Quantitative analysis of fluorescent image – from descriptive to computational microscopy

Michal Marek Godlewski[1,2], Agnieszka Turowska[3], Paulina Slazak[2], Daniel Martinez Puig[4] and Helena Nevalainen[1]

1. Chemistry and Biomolecular Sciences, Division of Environmental and Life Sciences, Macquarie University, Sydney, Australia
2. Department of Physiological Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences-SGGW, Poland
3. Department of Preclinical Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences-SGGW, Poland
4. Bioiberica S.A., Pol. Ind. "Mas Puigvert" Ctra., Palafolls, Barcelona, Spain

Application of fluorescence in microscopy led to a considerable breakthrough in biochemical sciences by facilitating a new way of observing complex biological processes, structures and interactions and locating them in particular parts of the cell. Today, fluorescent imaging is widely applied in published literature to confirm research hypotheses. However, about only one of ten publications provides supporting information for the observations based on fluorescence through a computational analysis of the acquired images.

"One picture is worth ten thousand words" was once stated by Frederic R. Bernard, however, images alone may not be enough to provide reliable information. For example, given population diversity, it is very likely that a selected cell ('the cell') will be found that confirms a particular theory, while most others will refute it. In addition the validity of observations conducted on cell cultures may be disputable, as these are usually cancer or immortalized cells and therefore differ significantly from the original tissue. It is known, for example that cultured cancer cells easily evolve into a variety of clones that may not differ morphologically, but when stimulated to undergo differentiation or cell death they tend to react differently to normal cells at the biomolecular level. While cellular analysis requires a great deal of caution, descriptive tissue analysis is even more subjective and interpretations are highly dependent on personal experience of the observer. Baak, in his article [Baak 2002] addressed difficulties in qualitative assessment of microscopic samples in pathology. The reliability was acceptable when an observation was assessed by at least three specialists, but consistency was low especially in more complex analysis. Comparison of the data between

different laboratories is also difficult considering the simplicity of observations made and the complexity of the pathological processes.

Modern imaging methods which use charged-coupled devices (CCDs) or photomultipliers (PMTs) provide a possibility to capture the visual observations in a numerical, digitalized form. In this format reliable quantitative data can be extracted which open new ways of understanding and supporting an observation process. In this chapter, various methods of quantitative analysis will be presented and discussed, applicable to studies of a single cell as well as whole populations and tissues.

Advantages of quantitative analysis

Descriptive microscopy is able to provide limited information only, such as for example on the presence or absence and localization of a particular process or phenomenon. This relative quantification, often expressed as –/+/++ (as accepted in pathology) can be easily improved through quantitative analysis. The diagnostic possibilities that lie in the combination of descriptive and quantitative assessment of biological samples are best seen in cancer medicine. Identification of cancer is mostly carried out by the means of descriptive analysis, however, in the case of leukemia, scanning cytometry provide an alternative with its ability to rapidly differentiate the cancer type based on 16 tumor-specific antigens [Clatch et al. 1996 & 1998]. The amount of biological material needed for quantitative assessment by this method is minimal when compared to other techniques, including flow cytometry, thus allowing the whole analysis to be carried out on a single thin-needle biopsy. Finally, as the sample is analyzed on a microscope slide, the same material can be later used for traditional staining procedures.

Cancerogenesis is a very complicated process, where multiple mutations are generated in various cells. During growth and spread of the disease, the cells differentiate into a variety of phenotypes that form the tumor. These clones vary, often significantly, in the expression level of various proteins and therefore their response to an antitumor agent may be different. To prevent selection of the resistant and thereby more dangerous cancer clones in the course of therapy, simple detection of the presence of antigens is insufficient. As indicated by Preston [Preston 2002], traditional approaches to quantify the level of protein expression, such as real-time PCR or Western and Northern blotting, give only the mean value of expression for the whole sample. The variability in the phenotypes of cells forming the tumor may introduce a serious possibility of misinterpretation when using these methods, as a small amount of highly-expressing cells may (sometimes significantly) increase the mean. In the

worst case scenario the majority of cells with low or no expression decrease the overall expression mean to an insignificant level, hiding the small, highly aggressive population of cancer cells. This is dangerous especially in the course of assessment of cell susceptibility to therapeutic agents and monitoring during the course of therapy.

Quantitative analysis based on a selection of markers answers several fundamental questions: What percent of the tumor cells do express these markers? What is the level of expression? Is there an association between the expression of different proteins in various cell phenotypes? In physiological studies, the aim is to observe and relate a process/pattern representative of the whole population or compare acquired results with the population average established during different experiments. Automated quantitative analysis of the data allows rapid and precise assessment of the development process (understanding growth, differentiation and maturation of complex tissues), also providing the possibility to easily compare data between experiments and laboratories. Figure 1 shows examples of quantitative evaluation of fluorescent data in a cell culture and in tissue analysis. Quantification based on parameters of a large number of individual cells (by scanning cytometry, discussed below) showed significant differences ($p<0.01$) between proapoptotic protein (Bax) expression levels indicating that there were two phenotypes present in otherwise uniform COLO 205 cells (Fig. 1 a). On the other hand, there were no differences in Bax aggregation, the parameter describing protein oligomerization and aggregation on cell organelles. A further experiment with an anticancer agent showed that response of COLO 205 cells to proapoptotic treatment was bimodal, with more sensitive cells dying earlier. Interesting results were obtained when the extent of apoptotic process in the duodenum of healthy young wistar rats was analyzed (Fig. 1 b). Variations between groups with high and low apoptosis were significant ($p<0.05$, asterisks). These variations were related to changes in the digestive juice flow and acidity (pancreatic juice, bile and hydrochloric acid) as they disappeared further along the gut [Godlewski et al. 2006a].

Methods of quantitative analysis

Single cell analysis usually carried out by the means of confocal microscopy provides deep insights into various processes occurring in the cell. However, observations performed on a single object cannot be assumed to be representative for whole population. Thus repetitive experiments are required, when a number of (randomly selected) cells are observed and the acquired data compared. Quantification of the data facilitates the comparison process,

as sets of numerical data is easier to manipulate than a collection of images. Furthermore, statistical data can be easily acquired from such quantitative measurements.

Today, an increasing number of researchers create and validate methods for data processing and use them in their experiments.. Our analysis of protein translocations within the cell provides a good example. We analyzed interactions of the proapototic protein (Bax and Smac/DIABLO) with mitochondria [Gorka et al. 2004; Godlewski and Gorka 2006] and their translocation between cytoplasmic and nuclear compartments of the living cell (Fig. 2). Among different approaches we found out that only the whole volume cell analysis (3-dimensional in time) gives accurate description of the pattern of changes. The analysis of a single cell cross-section, although quick and relatively easy, was not very accurate, as the expression varied in different layers of the cell and living cells were never entirely stationary throughout the entire experiment. Quantification of the data on the basis of fluorescence integrated optical density (IOD: a parameter describing mean fluorescence intensity multiplied by fluorescence area) in the cell volume made it possible to compare the results between a large number of experiments and present them graphically [Godlewski and Gorka 2006]. By comparing the temporal evolution of changes, we postulated the coincidence between Bax translocation and Smac/DIABLO efflux to and from the mitochondria, which was later confirmed by immunogold electron microscopy. Digitalization of the data also made it possible to compare experiments performed in living cells with those carried out on fixed cell populations which can be seen by comparing Fig. 2 with Fig. 3.

Another challenge in the analysis is to relate fluorescence intensity to protein concentrations in the cell. A recent technique proposed for data interpretation uses an external calibration and is based on the intensity curves for a series of dilutions of recombinant proteins, which is then compared to the fluorescence intensity observed in the cell [Fernandez-Gonzalez et al. 2006; Schwartz et al. 2006]. This approach has given satisfactory results in studies with YFP-tagged fusion proteins and transporter molecules, however, the data needed to be averaged over a relatively large population of examined cells.

Light intensity and the speed of the observation process are important parameters for a biologist as they can provide information of the intracellular transport patterns and the *de novo* generation of proteins in the cell. Kenworthy [2006] proposed the application of quantitative FRAP (Fluorescence Recovery After Photobleaching, described elsewhere in this volume) for a study of the role of palmitoylation in protein trafficking and recycling within the cell. Her results showed that protein recirculation was a dynamic process, as it took less than a minute for a full recovery of the fluorescence in the photobleached area. Furthermore,

experiments conducted with cycloheximide (a nonspecific inhibitor of protein synthesis) showed that proteins were recirculated to the Golgi complex from the cell membrane. A different approach to the quantification of protein dynamics was proposed for the study of association and disassembly of actin filaments within the cell. For this purpose, Fluorescence Speckle Microscopy (FSM) was combined with mathematical computational models to recognize different patterns of fluorescence of the actin filaments which were later associated with biological activity [Adams et al. 2003; Danuser and Waterman-Storer 2006]. The movement of the molecules could also be analyzed by tracking the speckle flow through multi-frame correlation [Danuser and Waterman-Storer 2006].

Various computational models based on Monte Carlo simulations have been implemented to calculate protein folding, conformation and interactions with other proteins or intracellular membranes, as in case of Bid (an important proapoptotic factor active at the early stages of programmed cell death) [Veresov and Davidovskii 2007]. These models are usually used to theoretically explain the biochemical and biophysical processes on which the patterns observed in fluorescent microscopy analysis were based.

Automated, fluorescence-based analysis of large populations of cells is the backbone of a computational approach in biology. The most popular and widespread tool in this field of research is a flow cytometer, however, its uses are limited to cells in suspension. The need to prepare cell suspensions is a major disadvantage of the method, as the majority of cells grow attached to a surface, and when removed tend to undergo considerable structural changes; in some cases the detachment alone can trigger apoptosis. Tissue analysis is also very difficult as the tissue needs to be digested before experiments, leaving cells suspended but undamaged. Another problem is that cells in suspension (even dead, fixed ones) tend to aggregate and this may produce a misleading results. Finally, as the cells after experiment can not be retrieved this eliminates the possibility for time-based studies of the dynamic processes in the living cell population since single cells are identified separately. Two methods of analysis are proposed to overcome the problems: one based on digital imaging from a confocal or fluorescent microscope, the other is scanning cytometry. Both are used to analyze cells or tissue cross-sections attached to the surface of a microscope slide or placed in a special chamber, where live-cell analysis can be performed. In scanning cytometry the slide is placed in a fluorescent microscope with a motorized stage which enables the entire slide to be scanned automatically with repeated focusing in each scanning steps. The objects are identified either based on their fluorescence intensity or by their edges. This produces a set of cellular images accompanied by cell coordinates and a number of quantitative parameters

such as cell number and distribution combined with average, maximum, minimum fluorescence intensities in designated observation areas. The fluorescence parameters may be measured separately over the nucleus and cytoplasmic area in each cell. Other analyses such as Fluorescence In-Situ Hybridisation (FISH) is also possible, as well as the colony cell counts. The most popular form of studies by scanning cytometry performed on cell cultures is the cell cycle analysis. The cell position in the proliferation cycle can be established from the three measured parameters: the nucleus size, DNA-related fluorescence and DNA aggregation pattern [for ref see Godlewski et al. 2001 & 2002; Kolek et al. 2003a]. Another possibility is the observation of protein-related fluorescence. Compared to flow cytometry, the scanning cytometry produces much more data concerned with every single cell. Not only the protein content and dispersion can be measured, but we can also distinguish between subcellular compartments. Usual targets for analysis are the nuclear and cytoplasmic compartments of the cell, however, when specific organelle-targeting dyes are applied there is also a possibility to differentiate between them and the rest of the cell [details in Godlewski et al. 2006b]. On this basis, protein expression, localization and translocation between different cellular compartments can be quantitatively analyzed in large populations (Fig. 4) [Godlewski et al. 2001 & 2002]. Based on the fluorescence intensity in a single pixel and differences between neighboring pixels, the pattern and intensity of protein aggregation within the cell can also be studied [Godlewski et al. 2001 & 2002; Kolek et al. 2003b]. Another unique feature of scanning cytometry is the ability to store X and Y position of each cell along with the fluorescence-related data. It allows to localize and identify them on a slide for further comparison between the biochemistry and morphological features of the cell (Fig. 4 – b: X-Y map and c).

In contrast to the “tidy” and “well-organized” cell cultures where all cells and nuclei are localized in the same plane, the analysis of tissues presents a major obstacle. Tissue architecture is much more complex and compact than that of individual cells. The nuclei are usually smaller and arranged randomly with respect to the dissection plane, often overlapping one another. The tissues are also composed of variety of cell types with different characteristics and these assemblages can further be embedded in different types of extracellular matrices. (Fig. 5). For the optimal observation conditions, a tissue sample should be cut in the way that it consists only of a single layer of whole nuclei – a task which is near impossible. Therefore the common practice is to cut the slice as thin as possible (4-5 μm for paraffin-embedded and 10 μm for frozen tissues), to minimize the occurrence of overlapping nuclei (see Fig 6 a and Fig. 9 a). This is crucial for quantitative analysis, where

nuclei-related fluorescence is the only means to distinguish between cells. Unfortunately, thin slices only minimize the problem but. there are two ways in which it can be overcome..The first option is to increase the analyzed area and lens magnification, so that a larger number of cells can be scanned with a greater precision (Fig. 9 b). The second option is to use confocal aperture to dissect the tissue section into even thinner slices (Fig. 6 b and Fig. 8 a). Confocal imaging greatly increases the readout accuracy but at a cost of considerable increase of the time of analysis. In our practice, we carry the analysis of complicated and variable tissues, such as gut cross-sections [Godlewski et al. 2006a; Strzalkowski et al. 2007] and complex tumors (e.g. head and neck carcinomas) by the means of confocal imaging, while the uniform tissues (e.g. pancreas, solid tumors) are being analyzed with great accuracy by scanning cytometry.

Another source for serious problems in tissue analysis originates from the preparation and fixing method. Figure (Fig. 7) shows three intestine cross-sections labeled against MAP I LC3 (programmed cell death II marker) and DNA. In this image the staining and imaging procedures were the same, the only difference was the preparation technique. The high background to signal ratio observed in the case of Bouine solution fixing (Fig. 7 b) obscured the image, rendering the automated analysis extremely difficult if not impossible. In contrast, a perfect background to signal ratio was obtained with the frozen tissues (Fig. 7 c). This kind of preparation requires confocal-based imaging as the frozen specimens cannot be cut as thin as paraffin-embedded ones. For scanning cytometry and fluorescent microscopy however the best method seams to be Para-formaldehyde fixing combined with paraffin embedding (Fig. 7 a). This method allows thin cross-sections to be made from the fixed tissue (4-6 μm) minimizing the possibility of nuclei overlap, while the immuno-specificity of the tissue is just slightly altered.

For confocal-based image analysis of protein expression indices (percentage of cells expressing the studied protein), at least 14 images were randomly acquired from the mucosa of every single intestine cross-section and at least three cross-sections per specimen were quantified. Random image selection of different areas in tissue analysis is crucial since the areas may vary significantly in the protein expression level. This can be seen in Fig 8 a where we can compare active caspase 3 expression on the villi (top) and in the crypts (low).. In this case, object recognition was performed on the basis of fluorescent intensity; objects under 50 pixels in size were automatically eliminated and the remaining cells were counted (Fig. 8). The number of objects, total fluorescence IOD and fluorescence area were acquired for the index, total protein expression level and process intensity, respectively. The staining and

image acquisition parameters were kept uniform in all experiments to achieve reproducibility between different specimens and experiments. The same intestine cross-sections were also analyzed by scanning cytometry. At least three whole cross-sections were analyzed per specimen with nuclei recognition based on the DNA-related fluorescence intensity. The system implemented the watershed method of mathematical separation which was carried out to separate overlapping nuclei. All the remaining overlapping conglomerates of nuclei were automatically eliminated from the readout by gating (see Fig. 9 b – DNA-spread cytogram). From the tissue map reconstructed from the X and Y positions of the nuclei, all areas with connective tissue were manually gated out (see Fig. 9 b – tissue map) and active caspase 3 expression was measured in the remaining cells (Fig. 9 b – active caspase 3 expression). The picture gallery (Fig. 9 c) shows the cells relocated from the slide on the basis of their stored X-Y coordinates from the regions of high and low caspase 3-related fluorescence. The results obtained by the two methods discussed above were comparable, although the actual readout was higher in the case of scanning cytometry. Overall, no significant differences were found between the methods (Tab.1).

Image processing

The digital form of acquired image presents a great temptation for post-capture modification by various software packages. Most commonly, the authors tend to enhance the image intensity and correct the background to signal ratio to make sure that the presented data are clearly visible and optimised for the printing process. While in the case of descriptive microscopy this kind of image processing may be justifiable, there are some issues concerning analyses carried out based on the processed image. The question one must ask is ‘what kind of information is to be gathered and how does image processing affect interpretation of the data?’. Image processing greatly influences the brightness level of each pixel thus altering fluorescent intensity readouts. Furthermore, the ability to vary the change between color ranges and tones to improve the image quality results in an even greater modification of the original data and significant loss in the data fidelity. Thus the images processed in any manner cannot be used for expression level analysis based on optical density (Fig.10 – table). However, in the case of index analysis where simply the number of objects matters, the image processing can be used to some extent to facilitate the recognition based on the difference between background and signal. Post-capture modification of the data in the case of index analysis offers significant advantages over pre-capture offsetting. Pre-capture offsetting removes all weak fluorescence from the image as a background regardless of its localization.

Thus, a low protein expressing profile, manifested by this weak, yet specific fluorescence may be irretrievably lost from the image.

In the case of tissue analysis where uniformity in sample preparation as well as staining and scanning protocols is critically required to allow comparison between specimens, the differences in the expression level are often clearly visible (compare Fig 10 a and 10 c). Both background and intensity correction, as presented in Fig. 10 b and d provide some advantages for the intensity histogram-based automated object recognition (Fig. 10 – white arrows). However, some artifacts are also generated (Fig. 10 – red arrows) and this fact needs to be strongly emphasized.

The volume analysis of the reconstructed confocal image presents different kind of problems. Due to optical limitations, the acquired voxel (three-dimensional pixel) is different from its original form. An image is always distorted along the Z axis and the fluorescence intensity is also slightly altered. Moreover, the smaller is the object being analyzed, the more it is distorted along all three axes (Fig. 11) [Godlewski et al. 2006b]. The interpretation of these images may be misleading, especially when the analysis is carried out on the basis of fluorescent spectra overlap during protein colocalization studies. The imperfections in fluorescent image may sometimes generate the overlap pattern that may sometimes be mistaken with the actual result. This is the reason why the colocalization experiments need to be always validated by another experimental techniques (FRET, co-immunoprecipitation or immunogold electron microscopy). Some authors have suggested the use of mathematical spatial deconvolution models to correct the problem [Roux et al. 2004]. Deconvolution is widely used as a means to correct the image clarity especially in 3D reconstructions. However, along with the increase of the number of deconvolutions there is a danger to alter the image far beyond the original, and obtain the data that are simply not real. We therefore suggest restraint in any image correction before the analysis to minimize the risk of data misinterpretation.

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Table 1

	confocal-based image analysis [%]	scanning cytometry [%]
1	22.48	25.17
2	19.03	20.02
3	7.70	15.3
4	20.83	36.30
5	15.09	15.04
6	23.96	30.47
Geomean	17.08	22.46
SEM	2.68	3.83

t student	0.111586444

Figure captions

Fig.1. Phenotype diversity in the expression of the proapoptotic protein Bax in genetically uniform human adenocarcinoma COLO 205 cell line, measured by scanning cytometry (a). On the cytogram of Bax expression vs. aggregation (top left), the two sub-populations of cells were differentiated on the basis of Bax expression level. Subpopulation with a higher expression has proven more susceptible to apoptosis induction (as confirmed by later experiments). Bax aggregation, a parameter describing protein oligomerization and translocation to cell organelles, however, remained similar. Bax expression histograms (red for low and green for high expression levels) were plotted against the mean (black line) of the population (top right). The table shows numerical data from the analysis. The expression levels in single cells were statistically evaluated by nonparametric Mann-Whitney test and significant difference ($p<0.05$) was found between low and high expression groups.

Differences in the apoptosis index (percent of apoptotic cells) evaluated by confocal-based image analysis of active caspase 3 expression (major executor caspase in late, irreversible phase of apoptosis) in duodenum mucosa of healthy young (8 week old) wistar rats. There were significant differences ($p<0.05$ by nonparametric Mann-Whitney test) between groups with low (light grey) and high (dark grey bars) apoptotic index. Inserted table shows the average results for different rats (± SEM) and the black bar represents the mean from the entire experiment.

Fig. 2. Four-dimensional (3D in time) analysis of Bax-GFP translocation to the nucleus in living human adenocarcinoma COLO 205 cell. Subsequent images show 3D reconstruction of a single COLO 205 cell incubated with the anticancer agent nimesulide (1 μm) progressing by 5 minutes intervals. The graph shows the results of integrated optical density (IOD) volumetric analysis of Bax in the nuclear area, showing the peak of translocation at 30 minutes after proapoptotic stimuli.

Fig. 3. Differences in the distribution of active (left) and inactive (middle) form of Bax within human adenocarcinoma COLO 205 cells stimulated to apoptosis by a 30 minute incubation with anticancer agent camptothecin (0.15 μm), measured by confocal microscopy. Active form of Bax was labeled with antibodies recognizing the #44-63 amino-acid (AA) sequence within the death domain responsible for protein oligomerization and activity. Inactive form of Bax was labeled by antibodies against #11-30 AA sequence of n-terminal part of protein that

is cleaved upon Bax activation. The graph (right) shows differences in the active and nonactive Bax-related IOD in the nuclear and cytoplasic compartments of the cell, designated upon DNA-related fluorescence area (not shown).

Fig. 4. Equine herpesvirus (JanE EHV-1)-induced changes in the actin cytoskeleton of the horse epithelial ED cells, visualized by confocal microscopy (a) and quantitatively analyzed by scanning cytometry (b). F-actin was labeled with Alexa Fluor 633 (red fluorescence), virus antigen by FITC-conjugated gamakon and cell nuclei were counterstained by HOECHST 33342. Confocal images present the pattern of F-actin organization in healthy cells where no virus antigen-relater fluorescence was observed (arrow 1) and in cells infected by the virus with low (arrow 2), normal (arrow3) and increased (arrow 4) actin-related fluorescence intensity (a). Scanning cytometry analysis (b) performed on 195 randomly selected fields of view (b – top left) of the same slides showed that the majority (over 98%) of the cells remained uninfected (b – top right quadrants and the table), and only around 2% of the infected cells remained attached to the slide. Cells with high gamakon and actin-related fluorescence are of the lowest abundance (only 0.01%). Two cells from healthy (green crosshair on the quadrant cytogram) and virus infected (red crosshair) were randomly selected and relocated (b – bottom) showing cell recognition (green outline) based on high intensity DNA-related fluorescence with the related actin pattern (blue outline). Gallery (c) shows randomly selected cells from the cytogram areas, labeled according to the region quadrant.

Fig. 5. Confocal Nomarski contrast and fluorescent microphotographs of a human head and neck carcinoma cross-section labeled against Bcl-2, a major antiapoptotic protein over-expressed in a variety of tumours (green fluorescence); DNA counterstained with 7 aminoactinomycin-D (red fluorescence). Letters indicate the different cell types and cell architecture within a tissue: a) blood vessel with endothelial cells surrounded by fibroblasts and connective tissue; b) necrotic loci of a few cells of high Bcl-2 expression and characteristic distorted arrangement under Nomarski contrast; c) solid tumour area with large number of compacted cells with variable Bcl-2 expression.

Fig. 6. Schematic view showing the advantage of confocal imaging over fluorescence imaging in tissues. Cell nuclei are aligned in a relatively random pattern on different depths in the slice. In the fluorescent microscopy, they often overlap providing misleading information about the DNA content and nucleus area (a). By “dissecting” the tissue into even thinner

slices (focal planes) by the use of confocal aperture, the architecture of the tissue can be resolved in much greater detail (b).

Fig. 7. Cross-sections of the piglet mid jejunum labeled against MAP I LC-3 protein, the marker of programmed cell death II (green fluorescence); nuclei were counterstained by 7 aminoactinomycin-D (red fluorescence). Tissues were prepared by three different techniques. a) Para-formaldehyde fixed, paraffin-embedded tissue shows good nuclei counterstaining, but the antibody-related labeling gives a fine background fluorescence. The advantage of the method is that samples can be cut into very thin (4-6 μm) slices minimizing the risk of nuclei overlap in the acquired fluorescent image. preferred method for scanning cytometry and fluorescent microscopy-based image analysis. b) Bouine-fixed, paraffin embedded tissue (the technique widely used for morphometry studies and classical histochemical staining, because it does not alter the tissue dimensions) gives huge background against both fluorescent channels, simultaneously significantly decreasing specific binding of both antibodies and DNA probes due to the presence of Picric acid, rendering it unsuitable for image analysis. c) Liquid nitrogen-frozen tissue gives the best background to signal ratio, however it requires confocal-based image acquisition because frozen samples cannot be cut into as thin slices as paraffin-embedded ones and the risk of nuclei overlap increases.

Fig. 8. Confocal-based apoptosis index analysis in the mucosa of the piglet mid jejunum labeled against active caspase 3 with Alexa Fluor 488 (green fluorescence); DNA counterstained with 7 aminoactinomycin-D. Photo-mosaic reconstruction showing the whole depth of the mucosal layer from villi on the top to the crypt region on the bottom (a). Automated, fluorescence intensity-based recognition of cell nuclei (yellow outline) and active caspase 3 expressing cells (blue outline)carried out with the image analysis system (b). Masking of cell nuclei (yellow) and caspase-positive cells (blue) as seen by counting software (c).

Fig. 9. Scanning cytometry analysis of the same cross-sections of piglet mid jejunum as presented on Figure 8. Alexa Fluor 488 anti-active caspase 3 labeling (green fluorescence) combined with 7 aminoactinomycin-D DNA counterstaining (red fluorescence). a) Fluorescent intensity-based automated nuclei recognition. Observe the large number of overlapping nuclei counted as single object that had to be later removed by gating of the DNA cytogram. b) Typical scanning cytometry panel for tissue analysis. DNA spread cytogram was

used to eliminate the large objects generated from overlapping nuclei, tissue map of the whole scanned cross-section was gated to avoid connective tissue. Also showing are the active-caspase 3 cytogram gating cells with low (region in bottom right corner) and high caspase-related fluorescence. Red crosshair indicates the cell with high caspase 3 expression relocated in bottom left panel. Gallery of randomly selected cells with high (upper) and low (lower) active caspase 3 expression (bottom right).

Fig. 10. Influence of post-acquisition image processing on the image analysis data. The background correction (a-b) increased the fluorescent intensity-based object recognition due to better resolved differences between objects (white arrows), but it also generated some artifacts (red arrows). The enhancement of very dim positive fluorescence (c-d) reduced the number of unspecific objects found and increased resolution between cells (white arrows) at a cost of some positive objects (red arrows). Both methods altered the integrated optical density of analyzed images significantly making it unsuitable for protein expression analysis (see the table).

Fig. 11. Differences between physical object dimensions (on the left) and its optical representation (right) when scanned in X-Z axes.