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Dissecting the pathways of doom – application of spectroscopy in the study of cell death and survival

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Biological studies rely on measurements performed on large populations, which allow the statistic evaluation of gathered data. On the other hand, the need to precisely localize observed processes and the facts occurring in the microscale results in the need of high resolution qualitative imaging. These two approaches are complementary and without anyone of them no valuable experimental data can be gathered from studies of fluorescent dyes. Both approaches are based on common origin of laser-induced fluorescence, but due to the method of analysis they split into the quantitative (laser scanning cytometry) and qualitative (confocal microscopy) fields of research.

Keywords: spectroscopy, laser scanning cytometry, confocal microscopy, programmed cell death.

1. Introduction: mitochondrial pathway of cell death

Cell death is induced by many different factors such as gamma and UV irradiation [1], hyperthermia and hydrostatic pressure, cytotoxic drugs and compounds [2, 3], lack or deficiency of trophic and growth factors [4]. The term apoptosis is used to describe a pattern of biochemical and morphological changes occurring in a dieing cell. One of the first features is destabilization of a cell membrane asymmetry and loss of mitochondrial membrane potential. In the next step, the cell shrinks and characteristic changes in the nucleus occur. Chromatin condenses and marginates near the nuclear envelope. This is followed by fragmentation of the nucleus and formation of apoptotic bodies. A variety of factors inducing apoptosis suggest the existence of many different induction paths merging into one common pathway of doom, finalized in the death and degradation of the cell.

Two distinct pathways of apoptosis induction are known: receptor and mitochondrial. The mitochondrial pathway of cell death originates in activation and hetero-

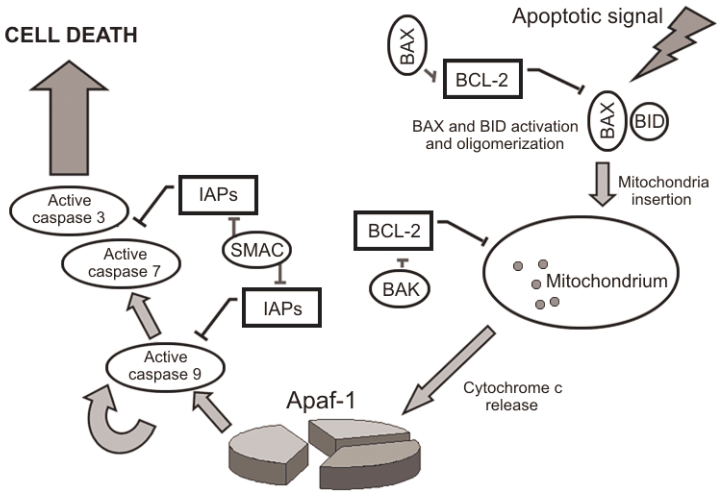


Fig. 1. Mitochondrial pathway of apoptosis.

oligomerization of proapoptotic proteins belonging to Bcl-2 family, such as BAX and BID. BAX-BID complexes translocate to mitochondria where they induce the release of cytochrome *c*, either directly or through complexes with membrane proteins [5–7]. Cytochrome *c* together with Apaf-1 and procaspase 9 forms a complex called apoptosome where activation of caspase 9 takes place [8–11]. Together with cytochrome *c* the secondary activators of apoptosis, like Smac/DIABLO proteins, and procaspases are released from mitochondria. Caspase 9 participates in activation of other caspases: 3 and 7, which are responsible for degradation of cellular proteins [12]. Caspase 9 is thus called a regulator caspase whereas caspases 3 and 7 – executor caspases. Interactions between different proteins involved in the process of apoptosis are presented in Fig. 1. There are two major pathways of apoptosis inhibition. The first one is mediated by anti-apoptotic members of Bcl-2 family, the second by inhibitors of apoptotic proteases (IAP's). IAP's mechanism of action consists of direct inhibition of the regulatory and executory caspases [11, 13]. In turn their action can be diminished by Smac/DIABLO. The Smac/DIABLO's N-terminal domain interacts with sequences characteristic of all members of IAP family and thus neutralizes their inhibitory potential [14]. The Bcl-2-mediated pathway of apoptosis inhibition is based on anti-apoptotic proteins that belong to Bcl-2 family with Bcl-2 and Bcl-xl considered to be the most potent. Their potential lies in heterodimerization with pro-apoptotic Bcl-2 family members (mainly BAX) [15]. It was also suggested that Bcl-2 can saturate membranes of the organelle leaving no space for BAX oligomers to insert [16]. Bcl-2 action can be diminished by other members of Bcl-2 family, *i.e.*, BAK or BID. They substitute BAX in BAX-Bcl-2 heterodimers and inflict structural changes in Bcl-2 molecule that prevents membrane anchoring [11] (see Fig. 1).

2. Quantitative approach: laser scanning cytometry

2.1. Basics

Biological studies apart from finding and localizing the process in single cell require the quantitative analysis carried out on large-size cell populations in cell cultures and tissues. The problem was to create the automatic means of approach that could either eliminate or significantly reduce the human factor from acquired results and simultaneously concern the biological diversity of cell phenotypes. The laser scanning cytometer (LSC) (CompuCyte Corp., Boston, MA, USA) is an instrument designed specially for analysis of cells growing attached to the surface. The unique element of this system is motorized stage which moves specimen under the laser light, thus enabling automatic analysis of large areas. The excitation provided by laser beam is

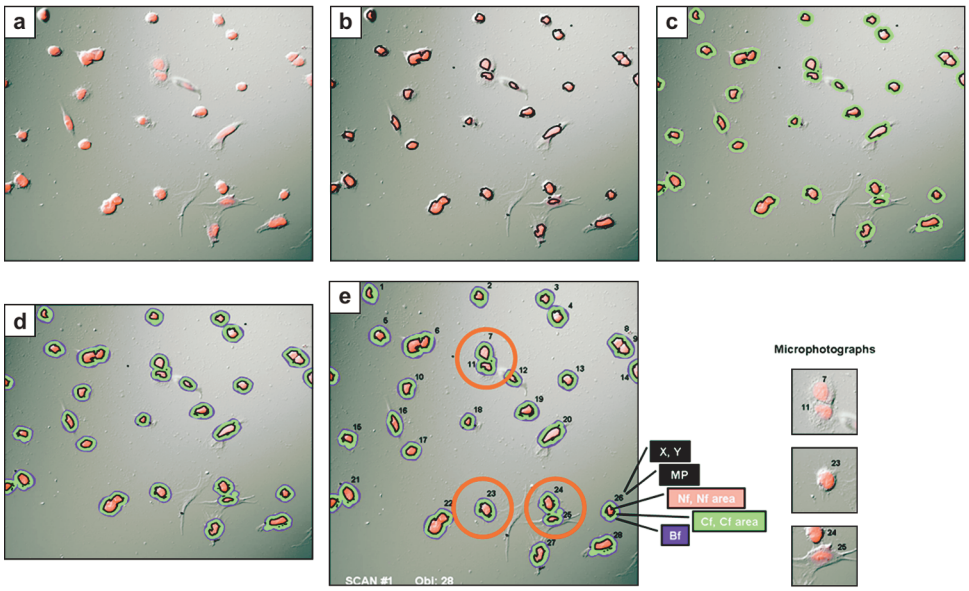


Fig. 2. LSC measurement: **a** – typical image of human glioma Ln229 cells with DNA visualized with the use of 7-aminoactinomycin D (7-AAD); **b** – nuclear contouring (black) created on the basis of 7-AAD threshold fluorescence; **c** – the cytoplasmic contour (green) around the nuclear area; **d** – background contour (blue) outside the cytoplasmic area; **e** – final elements of LSC protocol: time point of analysis, number of cells counted, *X* and *Y* coordinates, maximal pixel, nuclear fluorescence (Nf) value and area, cytoplasmic fluorescence (Cf) and area and background fluorescence value for each and every cell. Fluorescent data are acquired separately for each fluorescence channel. Cells with interesting features have been relocated and their microphotographs taken (red circles). Unfortunately, cells tend to grow in groups, and the situations in which clusters of cells with overlapping nuclei (like No 6 and 22) are recognized as one object with increased DNA content are common. Thus, it is crucial to preset the area of interest based on DNA content to eliminate these clusters from experimental data (see Fig. 4**a**).

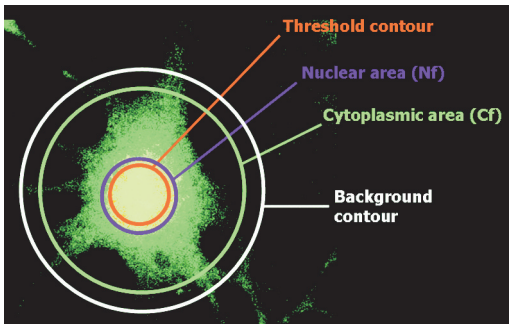


Fig. 3. Schematic contouring of the cell during LSC analysis. Precision of analysis depends on the closest possible fitting of nuclear and cytoplasmic contours. Fluorescence gathered from nuclear area is slightly overestimated by thin layers of cytoplasm under and over the nuclei and the need to confirm nuclear localization of fluorescence emerges (see Figs. 6**b** and 6**c**). Cytoplasmic fluorescence, on the other hand, is slightly underestimated, especially in elongated or astral-shaped cells, because of the inability to fit all of the cell cytoplasm under the cytoplasmic area. This setbacks are minimal compared to other LSC properties and advantages and the error is constant throughout the experiments carried out on a single experimental preset. Furthermore each cell type has its unique properties and requires different data gathering protocol, especially in the case of fitting nuclear and cytoplasmic contours.

focused on the specimen and triggered fluorescence was measured using a combination of dichroic mirrors and filters transmitting at desired wavelengths. Automatically generated experimental report consists of:

– number of objects scanned and analyzed;
– *X* and *Y* coordinates for every object;
– value of fluorescence intensity and maximal pixel in every area of the object for all fluorescence channels;
– fluorescent area for all channels;
– time point of analysis;
– mean, minimum, maximum, percentage and basic statistics for all objects analyzed.

Measured cells can then be relocated based on their stored *X* and *Y* coordinates and their microphotographs included in the report (Fig. 2). Fluorescent intensity and the area of fluorescence are gathered separately over the nuclear and cytoplasmic areas of the cell (Fig. 3). They provide information on the content of proteins or DNA within the cell. The maximal pixel demonstrates the highest value of fluorescence. It is measured over the whole cell regardless of the nuclear or cytoplasmic compartments and provides information about the aggregation of measured molecules. The background fluorescence value is automatically omitted from the acquired data.

2.2. Cell death and cell cycle analysis

Analysis of cell death and cell cycle based on DNA content is relatively simple. Acquired data is presented in the form of DNA content versus DNA aggregation

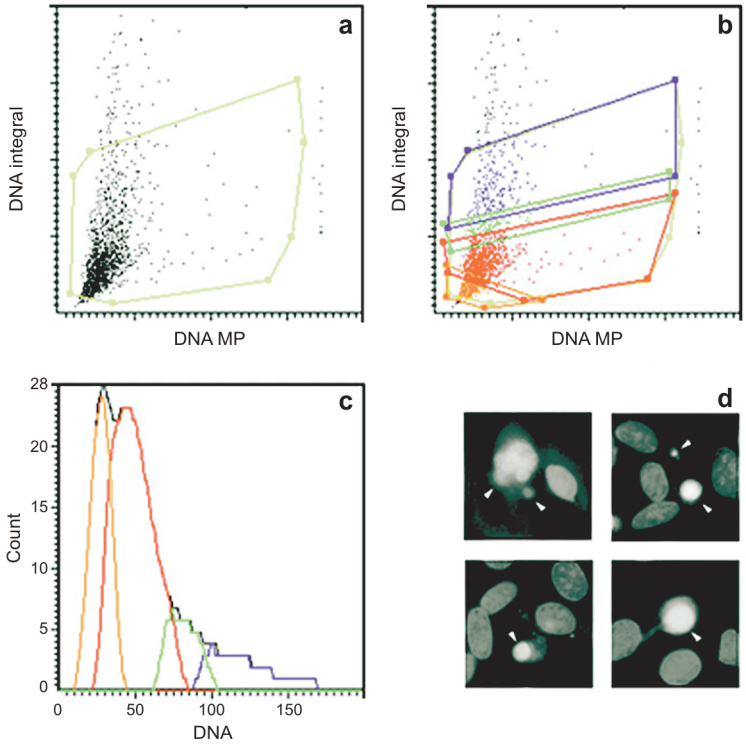


Fig. 4. Cell cycle analysis of human adenocarcinoma COLO 205 cells conducted with the use of LSC: **a** – typical cytogram of DNA content (DNA integral) versus aggregation (DNA MP). Yellow area designates the whole cell cycle drawn based on DNA content within the cells. Objects above the yellow area have been identified as clusters of cells with overlapping nuclei and their data was omitted from the experiment. **b** – Different phases of cell cycle designated on the basis of DNA content (G1 phase – red, S phase – green, G2M phase – blue). Cells with less than 2n DNA are localized in the subG1 area (orange). **c** – DNA-spread histogram with quantitative analysis of cell cycle. **d** – Microphotographs of cell relocated from subG1 area, showing typical features of apoptosis fragmentation of the nucleus, chromatin condensation and formation of apoptotic bodies.

cytogram (Fig. 4**a**). The mean content of DNA in the cells is then used to divide the whole population analyzed to the different phases of the cell cycle: postmitotic G1 phase (2n DNA) – red; DNA synthesis S phase (between 2n and 4n DNA) – green; and G2M phase of premitotic rest and mitosis (4n DNA) – blue. The cells with less than 2n DNA content are considered apoptotic and are localized in subG1 phase – orange (Fig. 4**b**). The data from the cytogram can then be presented as DNA spread histogram (Fig. 4**c**), the percentage and total number of the cells in different phases can be counted, and cells from different phases can be relocated for microphotography which is included into the report (Fig. 4**d**). Some problems occur in analyses of different forms of cell death, like autophagy [3, 17–19]. In this case, the nuclei do not shrink and, instead of fragmentation, the increased aggregation of the chromatin is

observed in the nuclei. This results in the increased DNA maximal pixel value with no changes in the subG1 area (Fig. 5).

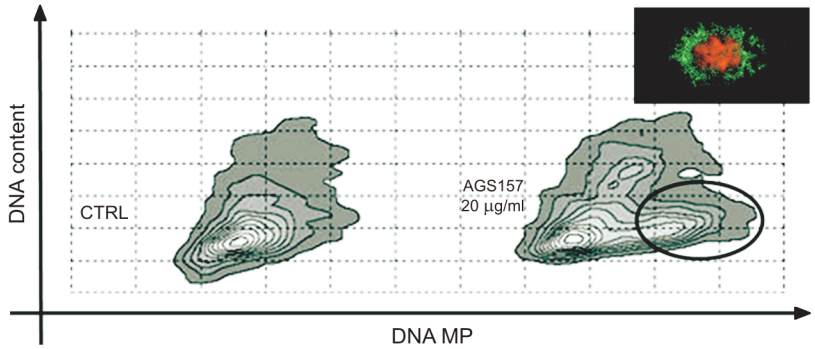


Fig. 5. DNA content versus aggregation map of human breast cancer MCF 7 cells showing changes in DNA aggregation (circle) induced during atypical form of cell death – autophagy. This form of cell death presents difficulties in automatic analyses, especially based on changes regarding DNA. Up to date the only reliable marker of autophagy remains the expression of MAP I LC3 protein – molecule involved in formation of autophagosome vesicles where cellular content and organelles are digested (insert).

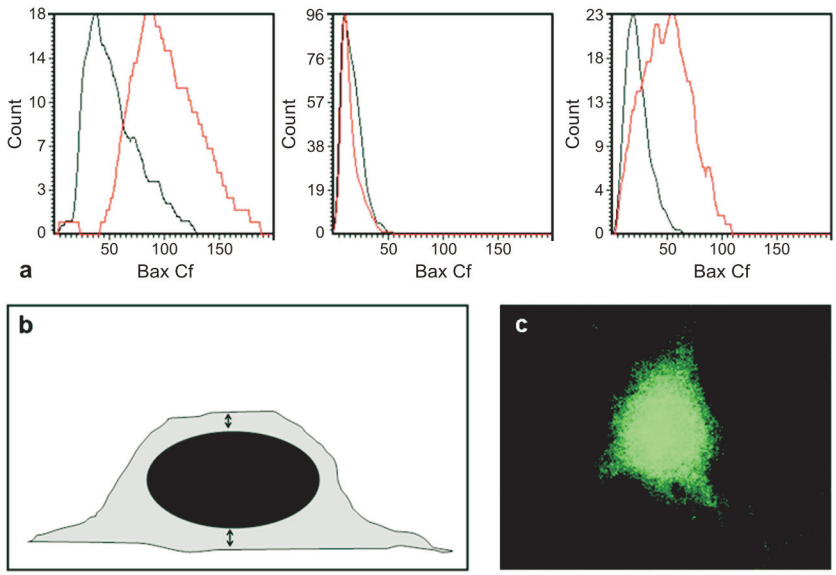


Fig. 6. **a** – BAX nuclear (Nf) and cytoplasmic (Cf) content in mouse mammary epithelial HC11 cells prior to (black line) and after 1 hour treatment with proapoptotic cytokine TGF-β1. An increase in BAX Nf/Cf ratio indicates the translocation of BAX into the nucleus in the course of apoptosis. **b** – Schematic cell cross-section showing the layers of cytoplasm (arrows) that could overlap the fluorescence from the nucleus (dark grey) during the LSC analysis. **c** – Cross-section of the HC11 cell acquired with the use of confocal microscopy confirming BAX localization (green fluorescence) within the nucleus.

2.3. Protein translocation and aggregation

Apart from cell cycle studies the LSC allows measurement of protein localization and content within the cells. During the apoptosis process various proteins tend to translocate through various cellular compartments, to the nucleus [2], or organellar membranes (mainly mitochondria) [20]. LSC analysis enables gathering of quantitative data not only from the cell as a whole, but separately from nuclear (Nf) and cytoplasmic (Cf) areas (Fig. 6**a**). An increase in Nf to Cf ratio tells about the translocations of proteins to the nucleus occurring in the course of apotosis. The importance of qualitative confirmation of the translocation with other analytical methods comes from LSC inability to view the 3-dimensional image of the cell. The proteins present within the thin layers of cytoplasm over and under the nucleus (Fig. 6**b** – arrows), may overlap the fluorescence of nuclear area. Thus the cross-section of the cell needs to be evaluated and the presence of proteins within the nucleus confirmed (Fig. 6**c**). The aggregation of proteins is the second major area of interest for the biologists. Changes in the aggregation result from protein binding on organellar membranes, formation of multimeric complexes or convergence around

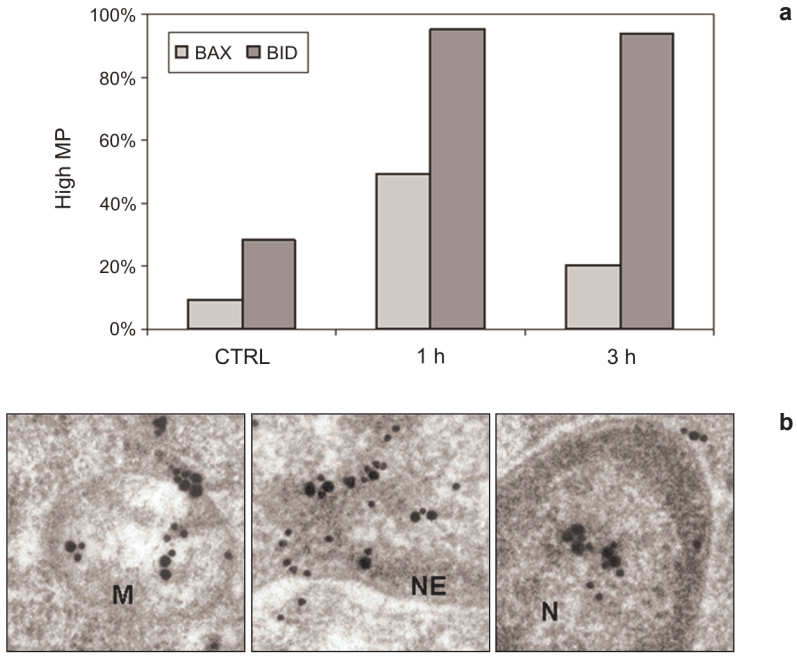


Fig. 7. Changes in BAX and BID aggregation (MP) in human adenocarcinoma COLO 205 cells after incubation with nimesulide (nonsteroic antiinflamatory drug that proved to be potent inducer of apoptosis in several colorectal cancers) – **a**. The similarities in time pattern of BAX and BID aggregation suggested interactions between these proteins in the course of apoptosis. This was confirmed with the use of immunoelectron microscopy, where BAX was labeled with 30 nm gold particles and BID with 20 nm gold – **b**. BAX-BID heterooligomers are visible on mitochondria (M), nuclear envelope (NE) and within the nucleus (N).

activation centers. The first quantitative observations of BAX and BID aggregation in the course of apoptosis have been conducted with the use of LSC [2, 21]. The similarity in the time pattern of BAX and BID aggregation suggested some sort of interactions between these proteins (Fig. 7**a**). Immunoelectron microscopy observations confirmed the creation of multimeric complexes on various cell ultrastructures including mitochondria, nuclear envelope. Their presence was also shown within the nucleus (Fig. 7**b**) [21]. Furthermore, tumor cells that survived initial apoptogenic treatment were observed to increase both in the level and aggregation of antiapoptotic Bcl-2 protein, as a means of resistance to the drug-induced cell death [21].

2.4. Enzyme kinetics

The ability of LSC to relocate cells, to return and rescan the analyzed area in preset time-intervals is taken advantge of for measurement of enzyme kinetics. One of

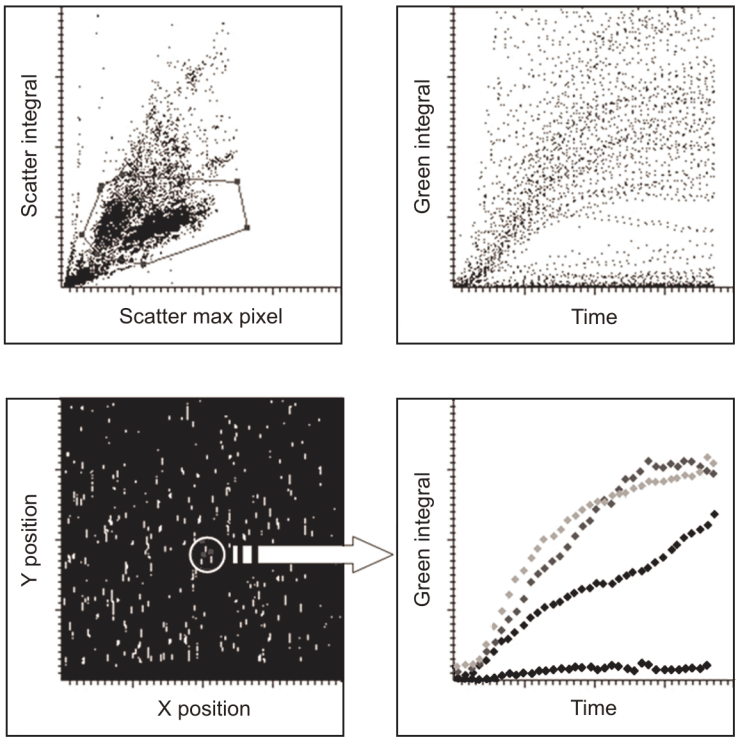


Fig. 8. Dynamics of free radicals (ROS) creation in living mouse L1210 leucemia cells. Kinetics of ROS generation are presented for whole population (top right) and for 4 cells that represent subpopulations with very different reactivity (bottom right). The unique ability of LSC allows relocation of the cells with different reactivity and correlation between biochemistry and cell morphology. Furthermore, after gathering the kinetic data the cells can be fixed and different molecules or processes visualized. Due to *X* and *Y* positioning new parameters can be correlated and compared to stored enzyme kinetics of each and every cell. Typical cytogram and map of measured area are presented in left panels.

the most common inducers of apoptosis is oxidative stress induced in the cells. Generation of free radicals (ROS) is a result of many intercellular processes involving oxygen. Free radicals induce a cascade of reactions in which single ROS can generate a multiple of oxidations. Cells protect themselves from oxidative stress by employing the ROS-silencing mechanisms, thus developing the resistance to apoptogenic stimuli. The LSC enables not only the analysis of mean oxidative status of the whole culture of tumor cells, but also differentiation between cell populations with different drug response and, what is very important, the comparison of cell biochemistry with its morphology (Fig. 8).

2.5. Tissue mapping

As a tool that analyzes the cells attached to the microscopic slide, LSC offers great opportunity for measuring tissue sections. The ability to store the *X* and *Y* coordinates of the cell enriches the gathered data with the tissue map. Correlation of the map to the biochemical status, fluorescence-related protein analysis and cell cycle allows differentiation between cell subpopulations (Fig. 9). These parameters are crucial in analysis of solid tumors and may influence the choice of adequate therapy. Nowadays the search focuses on correlating the expression of pro- and anti-apoptotic proteins with the survival prognosis and the type of therapy proposed for patients. Unlike other techniques of molecular biology used in biological analysis, the LSC enables automatic identification of molecules (mainly the proteins) in complexed and unequal cell populations which form tissues. This allows identification and analysis of bioactive molecules in certain subpopulations of cells, even if their content in the tissue is very low. Furthermore stored experimental presets can be transferred to other laboratories enabling high repeatability of results.

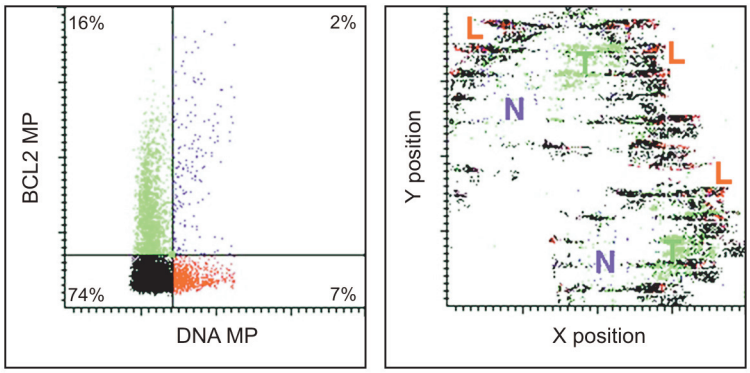


Fig. 9. Tissue map of human head-and-neck carcinoma showing cell subpopulations differentiated on the basis of Bcl-2 and DNA aggregation. Carcinoma cells with high aggregation of Bcl-2 are localized in solid tumor (T – green) and necrotic area (N – blue), while leukocytes with high DNA but low Bcl-2 aggregation are present on the rim of the tumor as they try to defend surrounding tissue from the tumor (L – red).

3. Qualitative approach: confocal microscopy

3.1. Basics

The idea behind the confocal microscope is the use of confocal pinhole, which blocks the fluorescence triggered from below and over the focal plane (Fig. 10). By moving the specimen along the *Z* axis under the microscope, series of cross-sections can be gathered, enabling the 3D reconstruction of the cell. Although the measurement of

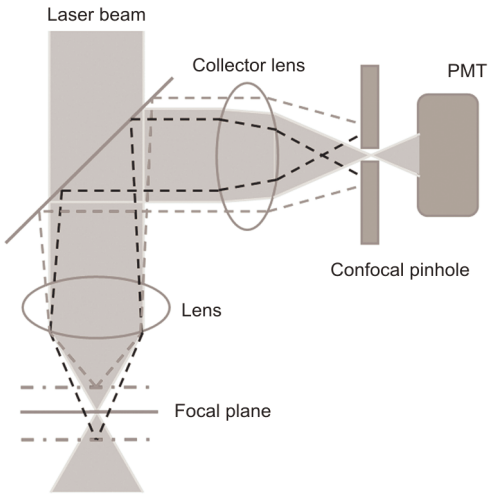
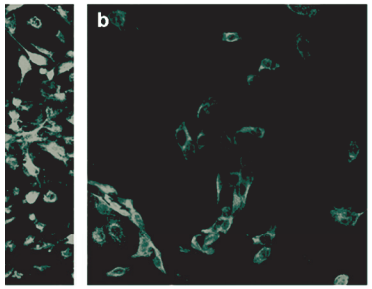


Fig. 10. Schematic optical pathway of confocal microscope showing the idea behind the use of confocal pinhole.

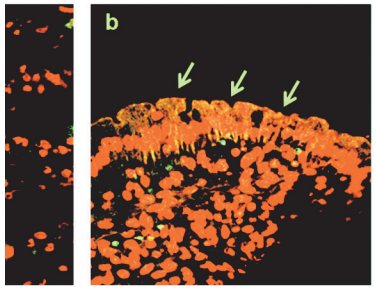
large populations is possible, it is a time-consuming and difficult process. Thus, the confocal microscopy is commonly used for precise dissection of a small number of cells and localization of processes within their ultrastructure.

3.2. Imaging and 3-dimensional reconstruction

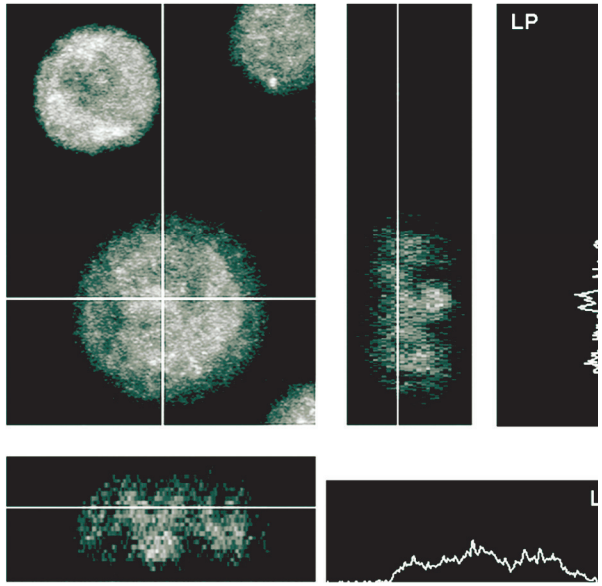
The confocal microscopy is mostly considered as a means to acquire the microphotographs of superb quality. A single cross-section of the cell remains the best way of visualizing various processes, it is also required for confirmation of results acquired by different experimental methods (Fig. 8**c**). Confocal imaging allows correlation between changes occurring in the cell morphology and localization of the proteins or the processes within it (Fig. 11). During tissue section analysis confocal imaging can be used to confirm the presence of apoptotic cells and to evaluate the extent of the process (Fig. 12). The 3-dimensional reconstruction, on the other hand, allows insight into processes occurring within the cell from different angles, with simultaneous analysis of the cell composition (Fig. 13). This is especially important for colocalization studies, as the stack of thin cross-sections tells about the difference between overlapping of fluorescence and colocalization in the final image. Acquired



◀ Fig. 11.



◀ Fig. 12.



▲ Fig. 13.

Fig. 11. Actin filaments in a single cross-section of the H9C2 heart myoblasts in normal conditions (**a**) and after hypoxia (**b**) visualized with the use of confocal microscopy. Studies of cell ultrastructure and cytoskeleton are extremely difficult with other experimental techniques, as no other method allows dissecting the cell into series of thin cross-sections and omits the problems with overlapping of fluorescence triggered on different layers of the cell.

Fig. 12. Confocal image of intestinal villus of 7-day old piglet. Apoptotic cells with high expression of caspase 3 were localized in groups in the middle section of the villus (**a**) and as a whole shedding top of the villi (**b**) – arrows. Analysis done with confocal microscopy allowed the identification of packet pattern of apoptosis occurring in the intestinal epithelium, where groups of neighboring cells die simultaneously. This sugests that auto- or paracrine-factors are involved in induction of cell death.

Fig. 13. Typical confocal analysis of BAX-related fluorescence distribution and intensity in series of cross-sections of human adenocarcinoma COLO 205 cell stimulated to apoptosis. Line profiles of fluorescence (LP) can be acquired along the lines dividing the cell from every single cross-section. This form of analysis informs us about distribution of molecules within the cell.

data can be semiquantitatively analyzed with the use of image-analysis systems allowing IOD values, or fluorescence profiles to be gathered (Fig. 13, LP).

3.3. Colocalization of the proteins

Colocalization studies in confocal microscopy are based on the shift of fluorescence spectrum of the dyes excited in close vicinity. It was proven that proteins that localize separately within the cell in normal conditions (like BAX and BID), tend to form

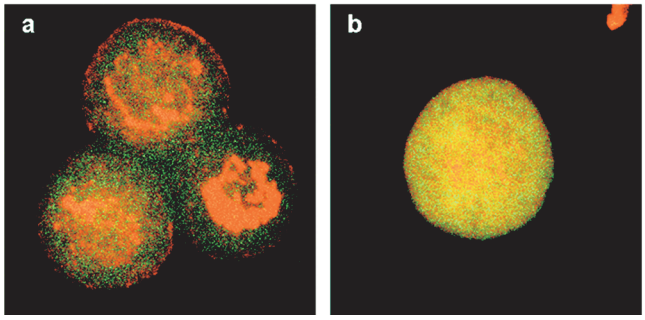


Fig. 14. BAX- and BID-related (green and red, respectively) fluorescence in the 3-dimensional reconstruction of human adenocarcinoma COLO 205 control cells (**a**) and cell stimulated to apoptosis with nimesulide (**b**). Shift of fluorescent spectrum from separate red and green towards yellow indicates colocalization of proteins examined in the course of cell death. Formation of BAX-BID heterooligomers was later confirmed with the use of immunoelectron microscopy (see Fig. 7**b**).

the heteromultimeric complexes after apoptogenic stimuli [21]. Their fluorescence spectrum shifts from "separate" red and green to "colocalized" yellow (Fig. 14).

3.4. Homeostatic confocal microscopy

The shift of fluorescent spectrum forms the basis of homeostatic confocal microscopy (HCM). This method was developed especially for analysis of protein translocation to, or from, the organelles in the living cells. To visualize the proteins and organelles the cells are transfected with fluorescent proteins. The shift of fluorescence spectrum occurring in the course of apoptosis can then be analyzed and visualized in the real time. This gives a precise time-analysis of events, correlated with changes in the cell morphology [20]. A combination of the HCM and image-analysis system enables semiquantitative analysis of observed translocations, making statistical evaluation of the experiments possible. It also provides the time-bases for experiments conducted with the use of different analytical methods [20, 22].

3.5. From colocalization to interactions

Verification of the colocalization data acquired by means of confocal microscopy is very important. The most popular way of evaluation is the immunogold electron microscopy, where different proteins are visualized by antibodies conjugated to

the gold particles (Fig. 7**b**). Clusters of gold particles of different size confirm that the creation of BAX-BID heteromers occurs in the course of apoptosis and localizes the process on several organelles and intracellular membranes [21].

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