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Near-Clinical Applications of Laser Scanning Cytometry

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Summary

Biological samples from human tissues are characterized by complexity and heterogeneity. The ability to make rapid, reliable, quantitative fluorochromatic measurements on clinical samples allows the development of new and practical assays that could influence diagnosis and treatment in a variety of clinical applications. Laser scanning cytometry (LSC) is a very versatile and adaptable technology that allows for the quantitative analysis of cell samples that are unsuitable for flow cytometry by virtue of their presentation and context. Crucially, it allows the direct visualization of cells and rare events and the correlation of imagery with fluorochromatic measurements. In this chapter, we describe early experiments in the study of cytotoxic drug uptake and resistance in human tumor cells and in the study of sputum cells from asthmatic patients, which harness the specific capabilities of LSC to practical clinical problems.

Key Words: Laser scanning cytometry; drug resistance; cancer; doxorubicin; breast cancer; asthma; sputum; eosinophils; cytospins; bronchial epithelium cells.

1. Introduction

This chapter introduces some applications of laser scanning cytometry (LSC) in the hospital-based clinical laboratory. LSC has proved to be a powerful research tool for cytometric studies with practical clinical applications, which are beyond the capability of flow cytometry (FC).

The key advantage of LSC over FC is that in LSC, the laser beam is scanned over the surface of the sample, constrained on a planar microscope slide or derivative presentational surface ***(1)*** (*see* **Fig. 1**). In FC, the individual particles move past the beam and are lost to further specific analysis. The LSC allows light excitation of the sample by a 488-nm argon laser, by fluorescence excitation from an Olympus epifluorescence system with a mercury arc lamp, and by normal illumination in conventional or dark-field modes. The direct visualization of cells, tissues, or fluorescent subcellular elements on the constrained

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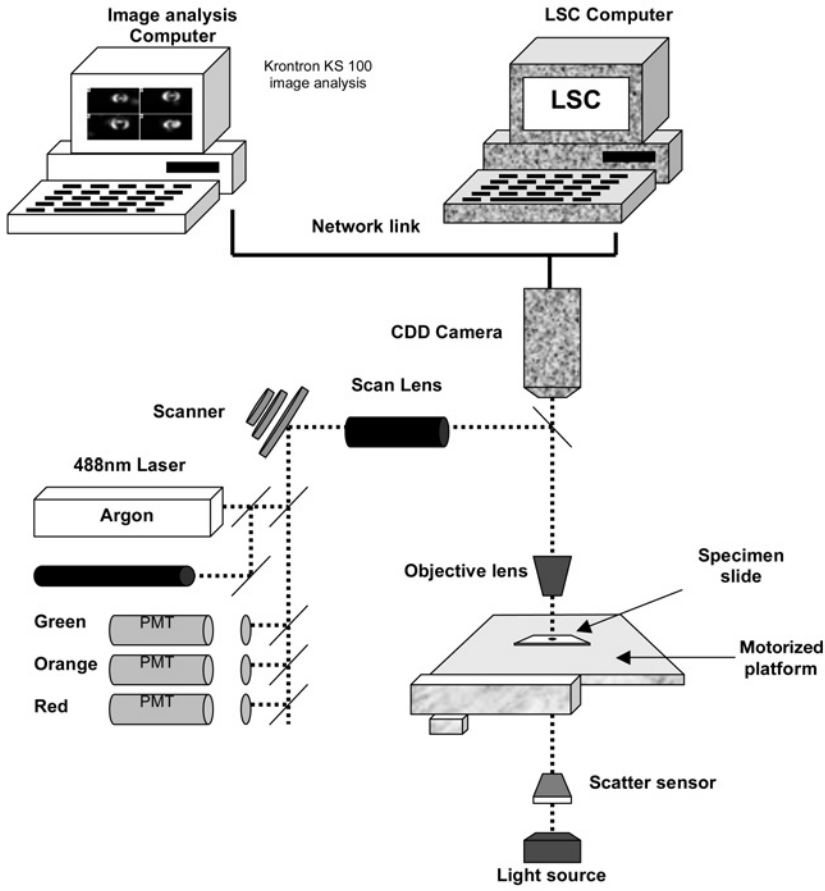

Fig. 1. Setup of the LSC.

surface is thus possible, using either binocular optics or a video digitizing camera.

The microscope slide is moved on the stage in precisely controlled steps by the computer-controlled motor. Each cell image comprises some 100 pixels with a 0.5-μm resolution. Measurements within each array include cell position on the slide, signal area, signal perimeter, peak fluorescence, the time of measurement, and texture. Data are collected and displayed in various graphical forms. Moreover, in LSC, a single cell can be scanned numerous times by the laser beam, with more uniform illumination than is possible with FC and a single light pulse. The speed of scanning of the LSC (1000 cells/min) allows large datasets such as are generated in the study of human tumors and tissues to be collected and analyzed in a realistic time frame.

Use of the microscope slide as the examination plane offers advantages in sample preparation. Whole cells, intact membranes, and normal cell physiology can be studied using chamber slides. The microscope slide allows a broad range of clinical sample types to be studied, including fine-needle aspirates, swab and brushings cytology, smear cytology, viscid and mucoid samples such as sputum, imprint specimens (such as from the cut surface of a carcinoma), and suspensions prepared as for FC. The scanning process allows subcellular fluorescence peaks to be identified and analyzed, such as fluorescence *in situ* hybridisation (FISH) markers *(2)* and micronuclei *(3)*. The instrument is also a powerful tool for immunophenotyping *(4,5)*.

The LSC only contains one light forward-scatter detector but can contain up to three fluorescence photomultiplier tubes (PMTs): channel 1, 530 ± 15 nm (green); channel 2, 570–630 nm (red/orange); channel 3, >650 nm (far red). The position of the cells can be recorded and the cells of interest can successfully be relocated for reanalysis by storing the information on disk *(6–9)*. A schematic representation of the LSC is illustrated in **Fig. 1**.

The X-Y coordinates of the cell position on the slide allow recall, visual, or camera inspection of specific cells and the reevaluation and repositioning of cells after further staining. Rare events can be validated within complex cell populations. Cells can be restained and remeasured in studies with additional fluorochromes in multiparameter studies. The sample can be remeasured to generate time-sequenced data using the integral clock and time stamp on each measurement.

More advanced instruments and software now offer up to three coaxial violet, blue and red/infrared lasers, more detectors, and more versatile image analysis software, including the capability for tissue section and FISH spot and multiparameter, high-content analysis.

The methodologies that we describe were developed on a first-generation instrument from Compucyte (Cambridge, MA; www.compucyte.com). This was acquired in 1996 for a small, specialized cytometry research facility within a district hospital, for near-clinical and cytopathological research. The instrument was equipped with a single argon laser (488 nm) and a mercury lamp for excitation, an Olympus microscope, light collection optics, three color PMTs, and forward-scatter detector, and an early version of the Wincyte proprietary analysis software on a Windows-based Pentium computer. Its image analysis capabilities were enhanced through a charge-coupled device (CCD) camera linked to a Kontron image analysis system *(8)*.

1.1. Specific Applications

Our studies have addressed a range of these capabilities, including single- and dual-parameter DNA content analysis, the measurement of cell proliferation in

human tumors following in vivo bromodeoxyuridine labeling *(9,10)*, viability studies of human tumor cell lines and of cells extracted from human tumors, chamber slide studies, micronucleus genotoxicity assays *(3)*, rare-event analysis on asthmatic sputum samples, and studies on the subcellular distribution, metabolism, and drug-resistance mechanisms in breast cancer cells *(11,12)*. In this chapter, we will describe our studies on intracellular cytotoxic drug distribution in encapsulated microspheres and on the detection of dispersed eosinophils in asthmatic sputum.

1.2. Laser Scanning Cytometry Studies of Fluorochromatic Drug Uptake in Human Tumor Cells

Treatment strategies for cancers are often inadequate. We hypothesized that quantitative cytometry and, in particular, LSC can provide useful information about tumor biology and treatment sensitivity not otherwise available to the clinician. Specifically, the decision to use adjuvant chemotherapy for primary, metastatic, or recurrent tumors and the selection of particular cytotoxic agents thereafter is much influenced by empiricism in clinical oncology. This might be depriving some patients of benefits from individually targetted adjuvant chemotherapy. Multidrug-resistance (MDR) mechanisms are a potent cellular source of treatment failure, and other patients might be being treated with ineffectual agents.

Tumor cells in suspension in blood such as leukemias and in malignant ascites are easy to study using FC *(13)*. White blood cells in particular have specific size, scatter and phenotypic characteristics. Fresh and archival samples of solid tumors pose problems to FC by virtue of their heterogeneity of size, scatter, viability, and biomarker preservation *(11)*. In contrast, visualization of complex populations and of individual cells by LSC allows verification of features and rare events in complex populations during quantitative cytometry.

The anthracycline and anthraquinone families of cytotoxic agents include adriamycin (doxorubicin), daunorubicin, mitozantrone, epirubicin, and idarubicin. They have a number of potent cytotoxic actions, including intercalation of DNA and inhibition of DNA repair, and are widely used in oncology practice. They possess intrinsic fluorescence and absorb light around 470 nm and emit around 560 nm. They are expelled from the cell by the p170 glycoprotein MDR pump.

Laser cytometry thus offers opportunities to assay the MDR capacity of cancer cells and its modification by MDR blocking agents *(14)*, to identify those tumors that fail to concentrate the agents and spare their owners from ineffective therapy, to identify those drugs most reliably concentrated in cells from tumor biopsies from a panel of agents, and to study new drug delivery vehicles such as drug-loaded albumin microspheres. The assay of drug uptake alone is

insufficient to indicate cell killing. For example, a fluorescent drug might be metabolised, sequestered, or otherwise rendered inert within target cells. Additional assays of drug efficacy are needed to show evidence of induced cell death or irreversible disruption of other vital functions, such as evidenced by mitotic disruption or apoptosis ***(15)***.

We first describe methods to evaluate the binding and internalization of doxorubicin-loaded albumin microspheres to breast cancer cells in vitro using LSC. Doxorubicin microcapsules are a drug delivery system comprising 3-µm human serum albumin (HSA) microcapsules with doxorubicin covalently linked to their outer surface. They are available commercially or they can be produced in the laboratory. In this experiment, we investigate the binding of doxorubicin microcapsules and uptake of doxorubicin in both the wild-type (WT) and doxorubicin-resistant (R) MCF-7 breast cancer cell lines using an in vitro model and LSC.

2. Materials

2.1. Cytometric Reagents

2.1.1. Doxorubicin

We use doxorubicin (Sigma-Aldrich Ltd, Nottingham, UK) as the anthracycline reporter molecule. The drug is used in lyophilized form, obtained in vials containing 10 mg doxorubicin hydrochloride. Each vial is reconstituted under sterile conditions in double-distilled sterile water (ddH_2O) to create stock solutions of 1 mg/mL, which can be stored in polypropylene screw-top vials and frozen at –70°C.

2.1.2. Reagents Used to Bind Doxorubicin to HSA Microcapsules

1. 1-Ethyl-3 (3-dimethyl aminopropyl) carboiimide (EDC) linker, mannitol, pepsin, and Tween 80 (Sigma-Aldrich Ltd).
2. Hydrochloride acid (32%) (Fisher Scientific Ltd, Loughbrough, UK).
3. HSA microspheres.

2.1.3. Selection of Breast Cancer Cell Lines and Culture Conditions

The reagents needed as follows (all purchased from Life Technologies Ltd [Paisley, Scotland]):

1. L-Glutamine.
2. Fetal calf serum (FCS), heat-activated, mycoplasm virus-free.
3. HEPES buffer: 1 *M* L-glutamine (2 m*M*).
4. Phosphate-buffered saline (PBS) without calcium magnesium or sodium biocarbonate.
5. RPMI 1640 medium without L-glutamine or phenol red.

6. Trypsin solution containing 0.25% trypsin.
7. 1 m*M* Ethylenediaminetetraacetic acid (EDTA).

3. Methods

3.1. Source and Production of HSA Microcapsules

The HSA microcapsules have a mean diameter of 3.3 µm. Ours were a gift from Andaris Healthcare Ltd, as a 1 : 2 (w/w) suspension with mannitol (7.5×10^9/mL: number of microcapsules). The microcapsules are washed free of mannitol prior to crosslinking the doxorubicin to the microcapsules as follows.

Microcapsules are suspended at 100 mg HSA/mL in a 1% (v/v) solution of Tween 80 in ddH_2O, vortexed once for 30 s and then left to stand at room temperature for 30 min. The suspension is then centrifuged at 875*g* for 2 min; the supernatant is removed and discarded. The microcapsules are resuspended in 1 mL of ddH_2O (50 mg/mL), vortexed for another 30 s, and centrifuged at 875*g* for 2 min. The supernatant is removed and discarded. This washing step is repeated twice.

3.2. Preparation of Doxorubicin Microcapsules Using 1-Ethyl-3 Carboiimide Linker

All procedures are carried out under sterile conditions. A solution of doxorubicin (3 mg/mL ddH_2O) is added to 100 mg of washed microcapsules; then the mixture is vortexed for 30 s. A solution of EDC (6 mg in 500 µL of ddH_2O) is then added and the mixture is revortexed for 30 s. A magnetic stirrer bar is placed in the reaction vessel and left stirring at 37°C overnight, in the dark. The doxorubicin microcapsules are then collected by centrifugation at 875*g* for 5 min and the supernatant is removed and discarded. Unreacted doxorubicin is removed by resuspending the doxorubicin microcapsules in 5 mL ddH_2O, vortexing, and then centrifuging the suspension at 875*g* for 5 min, with the supernatant then being discarded. This step is repeated until the supernatant appeared colorless to the eye. The drug-loaded albumin microcapsules are then resuspended in 1 mL of ddH_2O and stored at 20°C in the dark.

3.3. Selection and Subculture of MCF-7/WT Cells and MCF-7/R Cells

The MCF-7/WT human breast adenocarcinoma cell line no. 86012803 ***(16)*** can be obtained from the European Collection of Animal Cell Culture (Porton Down, UK). Cells are maintained in culture medium (RPMI 1640 without phenol red, supplemented with 10% [v/v] FCS, 2 m*M* L-glutamine, and 0.04 *M* HEPES buffer) and cultured at 37°C in a humidified incubator containing 5% CO_2.

Confluent cultures of MCF-7/WT cells are subcultured as follows. Cells are washed with PBS once, then trypsin/EDTA 0.1% is added (0.5 mL for a 25-well

plate, 1 mL for a 6-well plate, 3 mL for a 25-cm^2 flask [T_{25}], and 5 ml for a 75-cm^2 flask (T_{75}). The culture vessels are incubated in trypsin/EDTA for 3–5 min at 37°C until the cells become detached. The action of trypsin/EDTA is denatured by the addition of an equal volume of culture medium containing 10% FCS. The cells are then aspirated and placed into sterile 15-mL tubes, centrifuged at 450*g* for 8 min, resuspended in 1 mL of culture medium, and then counted using a hemocytometer.

Our MCF-7/R cells *(17)* were kindly provided by Dr. Tim Gant (MRC, Toxicology Unit, University of Leicester). These cells can be cultured in the same way as the MCF-7/WT cells. Additionally, they are maintained throughout in the presence of 0.5 μ*M* doxorubicin. This should be added to the culture medium and filter-sterilized at the time of subculturing.

Both the MCF-7/WT and MCF-7/R cells are normally seeded at 4×10^4 cells/cm^2, equivalent to 1×10^6 cells per T_{25} flask.

3.4. Drug Uptake Studies

The cell lines can then be used to investigate the rate of uptake of free doxorubicin and the binding of doxorubicin microcapsules to the target cells, the time-course of uptake, the patterns of intracellular distribution, the dynamics of drug exclusion and microcapsule metabolism, and the effects on cell viability, cytostasis, and apoptosis over various time periods and using various incubation doses. One can further quantitate the handling of different fluorochromatic drugs and reagents by different cell types. The following subsections illustrate our experiments in this context.

3.5. Measurement the Uptake of Free Doxorubicin and Doxorubicin Microcapsules

Cells are trypsinized (MCF-7/WT, MCF-7/R) and incubated at a cell density of either 2×10^5 or 5×10^5 cell/mL in serum-free culture medium in 5-mL polypropylene tubes at 37°C in a CO_2 incubator for 1 h with 0.5 μ*M* of either free doxorubicin or doxorubicin microcapsules *(15)*. At 10-min intervals during the incubation, the cell suspensions should be mixed manually. After incubation, they should be washed twice in PBS, resuspended in 500 μL PBS, and then visualized using the LSC.

Time-course experiments can be conducted to establish the rate of uptake of either free doxorubicin or doxorubicin microcapsules after the cells are seeded at 1×10^6 into the T_{25} flask incubated in 3 mL of culture medium at 37°C in CO_2, and incubated for a further 2–96 h.

Following incubation, adherent cells are washed with PBS and detached from the flasks with trypsin. The cell pellet is then resuspended in 1 mL culture medium, cell viability counts are performed, and the cell density are adjusted to

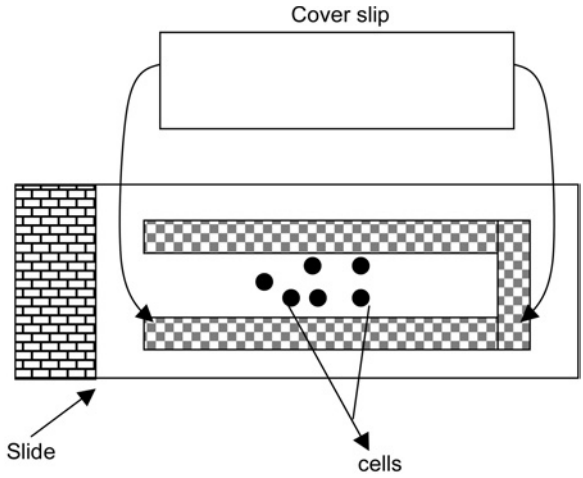

Fig. 2. Construction of a chamber slide.

5×10^5 cells/mL in all cells. Samples are then analyzed and visualized by the LSC to capture the uptake and internalization of the doxorubicin microcapsules by the target cells.

3.6. Slide Preparation for LSC and Fluorescent Microscopy of Labeled Cells

Chamber slides (*see* **Fig. 2**) (*see* **Note 1**) allow the study of spatially fixed but viable cells in fluid media under the microscope of the LSC. This allows a wide range of experimental options in the study of the response of cells to stimulants, toxins, or the binding of additional antibodies and fluorochromes.

3.7. Examination of Samples Using the LSC

The 488-nm argon laser provides excitation. Proprietary Wincyte™ software (Compucyte) is used to control the instrument. The target cells are identified and selected by contouring on laser light scatter and red fluorescence (as determined by the presence of doxorubicin and doxorubicin microcapsules). The integrated laser light scatter and red fluorescence of each cell are displayed in a dot plot of area vs red maximal pixel. The dot plot is very similar in concept and presentation to those familiar to flow cytometrists (*see* **Fig. 3**; Color Plate 7, following p. 274). The signal from each cell comprises some 200 pixels in 0.5-µm increments. The area measures the number of pixels in the field of interest, usually a single cell; thus, area represents the size of the cells or microcapsules. The maximum pixel value is represented the highest fluorescence signal from within the data contour of the individual cell. Each cell can be rescanned

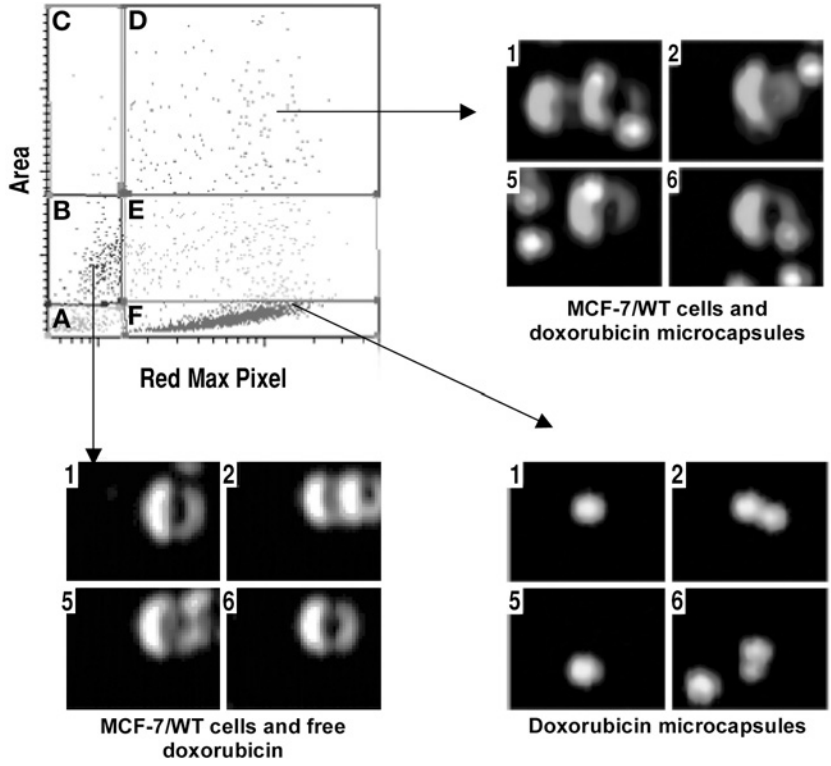

Fig. 3. Assay of cells incubated with either free drug or drug-encapsulated microspheres by LSC. The size of the cells or particles is denoted on the *Y*-axis (Area). The intensity of red, doxorubicin-associated fluorescence, is denoted on the *X*-axis. (*See* Color Plate 7, following p. 274.)

numerous times, because the LSC records the X-Y position of each cell on the slide. Rescanning each cell from any selected dot plot region creates laser light-scatter images and red fluorescent images, which can be stored separately and later merged. This allows us to produce galleries of cells images (*see* **Note 2**).

The specific channel-splitting facility of this program allows each image to be split into three 8-bit images comprising red, green, and blue color components. The coassembly of the laser scan and red fluorescent images of the cells allows false color to be produced.

3.8. Technical Observations

These studies demonstrate the utility of LSC in correlating quantitative data on complex cell populations equivalent to that generated by FC, with highly informative qualitative imagery of the cells of interest.

Our work has been conducted on a first-generation commercial instrument (circa 1996) and early versions of the proprietary Wincyte software. Our system has been adapted for advanced image analysis using a simple link to the Kontron KS100 image analysis system. Our system has been robust, reliable, and straightforward to use. We understand that more recent versions of the instrument offer considerable advances in capability.

We consider that LSC offers a considerable advance in many respects over conventional FC:

- It allows for the visualization of cells of interest and the quantitative fluorometric analysis of cell, subcellular, and particulate fluorescence to the limits of resolution of the instrument. This allows validation of rare events and the study of spatially related and temporally discrete events.
- It allows the study of viable and whole-cell preparations.
- It allows the retention, reexamination, reinterrogation, and reexperimentation of samples and cells of interest.
- It allows for novel forms of presentation of samples to the instrument, as, for example, in fluidic chamber slides.
- It can be made interoperable with other compatible instruments, such as confocal microscopes made by Olympus Optical, whereby the coordinates of cells of interest can be transferred to the stage of the confocal microscope.
- It makes possible broader forms of experimentation that are not possible on flow cytometers.

Specifically, in these studies, we have demonstrated, among others, the following:

- The normal operational mode of the LSC, using single-laser excitation, forward scatter, and two-color analyses
- Techniques of sample presentation on the microscope slide
- The use of the instrument to create galleries of images

3.9. Specific Biological Observations

Our ongoing series of studies have revealed the following:

- The kinetics of uptake, distribution, and elimination of free doxorubicin and encapsulated doxorubicin in wild-type (drug-sensitive) and resistant breast cancer cell lines. Such studies can clearly be extended to any experimental cell type or any fluorochromatic drug or reagent handled by cells (*see* **Fig. 4**; Color Plate 8, following p. 274).
- The facility to discriminate between single cells and aggregates of cells, and subcellular particles—in this instance, drug-loaded albumin microcapsules.
- The use of fluorochromatic cytotoxic drugs as their own biomarkers of binding, uptake, distribution, exclusion, and cell damage in cancer cell lines in vitro.
- The workings of drug-resistance mechanisms in cancer cells.

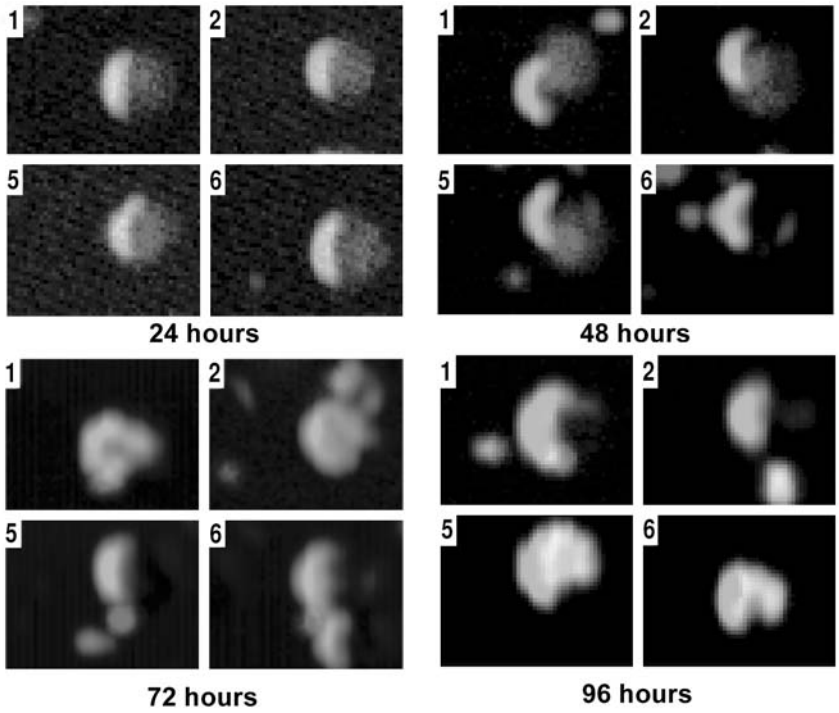

Fig. 4. Time-course experiments of doxorubicin-encapsulated microspheres. Microspheres were incubated with the target cells for 1 h under standard conditions. Cells were analyzed at various time intervals over 96 h. The images were captured on the LSC and then manipulated to produce the image galleries. The illustrations show the binding, uptake, and intracellular distribution of the bright red cytocaps and the gradual release of free doxorubicin into the cells. (*See* Color Plate 8, following p. 274.)

- The induction of cytostasis and apoptosis in target cells.
- The biological complexity of homogenous cell lines with respect to drug uptake and metabolism.

3.10. Quantitative Analysis of Bronchial and Peripheral Blood Eosinophils and Respiratory Epithelial Cells Using LSC

The objective, quantitative measurement of specific cell types in complex samples of clinical material has important diagnostic and therapeutic applications. Microscope-based studies with trained technical observers, such as used in the cervical smear cytology program or in genotoxicity testing, are laborious, inefficient, subjective, and expensive. Automation would offer considerable advantages. The quantitative measurement of eosinophils in the sputum of asthmatic patients has valuable research and diagnostic functions. Cellular and fluid

markers of inflammation in sputum have been assessed by a number of investigators, and their validity, repeatability, and responsiveness have been demonstrated in small-scale studies. Sputum eosinophil counts as a marker of asthmatic airway inflammation have the potential for aiding diagnosis and monitoring treatment response to steroid medication in clinical asthma. Sputum cytology also aids the diagnosis of other diseases, including lung cancer. Sputum is a viscid and heterogeneous substance that is unsuitable for FC analysis. Cell preservation is poor and relatively few cells are found in a unit volume of sputum. Manual analysis is very tedious. We thus devised a procedure for objective measurement of sputum eosinophils and bronchial epithelial cells using the planar analysis system of the LSC.

3.10.1. Human Sputum Samples

Samples of human sputum suitable for analysis are obtained in specialist respiratory practice by sequential saline aerosol inhalation at concentrations of 3%, 4%, and 5% for 5 min each. Subjects expectorated for approx 2 min and sputum samples were collected in a sterile 30-mL universal container on ice ***(18)***.

Sputum was processed within 2 h of expectoration. Macroscopically visible sputum plugs were manually selected from saliva using blunt forceps and a Petri dish to minimize oral squamous cell contamination. The selected sample was weighed and processed on ice in 4 vol of 0.1% dithiothreitol (DTT) using gentle aspiration through a Pasteur pipet and subsequent vortexing for 15 s. Following this, the sample was rocked on a bench rocker on ice for 5 min and mixed thoroughly with equal volumes (to DTT) of Dulbecco's phosphate-buffered saline (D-PBS). The cell suspension was filtered through 45-µm nylon mesh and centrifuged for 10 min at 800*g* at 4°C. The cell pellet was resuspended in D-PBS to determine cell viability, oral squamous cell contamination, and absolute cell numbers. The volume of the cell suspension was adjusted for optimal cytospin dispersion with D-PBS to 0.25×10^6/mL. Cell samples can be extracted from sputum by processing on ice in 4 vol of 0.1% DTT, followed by 4 vol of D-PBS, filtering through a 45-µm nylon gauze, and centrifuging for 10 min at 800*g* at 4°C. Suspensions should be adjusted for optimal cytospin dispersion with D-PBS to 0.25×10^6/mL.

3.10.2. Use of the Octospot Cytospin System

To accomplish time-efficient analysis by LSC without the need for frequent slide replacement, we used the novel Octospot® cytospin system (*see* **Fig. 5**). This system is compatible with the Shandon cytocentrifuge and allows transfer of cell suspensions (40–80 µL per well) from eight-well microtiter plate strips onto microscope slides, thus generating eight separate cytospins on a single slide. Octospot slides are covered with a solvent-resistant hydrophobic coating surrounding each well, allowing differential immunostaining of adjacent

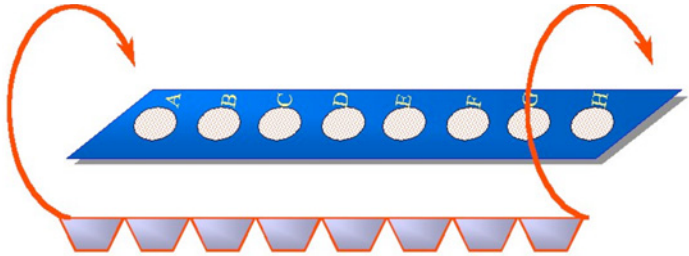

Fig. 5. The octospot® slide system allows the generation of eight individual cytospins on a single microscope slide using the Shandon microcentrifuge and standard eight-well microtiter plate strips.

cytospins. A 40-µL sputum cell suspension (0.25×10^6/mL) was added to each well and spun onto slides at 450 rpm for 6 min in a Shandon cytocentrifuge. Cytospins were air-dried and frozen at –20° until further analysis. After thawing, slides were fixed in high-grade acetone/methanol (50/50). Individual cytospins on nonproprietary standard microscope slides were circled with a hydrophobic wax marker to allow immunostaining of adjoining cytospins with 50 mL of antibody solutions (*see* **Note 3**).

Slides were incubated at room temperature for 1 h with mouse a human-MBP monoclonal antibody (Ab), mouse and human-cytokeratin Ab, or isotype-matched control Ab in PBS + 0.1% BSA. Each cytospin was washed with 20 vol (1 mL) of PBS using simultaneous suction and pipetting to avoid overflow and cross-contamination between adjacent cytospins. To reduce nonspecific binding of fluorochrome-conjugated second Ab to highly charged eosinophil proteins, cytospins were blocked with Chromotrope2R for 15 min, followed by a further washing step and incubation with goat and mouse–Oregon Green®-conjugated second Ab (20 mg/mL) and 0.2 mg/mL propidium iodide for 1 h at room temperature (*see* **Fig. 6**) (*see* **Notes 4–6**).

3.10.3. Analysis Using the LSC

Slides are scanned on the LSC stage using the 20× objective and argon laser light stimulation at 5 mW. Wincyte software is used to draw contours around areas of specified fluorescence intensity, relating to specific staining protocols. For all of the experiments, the red fluorescence channel was used for segmentation around propidium iodide (PI)-stained cell nuclei. Only the red and green sensors were used in channel 1 and 4, respectively. The scan data display in conjunction with scattergram windows for peak fluorescence (red and green max pixel) plotted against area was generally used for optimizing detector voltage and gain as well as background values (all set in *Setup* → *LSC settings*). Offset

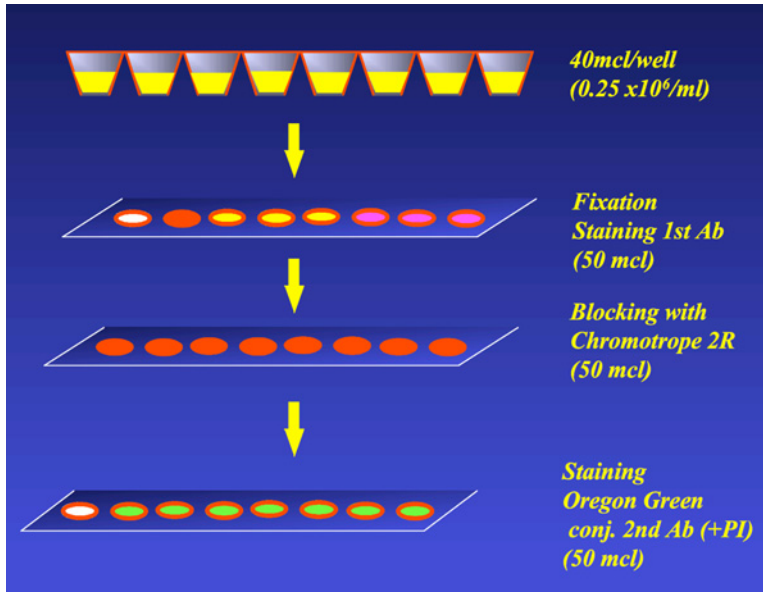

Fig. 6. Overview of indirect immunostaining protocol of sputum cytospins using the Octospot® slide system. Immunostaining with primary antibody was followed by a block step with Chromotrope 2R to reduce unspecific binding of conjugated secondary antibody to highly charged eosinophil-derived proteins. Finally, secondary antibody solution (Oregon Green® conjugated) is applied. Note that negative controls and differential antibody staining is performed on the same slide, thereby reducing between-slide variability.

values of 2000 for red and 2035 for green fluorescence were used. The PMT power setting ("gain") was varied depending on fluorescent-staining intensity between 15 and 60 for red and green fluorescence aiming to utilize the dynamic range of the instrument optimally for individual experiments. For sputum cytospin analysis, red gain was adjusted to achieve mean area values for single cells of 100–200 (25–50 mm^2 for scanning at ×20 magnification).

To allow accurate immunophenotyping of sputum cytospins, detection of a maximal number of single-cell events within the initial cell suspension was critically important. To minimize software contouring around cell clumps and overlapping cells, great care was taken during cytospin preparation to achieve adequate cell dispersion on the glass slide. Preliminary experiments established that cell dispersion for sputum cytospins was optimal at initial cell concentrations of 0.5 × 106/mL. Acytospin index (CI),

$$CI = \frac{SCC}{TCC}$$

where SCC is the total number of single-cell events contoured and TCC is the total number of events contoured, was determined for all cytospins to monitor cell dispersion. Single-cell events were determined by combining the software algorithm for single-cell detection with a gating step based on nuclear size, as PI stained nuclei were used as contouring parameter. Cell dispersion within single-cytospin areas was variable but could be optimized by adding a second cardboard filter to the clip mechanism during cytocentrifugation. In the case of the Octospot slide, eight regions can be set around the eight octospots, thus allowing for automated analysis and comparison of the entire content of each cytospin on the slide. Thus, for example, controls can be analyzed alongside the test samples to minimize experimental variation and error (*see* **Note 7**).

3.10.4. Results

In order to calculate the proportion of a particular cell subset as a percentage of the total blood and sputum samples were labeled with PI to utilize its bright red nuclear staining pattern for software contouring and, hence, capture of all nucleated cells or nucleated cell remnants. The WinCyte software that controls the LSC draws contours around areas of specified fluorescence intensity, if sufficiently contrasted from the background, and registers these contours as objects. Within the area of an object and within a customizable distance surrounding it, other cell parameters can be sampled. The software program distinguishes and optionally excludes cell aggregates based on bit-pattern data. By using this exclusion algorithm and, in addition, setting a nuclear size gate based on the measured area or total integrated red fluorescence of captured events, it was possible to focus on a relatively pure single-cell population within each cytospin (*see* **Figs. 7B**, **8A**, and **9A**). Green fluorescence was sampled within this single cell gate.

For sputum eosinophils, the peak fluorescence signal within each data contour was plotted against contour (nuclear) size (*see* **Figs. 7** and **8**), whereas the more homogenous and generally brighter staining of sputum epithelial cells was plotted as integrated green fluorescence within each data contour against peak green fluorescence signal (*see* **Fig. 9B**; Color Plate 9 following p. 274). To exclude oral squamous epithelial cells, events within the high green peak/high green integrated fluorescence, gate were plotted in addition for their red peak and integrated fluorescence, as bronchial epithelial cells feature more uniform and generally brighter nuclear staining than contaminating oral squamous epithelial cells (*see* **Fig. 9B**). For all sputum samples, at least three cytospins per slide and antibody were analyzed and specific mean percentages were calculated after subtraction of negative controls.

More detailed results and a method comparison using this method and traditional manual differential cell counting are presented elsewhere ***(18)***.

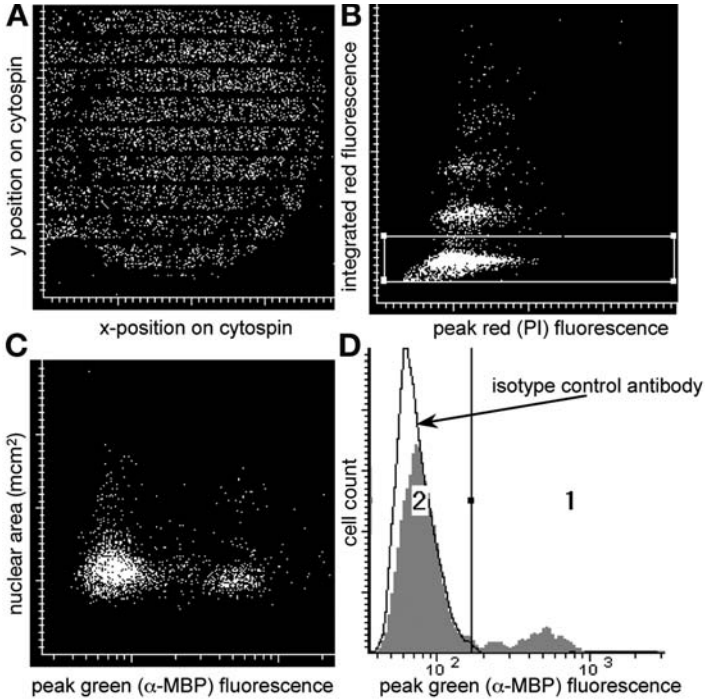

Fig. 7. Determination of blood eosinophil count by LSC. **(A)** *x*/*y*-position of propidium iodide labelled cells detected in a single cytospin. **(B)** Multiple cell objects are excluded on the basis of their higher integrated propidium iodide fluorescence. **(C)** Single cells are scanned for specific green fluorescence after labelling with α-MBP antibody and FITC-conjugated secondary antibody. **(D)** Histogram of plot in C with isotype matched control antibody overlaid.

3.10.5. Technical Observations: LSC in Sputum Analysis

- Octospot cytospin slides are a valuable tool for the presentation and analysis of samples to the LSC.
- Selective gating on peak and integral fluorescence and nuclear and whole-cell fluorescence allows discrimination of cell populations of interest, for both green and red fluorochromes.
- Gating strategies can be validated by direct visualization of the selected cells.
- Perseverance and attention to detail can allow quite challenging sample presentation problems to be overcome by LSC.
- Sample dispersion and cytospin density are critical to the optimal analysis.
- Propidium iodide staining for DNA is a valuable technique for gating on dispersed cells using their nuclear fluorescence.
- Repeated scans are simple to perform and overcome the problem of interobserver error, which complicates manual counts.

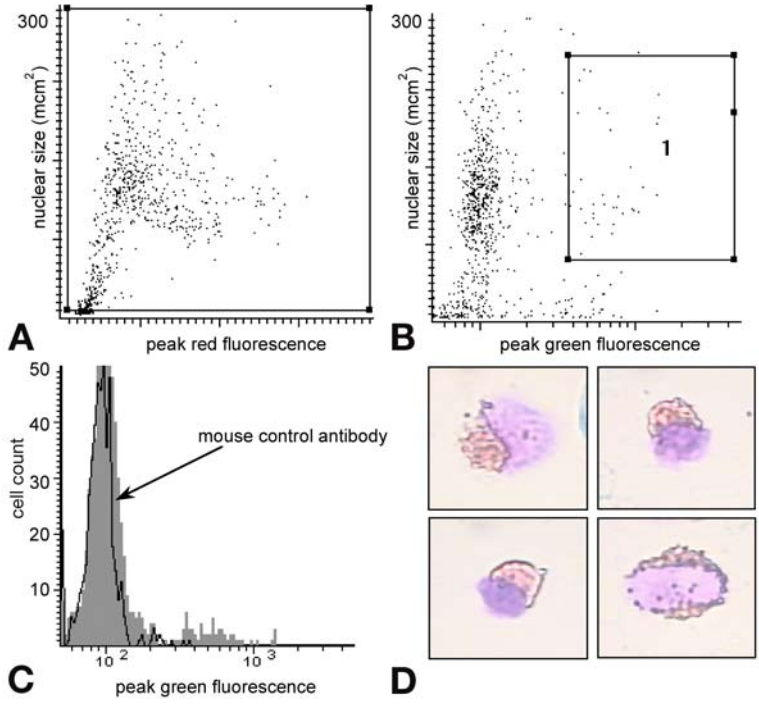

Fig. 8. Gate settings for the analysis of sputum eosinophil cells by LSC. **(A)** Single cells gated by detection of niuclear staining with propidium iodide. **(B)** Eosinophil gate (major basic protein positive cells in gate 1). **(C)** Histogram of plot B with isotype matched control antibody overlaid. **(D)** Brightfield microscopy video captures of relocated cells within eosinophil gate restained with Romanowski stain. More than 90% of cells within gate displayed eosinophil morphology. Outside this gate cells of this morphology were rare.

3.10.6. Biological Observations: LSC in Sputum Analysis

- Eosinophils and bronchial epithelial cells can be successfully identified and quantified on the LSC.
- Compared to manual counting—the current gold standard—estimation of the sputum eosinophil count by LSC was equivalent.
- The assay has demonstrated higher counts of eosinophils in the sputum of asthmatic than in normal subjects.
- Immunophenotyping using techniques demonstrated in this study could be extended to other cell types in complex clinical samples.
- The addition of a third photomultiplier tube to the instrument would allow double staining of other intracellular and extracellular antigens, such as is now possible.

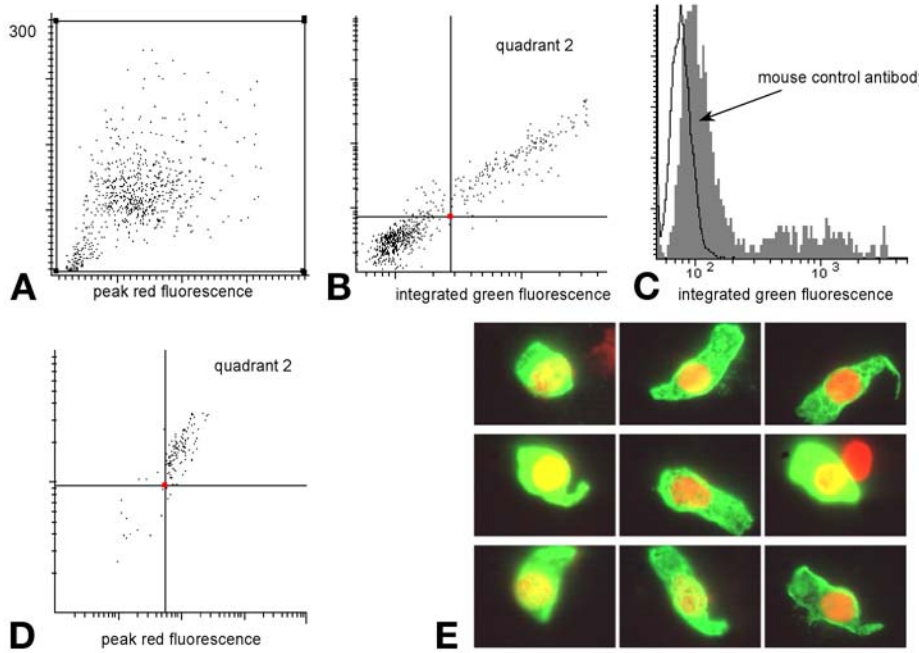

Fig. 9. Gate settings for the analysis of sputum bronchial epithelial cells. **(A)** Single cells gated by detection of nuclear staining with PI. **(B)** Peak green fluorescence plotted against integrated green fluorescence for single cells stained with α-cytokeratin antibody (labeled with Oregon Green-conjugated secondary antibody. **(C)** Histogram of B with isotype-matched control antibody overlaid. **(D)** Cells within quadrant 2 of plot B are plotted for red fluorescence to exclude oral sqamous epithelial cells; **(E)** Epifluorescent video captures of relocated cells within quadrant 2 of plot D (typical of bronchial epithelial cells). In excess of 95% of cells within this gate had this morphology. Outside of this gate, cells with this morphology were very rare (*See* Color Plate 9, following p. 274.)

4. Notes

1. Chamber slides can be constructed from normal glass microscope slides. Multilayered adhesive tape is aligned across the two faces of the slide with a shorter piece of tape joining the two at the end. A glass cover slip is positioned carefully on the tape and the edges are sealed. A 20-µL aliquot of sample is pipetted onto the chamber slide at the end of the cover slip that is not sealed. The cells are allowed to settle for 5 min. The slide should be then sealed with transparent nail varnish before analysis.
2. To improve graphical presentation on older LSC instruments, digital color images of the cells can be captured in WinCyte and copied to a graphic package such as Paint Shop Pro 5™ for Windows as bitmap (bmp) images for display. There, the images of red fluorescence can be superimposed onto the laser light-scatter

images. False colors can be used to identify the cells (green or blue), doxorubicin (red), and doxorubicin microcapsules (red).

3. Samples can be stored for later analysis by freezing at –20ºC. Cytospins can be circled with a hydrophobic wax marker to allow immunostaining with 50 µL of antibody solutions.
4. Oregon Green gives brighter staining and less photobleaching than FITC conjugates.
5. Each cytospin can be washed with 1.0 mL PBS with simultaneous microsuction to avoid overflow and cross-contamination between adjacent cytospins. Slides should be cover-slipped with glycerol 25% in PBS.
6. To reduce nonspecific binding of fluorochrome-conjugated secondary antibody to highly charged eosinophilic proteins, cytospins can be blocked with Chromotrope R (Sigma) for 15 min.
7. There must be sufficient contrast between the cells of interest and the background to achieve satisfactory contouring. By focusing on the cell nucleus rather than the entire cell, better contrasts can be achieved.

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