IMAGE ANALYSIS COMBINED FLUORESCENCE MICROSCOPY

Examples of ImageJ Software Application in Yeast Studies

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Abstract: For *Saccharomyces cerevisiae* yeast studies, three approaches have been developed. They are based on the image analysis (ImageJ software) application for the fluorescence microscopy data treatment. The first is a computer-aided fluorescence microscopy procedure for quantifying of the damaged cells in the ethanol-producing yeast culture. It was shown to be applicable for the assessment of the culture viability. The second is a means of characterizing Brownian motion of the insoluble polyphosphate complexes in the vacuoles. Using this approach, the apparent viscosity in the vacuoles was measured. The third is a method for locating intracellular sites/targets of the nucleic acid intercalators. This method may be of help in designing of new DNA-targeted drugs and in preliminary studies of their interaction with eukaryotic cells.

1 INTRODUCTION

The yeast *Saccharomyces cerevisiae* is an object of a large research interest for at least two reasons. First, it is widely used in food industry, including baking and production of alcoholic beverages, ethanol and food additives. Second, it serves as a model system for studies of eukaryotic cells since many of the basic cellular properties between yeast and humans are highly conserved. This is also due to the availability of the DNA sequence of the complete genome and biochemical data, convenience for molecular manipulations and the ease of handling. The yeast cells are shown to be a good model in many areas of cancer research and for new drug discovery. Hence, the development of new techniques and approaches for the studies of the yeast *S. cerevisiae* is an important task.

Fluorescence microscopy is a very informative method for single cell studies. Potentialities of the method can be significantly improved by combining it with the digital photography and computer image analysis.

In this paper, three approaches based on the ImageJ software (National Institute of Health, USA, http://rsb.info.nih.gov/ij) application for the fluorescence microscopy data treatment are described. The first approach was developed for the assessment of the yeast culture viability; the second for to evaluate the apparent viscosity in the vacuoles of the individual yeast cells; and the third for locating intracellular sites/targets of the nucleic acid intercalators.

2 ASSESSMENT OF THE YEAST CULTURE VIABILITY

This study was undertaken to develop rapid computer-aided fluorescence microscopy procedure for the assessment of the ethanol-producing yeast culture viability.

The fluorescence microscopy of rehydrated *S. cerevisiae* cells from a dry commercial Fermiol preparation (DSM Food Specialties Beverage Ingredients, The Netherlands) stained by a combination of ethidium bromide (E) and 4,6-diamidino-2-phenylindole dilactate (DAPI) revealed two types of cells. The cells of the first type showed blue-green fluorescence (DAPI), whereas the cells of the second type showed bright orange-red fluorescence (E). In special experiments, it was shown that the first type of fluorescence appearance was a characteristic of the intact cells and the second type was a characteristic of the damaged cells.

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Using ImageJ "RGB Split" option, the initial fluorescent images of the stained cells were separated into three images containing red, green and blue components of the fluorescence. This procedure enabled to establish that, in the ethidium stainable cells, along with the red component, there was well expressed green component of the DAPI fluorescence, too. This was an indication of the fact that DAPI stained all the cells. So, RGB split of the fluorescent image provided a means for assessing a fraction of the E stainable/damaged or E unstainable/undamaged cells. To this end, after an appropriate threshold adjustment, "Analyse Particles" option could be used for automated counting of the fluorescing cells ("particles") in the "red" and in the "green" parts of the RGB-splitted image.

A comparison of the viability rates of cultures, determined by the plate count method, and the relative numbers of intact cells, determined in the same cultures by the developed procedure, showed a good correlation between these parameters, thereby indicating a possibility of using this procedure for the assessment of the viability of rehydrated Fermiol preparations. It was noted that fluorescence microscopy underestimates the viability of yeast populations by 10–15%, compared to cultural methods. This disagreement can probably be explained by the fact that some damaged cells may recover during the subsequent cultivation of rehydrated cells.

The main advantage of the proposed approach is that it allows more rapid assessment of cell viability as compared to not only the cultural methods, but to the "manual" microscopy as well. Also, this approach opens a way to the automation of the analysis. For more details see (Puchkov, 2006).

3 MEASUREMENT OF VISCOSITY IN VACUOLES

In the *S. cerevisiae* cells, at some cultivation conditions, appear vividly moving particles, <1 μm in size, known as 'dancing bodies'. They were shown to be insoluble polyphosphate complexes (IPCs) localized in the vacuoles. Upon staining of the cells by DAPI, IPCs acquire a bright yellow fluorescent colour, while the nuclei and mitochondria fluoresce blue.

The aim of this study was to quantitatively characterize, by fluorescence microscopy combined with computer image analysis, the movement of IPCs in the vacuoles of *S. cerevisiae* VKM Y-2549 cells and to evaluate the apparent viscosity in the vacuoles, using the obtained data.

The immobilized cells were photographed in a Speed Burst regime of the Sony DSC-V3 digital camera. It gave a series of eight frames at intervals of 0.43 s and an interval between series of 2–3 s. Using ImageJ, in a frame of a series, a fluorescing particle was selected as a region of interest (ROI) by "Oval Selection" option. "ROI Manager" option was switched on to get the same ROI area in other frames, although position of the ROI was changed according to the position of the particle in a new frame. The locations of the IPCs in the two dimensional space (X and Y locations) were evaluated using "Center of Mass" option.

The results of this analysis indicated that IPC movements were chaotic or, as it is often referred to, were random walks, or Brownian motion.

The Brownian motion of particles obeys the Einstein–Smoluchowski equation, which for two-dimensional (2D) movement is as follows:

$$\langle s^2 \rangle = 4\kappa Tt/3\pi\eta D, \quad (1)$$

where $\langle \boldsymbol{s}^2 \rangle$ is the average of the square of displacement, $\boldsymbol{\kappa}$ is the Boltzmann constant, $\boldsymbol{T}$ is the thermodynamic temperature, $\boldsymbol{t}$ is the elapsed time, $\boldsymbol{\eta}$ is the viscosity and $\boldsymbol{D}$ is the diameter of the particle.

To evaluate the apparent viscosity in the yeast vacuoles via Brownian motion of the IPCs, the average displacement in 2D space and the diameters of the moving particles need to be estimated [equation (1)]. As IPCs move in three dimensions, a criterion for selecting the 2D displacements in the photorecords, which are in the focusing plane or at least close to it, must be found. To learn how this could be done, experiments were performed on suspensions of fluoresceinisothyocyanate-labeled latex microspheres of 2.1 μm and 3.1μm diameter in water. In this model, two parameters were known, the diameters of the microspheres and the viscosity of distilled water in which they were moving.

Using photorecords similar to those of IPC, two parameters of Brownian motion of fluorescing microspheres were estimated for each series of the eight speed regime shots – the consecutive two-dimensional locations and the mean fluorescence intensities ("Mean Gray Value" option) at these locations. The normalized fluorescence intensity served as a quantitative measure of the microsphere shift from the two-dimensional motion in each eight frames series of the speed regime shots. It was tested whether the fluorescence decrease of no more than 15% may be used as a criterion for taking

displacements of the microspheres close enough to the two-dimensional space for viscosity assessment by formula (1). Upon selecting "appropriate" displacements by this criterion from photorecords of the series of speed regime shots, computation of the apparent water viscosity using formula (1) has been made. The viscosity values of water estimated by these measurements agreed with 30% accuracy with the value (0,89 cP at 25 °C, the temperature of measurements) obtained by other methods. This result indicated that the developed approach may be used for viscosity assessment.

It was also found by analysis of the fluorescence intensity profile ("Line selection" across "Center of Mass" and "Plot Profile" options) of the microspheres that the level of the 85% fluorescence intensity corresponds to their outer borders. That gave the means of estimating the size of the fluorescing IPC particles by their fluorescence intensity profiles.

Using methodology developed on the latex microspheres, Brownian motions of the IPCs in four cells were analyzed. Displacements for each IPC in the two-dimensional space in the series of the eight speed regime shots were estimated. Outer borders of IPC were determined by estimating 85% level of fluorescence intensity in the IPC fluorescence profile. That gave the dimensions of IPC. Since the shape of IPC is not known, this parameter was assumed to be corresponding to the diameter of the microspheres.

In four yeast cells, the 2D displacements and sizes of the IPCs were evaluated. Apparent viscosity values in the vacuoles of the cells computed by the Einstein-Smoluchowski equation using the obtained data, were found to be of 2.16 ± 0.60; 2.52 ± 0.63; 3.32 ± 0.9; 11.3 ± 1.7 cP. The first three viscosity values correspond to 30 – 40% glycerol solutions. The viscosity value of 11.3 ± 1.7 cP was supposed to be an overestimation caused by the peculiarities of the vacuole structure and/or volume in this particular cell. This conclusion was supported by the particular quality of the Brownian motion trajectories set in this cell as compared to the other three cells. For more details see (Puchkov, 2010).

4 INTRACELLULAR LOCATION OF INTERCALATORS

The aim of this study was to test if intact (not fixed) yeast cells of *S. cerevisiae* can be used as a model for locating intracellular sites/targets of the nucleic acid intercalators (NAI). To this end, intracellular distributions of three fluorescing NAI – the anthracycline anticancer drug doxorubicin (DR) (trade name adriamycin; also known as hydroxydaunorubicin) along with nucleic acid dyes ethidium (E) and 4',6-diamidino-2-phenylindole (DAPI), were investigated using fluorescence microscopy combined with computer image analysis.

Upon incubating for at least 20 h, all the cells of *S. cerevisiae* VKM Y-2549 appeared to be stainable by the NAI. In the cells stained by DAPI, there were clearly visible fluorescing blue spots of the nuclei and small dots of the mitochondria. As compared to DAPI, intracellular fluorescence distribution of DR and E did not have the clear "spot and dot" appearance. Although nuclear region could be distinguished, even distribution of the DR and E fluorescence was observed in the regions where mitochondria should be located. There was heterogeneity in the expression of staining by DR and E. The observed difference in this feature of the individual cells may be a reflection of a specific combination of the cytoplasmic membrane diffusion barrier properties and of the presence/activity of drug export permeases.

Combined application of DR or E with DAPI visualized the location of the nuclear and of the mitochondrial DNA. However, visual analysis could not answer the question whether DR and E were located in the sites of the DAPI-stainable DNA.

To investigate the potential location of DR and E in the nuclei and in the mitochondria marked by DAPI, distribution of the red, green and blue components of the fluorescence ("pseudospectral analysis") in these regions was assessed quantitatively using ImageJ "Measure RGB" option of the "Analyze" plugin after appropriate ROI selection.

The obtained data indicated that, as it was expected, Red/Green ratio of DR and E were significantly higher than that of DAPI. In contrast to E, DR fluorescence had appreciably higher the Red/Green ratio in the nuclei than in the mitochondrial region. These data indicated that at least a fraction of DR molecules bond in the nuclei had more expressed red component of their fluorescence spectra than those in the mitochondrial region. Interaction of DR with nucleic acids is known to increase the "red" maximum in its fluorescence spectrum. So, the less expression of the red fluorescence in the mitochondria may be explained assuming that, along with binding to DNA, some molecules of DR are bound to the

membranes. Binding to the membranes may also be a plausible reason for visible even fluorescence distribution in the mitochondrial region in the cells stained by DR as compared to "dot"-like staining of the mitochondrial DNA by DAPI. There may be at least two binding sites for DR and E in the mitochondria: DNA and the membranes. Ability of E to interact with the mitochondrial membranes was demonstrated by others. Potential ability of DR to interact with biomembranes was shown on artificial membranes.

Upon combined addition of DR+DAPI simultaneously, in both the nuclei and in the mitochondria, the Red/Green fluorescence ratio was higher in comparison with the application of DAPI alone, but it was lower as compared to exposing the cells to DR only. If the cells were first incubated with DR, and DAPI was added later, the Red/Green ratio in the nuclei was higher than in case of simultaneous addition of the NAI, but still lower than after the addition of DR alone. Similar results were obtained for the pair E+DAPI with the difference that the order of the NAI addition did not significantly influence the final result. At the same experimental conditions, there were no appreciable changes in the Blue/Green ratio. So, quantitative image analysis revealed appearance of the red fluorescence component in the regions of DAPI stained DNA upon combined application of DAPI with DR and E. This is an indication of a colocalization of DAPI with DR and E in the nuclei and in the mitochondria.

In the sites of the colocalization of DR and E with DAPI, the red component of their fluorescence was less as compared to the application of DR and E alone. These data can be interpreted as a competition of DAPI with DR and E for the same DNA binding sites. It should be mentioned here that, although NAI can intercalate between base pairs, they may also be bound to some other parts of DNA, e.g. in the minor groove. The detailed mechanism of the competition of DAPI, DR and E for binding sites in DNA is not known as of yet. For more details see (Puchkov, 2011, in the press).

5 CONCLUSIONS

1. Three computer image analysis algorithms of the ImageJ software for the fluorescence microscopy data treatment have been developed.
2. The first, based on the use of "RGB-split" and "Analyze Particles" options, was shown to be successfully applied in a new method for rapid yeast viability assessment. This method could be of potential use for rapid viability evaluation of other microbial cultures, too.
3. The second enables the determination of the size and of the center of mass locations of the fluorescing particles, <1 μm in size, by their space fluorescence intensity distribution assessment. Using this algorithm, the apparent viscosity in the vacuoles of the individual yeast cells was evaluated by Brownian motion measurements. The methodology developed in this work may be of use for some other studies of moving microscopic fluorescing particles.
4. The third algorithm, the "pseudospectral analysis", was designed for locating intracellular sites/targets of fluorochromes in the yeast cells. The intact (not fixed) yeast cells of *S. cerevisiae* can be used as a model for intracellular locating of the nucleic acid intercalators by fluorescence microscopy combined with this computer image analysis algorithm. The model and the approach presented herein may be of help in the new DNA-targeted drug discovery and in preliminary studies of their interaction with eukaryotic cells.

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