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Sleuthing the Virus …

Widefield Image Acquisition and Analysis

The Application

One aspect of research on the human AIDS disease is the question how the HI virus proliferates in the cell. The REV protein of HIV plays an important role in the HIV-life cycle. As part of its function it constantly moves between nucleus and cytoplasm and therefore is called a "shuttle protein". Acquiring quantitative information about this kind of transport helps to understand HIV and AIDS and to develop novel therapy concepts.

An experiment [1] was designed to determine the dynamics of the shuttling of Rev between nucleus and cytoplasm by measuring fluorescence signals. The fast exchange of proteins leads to a steady state equilibrium. This process cannot be easily measured directly so that a mutant of the Rev protein is used, which unlike the wildtype protein does not localize in the nucleus. The experiment shall clarify whether the nuclear import capability is affected by the mutation. Therefore Leptomycine B (LMB) is used to block the protein's "way back" from the nucleus to the cytoplasm. If the import mechanism has been affected by the mutation nothing will change in the localization. If – on the other hand – the balance between import and export has been disturbed, the mutant protein should be accumulating in the nucleus over time after treatment. Also, the concentration of protein in the nucleus should increase inversely to its decrease in the cytoplasm. A measure for the concentration is the fluorescence intensity of the Rev protein in both nucleus and cytoplasm which is marked with yellow fluorescent protein (YFP). To discriminate nucleus from cytoplasm all nuclei are marked with red fluorescent protein (RFP) and the cytoplasms of those cells which produce Rev are marked with cyan fluorescent protein (CFP).

Image Acquisition and Analysis

Both acquisition and analysis of the experiment were performed with AxioVision, the widefield image acquisition and analysis software package from Carl Zeiss.

In this experiment AxioVision controls a Carl Zeiss AxioCam HRm CCD camera and the inverted research microscope AxioVert 200 M including Incubator XL and a motorized scanning stage. A Carl Zeiss 40x Plan Neofluar NA 1.3 Phase contrast objective and the Carl Zeiss filter sets No. 47 (CFP), No. 46 (YFP) and No. 00 (RFP) were used. The cells for this time lapse experiment were cultivated in 35 mm glass-bottom petri dishes (MatTek).

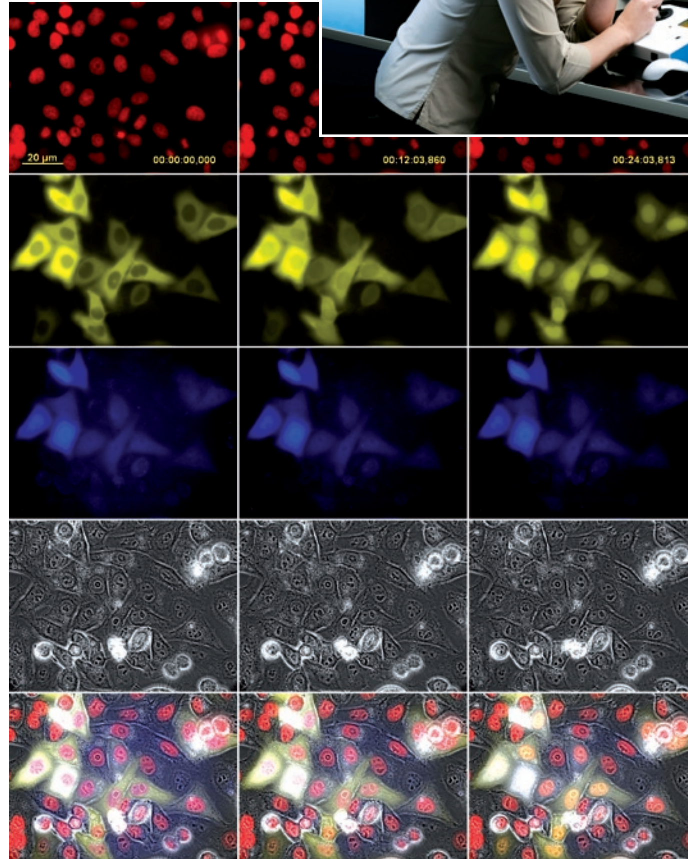

Fig. 1: Leptomycine B Experiment, 3 time points
Red – DsRed, Yellow – YFP, Blue – CFP, Grey – Phase contrast, Overlay of all pseudo-colored channels
(Image courtesy of Dr. Horst Wolff)

For analysis the AxioVision modules "AutoMeasure Plus" and "Commander" were used. All steps of processing, analysis and evaluation are stored in a script, which can be applied automatically e.g. on a whole folder of images.

Segmentation

In a first step the „objects" (nucleus, cytoplasm) in each fluorescence channel are discriminated from background using a threshold operator. The resulting images show the background in black and the objects in white. These images with only two gray values are called "binary" images.

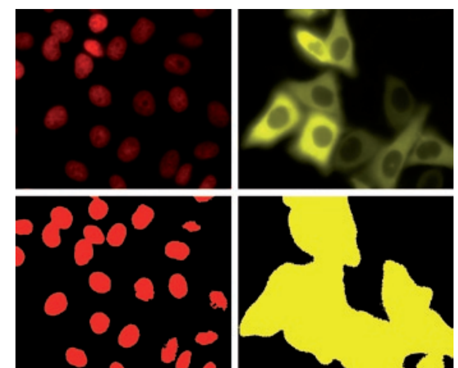

Fig. 2: Pseudo-colored original images and pseudo-colored results of threshold segmentation

Cleaning the Binary Image

The next step removes all objects below a minimum size. Also, holes in single objects are filled.

Following this procedure all nuclei still touching each other are separated using a watershed algorithm. For those cells (i.e. cytoplasms) which could not be separated during segmentation a "cut" line is calculated by a so called exoskeleton of the nuclei.

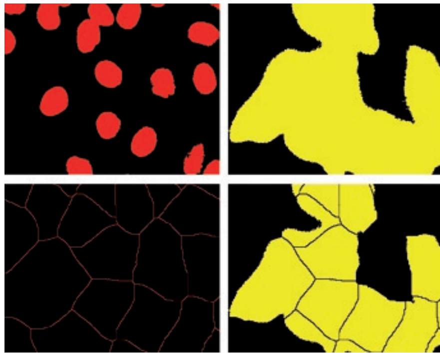

Fig. 3: Exo-skeletonized image of nuclei is used to separate the cytoplasm.
Top left: Segmented nuclei, Bottom left: Exoskeleton of nuclei
Top right: Segmented cytoplasm, Bottom right: Separated cytoplasm

The remaining cytoplasm then is masked with the nuclei so that only those parts of the cytoplasm are conserved which overlay a nucleus.

Masking

The next important step is to identify those nuclei which are encompassed by labelled cytoplasm to avoid measuring non transfected cells. This is done by "masking" the binary image of the nuclei with the binary image of the cytoplasm: only those nuclei remain which are "overlaid" by at least one pixel of cytoplasm. Finally the area of the nuclei is cut out of the area of cytoplasm by subtracting the binary image of the nuclei from the binary image of the cytoplasm.

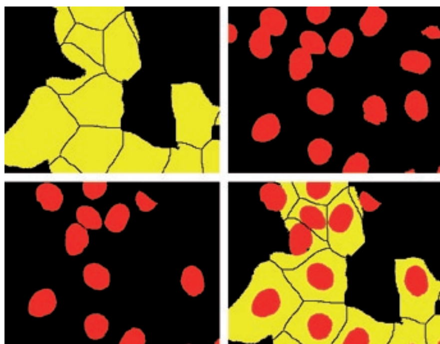

Fig. 4.: Nuclei marked with cytoplasm
Top left: Separated cytoplasm, Bottom left: Masked nuclei
Top right: All nuclei, Bottom right: Final mask

Calculation of Measurement Values

The cleaned and masked binary images are now used to measure the YFP labelled channel.

The following parameters are measured:
Parameters calculated for each nucleus and its surrounding cytoplasm (object specific parameters)

- Image's relative acquisition time
- Average of gray values of an object
- Highest gray value of an object (indicator, whether pixels were saturated during acquisition)
- Sum of gray values of an object
- Standard deviation of gray values of an object
- Area of an object

Parameters calculated for the whole image (field specific parameters)

- Average of all gray values of all objects
- Sum of densitometric gray values of all objects
- Area of all objects
- Number of objects

The calculations are performed for all cells and for each time point of the time series both in cytoplasm and in the nucleus.

The exemplary evaluation shown here uses only the sum of densitometric gray values for all objects. This is a measure for the concentration of the fluorescence signals.

Before evaluation the image background should to be subtracted. This is done by calculating the average gray value of the background of the YFP channel for each time point. This value is weighted by the area of all objects and then subtracted from the fluorescence intensity values.

In addition the following parameters are calculated:

- Sum of the intensities in nucleus and cytoplasm. This value shall stay constant over time and is a measure for the bleaching of the specimen over time. The potential of synthesis of new YFP labelled protein can be neglected due to the short total duration of the experiment.
- Intensity ratio of cytoplasm over nucleus.

Evaluation of Data

The calculated measurement values are stored in a data table and are used for a spreadsheet analysis which plots the relation between cytoplasmatic and nuclear fluorescence over time.

The Modularity of AxioVision

AxioVision is a modular software program. It's functionality covers all areas of

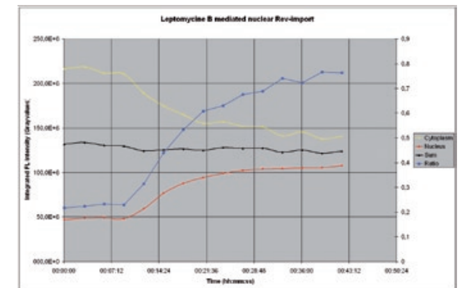

Fig. 5: Chart of Leptomycine B mediated nuclear Rev-import

microscopic imaging from image acquisition over image processing to image analysis and data evaluation.

The program controls all motorized microscopes from Carl Zeiss and a variety of external components like e.g. fast light sources. Together with the Carl Zeiss AxioCam cameras and some specialized high end cameras of other manufacturers simple and highly sophisticated acquisition experiments can be configured and reproduced fast and easily.

In adition to the image processing functions of the core package many modules are available for postprocessing and enhancing 2D and multidimensional images.

The modules for interactive and automatic measurement allow a wide spectrum of analysis.

Functions to handle and present the measurement results in data lists and reports and to archive images and data are available as well as modules to create dedicated programs as AxioVision scripts or using VisualBasic for Applications.

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References

[1] H. Wolff *et al.*: Experimental Cell Research 312, 443–456 (2006)

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