

MINIREVIEW

Laser-Scanning Cytometry: A New Instrumentation
with Many ApplicationsZbigniew Darzynkiewicz,^{*1} Elzbieta Bedner,^{*†} Xun Li,^{*} Wojciech Gorczyca,[‡] and Myron R. Melamed[‡]^{*}The Brander Cancer Research Institute and [‡]Department of Pathology, New York Medical College, Valhalla, New York 10595; and [†]Department of Pathology, Pomeranian School of Medicine, Szczecin, Poland

The laser-scanning cytometer (LSC) is a microscope-based cytofluorometer which has attributes of both flow and image cytometry. Laser-excited fluorescence emitted from fluorochromed individual cells on a microscope slide is measured at multiple wavelengths rapidly with high sensitivity and accuracy. Though the instrument has been available commercially for only 3 years, it is already used in a variety of different applications in many laboratories. This review focuses on the following unique analytical capabilities of LSC which complement those of flow cytometry and fluorescence image analysis: (a) the cells are positioned on slides during measurement so they may be examined repeatedly over time, a feature useful for studies of enzyme kinetics and other time-resolved processes; (b) sequential analysis of the same cells can be carried out using different immuno- or cytochemical stains or genetic probes, merging information on cell immunophenotype, cell functions, expression of particular proteins, DNA ploidy and cell cycle position, and/or cytogenetic profile for each measured cell; (c) any of the cells measured can be relocated to correlate with visual examination by fluorescence or brightfield microscopy or with any other parameter; (d) topographic distribution of fluorescence measurements within the cell, in cytoplasm vs nucleus, permits analysis of the translocation of regulatory molecules such as NF κ B, p53, etc., and is essential for FISH analysis; (e) hyperchromicity of nuclear DNA as measured by maximal pixel fluorescence intensity allows one to identify cell types differing in degree of chromatin condensation such as mitotic or apoptotic cells; (f) analysis of tissue section architecture and of the constituents in transected cells within tissue sections by ratiometric assays normalized to DNA content extends applications of LSC in clinical pathology; (g) because cell loss during sample preparation and staining is minimal,

samples with a paucity of cells can be analyzed; and (h) analyzed cells can be stored indefinitely, e.g., for archival preservation or additional analysis. Potential future applications of LSC are discussed. © 1999 Academic Press

INTRODUCTION

Progress in cell biology is being driven by the development of quantitative analytical methods applicable to individual cells or cell organelles. It began in the 1950s with the development, by Caspersson and his colleagues at the Karolinska Institute in Stockholm, of microspectrophotometry, followed by microfluorometry and microinterferometry [1, 2]. These methods, which provided a means of measuring DNA, RNA, and proteins in the cell, initiated the modern era of cell biology, based on quantitative rather than qualitative visual cell analysis. The next methodology significantly contributing to progress in cell biology was autoradiography [3]. Applications of autoradiography were widespread and particularly fruitful in studying cell reproduction, where they provided a foundation for the modern concept of the cell cycle.

The introduction of flow cytometry (FC) initiated a third phase in methods development [4–6]. Although FC is still a relatively young methodology, it is already in wide use and has found myriad applications in basic and clinical research as it has in the diagnostic clinical laboratory [7–9]. FC offers several advantages over the methods used before. One is the rapidity of cell measurements, allowing one to analyze large populations of cells, detect rare cells, and distinguish subpopulations of cells according to their different characteristics. Also attractive is the possibility of multiparametric analysis to quantify relationships among several cell constituents in particular cell subpopulations selected by some other feature. Sorting, another valuable function of FC, has been used to select clones of live cells for propagation and even to sort individual chro-

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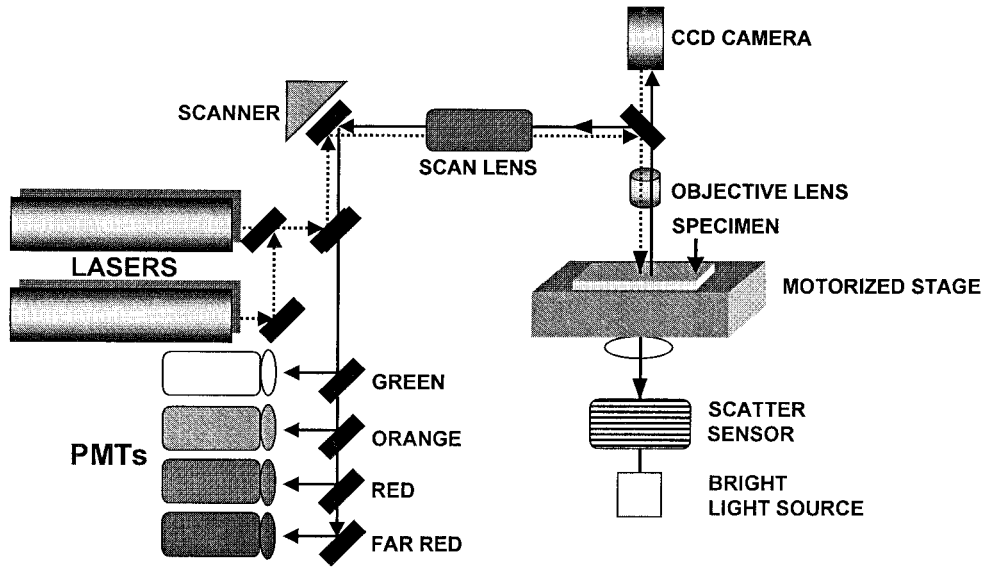


FIG. 1. Scheme representing major components of the LSC. See text for explanation.

mosomes for development of chromosomal DNA libraries. Flow cytometry with cell sorting is now indispensable in immunology, molecular and cell biology, cytogenetics, and the human genome project.

There are some limitations of FC, however, which restrict its usefulness in certain applications. Among these one can list the following:

(a) The individual cells are measured only once. Thus, FC does not allow one to analyze time-resolved events on the same cells, e.g., to measure enzyme kinetics;

(b) Although, in principle, single cells can be sorted according to their measured cell parameters and their morphology examined, this procedure is cumbersome and rarely used. As a corollary, fixing the measured cells for archival preservation is also restricted;

(c) Cell analysis by FC is at zero spatial resolution; initial attempts to resolve cell structure by slit-scan illumination found little acceptance. Thus, FC cannot provide information on the spatial distribution of fluorochromes within the cell, i.e., nuclear vs cytoplasmic localization, uniformity of distribution, localization with another fluorochrome, etc.;

(d) FC does not allow one to restain the already-measured cells with another probe(s) and merge the results on a cell-by-cell basis;

(e) Analysis of solid tissue by FC is based on measurement of dissociated cells or nuclei, and the isolation procedure, whether mechanical, enzymatic, or with detergents, produces a plethora of undesirable effects. Needless to say, the information on tissue topography, e.g., relationship of tumor cells to host stromal or infiltrating cells, blood vessels, the presence of

islands of proliferating or quiescent cells, etc., is lost after cell dissociation;

(f) Since sample preparation for FC often requires repeated centrifugations, significant cell loss occurs. The loss must be compensated by starting with large numbers of cells per sample. Therefore, small-size samples (e.g., fine needle aspirates; spinal fluid) are seldom analyzed by FC.

The newly developed, microscope-based laser-scanning cytometer (LSC) offers many of the advantages of FC and few of the limitations listed above [10, 11]. Although LSCs, which are manufactured in the United States (CompuCyte Corp., Cambridge, MA) and in Japan (Olympus Optical Co., Tokyo), became commercially available just a few years ago, numerous reports have already been published describing their capabilities and numerous applications. This review, while discussing much of the published data on LSC, is focused on applications that are unique to this instrumentation. An excellent review article describing the instrument itself, its analytical capabilities, and similarities and differences between LSC vs FC vs automated fluorescence image analysis (FIA) systems was recently published by Kametsky *et al.* [11]. It should be noted that because of similarity in name, the laser-scanning microscope, a confocal microscope illuminated by a scanning laser beam, and other instruments having a scanning laser as an illumination source happen to be confused with LSC.

PRINCIPLES OF CELL MEASUREMENT BY LSC

Figure 1 presents a diagrammatic scheme of the LSC. The microscope (Olympus Optical Co.) is an inte-

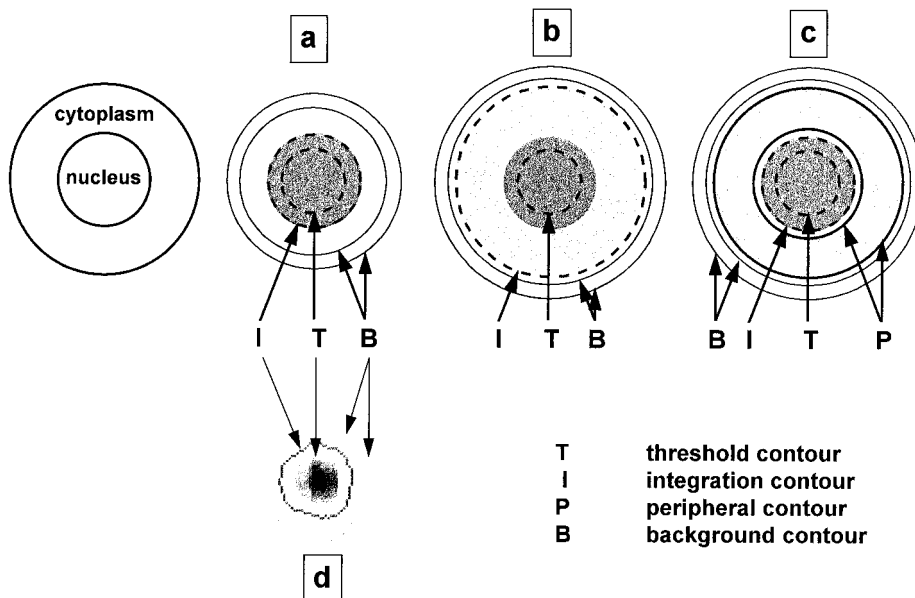


FIG. 2. Different settings for analysis of nuclear, total, and/or cytoplasmic fluorescence. When nuclear DNA is stained with the red fluorescing dye (e.g., PI), the threshold contour (T) is set on red signal to detect the nucleus, e.g., as in a. The integration contour (I) is then set a few pixels outside of T to ensure that all nuclear fluorescence is measured and integrated (a). However, when cytoplasmic fluorescence is also measured, I is set far away from T to ensure that fluorescence emitted from cytoplasm is integrated as well (b). It is also possible to separately measure nuclear and cytoplasmic fluorescence as shown in c. The peripheral contours (P) are then set at the desired number of pixels outside of I and the fluorescence intensities emitted from both areas, within the I boundary and within the P torus, are separately measured and separately integrated. In each case the background contour is automatically set outside the cell and the background fluorescence is subtracted from nuclear, cytoplasmic, or total cell fluorescence. The actual cell contours, as they appear on the monitor, are shown in d.

gral part of the instrument and provides essential mechanical and optical components. The specimen which is on a glass slide on the stage of the microscope is excited by a laser beam that rapidly scans the microscope slide. In the current instruments beams from two lasers (argon and helium neon) spatially merged by a set of dichroic mirrors are directed onto the computer-controlled oscillating (350 Hz) mirror which directs the beams through the epi-illumination port of the microscope and images them through the objective lens onto the slide. The laser beams, thus, rapidly sweep the area of the microscope slide under the lens. Depending on the lens magnification the beam spot size varies from 2.5 (at 40 \times magnification) to 10.0 μm (at 10 \times magnification). The slide with its position monitored by sensors is positioned on the computer-controlled motorized microscope stage and moved, with the stage, at 0.5- μm steps per laser scan, perpendicularly to the scan. Light scattered by the cells is imaged by the condenser lens and recorded by scatter sensors. Fluorescence emitted by the specimen is collected by the objective lens partially directed to a CCD camera for imaging. Another part of fluorescence light is directed through the scan lens to the scanning mirror. Upon reflection, it passes through a series of dichroic mirrors and optical interference filters to reach one of the four

photomultipliers. Each photomultiplier records fluorescence at a specific wavelength range, defined by the combination of filters and dichroic mirrors. A light source, additional to the lasers, provides transmitted illumination which is used to visualize the objects through an eyepiece or the CCD camera. The measurement of cell fluorescence (or light scatter) is computer controlled and triggered by setting a threshold contour for the cell above background (Fig. 2). For each measured object the following parameters are recorded by LSC:

(a) Integrated fluorescence intensity over the integration contour which can be adjusted to a desired width with respect to the threshold contour, representing the sum of intensities of all pixels within the area (Fig. 2);

(b) The value of maximal pixel within this area, so called "peak" or "max pixel value";

(c) The area within the integration contour, representing the number of pixels within the contour area;

(d) The perimeter of the contour (in micrometers);

(e) Fluorescence intensity integrated over the area of a torus of desired width defined by the peripheral contour located around (outside of) the primary integration contour. Thus, if the integration contour is set for the nucleus based on, e.g., red fluorescence (DNA

TABLE 1
Differences and Similarities between Cell Analysis by FC and LSC

	FC	LSC
Cell staining and measurement	In suspension	On a slide
Correlation of cell measurement with analysis of cell morphology (image analysis)	Only after sorting	Possible
Analysis of differences in intracellular fluorochrome localization (e.g., nucleus vs cytoplasm)	Practically not possible	Possible
Analysis of highest local intensity of fluorochrome in a cell	Not possible	Possible (maximal pixel)
Number of measurements of a given cell	One	Several
Sequential measurements of a cell in time (kinetics)	Not possible	Possible
Sequential analyses of a cell with different probes	Not possible	Possible
Cell loss during staining and measurement	Significant, depends on number of centrifugations	Minimal (<5%)
Archival storage of the measured cells	Not possible	Possible
Analysis of tissue sections	Not possible	Possible
Analysis of neighboring cell-to-cell interactions; tissue architecture analysis	Not possible	Possible
Semiautomatic FISH analysis	Practically not possible	Possible
Analysis of cells by light scattering	In forward and side directions	Only in forward direction
Cell/chromosome sorting	Possible	Not possible
Speed of cell measurement	Up to 10,000 cells per second	Up to 100 cells per second
Multiparameter analysis	Possible	Possible

stained by propidium), then the integrated (or maximal pixel) green fluorescence of FITC-stained cytoplasm can be measured separately, within the integration contour (i.e., over the nucleus) and within the peripheral contour, i.e., over the rim of cytoplasm of desired width outside the nucleus. It should be noted that all above values of fluorescence (a, b, and d) are automatically corrected for background which is measured locally outside the cell, within the background contour (Fig. 2);

(f) The slide position on X and Y coordinates of the maximal pixel; and

(g) The computer clock time at the moment of measurement.

Ratios of the respective parameters are easily preset as a new parameter, and the ratiometric data are then collected or calculated during data analysis. The spectrum overlap measured by individual photodetectors can be electronically compensated during data analysis.

There are many similarities between LSC and FC (Table 1). The measurements by LSC are rapid and with optimal cell density up to 100 cells can be measured per second. The accuracy and sensitivity of cell fluorescence measurements by LSC are comparable to the most advanced flow cytometers [10, 11]. Other features that can be measured, such as integrated fluorescence intensity of the cells, time of measurement, and forward light scatter also are identical for both instruments. However, right angle (side) light scatter, common to FC, cannot be measured by LSC. LSC, on the other hand, measures individual pixel values, which cannot be measured by FC. This parameter re-

flects inhomogeneity of the fluorochrome distribution with the analyzed object, and the peak pixel value represents the maximal concentration per area imaged on a single pixel. In contrast, peak fluorescence measured by FC represents the peak value of the analog electronic signal from fluorescence integration of the cellular fluorescence. The possibility of differential analysis of fluorescence emitted from nucleus vs cytoplasm is another feature of LSC absent in FC.

The most characteristic feature of LSC distinguishing it from FC is that cell analysis is done on a slide. This offers the possibility of visual cell examination to assess morphology and correlate it with the measured parameters. It also allows cell image capture, analysis, and/or display. Furthermore, additional cytofluorometric analysis of the same cells is possible using new sets of markers or other contouring thresholds. The results of the sequential measurements can then be integrated in list mode fashion, using the merge capability of the instrument. Applications that descend from these unique features of LSC are discussed below.

CELL MORPHOMETRY

As mentioned, in contrast to measurements by FC which are at zero spatial resolution, LSC offers the possibility of analyzing fluorochrome localization within the cell and relating it to cellular structures. Applications of LSC exploiting this feature can be categorized in three groups.

Maximal pixel (fluorescence peak) analysis. In this first group are applications that utilize the maximal pixel measurement as a feature discriminating the cell.

Maximal pixel DNA-associated fluorescence is a sensitive marker of chromatin condensation. Namely, DNA in condensed chromatin, e.g., in mitotic or apoptotic cells, shows increased stainability (per unit area of chromatin image) with most fluorochromes. Thus, even if the integrated fluorescence of the analyzed cells (representing their DNA content) is the same, the maximal pixel values of the cell with condensed chromatin is greater compared to the cell with more diffuse chromatin structure. DNA hyperchromicity was used as a marker to distinguish mitotic and immediately postmitotic G_1 cells from interphase cells [12, 13]. Although mitotic cells can be recognized by FC using a variety of markers [reviewed in 14], the advantage of this approach by LSC is that a single fluorochrome is used to discriminate between G_1 vs S vs G_2 vs M phase cells. Therefore, additional color dye(s) can be used to detect other cell constituents, e.g., cyclins, cytokeratin, cytokines, or immunophenotype markers, on the same preparation. By applying this principle, pulse labeling of DNA-replicating cells with BrdU (detected with anti-BrdU mAb) was combined with identification of mitotic cells by LSC to study the cell cycle kinetics by the fraction of labeled mitoses (FLM) method [15]. The FLM method, originally developed for autoradiography [16], provides a wealth of information on cell cycle kinetics but is cumbersome and time consuming and therefore rarely used. Its adaptation to LSC dramatically simplifies the procedure and shortens time of analysis [15].

Similar to mitosis, chromatin condensation during apoptosis also manifests by DNA hyperchromicity. Apoptotic cells, therefore, can also be identified by their high value of maximal pixel of DNA-associated fluorescence, and their identification can be combined with analysis of the cell cycle distribution [Fig. 3; Refs. 17 and 18]. However, because both mitotic and apoptotic cells are characterized by high maximal pixel value, their distinction from each other is not possible by this method. This limitation is of particular importance when apoptosis is induced by mitotic inhibitors such as Taxol or vincristine and therefore the sample contains large proportions of both mitotic and apoptotic cells.

It should be noted, however, that several other methods of identification of apoptotic cells, including their recognition by the presence of DNA strand breaks, decreased mitochondrial transmembrane potential, or fractional DNA content [reviewed in 19], have been successfully adapted to LSC [18, 20–22]. The possibility to subject the measured cells to morphological examination provided by LSC is particularly important in studies of apoptosis. This is due to the fact that apoptosis was originally defined by morphological criteria [23] and cell morphology still remains the gold standard to identify this mode of cell death. Using LSC, for example, it was possible to discriminate between

the genuine apoptotic cells and “false-positive” cells in peripheral blood and bone marrow of leukemic patients undergoing chemotherapy [18]. The latter cells were monocytes/macrophages containing apoptotic bodies (probably ingested from the disintegrating apoptotic cells) in their cytoplasm. While both the genuine apoptotic cells and the false-positive cells contained numerous DNA strand breaks and were indistinguishable by FC, analysis of their morphology by LSC made possible their positive identification [18]. Based on this observation and other findings it was concluded that LSC is the instrument of choice in analysis of apoptosis [18, 24].

Early during apoptosis the proapoptotic regulatory protein Bax undergoes translocation into mitochondria [25], where, most likely, it is involved in facilitating release of cytochrome *c* and dissipation of the mitochondrial transmembrane potential [26]. Interestingly, the mitochondrial translocation of Bax, which is reflected by the increase in its local density (as a result of accumulation in mitochondria), can be detected by LSC also by maximal pixel of Bax immunofluorescence analysis (Fig. 4). Likewise, the translocation of cytochrome *c* from mitochondria into cytosol during apoptosis can be detected by a decrease in maximal pixel of its immunofluorescence (manuscript in preparation). The analysis of maximal pixel to detect translocation of macromolecules (when the translocation is associated with change in their local density) may find many other applications, e.g., to monitor activation or deactivation of the signal transduction molecules, receptor clustering, etc. (Table 2).

The maximal pixel value was also useful as a marker discriminating lymphocytes, monocytes, and granulocytes [27]. These cell types differ between themselves by the degree of chromatin condensation. Consequently, stainability of their DNA with propidium, reflected by maximal pixel value, is also different [27]. Another parameter measured by LSC that is correlated (inversely) with chromatin condensation is fluorescence area. This parameter reflects nuclear size and is also different for lymphocytes, monocytes, and granulocytes [27]. Single-color analysis by LSC, therefore, yields differential count of white blood cells similar to that provided by FC based on simultaneous analysis of forward and side light scatter [7–9].

Nuclear vs cytoplasmic fluorescence. The second group of applications of LSC is associated with its ability to spatially resolve fluorescence within the cell and can be applied to analyze transit of individual proteins, detected immunocytochemically, between different cell compartments, notably between nucleus and cytoplasm. Translocation of individual proteins from cytoplasm to nucleus often reflects activation, and a classical example of such a protein is nuclear factor

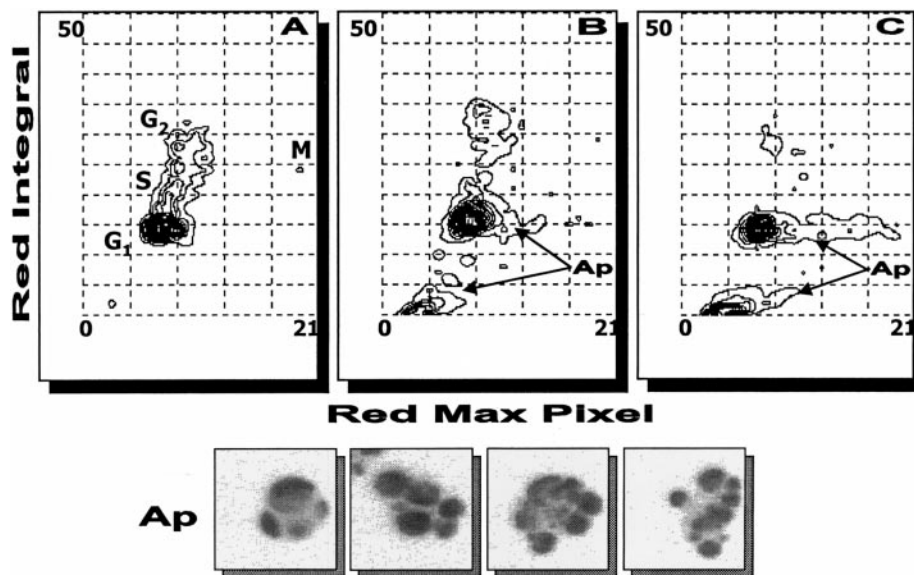


FIG. 3. Identification of apoptotic cells by LSC based on high values of maximal pixel detecting red fluorescence or fractional DNA content of propidium iodide (PI)-stained cells. Exponentially growing HL-60 cells, untreated (A) or treated with 0.15 μM DNA topoisomerase I inhibitor camptothecin (CPT) for 3 (B) or 4 h (C) to induce apoptosis, were stained with PI in the presence of 100 $\mu\text{g}/\text{ml}$ of RNase A [21, 22]. The contour maps represent bivariate distributions of cells with respect to their integrated red fluorescence (DNA content) vs maximal red fluorescence pixel value. Only mitotic cells (M) have high maximal pixel values in the untreated culture. Apoptotic cells (Ap) that are present in the CPT-treated cultures are characterized either by increased red fluorescence maximal pixel value or by a low (fractional “sub- G_1 ”) DNA content. The relocation feature of LSC allows one to observe morphology of the cells selected from particular regions of the bivariate distributions. The cells with high maximal pixel value or with fractional DNA content show chromatin condensation and nuclear fragmentation, typical of apoptosis (Ap; bottom four panels).

kappa B (NF- κ B). This ubiquitous factor is involved in regulation of diverse immune and inflammatory responses and also plays a role in control of cell growth and apoptosis [28]. In its inactive form NF- κ B remains

in the cytoplasm sequestered through interaction with I κ B protein. Rapid translocation of NF- κ B from cytoplasm to nucleus occurs in response to extracellular signals or DNA damage and is considered to be a hall-

TABLE 2
Major Applications of LSC

Applications	Advantages, examples
Immunophenotyping	Minimal cell loss (special utility for hypocellular samples), savings on reagents [38, 39]
Cell cycle analysis	Detection of mitotic and postmitotic cells by analysis of maximal pixel of DNA-related fluorescence [12, 13, 15, 17, 20, 30]
Detection of apoptosis	Examination of cell morphology, detection of apoptotic cells by analysis of maximal pixel of DNA-related fluorescence [17, 18, 48]
Analysis of enzyme kinetics, drug uptake, or ligand binding	“True” kinetics (or binding affinity) measured on the same individual cells (receptors) [37]
Analysis of activation or deactivation of macromolecules by their translocations	Cytoplasmic/nuclear translocations (e.g., activation of NF- κ B or p53) [29], mitochondrial translocations (e.g., Bax, detected by maximal pixel analysis, see Fig. 4)
FISH analysis	Rapid, semiautomatic, unbiased selection of cells [11, 32]
Correlation of cell function with morphology and other cell attributes	Unique to this instrumentation, offers a possibility to probe functions of live cells and directly correlate functional events with cell morphology and/or the changes that can be detected only on fixed cells
Studies of cell-to-cell interactions	Possible by analysis of the neighboring cells growing on the slide (identified by their recorded XY position)
Applications in pathology, tissue section analysis	Rapid analysis of expression of cell proliferation antigens, hormone receptors, etc., as prognostic markers, analysis of tissue (tumor) architecture [42–47]

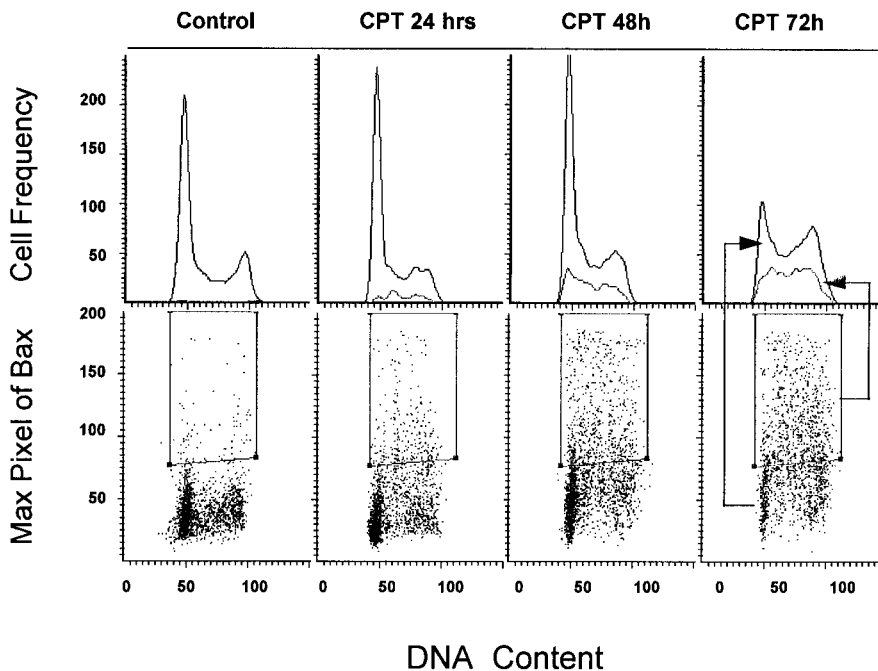


FIG. 4. Detection of Bax translocation from cytosol to mitochondria by analysis of the increase of maximal pixel of Bax immunofluorescence. MCF-7 cells were induced to apoptosis by their exposure to $0.15 \mu\text{M}$ camptothecin (CPT). Apoptosis of MCF-7 cells is observed with a delay (24–72 h) and is preferential to S-phase cells (52). Bax was detected in these cells immunocytochemically, with the FITC-conjugated Ab; DNA was counterstained with PI. Accumulation of Bax in mitochondria prior to and in early stages of apoptosis was revealed by the increased maximal pixel value; the integrated FITC fluorescence was also increased but to a lesser degree than the maximal pixel value (not shown). The DNA content frequency histograms of the gated subpopulations of the cells with low and high maximal Bax pixel values (as shown in 72-h CPT-treated culture) reveal that the latter consist of predominantly S-phase cells (manuscript in preparation).

mark of activation [28]. NF- κ B was detected immunocytochemically in several leukemic cell lines with FITC-tagged antibody, and its presence in the nucleus vis-a-vis cytoplasm was monitored by LSC measurements of green fluorescence (FITC) integrated over the nucleus vs over the cytoplasm, respectively [29]. Activation led to a rapid increase in NF- κ B-associated fluorescence measured over the nucleus concomitant with a decrease in fluorescence over the cytoplasm, which was reflected by a large increase in the nuclear to cytoplasmic fluorescence ratio. One of the virtues of this assay is that NF- κ B activation could be correlated with cell morphology, immunophenotype, or cell cycle position [29]. This application of LSC can be extended to monitor other factors that upon activation accumulate in cytoplasm and/or undergo translocation to the nucleus, such as tumor suppressor p53 and signal transduction or cell cycle regulatory molecules.

Since LSC allows one to integrate (merge) the results of two or more measurements, it is possible to measure the same cells twice, once with the contour setting to measure only nucleus and subsequently with a setting that measures both nucleus and cytoplasm. Such analysis revealed nuclear expression of cyclin B1 which could be compared with total cellular expression of this protein [30, 31].

Fluorescence in situ hybridization (FISH). FISH analysis represents the third type of LSC applications that are based on the capacity of this instrument to spatially resolve the distribution of fluorescent regions within the cell [11, 32]. The software developed for this application allows one to establish, within a primary contour representing nucleus stained with a particular dye (e.g., propidium), a second set of contours representing another color (e.g., FITC) fluorescence. Five secondary features are then measured in addition to the major features listed earlier, namely (a) number of secondary contours (i.e., FISH spots); (b) distance between the nearest spots; (c) integrated and (d) maximal pixel fluorescence; and (e) fluorescence area. The three last parameters (c–e) are measured for each secondary contour (Fig. 2).

An obvious advantage of LSC over visual analysis of FISH is the unbiased selection of the measured cells and semiautomated, rapid measurement. Furthermore, analysis of the integrated fluorescence intensity of the secondary contours may yield information pertaining to the degree of amplification of particular genome sections. However, as emphasized by Kamentsky *et al.* [11, 32], semiautomated FISH measurements by LSC are subject to potential traps and require high-quality technical preparations.

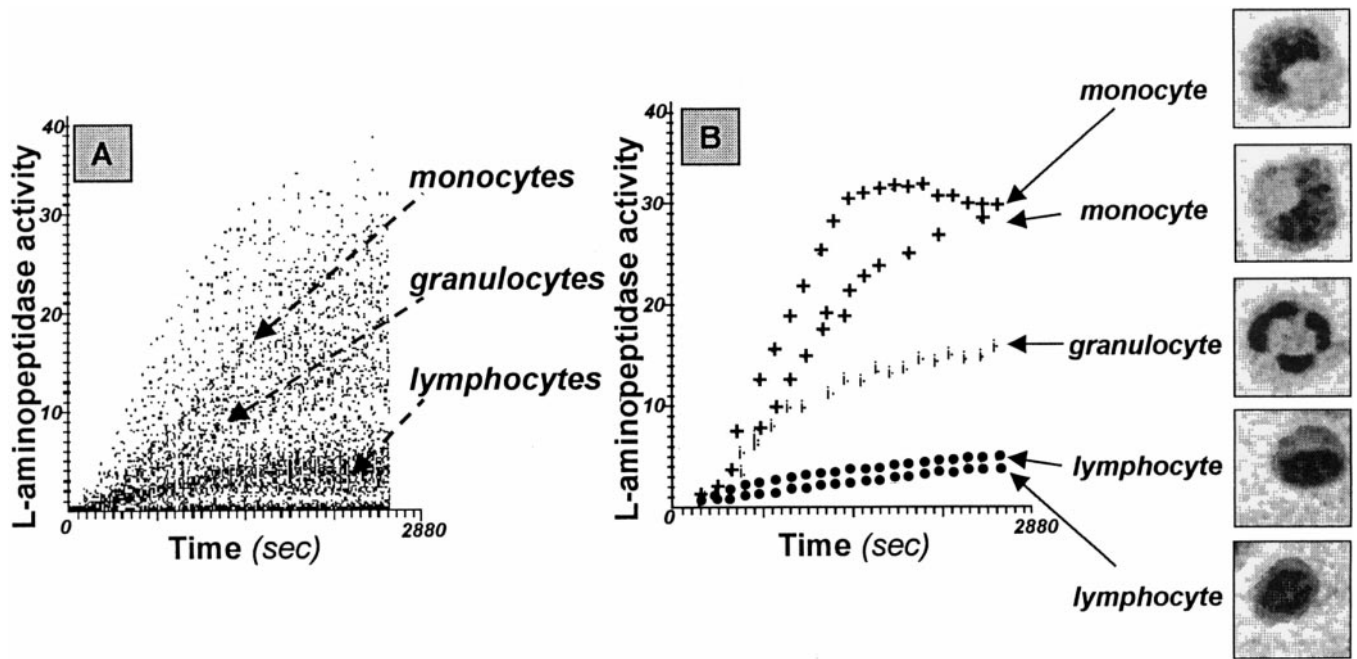


FIG. 5. Analysis of enzyme kinetics by LSC. L-Aminopeptidase activity was measured in white blood cells from human peripheral blood using a fluorogenic substrate, di-(leucyl)-rhodamine 110, and recording increase in fluorescence intensity of individual cells with time [37]. The slides were then stained with Giemsa and examined by light microscopy. Individual lymphocytes, monocytes, and granulocytes were identified (their image recorded in the cell galleries; right panels) and matched with their respective kinetic plots [37].

ANALYSIS OF TIME-RESOLVED EVENTS

Current methods assaying cellular enzyme activity suffer limitations. Biochemical assays of cell extracts, cell fractions, or isolated proteins in bulk provide no information on individual cells needed to assess the intercellular variability of cell populations, detect rare cells or cell subpopulations with distinct features, or relate the measured parameters to normal or abnormal cells, etc. Although individual cells are rapidly measured by FC, each cell is measured only once when in flow. Time-resolved events, therefore, cannot be measured on a cell by cell basis. The kinetics of enzymatic reactions [e.g., 33], change in pH [34], bursts of ionized intracellular calcium [35], or oxidative product formation [34] measured by FC are based on sequential measurements of single cells over time but not the same single cells.

LSC provides the means to measure kinetic reactions within individual cells in large cell populations (Fig. 5). Thus, repeated measurement of a group of cells within a selected area of the slide allows one to record all fluorescence parameters as a function of time [37]. Using the fluorogenic substrate di-(leucyl)-rhodamine 110, the kinetic activity of L-aminopeptidase was measured in several cell types by LSC [37]. The rate of fluorescein diacetate (FDA) hydrolysis by esterases as well as the rate of uptake of the lysosomotropic fluorochrome acridine orange (AO) was also assayed [37].

Several hundred cells per sample can be measured with a time resolution of 10 to 60 s. Since the time resolution is inversely proportional to the number of measured cells per sample, fewer cells can be analyzed if the time interval between measurements must be short. The kinetic curves constructed for individual cells can be matched with the respective cells, the latter identified by their position on the slide or classified by their fluorescence image or by light microscopy after staining with conventional absorption dyes [37].

Repeated scanning of the same cells causes fluorescence fading. The fading, which may be extensive when time intervals between scanning are short, unfortunately imposes a limitation on time resolution of the kinetic measurement. However, the fading rate as well as the fluorescence recovery rate can be measured in the same cells by LSC [37] and results corrected appropriately.

CELL IMMUNOPHENOTYPING

The usefulness of FC for immunophenotyping, especially in analysis of hematological malignancies, is indisputable. LSC can also be adapted to carry out routine immunophenotyping. Multichamber microscope slides were developed which can be used to automatically screen the cells against up to 36 antibodies on a single slide by LSC [38, 39]. The chambers are filled

with cell suspension by capillary action. In the absence of serum or other proteins in the suspension, the cells strongly attach to the floor of the chambers by electrostatic interactions [37, 38]. Various antibody combinations are then introduced into the chambers, the cells are incubated in their presence for 30–60 min, and, following the rinse, their fluorescence is measured. The rate of analysis is relatively fast, as it takes an overall ~20 min to screen the cells distributed in 12 chambers labeled with a panel of 36 antibodies, measuring 3000 to 5000 cells per chamber [38].

Although the rate of measurement by LSC is slower than FC, and the lack of side light scatter analysis impedes discrimination of lymphocytes from monocytes and granulocytes, certain advantages of LSC may outweigh these deficiencies. Thus, LSC is preferred for hypocellular samples which cannot tolerate repeated centrifugations that lead to cell loss. It must be stressed that loss of cells during centrifugations, as required for FC analysis, is not random but preferential to different cell types [27]. LSC is also economical: because of small sample size in LSC, the cost of the reagents (mAbs) is reduced by more than 80% compared to FC [38]. Furthermore, LSC provides the possibility to relocate immunophenotyped cells for additional analysis or archival preservation. This feature is discussed later in the article.

APPLICATIONS OF LSC IN PATHOLOGY

Cytometry still plays only a minor role in anatomic pathology. In the two most common types of preparations, histologic sections and fine-needle aspirates (FNA), diagnosis is greatly dependent on the judgment and experience of the examiner and is likely to remain so. However, by quantifying key attributes of selected cells in a specimen of known diagnosis, cytometry can contribute useful prognostic information and help guide therapy. LSC is particularly suitable for this task. FNA samples provide adequate numbers of cells for analysis by LSC and no significant cell loss occurs during the staining and measurement [40, 41]. In histologic sections, areas of interest that may be a minor component of the whole section can be selected to exclude extraneous tissues from measurement. As already noted, the slides can be destained and restained to measure additional attributes of the same cells; the relocation feature of LSC allows one to precisely identify each cell by its location on the slide. Several publications do account for the usefulness of LSC in analysis of tissue sections or FNA samples [40–46].

One of the drawbacks inherent in measuring constituents of the cells in histologic sections is that most of the cells are transected at different levels. Thus, because only a fraction of a cell or nucleus, unknown in size, is assayed, such measurement provides no infor-

mation about quantity of the measured constituent per cell. However, a ratiometric analysis, relating the quantity of the measured nuclear constituent per unit of DNA, normalizes the data and makes them comparable between sections of different thickness. Such normalization is easily accomplished by LSC, where contouring can be done on the DNA-associated fluorescence and another color fluorescence, representing the measured nuclear constituent and integrated within the same contour, is expressed as a ratio per DNA-associated fluorescence. This method of normalization was applied to a study of estrogen and progesterone receptors in human breast cancer [46].

LSC also offers advantages over current methods of image analysis that with few exceptions (e.g., Feulgen staining) rely entirely on light-absorbing dyes and are not quantitative. The basic approach of image analysis, to classify cells by their light microscopic morphology, fails to take advantage of important functional information inherent in the immuno- and cytochemical assays of LSC and FC.

Still to be worked out are the computer-assisted analytical methods that will be needed to fully exploit the information in histologic sections. In the case of solid tumors this includes the relationship between tumor cells and reactive host cells, stroma, proliferating vessels, etc. and the distribution of proliferating vs apoptotic cells within the tumor, the expression of growth factor receptors in tumor cells according to location and in relation to host cells and blood vessels, and the effect of drug therapies on the functional measurements of the cells. The number of measurable features is growing, providing new tools to characterize and monitor human tumors in ways not possible by conventional light microscopy.

The possibility of sequential cell measurements as offered by LSC has already been explored in analysis of the effect of infectious agents, such as human granulocytic ehrlichiosis or adenovirus, detected immunocytochemically within the cell, on proliferation and expression of the proliferation- or apoptosis-associated markers such as tumor suppressor p53 protein, cyclins, inhibitors of cyclin-dependent kinases, proapoptotic Bax, or anti-apoptotic Bcl-2 proteins, by the infected cells [47, 48].

FUTURE APPLICATIONS

Although only a few years have passed since LSC has become commercially available, numerous publications have already appeared describing a plethora of its applications (Table 2). This is an indication that LSC is a versatile, multitask instrumentation that immediately has found utility in many different fields. The unique capabilities, as discussed earlier, make LSC the instrument of choice in a variety of studies.

The major virtue of LSC, which will be the driving force for its future applications, is the merge capability, the possibility it offers to relocate the once measured cells for further analysis. As mentioned, this feature is essential in studies of the time-resolved events such as enzyme kinetics. It will be used to study metabolic changes, transmembrane transport rates of drugs, metabolites, etc., as well as other cell functions that can be probed by changes in time. Likewise, association constants of the fluorochrome-conjugated ligands with the respective receptors can easily be assessed for individual cells by LSC by repeatedly measuring ligand binding to the same cells as a function of increasing ligand concentrations. The relocation feature also allows secondary measurements of the once probed cells, using other markers. It also makes these cells accessible for visual examination and image analysis. Furthermore, their additional analysis in the future, after archival preservation, is feasible. Individual cells thus can be immunophenotyped and, when still alive, subjected to functional assays, e.g., for a particular organelle, oxidative metabolism, pH, enzyme kinetics, etc. Following fixation (~95% cells initially attached electrostatically remain attached after fixation), the very same cells can be probed for DNA content (to assess DNA ploidy and/or cell cycle distribution) or DNA replication (after prelabeling with BrdU), as well as for content of any intracellular constituent that can be detected immunocytochemically. To obtain their cytogenetic profile, the same cells may then be probed by FISH. Conventional staining with absorption dyes followed by microscopy can identify the measured cells and correlate their morphology with any of the measured parameters. If desired, a more sophisticated image analysis of the selected cells can follow: A simple linkage of LSC to the image analysis system (Kontron KS 100 system) through standard connections has recently been described [49]. The slide may be stored indefinitely, and then when a new probe is developed, this probe may be applied to the same cells and the results from early and late studies integrated. Large cell populations can be studied along the scheme described above to detect cell heterogeneity and identify cells with rare features or cell subpopulations with different features. Clearly, the relocation feature offered by LSC opens an infinite number of applications of this instrument in cell biology.

The factor that may limit sequential analyses of the same cells is the necessity of removal of the fluorescence from the earlier analyses prior to the next measurement. Currently available means of enzymatic or chemical removal of the fluorochrome, or its bleaching, may not always be effective and new methods must be developed. However, addition of uv light laser to LSC will enhance the possibility of sequential measurements with different color probes, without the neces-

sity for fluorochrome removal. Furthermore, a combination of fluorescence and time-delayed luminescence probes which are both color and time resolved [50] and which can be adapted to LSC can double the analytical capability of this instrument.

The capability of spatial localization of fluorochrome within the cell (nucleus vs cytoplasm, its highest local concentration by maximal pixel analysis, spatial distribution of FISH probes) is another feature of LSC that will attract new applications. One of the obvious uses of LSC will be in analysis of micronucleation, e.g., in mutagenicity or environmental studies [51]. The software that is already available is adequate to automatically identify and count individual nuclei and micronuclei within the cell. LSC is also expected to become useful for measurement translocation of different factors, such as NF- κ B [30], p53, components of the signal transduction pathway, etc., from cytoplasm to nucleus.

Spatial resolution of the fluorochrome and the possibility of localization of the measured cells on slides offer an opportunity for analysis of cell-to-cell interactions. Signal transfer between the cells, cell-to-cell transport of the fluorochrome-tagged molecules, and local differences in cell proliferative potential or apoptosis all can be studied on the cells attached to slides before and after their fixation. Likewise, the role of cytokines or other growth factors released from individual cells on proliferation or apoptosis of the neighboring cells, whether in cell monolayers or in tissue sections, also can be studied by LSC. This can be accomplished by analyzing localization of the cells probed with immunocytochemical markers detecting the presence of these factors vis-a-vis the localization of the affected cells probed with markers of proliferation or apoptosis.

LSC also has a potential to become an indispensable tool in the laboratory of every pathologist, providing quantitative data on FNA, tissue sections, or cytology smears. As new diagnostic and prognostic markers are rapidly being developed and their clinical utility becomes more and more evident, the need for quantitative assays of these markers also becomes evident. The capabilities of LSC predetermine it to serve this function.

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