THE LANCET

Volume 353, Number 9149 • Founded 1823 • Published weekly • Saturday 23 January 1999

Contents list continues inside

£4.50

10 Ramsay LE, Haq IV, Jackson PR, et al. Targeting lipid-lowering drug therapy for primary prevention of coronary disease: an updated Sheffield table. *Lancet* 1996; **348**: 387–88.

11 Lowensteyn I, Joseph L, Levinton C, Abrahamowicz M, Steinert Y, Grover SA. Can computerized risk profiles help patients improve their coronary risk? The results of the Coronary Health Assessment Study (CHAS) *Prev Med* (in press).

12 Avorn J, Monette J, Lacour A, et al. Persistence of use of lipid-lowering medications: a cross-national study. *JAMA* 1998; **279**: 1458–62.

Laser-scanning cytometry

Technology has driven research in clinical pathology since the optical microscope was introduced more than 200 years ago. The rapid, reliable, and quantitative assessment of biological events in large numbers of individual cells finds important applications in experimental systems and clinical analysis, such as in immunophenotyping, in counting of CD4 cells in HIV infection, in DNA ploidy studies, and in the quantification of cell-membrane, cytoplasmic, and nuclear molecules.

Samples from experimental systems, blood, tissue, and tumour samples are usually composed of large, heterogeneous populations of cells, of varying morphology, function, differentiation, cell-cycle phase, destiny, and viability. Conventional assessment by trained observers of slide-based samples is, despite histochemistry and image cytometry, primarily a qualitative exercise.

Light-scatter and fluorescence-based techniques offer considerable advantages in the cell-by-cell analysis of complex samples. One approach to quantification has been the analysis, by flow cytometry, of the optical characteristics of particles in suspension. The standard flow cytometer can quantify multiple fluorescence markers and light scatter in heterogeneous samples in fluid suspension at rates of several hundred particles (cells, nuclei, or chromosomes) or more per second.[1] These particles are streamed through a beam of coherent light of defined excitation wavelength, generally a laser beam. Laser-based flow cytometry has been the mainstay of quantitative cell research since the late 1970s. 100 000 cells per sample can be studied with ease. Data are presented as computer-generated histograms and dot plots.

Flow cytometry is particularly useful where cell size and scatter patterns are consistent, as in leucocyte immunophenotyping; where they are immaterial, as in the measurement of DNA content (ploidy) in tumours; or in chromosome sorting. However, the disaggregation of tissues and solid tumours into single-cell suspensions for analysis destroys information contained in the cell and tissue architecture, thus confounding interpretation of data. Analysis is also limited by sample size and consistency; fine-needle aspirates or viscid samples are generally unsuitable for analysis.

Fundamentally, the disadvantage of flow cytometry is uncertainty, because sample morphology cannot be correlated with light emissions on a cell-by-cell basis. Having to guess at the nature of the cells from the scatter and fluorochrome-labelling characteristics is generally acceptable in haematological studies, where leucocytes have precise and discrete physico-optical characteristics. It is not satisfactory for the study of cell disaggregates derived from tissues and solid tumours, where the variation in size and scatter defies analytical algorithms. Cell sorting helps overcome this limitation but does not allow consistent correlation of light measurements with every cell in the sample.

Laser-scanning cytometry integrates the capabilities of flow cytometry with optical microscopy. A prototype instrument was first described in 1991.[2] A refined model entered commercial production in 1996, and about 100 instruments have since been installed in research centres worldwide.

The laser-scanning cytometer includes many of the components and analytical processes of flow cytometry, integrated with a conventional, fluorescence-adapted microscope and imaging system. It allows for laser excitation of the sample from a 488 nm blue argon laser, and from an orange or red helium-neon laser at 546 nm or 633 nm where fitted. Ultraviolet laser excitation, which is of use in the study of features of cell physiology such as calcium fluxes, is not presently an option because of the optical characteristics of the microscope lenses. Light-collection optics and photomultiplier tubes record fluorescence emissions. An integral, standard epifluorescence illumination system using a mercury arc lamp further extends the range of excitation wavelengths.

The radical development is that, in laser-scanning cytometry, the laser beam is scanned over and through the sample on a microscope slide. The beam, steered by directional optics, moves in an axis at 90° to that in which the microscope stage is moved in 0·5 μm computer-controlled steps. Measurements of each cell thus include its precise position on the slide, its area, its perimeter, its peak fluorescence, and the time of measurement. Each cell can be recaptured and rescanned many times by the laser beam, subject to the limitations of photobleaching. Up to 1000 cells per min can be analysed. The instrument makes about 100 measurements on each cell at cell densities of 100–1000 cells per mm^2, using a two-dimensional array of values rather than the pulse-spot analysis of flow cytometry. Data are initially collected and displayed in the conventional graphical forms used in flow cytometry. A camera attached to the microscope allows digitisation, display, and recording of images, and the microscope allows direct inspection of each cell or field of interest at different magnifications, and thus direct correlation of cell-fluorescence signals with morphology.[3] A simple connection to a commercially available image-analysis system further extends the value of the optical system.[4]

Laser-scanning cytometry thus finds applications that derive from flow cytometry, including DNA ploidy and S-phase fraction analysis,[5,6] biomarker expression,[7,8] and tumour immunophenotyping.[9] The Chicago group[7,9,10] in particular has sucessfully exploited the technique such that its laboratory immunophenotyping service is now based on laser-scanning cytometry. Reported benefits include automation of the assay, savings on reagents, and accurate diagnosis on very small biopsy samples. The instrument can analyse six variables.[10] In these studies, instrument performance is reported to be similar to that in flow cytometry, except that the rate of the data acquisition is slower by a factor of 10, which reflects the large data-set collected on each cell.

At about £100 000 per machine, laser-scanning cytometry must provide a unique range of practical applications to attract scientists. These applications stem from the slide-based nature of the analysis. Key benefits include the fact that cells can be classified by their morphology and viability; that rare cells and events such as leukaemic blast cells, mitoses,[11] or apoptotic cells[12] can be visualised within complex cell populations; and that fine-needle aspirates, smears for cytology, viscid and mucoid

samples, and imprint specimens such as those from the cut surface of a tumour can be studied directly on the collection slide. In addition, the microscope slide provides considerable scope for development. It can be modified to allow for multiple experiments to be conducted on one slide, either serially or spatially.

Dynamic experiments on the physiology of intact membranes are possible—for example, measurement of the rate of intracellular accumulation or exclusion of fluorophores, and quantitative assessment of their translocation between cytoplasm and nucleus in signal-transduction studies.[13,14] Cell-surface and intracellular labelling of fluorochromes can be distinguished by use of selective permeabilisation protocols and morphological criteria. Chamber-culture slides allow direct analysis of cells grown to confluence without need for transfer.[15]

The pixelation of data-sets also offers new opportunities, because subcellular peaks of fluorescence can be identified and quantified. However, the limited subcellular resolution of the instrument limits fluorescence in-situ hybridisation to coarse FISHing of perhaps two bright probes per cell,[16] whereas confocal microscopy offers higher sensitivity and resolution. One practical example under development lies in the automation of the laborious mouse micronucleus assay used in genotoxicity testing.[17]

The extent of the capabilities and limitations of laser-scanning cytometry has yet to be fully explored. Further technical innovation seems likely as compact lasers and image-capture and processing techniques are added, and there remains scope for simplification and automation of software protocols. The system was originally designed for the study of cell isolates, but has already been modified for the study of confluent cells and thin tissue sections. A combination of optical microscopy and laser cytometry should be a useful system for clinical cytopathologists. Substantial developments in technology and applications can be expected, as has been the case with flow cytometry.

*David A Rew, Gerrit Woltmann, Andrew J Wardlaw

Advanced Laser Cytometry Facility, University of Leicester, Glenfield Hospital, Leicester LE3 9QP, UK

1 Watson JV. An introduction to flow cytometry. Cambridge: Cambridge University Press, 1992.

2 Kamentsky LA, Kamentsky LD. Microscope based multiparameter laser scanning cytometer yielding data comparable to flow cytometry data. *Cytometry* 1991; **12:** 381–87.

3 Woltmann G, Ward RJ, Symon FA, Rew DA, Pavord ID, Wardlaw AJ. Objective quantitative analysis of eosinophils and bronchial epithelial cells in induced sputum by laser scanning cytometry. *Thorax* (in press).

4 Woltmann G, Wardlaw A, Rew DA. Image analysis enhancement of the laser scanning cytometer. *Cytometry* 1998; **33:** 362–65.

5 Martin-Reay DG, Kamentsky LA, Weinberg DS, Hollister KA, Cibas ES. Evaluation of a new slide-based laser scanning cytometer for DNA analysis of tumors: comparison with flow cytometry and image analysis. *Am J Clin Pathol* 1994; **102:** 432–38.

6 Sasaki K, Kurose A, Miura Y, Sato T, Ikeda E. DNA polidy analysis by laser scanning cytometry in colorectal cancers, and comparison with flow cytometry. *Cytometry* 1996; **23:** 106–09.

7 Clatch RJ, Walloch JL, Foreman JR, Kamentsky LA. Multi-parameter analysis of DNA content and cytokeratin expression in breast carcinoma by laser scanning cytometry. *Arch Pathol Lab Med* 1997; **121:** 585–92.

8 Gorczyca W, Darzynkiewicz Z, Melamed M. Laser scanning cytometry in pathology of solid tumours: a review. *Acta Cytol* 1997; **41:** 98–108.

9 Clatch RJ, Foreman JR, Walloch JL. Simplified immunophenotypic analysis by laser scanning cytometry. *Cytometry* 1998; **34:** 3–16.

10 Clatch RJ, Foreman JR. Five colour immunophenotyping plus DNA content analysis by laser scanning cytometry. *Cytometry* 1998; **34:** 36–38.

11 Luther E, Kamentsky LA. Resolution of mitotic cells using laser scanning cytometry. *Cytometry* 1996; **23:** 272–78.

12 Bedner E, Burfeind P, Hsieh T, et al. Cell cycle effects and induction of apoptosis caused by infection of HL-60 cells with human granulocytic ehrlichiosis pathogen measured by flow and laser scanning cytometry. *Cytometry* 1998; **13:** 47–55.

13 Deptala A, Bedner E, Gorczyca W, Darzynkiewicz Z. Activation of nuclear factor kappa-B assayed by laser scanning cytometry. *Cytometry* 1998; **33:** 376–82.

14 Bedner E, Melamed M, Darzynkiewicz Z. Enzyme kinetic reactions and fluorochrome uptake rates measured in individual cells by laser scanning cytometry. *Cytometry* 1998; **33:** 1–9.

15 Musco ML, Cui S, Small D, Nodelman M, Sugarman B, Grace M. Comparison of flow and laser scanning cytometry for the intracellular evaluation of adenoviral infectivity and p53 protein expression in gene therapy. *Cytometry* 1998; **33:** 290–96.

16 Kamentsky LA, Kamentsky LD, Fletcher JA, Kurose A, Sasaki K. Methods for automatic multiparameter analysis of fluorescence in situ hybridised specimens with a laser scanning cytometer. *Cytometry* 1997; **27:** 117–25.

17 Styles JA, Rew DA. Automation of the mouse micronucleus genotoxicity assay by laser scanning cytometry . *Cytometry* 1998; suppl 9: 41 (abstr).

Headache relief or impotence?

See p 287

Nitric oxide really does seem to be the molecule of the moment. Its importance in regulating blood pressure, providing host defence, and inhibiting platelet function, and as a neurotransmitter has become much more widely known recently because of the award of the 1998 Nobel prize for medicine to three scientists who helped in revealing its biological importance. Drugs that work through the nitric oxide pathway—sildenafil (Viagra), for instance—are starting to be launched. This drug enhances the effect of nitric oxide by inhibiting the breakdown of the second messenger, cyclic GMP, synthesis of which is provoked by nitric oxide and which causes smooth-muscle relaxation.

Nitric oxide manufacture was first identified in endothelial cells,[1] and the chemical is now known to be continuously synthesised by a constitutive endothelial enzyme. It is also continually made by a different nitric oxide synthase (NOS) in autonomic nerve cells where, among its other actions, it is vital for genital erectile function. Its synthesis can be induced in most mammalian cells when they are exposed to inflammatory cytokines, which is when it plays an important part in host defence.[2] There is a large population of central neurons that also contain neuronal NOS, but the function of nitric oxide in the brain is not entirely clear. One suggestion is that this source of nitric oxide is important in maintaining long-term potentiation of neuronal depolarisation and therefore is involved in memory.[3,4] There has also been much interest in the possibility that nitric oxide may contribute to nerve-cell damage after stroke. Inhibition of nitric oxide synthesis in an animal model of stroke reduces infarct volume.[5] The third main strand of brain nitric oxide research has been concerned with the ability of centrally synthesised nitric oxide to modulate the sensation of pain. In particular, the suggestion from animal work is that nitric oxide inhibits the effects of endogenous opioid-like peptides and that inhibition of nitric oxide synthesis may have analgesic effects.[6]

The study described in today's *Lancet* examined the effect of monomethyl L-arginine (L-NMMA), a specific inhibitor of NOS, in patients with chronic pain due to tension-type headache. Intravenous infusion of 6 mg/kg L-NMMA resulted in a modest but definite reduction in perceived pain intensity compared with glucose placebo. Pain was measured by a visual analogue scale. The