during extracorporeal circulation such as cardiopulmonary bypass and renal dialysis; 3) platelets become activated during vascular damage, myocardial infarction and thrombosis, and 4) there is increased activation in patients at high risk for cardiovascular events.

The recent availability of immunological markers of platelet activation such as CD62, CD63 and CD69, combined with the unique capability of FCM to identify small subpopulations of platelets and to quantitate membrane glycoprotein levels, now allows us to assess platelet function in an entirely new way. The clinical and research potential of these developments is only beginning to be explored.

### *Conclusions*

The aim of the present review was to summarize what FCM is currently able to do in the clinical hematology laboratory and to suggest possible directions of future developments. The authors have tried to demonstrate that FCM is not simply a research tool, but a technique that will ultimately find its place in the mainstream of hematology laboratory practice.

Flow cytometric analysis will probably be indispensable for a rapid and objective assessment of the cell surface and different intracellular characteristics and, when combined with other clinical and morphometric data, this methodology will help to improve diagnosis and patient care. The quality control and standardization methods recently applied to the generation of flow cytometric data have greatly improved their reliability.

There are already several areas involving the diagnosis/prognosis of hematological diseases in which the use of FCM techniques is approaching a consensus, in particular the initial phenotyping of acute leukemias and lymphoproliferative disorders.[105] The FCM industry is rapidly evolving:[106] flow cytometers are becoming more user friendly, and there is much interest in the development of fluorochromes for multiparametric analysis[107] and the clinical application of new clusters of monoclonal antibodies.[108]

The next step will be to see that all clinical laboratories adhere to such rigid standards and that inter-laboratory quality control becomes likewise stringent, in order to verify the reproducibility of results obtained in the above mentioned fields of hematology. Thus FCM-derived data will acquire a more conclusive clinical impact.



### *References*


1. Andreeff M, ed. Clinical cytometry. Ann New York Acad Sci 1986; 468:1-512.
2. Laerum OD, Bjerknes R, eds. Flow cytometry in haematology. London:Academic Press, 1992.
3. Orfao A, Ruiz-Arguelles A. Citometria de flujo y su aplicacion en hematologia. In: Lopez Borrasca A, Arocha Pirango C, Campos Guerra C, Parreira A, Pavlovsky S, Ruiz-Arguelles G, San Miguel JF, eds. Enciclopedia de Hématologia Iberoamericana. Universidad de Salamanca, 1993:161-75.
4. Ault KA. Flow cytometric measurement of platelet associated immunoglobulin. Pathol Immunopathol Res 1988; 7:395-408.
5. Coon Js, Landay Al, Weinstein RS. Advances in flow cytometry for diagnostic cytopathology. Lab Invest 1987; 57:453-79.
6. Andreeff M. Flow cytometry of leukemia. In: Melamed MR, Lindmo T, Mendelsohn ML, eds. Flow cytometry and sorting. New York:Wiley-Liss, 1990:697-724.
7. Foon KA, Todd III RF. Immunologic classification of leukemia and lymphoma. Blood 1986; 68:1-31.
8. Hernandez JM, Gonzalez-Sarmiento R, Martin C, et al. Immunophenotypic, genomic and clinical characteristics of blast crisis of chronic myelogenous leukemia. Br J Haematol 1991; 79:408-14.
9. Hernandez JM, Sanchez I, Gonzalez M, et al. Acute lymphoid leukemias following either a previous chronic myelogenous leukemia or myelodysplastic syndrome: phenotypic and genomic differences. Am J Hematol 1993; 43:256-8.
10. Pui C-H, Raimondi SC, Head DR, et al. Characterization of childhood acute leukemia with multiple myeloid and lym-

phoid markers at diagnosis and at relapse. Blood 1991; 78: 1327-37.
11. Basso G, Rondelli R, Putti MC, et al. Incidence and prognostic significance of immunophenotypic subgroups in childhood acute lymphoblastic leukemia: the experience of the AIEOP cooperative study. Recent Results Cancer Res 1993; 131:297-307.
12. Basso G, Putti MC, Cantù-Rajnoldi A. The immunophenotype in infant acute lymphoblastic leukemia: correlation with clinical outcome. An Italian multicentre study (AIEOP). Br J Haematol 1992; 81:184-91.
13. Ferster A, Bertrand Y, Benoit Y, et al. Improved survival for acute lymphoblastic leukemia in infancy: the experience of EORTC-Childhood Leukemia Cooperative Group. Br J Haematol 1994; 86:284-90.
14. Reiter A, Schrappe M, Dieter Ludwig W, et al. Favorable outcome of B-cell acute lymphoblastic leukemia in childhood: a report of three consecutive studies of the BFM group. Blood 1992; 10:2471-8.
15. Sullivan MP, Pullen DJ, Crist WM, et al. Clinical and biological heterogeneity of childhood B cell acute lymphocytic leukemia: implications of clinical trials. Leukemia 1990; 4: 1000-11.
16. Rosanda C, Cantù-Rajnoldi A, Invernizzi R, et al. B-cell acute lymphoblastic leukemia (B-ALL): a report of 17 pediatric cases. Haematologica 1992; 77:151-5.
17. Cantù-Rajnoldi A, Rondelli R, Putti MC, et al. Co-expression of myeloid antigens in childood acute lymphoblastic leukemia: relationship with the stage of differentiation and clinical significance. Br J Haematol 1991; 79:40-8.
18. Pui C-H, Behm Fg, Crist WM. Clinical and biological relevance of immunologic marker studies in childhood acute lymphoblastic leukemia. Blood 1993; 82:343-62.
19. Pui C-H, Schell MJ, Raimondi DR, et al. Myeloid-antigen expression in childhood acute lymphoblastic leukemia. N Engl J Med 1991; 325:1378-81.
20. Wiersma SR, Ortega J, Sobel E, Weinberg KI. Clinical importance of myeloid antigen expression in acute lymphoblastic leukemia in childhood. N Engl J Med 1991; 324:800-8.
21. Sobol RE, Mick R, Royston I, et al. Clinical importance of myeloid antigen expression in adult acute lymphoblastic leukemia. N Engl J Med 1987; 316:1111-7.
22. Kuerbitz SJ, Civin CI, Krisher Y, et al. Expression of myeloid-associated and lymphoid-associated cell surface antigens in acute myeloid leukemia of childhood: a pediatric oncology child study. J Clin Oncol 1992; 10:1419-29.
23. Drexler HG, Thiel E, Ludwig WD. Acute myeloid leukemias expressing lymphoid-associated antigens: diagnostic incidence and prognostic significance. Leukemia 1993; 7:489-98.
24. Orfao A, Vidriales B, Gonzalez M, Lopez-Berges MC, Del Canizo MC, San Miguel JF. Diagnostic and prognostic importance of immunophenotyping in adults with acute myeloid leukemia. In: Ludwig WD, Thiel E, eds. Recent results in cancer research: recent advances in cell biology of acute leukemias. Impact on clinical diagnosis and therapy. Berlin:Springer-Verlag, 1993:369-79.
25. San Miguel JF, Gonzalez M, Canizo MC, et al. Leukemias with megakaryoblastic involvement: clinical, haematological and immunological characteristics. Blood 1988; 72:402-7.
26. San Miguel JF, Hernandez JM, Gonzalez-Sarmiento R, et al. Acute leukemia following a primary myelodysplastic syndrome: immunophenotypic, genotypic and clinical characteristics. Blood 1991; 78:768-74.
27. Geller RB, Zahurak M, Hurwitz C, et al. Prognostic importance of immunophenotyping in adults with acute myelocytic leukemia: the significance of the stem cell glycoprotein CD34 (My10). Br J Haematol 1990; 76:340-7.
28. Lee E, Yang J, Leavitt R, et al. The significance of CD34 and TdT determinations in patients with untreated *de novo* acute myeloid leukemia. Leukemia 1992; 6:1203-9.
29. Solary E, Casanovas R, Campo L and the Group d'étude immunologie de leucemies. Surface markers in adult acute myeloblastic leukemia: correlation of $CD19^+$, $CD34^+$, and $CD14^+/DR^-$ phenotypes with shorter survival. Leukemia 1992; 6:393-9.
30. Vidriales B, Orfao A, Gonzalez M, et al. Expression of NK and lymphoid-associated antigens in blast cells of acute myeloblastic leukemia. Leukemia 1993; 7:2026-9.
31. Matutes E, Catovsky D. The value of scoring systems for the diagnosis of biphenotypic leukemia and mature B-cell disorders. Leuk Lymphoma 1994; (suppl.1):11-4.
32. Bennet JM, Catovsky D, Daniel MT, et al. The French-American-British (FAB) Cooperative Group. Proposal for the classification of chronic mature B and T lymphoid leukemias. J Clin Pathol 1988; 42:567-84.
33. Campana D, Counstan-Smith E, Janossy G. The immunologic detection of minimal residual disease in acute leukemia. Blood 1990; 76:163-71.
34. Campana D, Otubo Freitas R, Coustan-Smith E. Detection of residual leukemia with immunological methods: technical developments and clinical implications. Leuk Lymphoma 1994; 1(suppl):31-4.
35. Orfao A, Ciudad J, Lopez-Berges MN, et al. Acute lymphoblastic leukemia (AL): detection of minimal residual disease (MRD) at flow cytometry. Leuk Lymphoma 1994; 13 (suppl.):87-90.
36. Pizzolo G, Vincenzi C, Nadali G, et al. Detection of membrane and intracellular antigens by flow cytometry following ORTHO permeafix TM fixation. Leukemia 1994; 8:672-6.
37. Orfao A, Gonzalez M, Ciudad J, et al. Cell cycle and DNA aneuploidy: biologic bases and terminology. In: Sampedro A, Orfao A, eds. DNA cytometric analysis. Servicio de Publicaciones, Universidad de Oviedo, 1993:13-24.
38. Merkel DA, Dressler LG, Mcguire WL. Flow cytometry cellular DNA content and prognosis in human malignancy. J Clin Oncol 1987; 5:1690-703.
39. Yen A, ed. Flow cytometry advanced research and clinical applications. Boca Raton:CRC Press Inc., 1989.
40. Darzynkyiewicz Z, Bruno S, Del Bino G, et al. Features of apoptotic cells measured by flow cytometry. Cytometry 1992; 13:795-808.
41. Huschtscha LI, Jeitner TM, Andersson W, Bartier WA, Tattersall MHN. Identification of apoptotic and necrotic human leukemic cells by flow cytometry. Exp Cell Res 1994; 212:161-5.
42. Bergamaschi G, Rosti V, Danova M, Lucotti C, Cazzola M. Apoptosis: biological and clinical aspects. Haematologica 1994; 79:86-93.
43. Bergamaschi G, Rosti V, Danova M, Ponchio L, Lucotti C, Cazzola M. Inhibitors of thyrosine phosphorylation induce apoptosis in human leukemic cell lines. Leukemia 1993; 7:2012-8.
44. Carbonari M, Cibati M,Cherchi M, et al. Detection and characterization of apoptotic peripheral blood lymphocytes in human immunodeficiency virus infection and cancer chemotherapy by a novel flow immunocytometric method. Blood 1994; 5:1268-77.
45. Oyaizu N, Mccloskey TW, Coronesi M, Chirmule N, Kalyanaraman VS, Pahwa S. Accelerated apoptosis in peripheral blood mononuclear cells (PBMCs) from human immunodeficiency virus type-1 infected patients and in CD4 cross-linked PBMCs from normal individuals. Blood 1993; 11:3392-400.
46. Andreeff M. Cell kinetics of leukemia. Semin Hematol 1986; 23:300-14.
47. Duque RE, Andreeff M, Braylan RC, Diamond LW, Peiper SC. Consensus review of the clinical utility of DNA flow

cytometry in neoplastic hemopathology. Cytometry 1993; 14:492-6.
48. Shankey TV, Rabinovitch PS, Bagwell B, et al. Guidelines for implementation of clinical DNA cytometry. Cytometry 1993; 14:472-7.
49. Mauer AM. Cell kinetics and practical consequences for therapy of acute leukemia. N Engl J Med 1975; 21:89-94.
50. Hall R, Kantarajian H, Keating M, Smith T, Freireich EJ. Pretreatment cytokinetics in acute myelogenous leukemia (AML): biologic and prognostic implications. Blood 1983; 55:474-8.
51. Lowenberg B, Van Putten WLJ, Touw IP, Delwel R, Santini V. Autonomous proliferation of leukemic cells *in vitro* as a determinant of prognosis in adult acute myeloid leukemia. N Engl J Med 1993; 328:614-9.
52. Orfao A, Ciudad J, Gonzalez M, et al. Prognostic value of S-phase WBC count in B-cell chronic lymphocytic leukemia. Leukemia 1992; 6:47-51.
53. Montecucco CM, Riccardi A, Merlini P, Mazzini G, Danova M, Ascari E. Plasma cell DNA content in multiple myeloma and related paraproteinemic disorders: relationship with clinical and cytokinetic features. Eur J Cancer Clin Oncol 1984; 20:81-90.
54. Barlogie B, Alexanian R, Dixon D, Smith L, Smallwood L, Desalle K. Prognostic implication of tumor cell DNA content and RNA content in multiple myeloma. Blood 1987; 66:338-41.
55. Tafuri A, Meyers J, Lee BJ, Andreef M. DNA and RNA flow cytometric study in multiple myeloma. Cancer 1991; 67:449-54.
56. Tienhaara A, Pelliniemi TT. Flow cytometric DNA analysis and clinical correlations in multiple myeloma. Am J Clin Pathol 1992; 97:322-30.
57. Riccardi A, Danova M, Montecucco CM, et al. Acute non lymphoblastic leukemia: reliability and prognostic significance of bone marrow S phase size determined with propidium iodide DNA flow cytofluorometry. Scand J Haematol 1986; 36:11-7.
58. Raza A, Preisler H, Day R, et al. Direct relationship between remission duration in acute myeloid leukemia and cell cycle kinetics: a Leukemia Intergroup study. Blood 1990; 76:2191-7.
59. Bokhari SAJ, Abbas A, Yousuf N, et al. Cell cycle parameters as biological predictors of prognosis in AML: a review and update of cell cycle kinetics and remission induction/duration in acute leukemia. Leuk Lymphoma 1992; 6:197-207.
60. Riccardi A, Danova M, Ascari E. Bromodeoxyuridine for cell kinetic investigations in humans. Haematologica 1988; 73:423-30.
61. Riccardi A, Danova M, Wilson G. Cell kinetics in human malignancies studied with *in vivo* administration of bromodeoxyuridine and flow cytometry. Cancer Res 1988; 48:6238-45.
62. Brons PPT, Haanen C, Boezeman JBM, et al. Proliferation patterns in acute myeloid leukemia: leukemic clonogenic growth and *in vivo* cell cycle kinetics. Ann Haematol 1993; 66:225-33.
63. Andreef M, Darzynkiewicz Z., Sharpless TK, Clarkson BD, Melamed MR. Discrimination of human leukemia subtypes by flow cytometric analyses of cellular DNA and RNA. Blood 1980; 55:282-93.
64. Preisler HD, Raza A, Gopal V, Banavali J, Bokhari J, Lampkin B. The study of acute leukemia cells by means of acridine orange staining and flow cytometry. Leuk Lymphoma 1994; 13:61-73.
65. Danova M, Wilson G, Riccardi A, et al. *In vivo* administration of bromodeoxyuridine and flow cytometry for cell kinetic studies in human malignancies. Haematologica 1987; 77:115-20.
66. Giordano M, Danova M, Pellicciari C, et al. Proliferating cell nuclear antigen (PCNA) expression during the cell cycle of normal and leukemic cells. Leuk Res 1991; 11:965-74.
67. Danova M, Riccardi A, Mazzini G. Cell cycle-related proteins and flow cytometry. Haematologica 1990; 75:252-64.
68. Casasco A, Giordano M, Danova M, et al. PC10 monoclonal antibody to proliferating cell nuclear antigen as probe for cycling cell detection in developing tissues. Histochemistry 1993; 99:191-9.
69. Giordano M, Riccardi A, Danova M, Brugnatelli S, Mazzini G. Cell proliferation of human leukemia and solid tumors studied with *in vivo* bromodeoxyuridine and flow cytometry. Cancer Detect Prev 1991; 15:391-6.
70. Riccardi A, Giordano M, Danova M, et al. Cell kinetics with *in vivo* bromodeoxyuridine and flow cytometry: clinical significance in acute non-lymphoblastic leukemia. Eur J Cancer 1991; 27:882-7.
71. Danova M, Giordano M, Mazzini G, Riccardi A. Expression of p53 protein during the cell cycle measured by flow cytometry in human leukemia. Leuk Res 1990; 14:417-22.
72. Danova M, Giordano M, Mazzini G, Riccardi A. Cell kinetics of human acute leukemia: *in vivo* study with bromodeoxyuridine and flow cytometry. In: Fleitcher S, ed. Leukemias. Berlin:Springer Verlag, 1993:32-6.
73. Giordano M, Danova M, Mazzini G, Gobbi P, Riccardi A. Cell kinetics with *in vivo* bromodeoxyuridine assay, proliferating cell nuclear antigen expression and flow cytometry: prognostic significance in acute non-lymphoblastic leukemia. Cancer 1993; 71:2739-45.
74. Riccardi A, Danova M, Paccagnella A, et al. Bone marrow myeloid cell kinetics during treatment of small cell carcinoma of the lung with chemotherapy not associated and associated with granulocyte-macrophage colony-stimulating factor. Ann Haematol 1993; 66:185-93.
75. Danova M, Lucotti C, Rosti V, Bergamaschi G, Riccardi A, Cazzola M. *In vivo* growth characteristics of human $CD34^+$ bone marrow cells after chemotherapy plus GM- or G-CSF. Blood 1993; 82:497a.
76. Bettelheim P, Valent P, Tafuri A, et al. Recombinant human granulocyte-macrophage colony-stimulating factor in combination with standard induction chemotherapy in de novo acute myeloid leukemia. Blood 1991; 77:700-11.
77. Tafuri A, Andreef M. Kinetic rationale for cytosine-induced recruitment of myeloblastic leukemia followed by cycle-specific chemotherapy *in vitro*. Leukemia 1990; 4:826-34.
78. Tafuri A, Lemoli RM, Chen R, Gulati SC, Clarkson B, Andreef M. Combination of hemopoietic growth factors containing IL-3 induce acute myeloid leukemia cell sensitization to cycle specific and cycle non-specific drugs. Leukemia 1994; 8:749-57.
79. Danova M, Riccardi A, Ucci G, Luoni R, Giordano M, Mazzini G. Ras oncogene expression and DNA content in plasma cell dyscrasias: a flow cytofluorimetric study. Br J Cancer 1990; 62:781- 5.
80. Trueworthy R, Shuster T, Look T, et al. Ploidy of lymphoblasts is the strongest predictor of treatment outcome in B-progenitor cell acute lymphoblastic leukemia of childhood: a pediatric oncology group study. J Clin Oncol 1992; 10:606-13.
81. Danova M, Riccardi A, Sacchi S, et al. New approaches to cancer chemotherapy. Hematol Rev 1994; 8:101-14.
82. Danova M, Giordano M, Erba E, et al. Flow cytometric analysis of multidrug-resistance-associated antigen (P-glycoprotein) and DNA ploidy in human colon cancer. J Cancer Res Clin Oncol 1992; 118:575-80.
83. Epstein J, Xiao H, Oba BK. P-glycoprotein expression in plasma cell myeloma is associated with resistance to VAD. Blood 1989; 74:913-7.
84. Hedley DW. Flow cytometric assays of anticancer drug resistance. In: Landay AL, Ault K, Bauer KD, Rabinovitch PS, eds. Clinical flow cytometry. Ann NY Acad Sci 1993; 677:341-53.
85. Michieli M, Damiani D, Geromin A, et al. Overexpression of multidrug resistance-associated p170 glycoprotein in acute

non-lymphocytic leukemia. Eur J Haematol 1992; 48:87-92.
86. Michieli M, Raspadori D, Damiani D, et al. The expression of the multidrug resistance-associated glycoprotein in B-cell chronic lymphocytic leukemia. Br J Haematol 1991; 77:460-5.
87. Paietta E, Andersen J, Racevskis J, et al. Significantly lower P-glycoprotein expression in acute promyelocytic leukemia than in other types of acute myeloid leukemia: immunological, molecular and functional aspects. Leukemia 1994; 8:968-73.
88. Sugawara I, Kodo H, Ohkochi E, et al. High-level expression of MRK16 and MRK20 murine monoclonal antibody-defined proteins (170,000-180,000 P-glycoprotein and 85,000 protein) in leukemias and malignant lymphomas. Br J Cancer 1989; 60:538-41.
89. Ucci G, Petrini M, Riccardi A, et al. Expression of p170 protein in multiple myeloma: a clinical study. Hematol Oncol 1992; 10:213-20.
90. Zhou DC, Marie JP, Suberville AM, Zittoun R. Relevance of mdr 1 gene expression in acute myeloid leukemia and comparison of different diagnostic methods. Leukemia 1992; 6: 879-85.
91. Kato S, Ideguchi H, Muta K, Nishimura J, Nawata H. Absence of correlation between cytotoxicity and drug transport by P-glycoprotein in clinical leukemic cells. Eur J Haematol 1991; 47:146-51.
92. Dolbeare F, Gratzner H, Pallavicini MG, Gray JW. Flow cytometry measurement of total DNA content and incorporated bromodeoxyuridine. Proc Natl Acad Sci USA 1983; 80:5573-77.
93. Major PP, Egan EM, Merrik DJ, Kufe DW. Effect of ara-C incorporation on deoxyribonucleic acid synthesis in cells. Biochem Pharmacol 1982; 31:2937-41.
94. Lacombe F, Belloc F, Dumain P, et al. Quantitation of resistance of leukemic cells to cytosine arabinoside from BrdUrd/DNA bivariate histograms. Cytometry 1992; 13:730-8.
95. Ross DD, Thompson BW, Joneckis CC, Arman SA, Schiffer CA. Metabolism of ara-C by blast cells from patients with ANLL. Blood 1986; 68:606-10.
96. Krishan A, Sauerteig A. Flow cytometric monitoring of cellular resistance to cancer chemotherapy. In: Bauer KD, Duque R, Shankey TV, eds. Clinical flow cytometry: principles and application. London:William & Wilkins, 1993:459-67.
97. Lacombe F, Belloc F, Dumain P, et al. Detection of ara-C resistance in patients with acute myelogenous leukemia using flow cytometry. Blood 1994; (in press).
98. Waldman F, Dolbeare F, Gray JW. Detection of ara-C resistant cells at low frequency, using the BrdUrd assay. Cytometry 1985; 6:657-62.
99. Kienast J, Schmitz G. Flow cytometric analysis of thiazole orange uptake by platelets: a diagnostic aid in the evaluation of thrombocytopenic disorders. Blood 1990; 75:116-21.
100. Ault KA, Rinder HM, Mitchell J, Carmody MB, Vary CPH, Hillman RS. The significance of platelets with increased nucleic acid content (reticulated platelets): a measure of the rate of thrombopoiesis? Am J Clin Pathol 1992; 98:637-46.
101. Ault KA, Rinder HM, Mitchell JG, Rinder CS, Lambrew CT, Hillman RS. Correlated measurement of platelet release and aggregation in whole blood. Cytometry 1989; 10:448-55.
102. Rinder HM, Munz UJ, Ault KA, Bonan JL, Smith BR. Reticulated platelets in the evaluation of thrombopoietic disorders. Arch Pathol Lab Med 1993; 117:606-10.
103. Shattil SJ, Cunningham M, Hoxie JA. Detection of activated platelets in whole blood using activation dependent monoclonal antibodies and flow cytometry. Blood 1987; 70:307-15.
104. Latorraca A, Lanza F, Moretti S, et al. Flow cytometric analysis of anti-platelet antibodies in idiopathic thrombocytopenic purpura. Haematologica 1994; 79:269-72.
105. Smith BR. Integrating flow cytometry in the hematology laboratory: a curmudgeon's view. In: Landay AL, Ault KA, Bauer KD, Rabinovitch PS, eds. Clinical flow cytometry. Ann NY Acad Sci 1993; 468:326-33.
106. Shapiro M. Trends and developments in flow cytometry instrumentation. In: Landay AL, Ault KA, Bauer KD, Rabinovitch PS, eds. Clinical flow cytometry. Ann NY Acad Sci 1993; 468:155-66.
107. Stewart CC, Stewart SJ. Immunological monitoring using novel probes. In: Landay AL, Ault KA, Bauer KD, Rabinovitch PS, eds. Clinical flow cytometry. Ann NY Acad Sci 1993; 468:94-112.
108. Lanza F, Moretti S, Papa S, Malavasi F, Castoldi GL. Report of the Fifth International Workshop on Human Leukocyte Differentiation Antigens, Boston, November 3-7, 1993. Haematologica 1994; 4:374-86.