

PERSPECTIVES

Laser scanning cytometry (LSC) in pathology – a perspective tool for the future?

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Abstract: Cytometry is becoming a standard method of examination not only in biology but also in various fields of experimental and clinical medicine. While in flow cytometry suspensions of cells are measured, laser scanning cytometers enable both the measurement of cells in single-cell suspensions (after immobilising the cells on a conventional glass slide) and in frozen or paraffin-embedded tissue sections. We discuss the possible fields of utilisation and future perspectives of laser scanning cytometry in medicine with special reference to clinical pathology and cytology (Fig. 3, Ref. 49). Full Text (Free, PDF) www.bmj.sk.

Key words: laser scanning cytometry, pathology.

Since microscopy has been introduced in biology and medicine as a routine tool of examination, its rapid advances in mid-nineteenth century promoted cytological and histological analysis of bioptic specimens to become a standard diagnostic approach also in clinical pathology and histopathology (1). Currently, there is an ever growing need for obtaining rapid, reliable, quantitative and highly reproducible methodologies and approaches not only on tissue level, but also on the level of cells and subcellular structures. Examination of biological events in large cell populations in formalin-fixed, paraffin-embedded tissue sections as well as in cell suspensions offers a number of applications in various experimental settings and clinical analyses.

Fluorescence microscopy is one of the methodological approaches of examination widely used in pathology. Although fluorescence dyes have been used in microscopy for the first time by August Köhler in 1904 (2), it was only in 1941 when the method of fluorescent-labelled antibodies was introduced by Coons (3). Since that time, fluorescence microscopy has witnessed an almost exponential development. The fluorescent microscope enabled to discriminate between morphologically similar cells based on their different reactivity with fluorescently labelled antibodies. However, the analysis of these populations under a fluorescent microscope was rather cumbersome and suffered from considerable subjective error. Moreover, interpretation of the results

of fluorescence microscopy was also hampered by non-specific binding of cells and by naturally occurring autofluorescence. Further development resulted in a plethora of novel methodological aspects supported by unprecedented technical progress that enabled the advent of flow cytometry and later of laser scanning cytometry.

Flow cytometry and laser scanning cytometry

The invention and construction of the flow cytometer in the late 1960s allowed, to a considerable extent, to overcome almost all of the problems encountered in fluorescence microscopy, in particular since the early 1970s, when the first commercially usable flow cytometers became available in clinical diagnostics (4). Flow cytometry not only enabled an objective and rapid analysis of large numbers of cells within a short period of time on the basis of laser beam scatter and intensity of fluorescence, but also offered insight into cell and gene function, molecules associated with these functions, and the understanding of the underlying mechanisms (5). Cytometry and multiparametric fluorescence analysis of cell populations are a perspective technique of cell analysis and currently belong to those methodological approaches used in pathology whose contribution may grow in the future.

In general, cell populations can be cytometrically investigated either by flow cytometry or by laser scanning cytometry. The principle of analysis is the same in both approaches, however, while the subject of flow cytometry is a single-cell suspension, laser scanning cytometry measures individual cells either within monodispersed cell populations cytospun onto a microscopic slide, or in formalin-fixed, paraffin-embedded tissue sections.

Laser scanning cytometry is a relatively new, microscope-based technology that generates laser-scanned images and quantitative measurements of cells deposited on glass slides. It combines both flow cytometry and fluorescence image analysis (6, 7)

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commercially introduced in the early 1990s enabling slide-based morphological measurements and multiparametric cytometry on a cell-by-cell basis (8). The most important feature of the LSC is the recording of the exact position of every cell (event) along with the fluorescence data. Upon re-localisation, LSC allows to determine whether an event is a single cell or a cluster of two and more cells, whether it is a whole cell, debris, or other artefact, and enables further studies of cell morphology of every recorded cell. Another important property of LSC, in contrast to flow cytometry, is its capability of measuring specimens containing as few as several hundred cells, and the possibility to re-stain any cell as many times as needed.

Malignant tumour progression and the response of the tumour to therapy depend on a number of clinical and pathological parameters reflecting the biological properties of any given tumour. In majority of solid malignancies the tumour stage, extent of the tumour mass, lymph node status, and the presence/absence of dissemination to distant sites are the most important determinants for assessing disease prognosis and determining therapy. This means that biological behaviour and growth rate of every individual tumour will depend on the number of proliferating cells on the one hand, and on the number of necrotic/apoptotic cells on the other (9, 10).

Applications of laser scanning cytometry in cytology and pathology

Cytology

At present, the biological characteristics (e.g. DNA content) in cytological specimens are mainly investigated by flow cytometry and image analysis (11). Both methods, besides their advantages, also have several disadvantages. Flow cytometry requires sufficient amounts of cytological material for analysis (12), therefore in many cases a reliable examination of cytological samples is not possible. Moreover, in flow cytometry there is no possibility of verifying the measured data. Image analysis, on the other hand, can also cope with the paucity of available cells but the method is, to a certain extent, biased by the experience of the analyst. LSC is a combination of the advantageous properties of both these approaches, measuring fluorescence in a broad range of wavelengths of emitted light, and analyses cells attached to a microscopic slide. As few as several hundred cells are sufficient for the analysis, and the cells can be re-localised for morphological verification. LSC can be advantageously used for analysing lavages from the bladder, pleura, peritoneum, pericardium, cerebrospinal fluid, cervical smears, material from FNAB, etc. (13). Especially FNAB is suitable for LSC analysis due to the usually limited amount of available material, and the method of material withdrawal is only less invasive (14).

Circulating tumour cells

Much attention is recently being paid to the detection of circulating tumour cells (15). The possibility to quantify such presumed tumour cells in the blood of oncological patients would make it possible to monitor patients with potential metastatic

involvement in early stages of their disease that in turn would enable to identify patients at risk for recurrent cancer (16). These cells, as soon as they are detectable in circulation, may be characterised in greater detail in relation to their sensitivity to therapy, which enables a better prediction of the therapeutic result.

Several methods have been developed for the detection of circulating tumour cells by LSC or by real-time polymerase chain reaction (RT-PCR). The sensitivity of both methods is comparable, however an advantage of LSC using fluorescently labelled antibodies is the identification and measuring of individual cells with the possibility of further characterise these cells (17) either in parallel or consecutively. Furthermore, cells attached to the slide can be further characterised also on the level of molecules (18).

Apoptosis

Apoptosis (programmed cells death) is one of the possible ways a cell can die that plays an important role in various physiological and pathological conditions (19). The apoptotic cell is characterised by characteristic morphological features, on the other hand in the apoptotic cell a number of changes occur on subcellular and molecular level such as nuclear fragmentation or DNA fragmentation. Both flow and laser scanning cytometry are suitable methods of examining apoptotic cells (20, 21). Several methods can be utilised, e.g. annexin V, loss of the transmembrane potential in mitochondria, analysis of DNA fragmentation, or alterations in DNA condensation (22).

Immunophenotyping of leukocytes

Immunophenotyping of leukocytes from peripheral blood by cytometry was among the first LSC methods of analysis of clinical samples (23). LSC analysis in clinical practice is in particular useful in those cases when the amount of the obtained samples for analysis is to be reduced to a minimum degree, e.g. in neonates or in patients in critical condition (24), or if the paucity of material would make other analyses impossible.

The first clinical tests (25) were performed in peripheral blood, in biopsies from bone marrow, in material withdrawn by fine needle aspiration from lymphatic tissue and in other body fluids. This method was later developed to that perfection (26) that it enables staining with up to five fluorochromes in a volume less than 15 µl of peripheral blood. The triggering signal is a direct scatter of the laser beam (forward scatter) and the cells are further analysed by specific immunostaining, usually for CD45.

Another method of peripheral leukocytes immunophenotyping by LSC is based on the measurement of fluorescence of the cell nucleus after staining for DNA with 7-aminoactinomycin D (7-AAD) (27). This method makes it possible to distinguish between neutrophils and mononuclear cells on the basis of intensity of 7-AAD fluorescence. Similar results were also obtained in the case of nuclear staining with propidium iodide (PI) (28). An advantage of DNA staining as the triggering signal with 7-AAD or PI is their relative inexpensiveness, as well as the fact that in LSC practically all nucleated cells will be contoured.

The current technical equipment of laser scanning cytometers enables parallel immunophenotyping by up to five antibodies labelled with various commercially available fluorochromes (29). By the introduction of tandem fluorochromes (PE-Cy7 and APC-Cy7) up to seven different fluorochromes can be used for immunophenotyping, although for flow cytometry some existing systems can utilise up to 11 fluorochromes (30). These methods can be adopted also for LSC and can be used for the detection of rare cells (31) and other functionally distinguishable leukocyte subpopulations (32).

Ploidy and DNA index

Chromosome numbers and the amount of DNA in malignant tumours may play an important role in the rise and/or development of cancer (33). An aneuploid number of chromosomes is one of the characteristic features of the malignant cells, although it is still unclear whether aneuploidy is the cause or a consequence of the malignant transformation of a cell. It was found that aneuploidy might be a prognostic marker in several tumour localisations (34).

In determining the DNA content in cells by LSC we should better speak of DNA index instead of ploidy because LSC does not count the number of chromosomes but measures the amount of DNA in relation to a diploid standard irrespectively of the number of chromosomes into which any given amount of DNA is divided.

The analysis of DNA content is an important parameter of tumour characterisation but with respect to the determination of its grade of malignity, as well as with respect to the prediction of tumour response to therapy. In clinical material originating from both normal and pathological tissues the staining of cell nuclei with fluorochromes specific for DNA (such as propidium iodide or 7-aminoactinomycin D) was the first and best documented application of LSC in clinical practice (23). Several studies have shown that the cytometric analysis of the same material by flow cytometry and LSC are highly comparable (35, 36). The amount of DNA can be determined either from archived histological material (from formalin-fixed paraffin embedded samples) or from freshly obtained bioptic material or from freshly frozen samples (37, 38). In all applications for determining DNA index, DNA may be stained with any fluorochrome although DNA may be stained with any fluorochrome that is excitable by blue or red laser beam (39, 40).

In several studies using LSC and clinical material from oncological patients it was shown that there were certain correlations between DNA content and the clinical characteristics of the tumour (41,42) or investigated DNA content together with genetic parameters such as comparative genomic hybridization, FISH or immunohistochemistry (43). In these studies LSC was used to determine ploidy in malignant tissues in patients with gastric cancer, colon, kidneys, urinary tract, head and neck, as well as patients with gliomas and pituitary gland adenomas.

S-phase of the cell cycle

Cytometric analysis of the ratio of cells actively synthesizing DNA may help in assessing the size of fraction of tumour

cells in S-phase of the cell cycle which at the same time permits to assess the proliferation activity of the tumour (44). The problem of measuring S-phase by flow cytometry or LSC and the search for relationships between S-phase and prognosis of the disease has been widely studied (45). The majority of investigators agree that a high S-phase value in general is associated with poor prognosis. Moreover, the mean value of S-phase in diploid tumours is usually lower than the mean values of S-phase in aneuploid tumours. S-phase may be a potential predictive marker for chemotherapy in patients with breast cancer. Several investigators found a better response to adjuvant chemotherapy and radiotherapy in patients with high values of S-phase (46, 47). However, according to the ASCO guidelines S-phase currently is not recommended as a routine clinical examination (48). Undoubtedly, standardisation and quality control both in flow cytometry and LSC should be improved (49).

Future perspectives of LSC in pathology

Laser scanning cytometry has been introduced to pathological practice, beside flow cytometry, as a promising method of examination and is expected to further improve routine clinical diagnostics. Currently, LSC is used largely in experimental pathology and cell biology, and, despite its undoubted contribution to this field, so far it does not seem to be a widely utilised methodological approach equivalent to flow cytometry. Nevertheless one of the advantages of LSC over FCM is its re-localisation feature and the possibility of multiple and repeated staining of the same cells with different fluorochromes without considerable loss of material. However, in other expectations, e.g. that LSC would be able of measuring DNA content and determine S-phase of the cell cycle directly from paraffin embedded tissue sections, flow

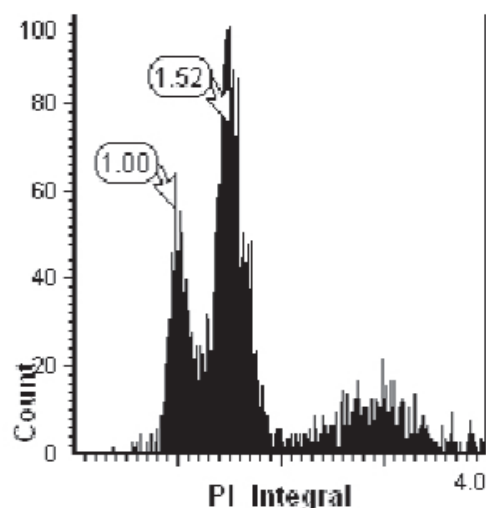


Fig. 1. Laser scanning cytometric DNA histogram showing a breast tumour with aneuploid cell population. Left peak (DNA index 1.00): normal cells (lymphocytes) at G0/G1 used as diploid control, middle peak (DNA index 1.52): aneuploid tumour cells at G0/G1, right peak: aneuploid tumour cells at G2/M of the cell cycle.

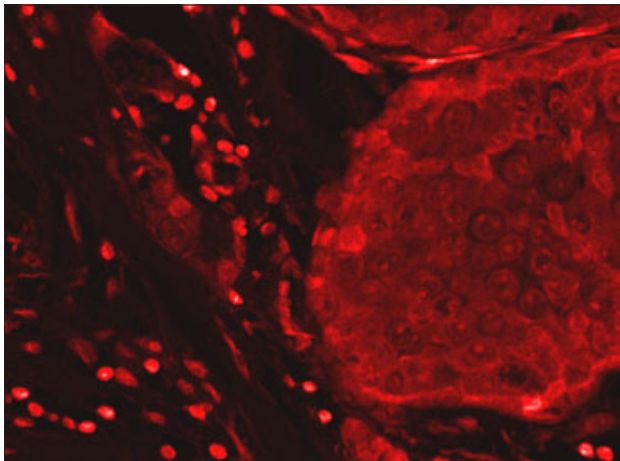


Fig. 2. Formalin fixed, paraffin embedded histological section of the same tumour. Left: stroma, right: tumour cells. Stained with propidium iodide (5 µg/ml, 5 min). x200.

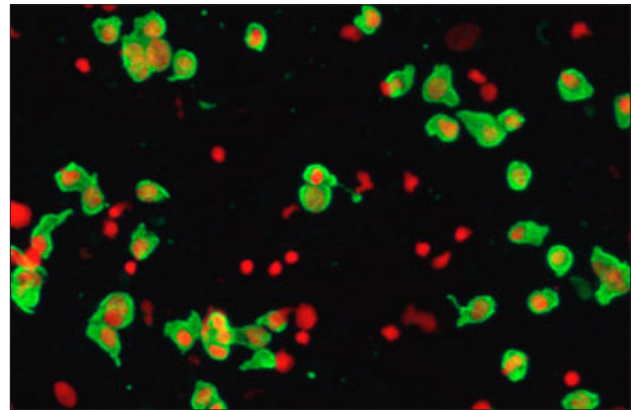


Fig. 3. Cell population prepared from the same tumour. Cells stained with propidium iodide for DNA (red cell nuclei, 5 µg/ml, 5 min) and with FITC-labelled cytokeratin (green cytoplasm, staining 1:10, 10 min). Cytokeratin positive cells are tumour cells, cytokeratin negative cells are stromal cells and lymphocytes.

cytometry seems to be clearly superior to LSC. A large proportion of cell nuclei in the paraffin sections are cross-cut, thus the real DNA content of such cell populations is difficult – if possible whatsoever – to assess objectively. Despite the fact that LSC is currently mostly used for research purposes, it has already found some applications also in oncological diagnostics (Figs 1–3).

On the other hand, LSC might be rather successfully employed in clinical cytology due to its relocalization feature as well as to the fact that the laser scanning cytometer enables to measure and evaluate also limited clinical materials (of the order of tens or hundreds of cells) e.g. from smears, fine needle aspiration biopsies, etc.

Last but not least, a certain disadvantage of laser scanning cytometry, as compared to flow cytometry, is the relative expensiveness of the LSC instrumentation, while the use of digital or confocal microscopy may yield equivalent information with a considerable lower input of investment.

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