3-D analysis of biomarkers and investigation of temporal series by Quant3D, a Linux/UNIX software package

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Sumary

Fluorescent Laser Scanning Confocal Microscopy (LSCM) and image analysis are the tools of choice to record and virtually manipulate the volumetric information collected by optical sectioning of multilabeled crypts or colonocytes primary culture. Rendering of biomarker distribution and relative quantification according to the tissue geometry have been achieved by writing a user-friendly Linux/ UNIX software (Quant3D). Image stacks are registered by confocal microscopy on 3 channels, Red Green Blue coded and indexed. The depth of 1 micron between two consecutive images was set to consider the 730 nm maximal depth resolution described for fluorescent LSCM (Brakenhoff et al, 1985). We used a Zeiss LSM 410 confocal microscope equipped with 2 lasers. Stacks of multiple labeling were assembled with a public available image analysis software (ImageJ : http://rsb.info.nih.gov/ij/) and analyzed with our Linux software, Quant3D, developed by the Biometry Unite of the INRA. We propose a microscopic multidimensional analytic system to provide quantitative recording in primary culture. The system is amenable to semi-automation by allowing a rapid extraction of intensities from image series of crypt cells multilabeled for nuclei, Epidermal Growth Factor-Receptors (EGF-R), gap-junction proteins, mitochondria activities, cells membranes. We have built a new graphical interface of a software developed for visualization and analysis of three dimensional multispectral data. Most of the software relies on two C++ libraries : Qt and Vtk. The tridimensional colorized views are illustrative of the qualitative and quantitative possibilities of this application. Quant3D will be improved to allow graphical and quantitative presentation of data obtained by recording biomarkers useful for chronobiological studies in fluorescent microscopy.

Key words: 3D imaging, biomarkers, confocal microscopy, Epidermal Growth Factor-Receptors, fluorescent laser microscopy, Linux/UNIX; Quant3D, software

Introduction

In Mammals, the intestinal epithelium is organized as independent pouches called crypts which can be studied by fluorescent laser confocal microscopy to record biomarkers with an accuracy sometimes higher than classical methods in histology (Konishi et al, 1996). Volumic rendering of multiple biomarkers along an intestinal crypt is a difficult task which can be simplified by user-friendly software.

We have chosen to develop our procedure under Linux, because this is a free UNIX-like operating system (UNIX is a fully multi-user and multi-tasking operating system), originally created by Linus Torvalds with the assistance of developers around the world. Developed under a GNU General Public License, the source code for Linux is freely available to everyone. Linux may be used for a wide variety of purposes including networking, software development and as end-user platform.

In this paper, we present Quant3D (Hoebeke et Trubuil, 1999): an interactive software tool designed to carry out

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intensity measurements on 3 dimensional multi-channel datasets. The measurements can be made on whole datasets or on subvolumes selected through user interaction. This software has 3D visualization functions, 3D segmentation functions and coalescent 3D object detection capabilities. Quant3D is written in C++ and relies on publicly available libraries, Vtk, Qt and LEDA, in particular.

Material and methods

1. *Biological material and fluorescent labeling:*

Quant3D is used by biologists who study the digestive tract (see this congress Kaeffer et al, 2002). So, to perform 3D analysis of biomarkers, colonic crypts were isolated by chelation (procedure adapted to rat from Whitehead et al, 1987), or after fixation steps in formaldehyde and alcohol by microdissection (Goodlad et al, 1991 ; Konishi et al, 1996), from the distal part of the colon of rat. Crypts obtained by chelation, unlike those microdissected, were free from stromal cell contamination and retained their in vivo organization. Then, the crypts cells were multilabeled for nuclei, Epidermal Growth Factor-Receptors (R-EGF), gap-junction proteins, cells membranes and/or mitochondria activities.

2. *Acquisition of image stacks:*

We acquired image stacks by a Zeiss LSM 410 confocal microscope equipped with 2 lasers (Argon and UV lasers), on 3 channels. Quantification was made at one byte per channel and per pixel, and the images were coded in 256 levels of gray. Conditions of acquisition were standardized (Good et al, 1992) and kept according to Pawley's (1990) recommendation for confocal microscopic analysis. Specimen were mounted in glycerol. The microscope acts as an "optical microtome" allowing the sectioning of fluorescent-labeled cells sequentially and storage of each optical section digitally. Preparations were observed under a X40 water-immersion objective with 1.2 aperture and captured with a 72 dot per inch resolution in frame of 512X512 pixels. Each confocal image was obtained by averaging 4 images (recorded in one second each) to reduce noise. The zoom factor was 1. The depth of 1 micron between two consecutive images was set to consider the 730 nm maximal depth resolution described for fluorescent LSCM (Brakenhoff et al, 1985). The (x,y) and z

resolution were 1.605 pixels/µm and 1 pixel/µm, respectively. The acquisition started at the deepest plane.

3. *Multidimensional image analysis :*

The 3D images were made by piling up all the consecutive confocal images of one sample, with publicly available image analysis software (ImageJ : http://rsb.info.nih.gov/ij/) and analyzed with our Linux written procedure : Quant3D.

The software has an ortho-slice interactive functionality which allows display in 3 dimensions of the image stacks acquired in the different channels. Indeed, it is a volume rendering ortho-slice for the displayed slices are composition of parallel slices in each direction. The composition depends on opacities chosen by the user.

1. Method to evaluate the cell number:

The cell number within a crypt was determined with help of nuclei detection. This procedure to detect the cell nuclei (figure 1d) is performed in three steps (Wang et al, 2000):

-   detection of a standard outline of nuclei by standard filters operators (figure 1a);
-   detection of germs inside nuclei (a germ corresponds to one point we are confident to be inside the nucleus), (figure 1b);
-   aggregation of germs according to nucleus membership. Different criteria are used like size of nuclei, homogeneity of biomarker for germs from the same nucleus, etc. (figure 1c).

2. Method to determine probe fluorescence intensity:

We assumed the data are corrected of all default in connection with depth acquisition of biological sample, and there was a linearity between brightness and local concentration of marked molecules. Biomarker fluorescence intensity was restricted to the crypt area by using masks. Masks are obtained with basic thresholding and filtering operators on the different spectral channels through 2 steps:

-   selection of pixels which intensity is higher than a given threshold, all the channels may be used;
-   filling "gaps" inside the crypt or inside cytoplasm with the help of classical morphological operators.

Figure 1 : Illustration of the procedure to detect cell nuclei. a) Detection of the outline, in one section. b) Detection of germs, in one section. c) Detection of germs neighborhoods in one section. d) Detection of cells nuclei shown by small white dots, in one section.
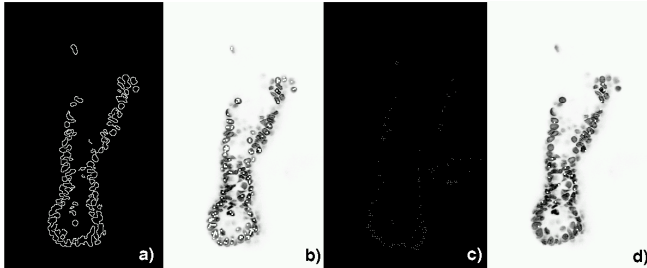
Once the layout of the crypt estimated inside the stack of images, the channel of acquisition to be quantitated is chosen and two possibilities are offered to the user:

- interactive specification of a polyhedral domain and quantification of biomarker fluorescence intensity inside the area defined by this domain and the mask (figure 2);

Figure 2: 3D illustration of quantification with mask (in translucent gray) into a polyhedral domain (yellow outline). The nuclei are shown in red, the membrane in green and the EGF-R in blue . Two orthogonal sections are visible.
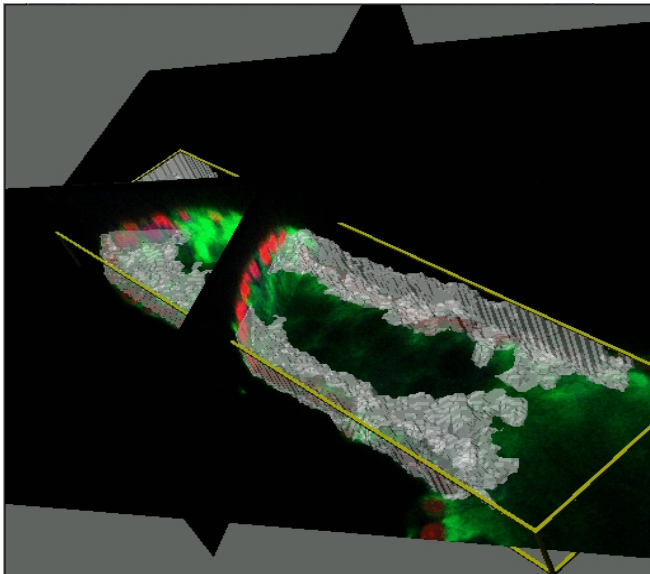
- estimation of the median axis of the crypt and quantification along this axis considering intersection of crypt layout and slices orthogonal to the median axis. This tool performs biomarker quantification in height

points (equally distributed along the axis) and displays the result as a curve representing the gradient of biomarker fluorescence intensity along the axis (figure 3).

Figure 3 : 3D illustration of a quantification mask area (translucent gray) along the crypt axis (yellow). The nuclei stained by Hoechst is shown in red, the membrane in green and the EGF-R in blue. Detected nuclei are labeled by small white spheres, near two orthogonal sections of the plane.
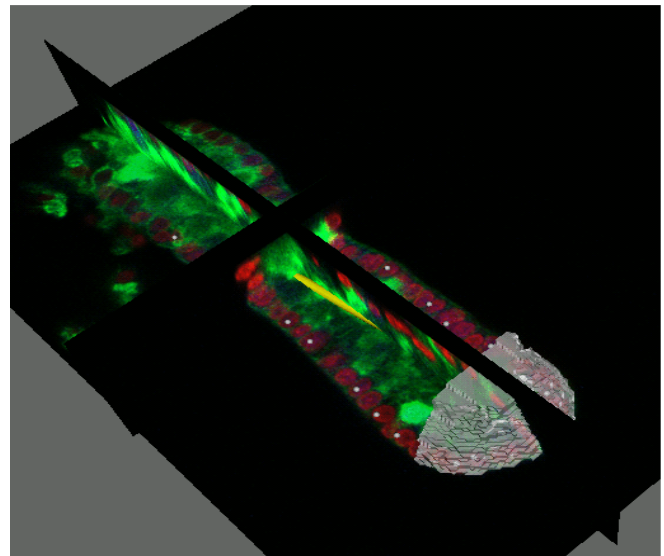
In any case, the quantification results are displayed in a table containing center coordinate, size and orientation of the polyhedral domain, the quantified volume, the total intensity of probe fluorescence (expressed as the total fluorescence intensity per voxel) and the fluorescence intensity - volume ratio. Because of crypt size heterogeneity, and in order to compare results between crypts, the fluorescence intensity results are indexed on the total fluorescence intensity of probe under study.

Result and discussion

1. Cell number evaluation

A manual quantification of the nuclei has confirmed the automated quantification.

Detection tools allowed us to model nuclei positions. From this whole detected nuclei and the images in the different stacks, the crypt orientation is evaluated, and its axis represented in 3D. The whole volume can then be rendered in 3D (figure 4).

In any case, the quantification results are displayed in a table containing center coordinate, size and orientation of the polyhedral domain, the quantified volume, the total intensity of probe fluorescence (expressed as the total fluorescence intensity per voxel) and the fluorescence intensity - volume ratio. Because of crypt size heterogeneity, and in order to compare results between crypts, the fluorescence intensity results are indexed on the total fluorescence intensity of probe under study.

Figure 4 : Volumic rendering with the principle axis (yellow) and the detected nuclei (hoechst stained nuclei is shown in gray and enumeration output by small spheres).
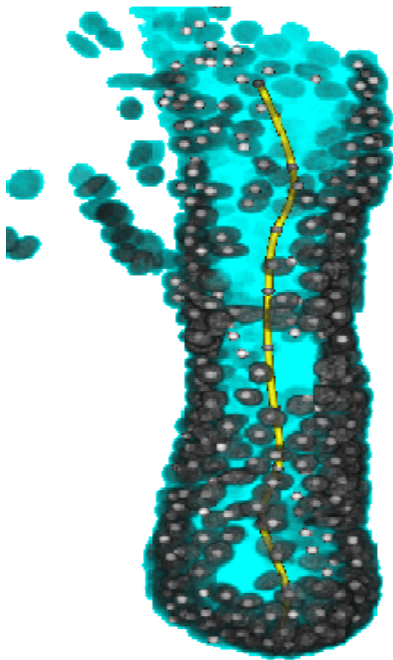
Meanwhile, the procedure can not exactly determine the number of nuclei for different reasons : a) some of nuclei were not labeled; b) there was some problem with image acquisition as attenuation in depth of fluorescence intensity occurred due to repetitive passes of the laser on the sample and photobleaching (Nagelhus et al, 1996; Rigault et Vassy, 1990), that do not allow us to have high quality nuclei imaging. Moreover, at this time, the procedure does not differentiate a cell in telophase of two cells. The nuclei detection uses only one channel (nuclei labeled by Hoechst), but it may be possible to extract information given by the other channels and to mitigate, in part, the consequences of these acquisition problems.

2. Biomarker fluorescence intensity estimation :

In the first stage, fluorescence intensity of a given probe was globally measured on the whole crypt or on specific areas of the crypt according to the region of interest, selected through user interaction. But, in certain cases, we cannot make a comparison between two crypts by only considering the fluorescence intensity on the whole crypt. For example, if we take two crypts of rats submitted to a fiber supply by continuous infusion and bolus respectively, and we study R-EGF expression, we have similar quantification results. But in paying attention to the 3D representation, we can see a difference in the location distribution of R-EGF between the two crypts, along the crypt axis. This difference in location distribution is appraised by fluorescence intensity quantification along the crypt axis.

When evaluating of biomarker fluorescence intensity, it is important to consider that the results obtained are strongly qualitative. Indeed, there are different defects in the sample quality and on acquisition of image stacks. First, the sample is not pure and contaminant may bring us to over-evaluate the fluorescence intensity. But we can consider that this noise is negligible if we take all precautions in sample preparation and if we determine the sample autofluo-rescence to correct the image stacks. In the second time, there is the problem of overlapping of excitation and emission spectra of fluorochromes. To limit this overlapping we must choose fluorochromes with very well defined excitation and emission spectra and use appropriate filters when acquiring image stacks.

Conclusion and perspectives

Currently, software packages exist which allow evaluation of biomarker fluorescence intensity and 3D representation of 3D data acquired in microscopy, and which are developed by microscope manufacturers or third party companies. This package often are trade marked and expensive and/or not customizable to the specific data analysis requirements of biologists. We are citing some companies here for information to the reader without the aim of being extensive: Amira (http://www.amira.zib.de), Imaris (http://www.imaris.com), Aphelion (http://www.aai.com:80/imaging/develpr.html).

Quant3D can be obtained from us as an RPM package provided the licensing terms for the included libraries apply (Quant 3D cannot be used for commercial purposes), and in

condition to quote Hoebeke and Trubuil (1999) and Pardini et al (this communication).

Recently we have built a new graphical interface of Quant3D (figure 5) which includes in one window the different windows of the application and integrate external tools (nuclei detection,mask production, volume rendering).

Figure 5 : Representation of the new user interface of Quant3D.
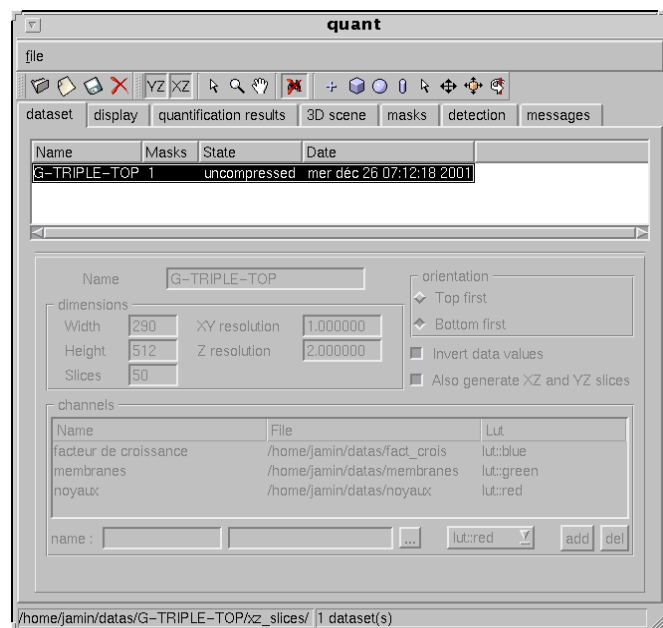
We have also developed other tools such as the selection of a quantification volume using an ellipsoid, and we will develop procedures to evaluate the number of mitotic cells. Moreover, algorithms, probably based on Rigaut et Vassy (1990) and Nagelhus et al (1996), will be included in the procedure in order to automatically correct acquisition defects resulting from photobleaching and fluorescence attenuation depth related. Quant3D is currently being used by biologists to study evolution of biomarkers along the crypt axis during the time and in function of survival environment, of Human colonic crypts isolated from biopsies.

In the future, Quant3D will be used to study spatial and temporal variations of biomarkers along colonic crypts recovered from human biopsies.

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