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Review

Use of laser scanning cytometry to study tumor microenvironment

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Summary. The study of phenomena occurring in the tumor microenvironment is a challenging task because of technical difficulties, particularly when dealing with hypocellular specimens. Laser scanning cytometry (LSC) is a new laboratory technology that has been recently introduced to overcome the limitations of other traditional technologies. By combining the properties and the advantages of flow cytometry (FC) and immunohistochemistry (IHC), LSC allows the investigator to obtain objective information on DNA content, protein expression and cellular localization is combination with morphological features. It has been already shown that LSC results are reliable compared to more traditional technologies, and its implementation in the clinical routine is under way. Its use in oncology, which is rapidly expanding, spans from apoptosis analysis to DNA content quantitation and tumor cell phenotyping.

Here we describe the technology underlying this novel fluorescence-based device, review its use in oncology by dissecting the phenomena occurring in the tumor microenvironment and propose its application for the immunological follow-up of malignant lesions undergoing immunotherapeutic manipulation.

Key words: Laser scanning cytometry, Tumor biology

Introduction

Studying the cellular and molecular phenomena occurring within the tumor microenvironment is obviously of paramount importance for the understanding of the mechanisms leading to tumor progression as well as tumor responsiveness/resistance to therapeutic agents. *In vitro* studies are biased by the over simplification of the models used that lack the complex network of cellular and molecular interactions occurring *in vivo*. On the other hand, *in vivo* studies are burdened by several difficulties. In fact, besides the difficulty in the interpretation of complex results resulting from a highly integrated and sophisticated system, investigators dealing with *in vivo* research must face several technical challenges. These are due to the limited amount of material available coupled with the need for minimum manipulation (e.g. cultures, conditioning) of the specimens for them to be truly representative of the *in vivo* conditions.

Flow cytometry (FC) and immunohistochemistry (IHC) are techniques traditionally used to characterize the expression of proteins in tumor specimens. At the gene level, a sensitive measurement of gene expression can be obtained by means of quantitative real-time PCR (qrt-PCR) (Kammula et al., 2000). Each of these techniques, however, presents intrinsic limitations. FC necessitates single cell suspensions and a relatively high number of cells. Although this is compatible with the study of haematological malignancies, it is inadequate for tissue sections and/or hypo-cellular specimens such as fine needle aspirates (FNA). Furthermore, FC does not allow direct correlation of the fluorescence emission signal from individual cells with their morphology. IHC is a semi-quantitative technique limited by the resolution power of the eye and the subjectivity of the operator. Finally, qrt-PCR provides accurate estimates of gene expression within a cell population but does not yield information about the cell source expressing the gene. Thus, the information obtained by qrt-PCR is often subjectively depended upon the guessed specificity of gene-expression of a given sub-population of cells. This limitation is shared by novel technologies such as high throughput cDNA microarray and proteomics.

Recently, some investigators have introduced laser scanning cytometry (LSC) as a new laboratory tool that could be particularly useful to study the tumor microenvironment (Kamentsky and Kamentsky, 1991; Rew et al., 1999; Gorczyca et al., 2001). LSC is a microscope-based cytofluorimeter that combines flow

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and image cytometry properties (Kamentsky et al., 1997a). The technology is similar to FC in that the measurements are based on the analysis of laser-excited fluorescence emitted by individual cells and detected by the scanner at different wavelengths. However, since the cells are collected on a microscope slide, it is possible to combine morphological evaluation of the events gated according to fluorescence intensity with light and/or fluorescence microscopy.

In this paper we will review the technology underlying LSC and its application for the dissection of tumor biology ex vivo.

The technology

Directing one, two, or three coaxial laser beams to a scanning mirror, LSC optics apply the scanned beam through the microscope objective lens onto the specimen (Kamentsky et al., 1997b; Darzynkiewicz et al., 1999). The specimen, which is mounted on a standard microscope slide, moves past the scanning laser beam on a motorized micro-stepping stage. As cells or other fluorescent objects pass through the scanning beam, the emitted photons return along the same path as the laser beam and are collected in the wavelength-specific photomultiplier tubes (Fig. 1).

The WinCyte® acquisition analysis software generates a bitmap image for every detector, consisting of 1000 by 768 pixels of 14-bit fluorescent or scattered-light data. Every 500 microns of stage movement generates a new set of bitmaps. Once a bitmap is generated, the software segments the cells, or events, from the background, and extracts high-content fluorescent data from each event, including morphometric features and localized subcellular characteristics. Recoding the event's position on the slide allows the investigator to visualize it later for morphological confirmation, or reanalyze it under different staining conditions for one-to-one comparison (e.g., kinetic experiments).

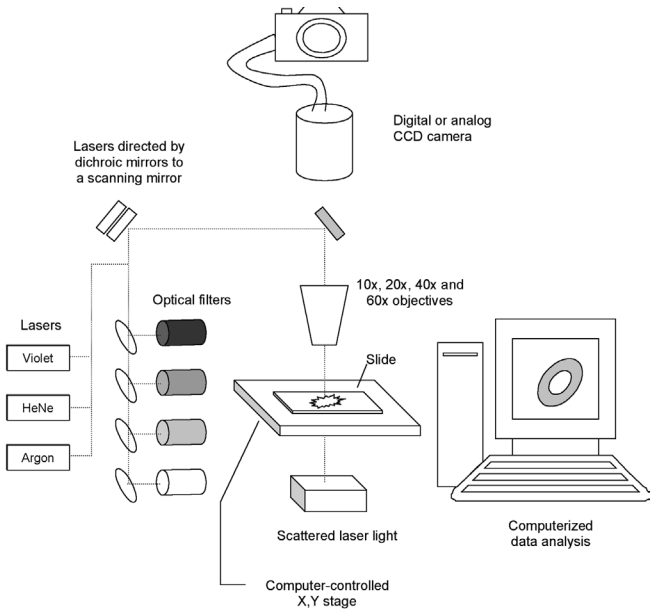

Fig. 1. Laser scanning cytometer: scheme.

Laser scanning cytometry synergistically combines and transcends the advantages of FC, image analysis, and automated fluorescence microscopy. Like FC, LSC uses lasers to excite fluorochromes in cellular specimens and detects the fluorescence in discrete wavelengths with multiple photomultiplier tubes. Also like FC, LSC yields high-content stoichiometric data on heterogeneous populations of small or large numbers of cells, offering software analysis tools to obtain statistical data on the population under examination.

Similarly to image analysis, LSC creates digital images of the specimens on microscope slides and employs image processing algorithms to automatically identify and segment the "events" (e.g., individual cells or other objects) on the specimen. LSC can additionally find and quantitate events within events, making it possible to count and measure the fluorescence during in situ hybridization or to discriminate around a segmented nucleus cytoplasmic from nuclear fluorescence.

Finally, like fluorescence microscopy, LSC generates high-resolution images of fluorescent and scattered light from specimens on microscope slides.

The combination of these properties makes LSC a versatile technology with which investigators can: 1) analyze cell suspensions as well as paraffin-embedded tissue sections. 2) have morphological and fluorescence-based information about individual events in a given cell population. As a corollary, a picture of the microscopic or fluorescent appearance of the selected event(s) can be taken. 3) select a set of cells on the basis of the fluorescence analysis and visualize them and *vice versa* (select the cells on the basis of their morphological features and get the statistical analysis of that set of cells). 4) study hypocellular specimens (e.g. fine needle aspirates, FNA) since few hundred cells are sufficient for a satisfactory analsyis. 5) store slides for future re-analysis under varying conditions. This allows the performance of kinetic experiments with merged data sets and/or re-stain specimens with additional reagents. 6) analyze cells in their natural environment (tissue section analysis) to study the crucial interactions between cells of different types. 7) assess simultaneously antigen expression and localization within sub-cellular components by separately measuring the fluorescence within the nucleus and the cytoplasm (Kakino et al., 1996; Buse et al., 1999; Juan and Cordon-Cardo, 2001). 8) perform fluorescence in situ hybridization studies (Kamentsky et al., 1997b)

The applications

DNA content

Measurement of DNA content represents an important aid in the diagnostic process of some

malignancies, especially when the sample is hypocellular (Decker et al., 1998). DNA ploidy status, together with cytopathological features, can help discriminate between benign and malignant cells and can provide clinicians with an useful prognostic tool (Friedlander et al., 1984; Teodori et al., 1984; Walle and Niedermayer, 1985; Vogt et al., 1995; Pilch et al., 2000). Like FC, LSC has been extensively employed to evaluate sample DNA content (Fig. 2). In this field, LSC results have been shown to closely correlate with those obtained with FC or IA (Sasaki et al., 1996; Clatch et al., 1997; Fattorossi et al., 2000). Furthermore, LSC-based DNA content analysis portrays well the genetic instability of tumor cells, which is often a sensitive indicator of tumor behavior and, therefore, clinical outcome (Harada et al., 1998; Miyazaki et al., 1999; Furuya et al., 2000). Since LSC features many useful properties of FC (e.g. automation, accuracy of quantitation, rapidity) and IA (e.g. minimal tissue requirement, no need for special preparation, visual selection of the cells measured), its use in clinical practice has been advocated to study the DNA content of both malignant cell suspensions and paraffin-embedded tumor specimens (Martin-Reay et al., 1994; Kamada et al., 1997; Kamiya et al., 1999; Tsukazaki et al., 2000; Hashimoto et al., 2000; Kawamura et al., 2000; Gorczyca et al., 2001; Wojcik et al., 2001).

Tumor phenotyping

Immunophenotypic analysis of haematological specimens is a useful laboratory adjunct to surgical pathology and cytology to confirm or further characterize diagnoses of leukaemia or lymphoma. Laser scanning cytometry has been applied to analyze excised lymph nodes, other tissue biopsies, peripheral blood samples, bone marrow aspirates, body fluids and FNA of lymphoid tissue (Clatch et al., 1996; Harada et al., 2001). Up to six fluorescent parameters, five representing binding of immunofluorescent antibodies and one for stoichiometric measurements of DNA content, can be used (Clatch and Foreman, 1998). In order to make full use of the LSC capabilities, Clatch el al. have developed a new method for the preparation of the immunofluorescent reactions (Clatch et al., 1998). The technique allows the use of panels of up to 36 different antibodies on specimens as small as 50,000 total cells. The laboratory methodology is simple and requires 85% less antibody than FC. In addition, it allows the study of cell morphology on a cell-by-cell basis. Once put to the test in the clinical setting, the method has proven remarkably useful, particularly for extremely small specimens such as FNA.

LSC-based phenotyping has been also adopted to measure a variety of tumor markers including cell proliferation indicators (e.g. Ki67) and estrogen/progesterone receptors in breast cancer biopsies or FNA (Gorczyca et al., 1998a; Zabaglo et al., 2000). Results correlated well with conventional techniques. In addition, the marked variation in relative number of ER binding sites per cell could be quantified by LSC and displayed by histogram distribution. Therefore LSC measurements can be considered fast and objective and can be carried out on sections of paraffin-embedded tissue after routine processing in the pathology laboratory. As an example, LSC data can provide relative densitity of hormone receptor binding sites per unit of DNA, which may reveal clinically significant skewed distributions in subpopulations of tumor cells.

Finally, LSC-based immunophenotyping has been also applied to the characterization of tumor infiltrating lymphocytes (Gniadecki, 2000).

Apoptosis

Tumor development and progression is often associated with defective regulation of apoptotic pathways, and measurement of apoptosis might be as important as the analysis of cell proliferation. Moreover, tumor cells die by apoptosis during chemotherapy or radiotherapy, so monitoring the level of apoptosis might prove useful in modulating treatment or in predicting the biologic behavior of the tumor. Flow cytometry, in conjunction with DNA strand-break labeling assays, is commonly used to assess apoptosis. However, unless combined with cell sorting, FC results cannot correlate with morphology. Also, analysis by FC is associated with cell loss during preparation of the cell suspension or tissue. Several authors have demonstrated that LSC provides data comparable to those obtained by FC (Li et al., 1996; Bedner et al., 1999; Darzynkiewicz and Bedner, 2000). Therefore, LSC, overcoming the limits of conventional techniques, could represent a useful tool to dissect the apoptotic process in the tumor microenvironment.

Thanks to the possibility of working over time with live cells, LSC-based *in vitro* studies have elucidated molecular trafficking proper of the apoptosis pathway, particularly when induced by anticancer agents (Bacso et al., 2000; Bedner et al., 2000a,b; Ercoli et al., 2000; Godlewski et al., 2001). *In vivo*, some authors have demonstrated that different morphologic variants of apoptosis might be common in different tumor types and that LSC analysis might provide biologically useful information not otherwise easy to obtain (Gorczyca et al., 1998b). According to Abdel et al., the implementation of LSC in routine specimen examination could be of clinical value for fast, objective analysis of apoptosis that, along with the determination of cell proliferation rate and DNA content, might represent a sensitive indicator of prognosis for patients with malignant tumors (Abdel-Moneim et al., 2000).

Immunological follow-up

Over the last decade, we studied tumor/host interactions in patients with metastatic melanoma undergoing immune-based treatments (Kammula et al.,

1999; Riker et al., 1999; Mocellin et al., 2001a; Wang et al., 2002). We recently proposed to follow the natural history of melanoma metastases by serial analysis of tumor marker expression within the same lesion by repeated FNA (Wang and Marincola, 2000). This strategy, by leaving tumor nodules in place, permits the

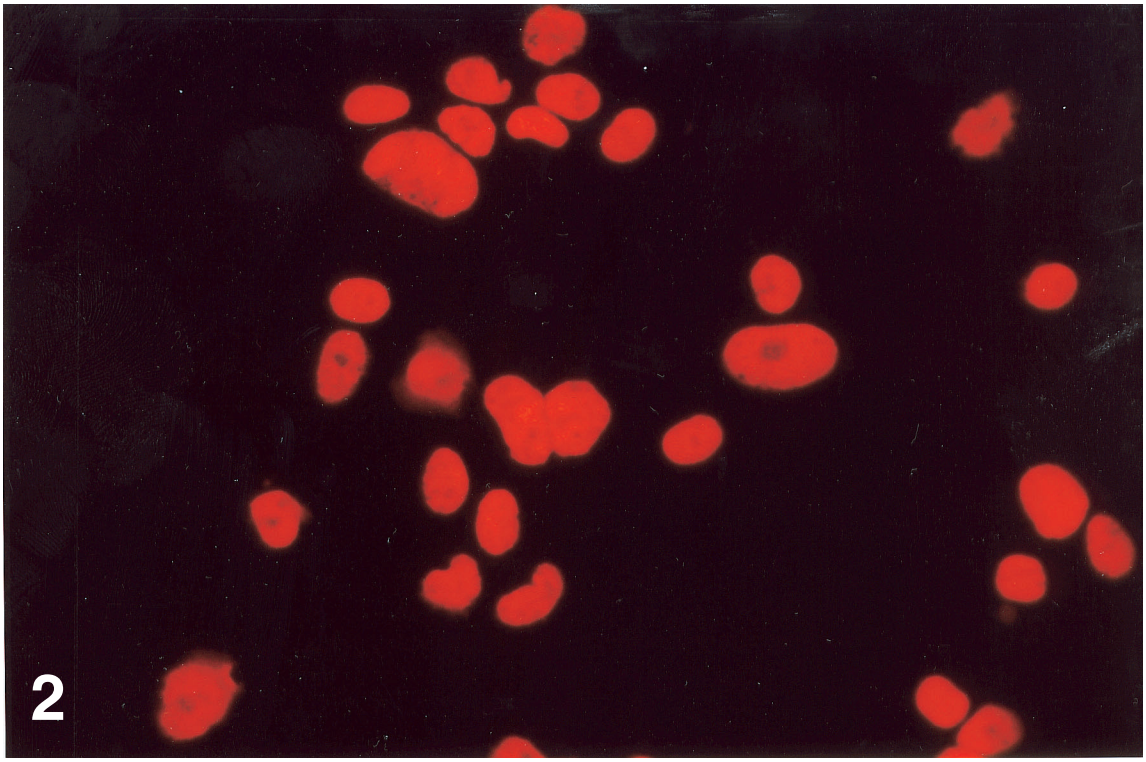

Fig. 2. DNA content of fine needle aspirate material from a melanoma metastasis of the skin. In order to avoid cell clumps, FNA material for LSC analysis was vortexed and appropriately diluted. Sample was stained with propidium iodide (2 mg/ml) in the presence of 0.1% RNase (Calbiochem, San Diego, CA) for 10 minutes at room temperature. This nuclear staining allowed for calculation of cellular DNA content.

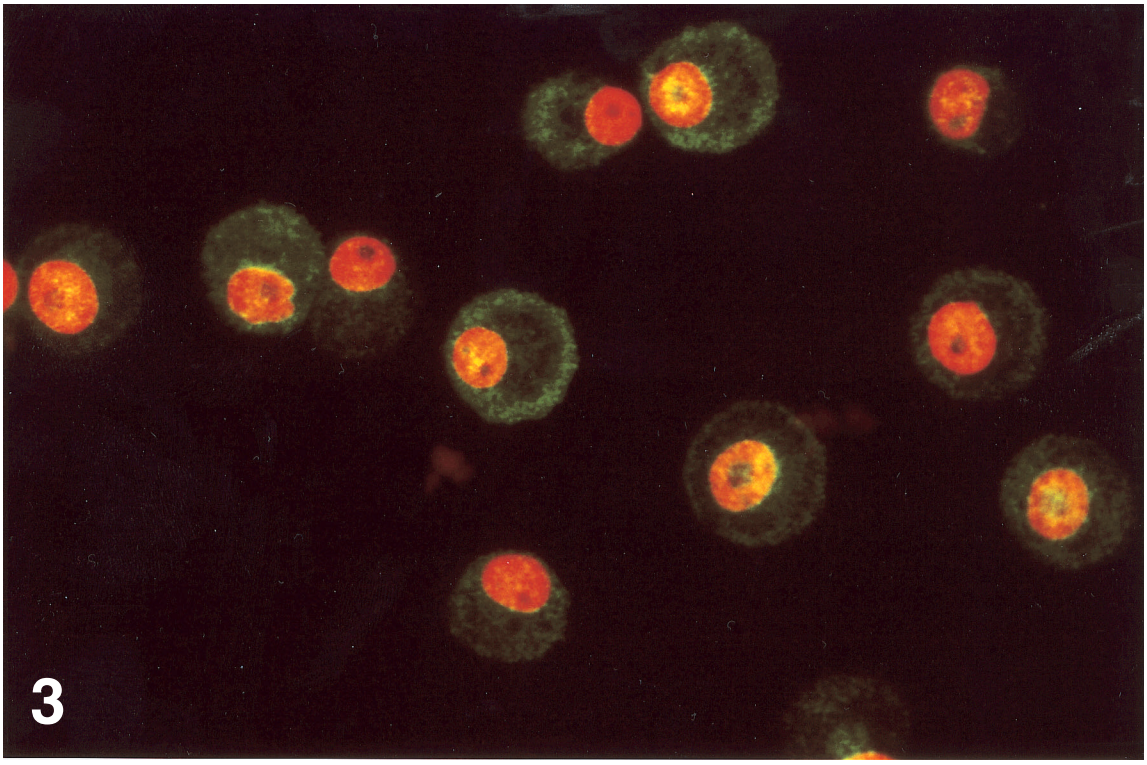

Fig. 3. Expression of an intracellular tumor associated antigen (gp100) by melanoma cells from a skin metastasis. Cytospins of fine needle aspirate material were air-dried for 30 minutes and then placed (unfixed) in a desiccated container until tasting, 12-24 hours later. Air-dried cytospins were fixed in acetone for 10 minutes and then stained with monoclonal antibodies to gp100, clone HMB45, 1:20 dilution (BioGenex, San Ramon, CA). A goat-anti-mouse FITC-labeled antibody (Becton Dickinson, San Jose, CA) was used for the secondary staining.

documentation of the kinetics of melanoma antigen (MA) expression during treatment and in relation to clinical behaviour.

We postulated that the limited success encountered by this therapeutic strategy may be related, at least in part, to fading in time of the expression of MA and/or the relevant HLA class I alleles (Ferrone and Marincola, 1995; Cormier et al., 1998). Since recognition of tumor cells by MA-specific T cells is quantitatively affected by the abundance of HLA class I alleles and MA, functional inferences about their expression can be best proposed when based on accurate measurements through time.

To this aim we found LSC a tool particularly suitable to analyze hypocellular specimens such as FNA. The use of LSC for FNA material analysis was already described by other authors (Clatch et al., 1996; Clatch and Walloch, 1997). However, we first adopted this technique for the immunological follow-up of malignant tumors treated with immunotherapy (Mocellin et al., 2001b). In that study we collected FNA of metastases from patients affected by melanoma and treated with IL-2/peptide based vaccination (Rosenberg et al., 1998). FNA material (on average $1x10^5$ cells) was split to perform quantitative real time-PCR, immuno-histochemistry and LSC analysis of tumor associated antigens and HLA-A2 gene/protein expression. Melanoma cells could be recognized by means of DNA content and visual confirmation. Overall we found a good correlation between IHC, qrt-PCR and LSC results, confirming the reliability of the novel technique. Moreover, LSC analysis showed that within the same tumor cell population MA expression is quite heterogeneous, which might explain at least in part the phenomenon of partial responses to the vaccination. Finally, we observed an iverse correlation between MA expression and aneuploidy (Fig. 3), suggesting that genetic instability may affect tumor responsiveness to MA-specific vaccination by decreasing the expression of target MA during the de-differentiation process.

Discussion

By combining the properties of traditional techniques such as FC, IHC and image analysis, LSC appears a promising device to study the tumor microenvironment. To date, LSC has been successfully used to perform up to six-colour immunophenotypic analysis of haematological specimens, single-colour immunophenotyping plus DNA content analysis of numerous specimen types, and automated analysis of fluorescence in situ hybridization specimens. Several other interesting applications have also been described such as apoptosis and cell cycle studies. In general, advantages of LSC include reduced specimen size requirements, simplified methodologies, and the ability to microscopically examine individual cells, thus allowing for the direct correlation of cytological morphology with objective fluorescence measurements. In addition, specimen preparation techniques are less restricted and more cost efficient, and even extremely small and/or hypocellular specimens (e.g. body fluids and FNA) can be successfully analyzed.

We believe that a particularly useful LSC application is for the analysis of hypocellular specimens such as FNA material for the monitor of intra-tumoral events in natural conditions or in response to therapy. In fact, FNA sampling gives investigators the unique opportunity to follow tumor microenvironment changes as the therapeutic agents are administered and act on malignant cells. As we and others have observed, in this setting LSC maintains its accuracy and reliability though only part of the FNA material is utilized, permitting to use the remaining cells for other analyses (e.g. genetic evaluation). This is of particular value after the introduction of RNA amplification technology (Wang et al., 2000), which allows to perform high throughput microarray analysis even from hypocellular starting material. Therefore, by combining these new technologies, we are currently potentially able to yield high density information on the molecular (RNA, DNA, protein) and cytological pattern of intratumoral environment components.

Other applications of LSC in oncology are under evaluation. For instance, LSC capability of working with extremely hypocellular specimens might be exploited to detect circulating tumour cells in the peripheral blood. Molecular genetic methods as well as FC lack the possibility to visualize the cell source of the selected tumor specific gene/antigen, therefore precluding any possibility of verifying the results. On the other hand, detection of disseminated tumour cells in the blood stream by immunocytochemistry would be exceedingly time-consuming. In an experimental breast cancer model, Pachmann et al. successfully employed LSC to detect very small numbers of tumor cells dispersed in blood samples (Pachmann et al., 2001). After staining with fluorochrome-conjugated anti-epithelial antibody, slides were screened for positive events directly or after enrichment with antibody-coated magnetic beads. One positive cell was unequivocally detectable in 10^4 cells and 50 out of 60 tumour cells were reliably recovered from a 20 ml blood volume, equal to 1-2 cells per 10^7, after magnetic bead enrichment.

Accordingly, LSC analysis may allow the enumeration of tumor cells in peripheral blood and bone marrow in a reasonable amount of time and may enable the investigation of tumor cell seeding during the metastatic process.

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