STATISTICAL VALIDATION OF A HIGH PRECISION CELL SIZE MEASUREMENT SOFTWARE

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Summary An important methodological element in the research of cell cycle regulation is the very accurate measurement of the cell size, which can be solved by measuring time lapse microscopic photographs. The model organism used for the measurement is Schizosaccharomyces pombe. The publication discusses the functional analysis and validation strategy of software designed to automate measurement. Based on the given methodology, an automated software testing and validation system can be created.

Keywords: ImageJ, cell size measurement, image processing, software validation, Akiake information criterion

Introduction

A very important area of research in bioinformatics is the study of the regulation of cell division. Within this, the regulation of cell size control is an interesting issue of which software side is addressed by the present research. *Schizosaccharomyces pombe*, a fission yeast was selected as model organism, because its growth is longitudinal, as growth is limited to the two cell ends. The cylindrical cells of the wild-type fission yeast are on average 7-8 μm long at birth and have a constant diameter during the whole cell cycle of about 3.5 μm. By the end of the cell cycle, near division the cell length becomes 13–14 μm [1]. The duration of the cell cycle is 2-4 hours, so 60-100 images are taken per cell with recording every two minutes. Division is usually regulated by the cell mass, or cell volume, which in this case is a linear function of cell length, so it can be measured fairly accurately. The measurement itself can be accomplished by evaluating a time-lapse microscopic series of live, unstained cells and followed with the processing by fitting the appropriate model functions.

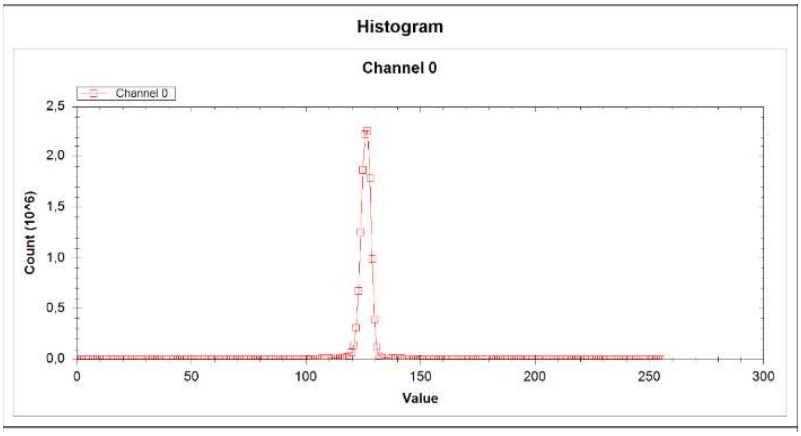

Figure 1 Histogram of a microscopic photo with unstained *Schizosaccharomyces pombe* cell culture. The photo was created with 8 bit grayscale coding, but the real width of the range of pixel colours is only 15.

Evaluation of the time-lapse recordings is a time-consuming task. In order to obtain meaningful information about the growth pattern of a culture, a representative number of cells must be monitored for growth during the cell cycle. The large number of recordings to be evaluated raises the need to develop an automated evaluation method that speeds up the process, thus facilitating the work of researchers involved in cell cycle studies. This paper presents the development of an automatic cell size determination algorithm and the automated validation of the first version of this software (later ASAP).

Nowadays automatic image processing and object detection are frequently occurring computer science problems. Algorithms using AI that implement different machine vision are able to accurately identify very complex objects in real time (e.g. YOLO, LENET). However, this is typically depending on the availability of image information (color components, normal histogram). Unfortunately, in this case, this condition is only partially met. Because we want to study living and natural cells, staining techniques to contrast the cells cannot be used. Thus, cells can produce contrast-deficient, grayscale images in which the detection of the cell contours is not trivial. Figure 1 shows a histogram of such a microscopic photograph. On the other hand, we expect the software to have an object detection accuracy close to 100%, as well as the appropriate measurement accuracy.

During the development of the software, the following research questions arose. At algorithm development, possible algorithms should be tested. The question is from what criteria can the software be qualified? What criteria system should be defined in the software requirements specification? It is worth formulating the requirements in such a way that validation can be automated based on them, so that it can be possible to test several target algorithms.

Proposal

The accuracy of the cell contour recognition must be specified in the software requirement specification. The theoretical goal is nearly 100% accuracy in the cell recognition. Although a semi-automatic cell detection algorithm would be acceptable at the end-user level of the software, it makes the automated validation impossible. In the case of cell contour recognition, the goal is to maximize the accuracy, and this step can be separated from the subsequent measurement, so a separate test should be suitable in the first phase of validation.

The second requirement concerns the accuracy of the measurements. Before assigning a completely arbitrary accuracy at 5% or 10%, it is worth considering this issue carefully. In essence, we want to replace expert measurements with automated software; hence the requirement of the software is to reach a similar decision with the expert at the end of the measurement process.

During the measurement, the cell length is determined regularly through the entire length of the cell cycle, and then a cell growth model is set up. The biochemical background is discussed in the relevant literature. During modeling, a linear, bilinear, or exponential model can be fitted to the measured data using statistical methods [2].

Fitting the models is a minimum search task, in which the values for the parameters of the functions to be fitted have to be found and at which the sum of the squared errors (SSE) formed from the difference between the values of the measurement points and the function is as small as possible. In the first approximation of the different functions, the one with the smallest value of SSE can be considered the most adequate. If we have more variable parameters, we have a better chance of achieving a better fitting with the functions fitted to the data points. Based on these principles the adequacy of the bilinear function against the exponential can be questioned. In many cases, an exponential function can be fitted with a similarly high correlation coefficient to the growth considered bilinear, but at the same time it does so with far fewer parameters. In this case, the model with the smaller number of parameters should be preferred to the model with the larger one. It can be seen from this that it is not enough to base the selection of the appropriate model only on the correlation coefficient; furthermore it is necessary to use more complex statistical measures, so-called model selection criteria. AIC (Akaike Information Criterion) and SBIC (Schwarz Bayesian Information Criterion) determine a quantitative measure of fitting quality based on the SSE value and the number of parameters used for matching.

The lower this value, the more adequate the model is. Increasing the number of parameters used for matching is said to penalize the model. The two

mentioned criteria differ in rigidity, because SBIC penalizes the increasing number of parameters more severely than AIC [3].

In this way, the selection of a particular model for the points obtained when examining the growth of a cell will be statistically better supported.

According to the linear growth model, the growth rate of the cells is constant throughout the studied phase. The model can be characterized by two parameters, one is the slope of the function, which is actually the growth rate of the cells (γ), and the other is the axis section (δ), which corresponds to the length of the cell at birth.

(1) $L(t) = \gamma * t + \delta,$

where $\boldsymbol{L}$ is the cell length [μm], $\boldsymbol{\gamma}$ is the slope [μm / min], $\boldsymbol{\delta}$ is the axis section [μm], $\boldsymbol{t}$ is the time [min].

According to the exponential growth model, a simple two-parameter exponential function is perfectly suitable for describing cell length.

(2) $L(t) = \kappa * e^{\mu t},$

where $\boldsymbol{L}$ is the cell length [μm], $\boldsymbol{\kappa}$ is the estimated cell length at birth [μm], $\boldsymbol{\mu}$ is the specific growth rate [1 / min], $\boldsymbol{t}$ is the time [min].

The third most important growth model for fission yeast is the bilinear model. According to the model, the growth pattern can be characterized by two sections with different slopes, which are separated by the gear shift point.

We fitted a bilinear function to the measurement results, in which the transition is not a sudden breaking point, but rather an exponential transition phase. The more accurate name of the model is linearized biexponential because it is obtained as the sum of two exponential functions. Far enough away from rate change point (RCP2), the value of one exponential term in the expression is negligibly small next to the other. For this we take the natural-based logarithm, thus obtaining the first or the second bilinear stage, depending on which exponential term in the sum is very small. In the transition range, the value of none of the exponential terms in the function is negligible. Its use is more advantageous than that of a bilinear function with a sudden breaking point, because, on the one hand, it can be continuously differentiated and, on the other hand, it is closer to reality, since the change in growth rate in cells is not necessarily instantaneous. The model has five parameters.

(3) $$L(t = \eta * ln\left[e^{\frac{\alpha_1(t-\tau_{RCP2})}{\eta}} + e^{\frac{\alpha_2(t-\tau_{RCP2})}{\eta}}\right] + \varepsilon$$

where, $\boldsymbol{L}$ is the cell length [μm], $\boldsymbol{t}$ is the time [min], $\boldsymbol{\alpha 1}$ and $\boldsymbol{\alpha 2}$ are the slopes of the two linear sections [μm / min] $\boldsymbol{\eta}$ is the parameter determining the width of the transition range [μm]. During the fit, its value was allowed to change between 0.01 μm and 0.5 μm. This is because above the interval, a significant part of the growth phase falls in the transition range and the fitted bilinear function already looks more exponential. And below the range, the

program cannot handle the huge numbers generated in this case, τRCP2 is the time of occurrence of RCP2 [min], ε is a constant additive term [μm].

The width of the transition range depends not only on the value of η, but also significantly influenced by the value of α1 and α2. The following formula can be calculated:

(4) $Transition\ range[min] = ln\left(\frac{7}{3}\right) * \frac{\eta}{\alpha_1 - \alpha_2}$,

where ***α1*** and ***α2*** are the slopes of the linear sections, ***η*** is the width parameter [3], [4].

Akaike information criterion at small sample sizes the second order AIC:

(5) $\mathrm{AIC} = \mathrm{n_{obs}} * \ln(\mathrm{SSE}) + 2\mathrm{n_{par}}$

(6) $SSE = \sum_i (y_i - y_{i,b})^2$

SBIC (Schwarz Bayesian Information Criterion)

(7) $\mathrm{SBIC} = \mathrm{n_{obs}} * \ln(\mathrm{SSE}) + \mathrm{n_{par}} * \ln(\mathrm{n_{obs}})$,

where n_{obs} the number of data points and n_{par} is the number of parameters.

The proposed validation process is as follows: Measurement of a representative amount of cells in the series of photos and calculation of the growth model, then the same is done automatically using the software. Validation can be traced back to an automatic measurement after modeling, and modeling and comparison of the resulting growth patterns.

Methods

Time-lapse images of fission yeast growth were taken by photographing every two minutes in the usual experimental setup and conditions[1], [2], [4].

Software development was the development of an algorithm containing elements of a standard image processing pipeline. Because multiple image enhancement procedures were required due to the poor histogram of the image, the use of neural networks in the recognition was discarded, and classical image processing methods were used instead.

Algorithm

During the algorithm development, we chose to use the classical image processing pipeline, in which the specific processing algorithms were optimized separately for each unit. Figure 2 shows the main processing steps. Gaussian Blur calculates the new value with a normal distribution. The sigma value of the distribution along the x and y axes can be specified separately with a parameter. The Gaussian filter was applied with a 5x5 kernel.

It has been suggested that since the dynamic range of the image, i.e. the difference between light and dark parts, is small, contrast compensation can improve this, but this did not change the efficiency of the further image processing functions, so we did not use this option.

An adaptive thresholding method was used for edge detection. This differs from simple thresholding in that it analyzes the image in details and makes a decision based on the value of the pixels within it. The evaluation function can

be a mean or a normal distribution. You can specify the size of the area to be examined at one time, or a constant that can be used to reduce the calculated value before evaluating the pixels.

The Satoshi Suzuki and Keiichi Abe algorithms were used for contour search. From the detected shapes the too small ones were deleted from the image.[5]

Post-processing: Two morphological functions of the library were used. The two basic operations of these algorithms are erosion and dilation. First an open operation was used to remove any residual noise from the image. It is a combination of an erosion followed by a dilation. The other operation used is the open, is the reverse of the close function, which is a dilation after an erosion. These are for close the tiny gaps between the outlines.

Contour search and sorting

Find an enclosing rectangle.

At the end of the process, a preprocessed image is available on which the automated measurement can be performed after accurate cell contour recognition (fig 3.).

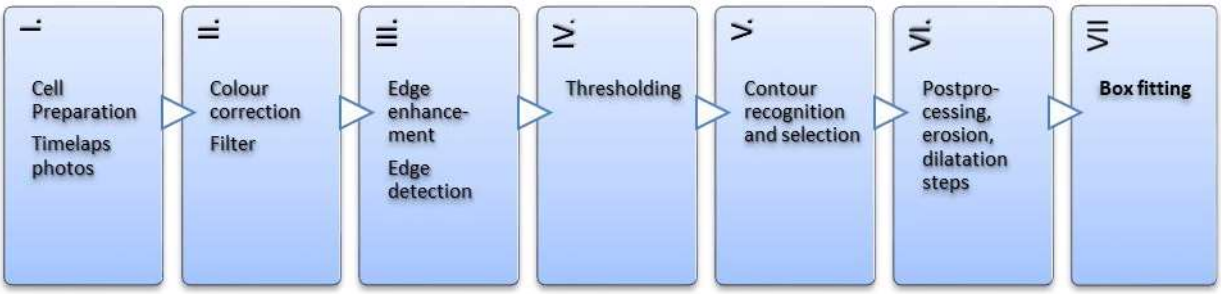

Figure 2 Applied image processing pipeline

The algorithm was implemented using the OpenCV image processing library.[6]

The ASAP software can be download from https://github.com/hajnaleva/ASAP.git [7]

Preparation for validation

The measurements of the automatic algorithm were executed and measured results of relevant cells from the image were collected. The selection of cells for measurement is an expert decision based on the viability of the cells and the cell density shown in the image.

The selected cells were measured with manual measurements using ImageJ software according to the previously applied protocol [8].

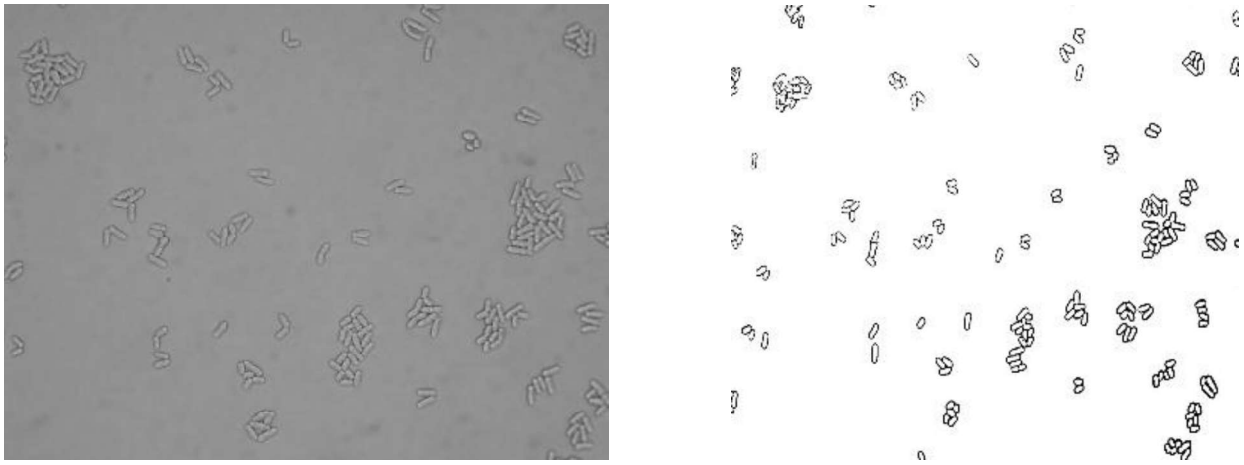

Figure 3 A, A microscopic image before processing, the edges of the image are darker than the central part which implicated the usage of the adapted thresholding method. B. Image after the image processing before the measurement.

Validation of the ASAP software is based on the statistical comparison of the obtained results.

The first phase of validation is to determine the accuracy of the automatic recognition of cells pre-selected by the expert. After counting the cells, the accuracy can be calculated.

The second phase of validation is the statistical analysis of cell length measurement. Since the same identified cells are measured with the automated software as with the manual determination, the deviation of the measurements can be checked by a pairwise T test. However, because we want to separate the two phases of validation, we used a two-sample T test instead of this one followed by regression analysis. In the regression tests, the null hypothesis was the 0 slope of the regression line; the obtained results were interpreted at 95% significance level. For each cell, for each measurement point the difference and the quotient of the results of the two programs were calculated. The obtained differences and quotients were then subjected to regression analysis. Linear regression was performed for each cell using Minitab, separately for differences and separately for quotients. It was examined, whether there is any correlation between the elapsed time and the differences and quotient.

The third phase of validation is the comparison of models fitted to cell lengths measured with ASAP and by an expert with help of ImageJ. The model fitting was established by a statistical software (Minitab), according to the proposal, however it can be automated later.

Results

The results show that the cells are misrecognized at a large number of measurement points. Table 1 shows the accuracy of automatic cell detection for 10 cell lines compared to expert evaluation. It is important to note that not all missing points were due to the ASAP error.

Table 1: First phase of validation, accuracy of cell detection during tracking the growth of ten cell lines. COUNTA the number of valid cell detection, COUNTE contains the number of photos, third row is the accuracy.

	1	2	3	4	5	6	7	8	9	10	TOTAL
COUNTA	66	77	65	63	72	75	58	66	70	62	**674**
COUNTE	67	79	69	69	74	78	70	78	75	71	**730**
ACCURACY	99%	97%	94%	91%	97%	96%	83%	85%	93%	87%	**92%**

In some images, the cells were not visible clearly due to a filming error. In comparison, 73 cell lines were measured with an expert and more than 5 consecutive missing values were measured in one case, 1 measurement point was missing in 32 cases, and all data could be successfully measured throughout the remaining 40 cells.

Overall, we can say that the accuracy of cell detection is more than 90%. Experiences have shown that recognizing the pattern of state around the cell division is a problem for ASAP software.

The second phase of the validation is the examination of the cell length measurements. Table 2 shows the differences between the ASAP and ImageJ measurements. The relative difference is ~4% which can be categorized theoretically into the excellent category. Table 3 shows the T probe and linear regression results between the difference of the two measurements, quotient of the two measurements and the elapsed time. Generally, the absolute difference can be constant, which means that it could be compensated with a fix factor, or in some cases it is negatively correlated with the elapsed time which means that it can be compensated with a linearly decreasing factor.

Table 2 The accuracy of cell length measurement between ASAP and ImageJ measurements

CELL NUMBER	MEAN DIFFERENCE BETWEEN IMAGEJ AND ASAP MEASUREMENTS IN PIXELS	MEAN RELATIVE DIFFERENCE BETWEEN IMAGEJ AND ASAP
1	4.79	5%
2	2.60	3%
3	3.63	4%
4	2.36	2%
5	5.64	7%
6	2.94	3%
7	3.85	4%
8	4.11	4%
9	4.35	5%
10	3.13	3%
MEAN	**3.74**	**4%**

Table 3 Linear regression results of the difference and quotient of the ImageJ and ASAP measurements with time. Null hypothesis is the 0 slope.

	NUMBER OF CELLS	DISTRIBUTION
CONSTANT DIFFERENCE	25	35%
NEGATIVE CORRELATION OF DIFFERENCE AND QUOTIENT	31	44%
OTHER	15	21%

The last part of validation is checking the model fit. Based on the measurements, a model was fit to 52 cells and Fig. 4 shows the distribution of the models fitted by the two methods. It can be seen that ASAP fitted a linear model to most of the cells, while expert evaluation chose the bilinear model in most cases. Unfortunately, the distributions of the models fitted by the two methods do not match.

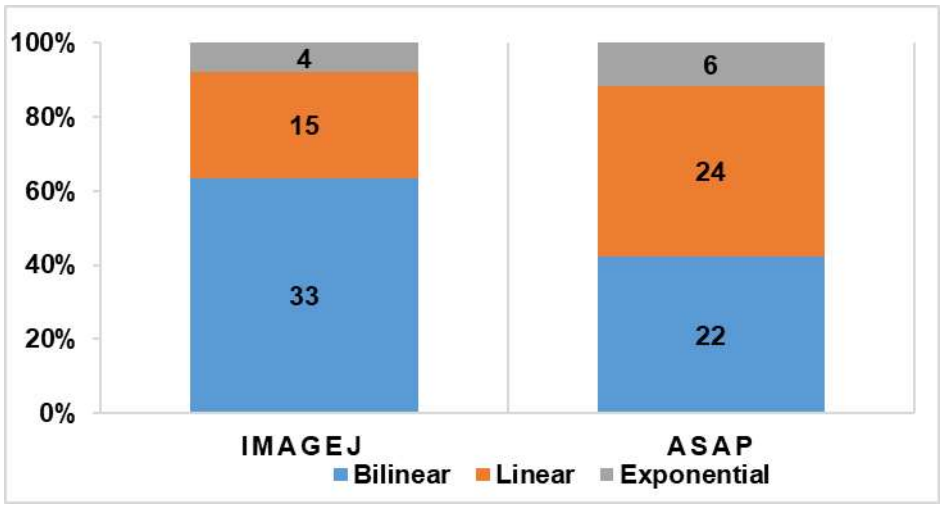

Figure 4 Results of the third phase of validation: the fitted models by manual expert measurements (ImageJ) and automated by ASAP software

Conclusion

It has become necessary to develop a software to facilitate the research of cell division regulation. As a result of the software development, the ASAP software were created, which is able to detect cells and automatically determine cell lengths on microscopic images of living, unstained cells. The software was validated using a complex statistical method. There were three phases of validation. The first phase evaluates the accuracy of cell recognition. Based on

this, it can be concluded that the software has a cell recognition accuracy of over 90%.

The second phase of the test is aimed at the accuracy of the cell length determination and the statistical analysis of the deviation. Based on this, the average error is within 5%, and this is likely to be further corrected by a correction factor to an even lesser extent.

The third phase is a model selection statistical study to clarify that according to the cell length measurements performed with the software and model fitting, which functions can be obtained.

Although the software gave a very good result for the superficial observer in the first and second studies, in the third phase it proved that it resulted in a completely different fitted model distribution compared to the expert measurements and model fitting. Based on this, it can be concluded that the software is not yet applicable for this research. However, the performed expert work and the developed validation methodology can be automated and used in the further development of the software. An opportunity for further development is to use a genetic algorithm to find a correction factor for which the deviation from the expert measurement is minimal, and to minimize the deviation in the distribution of the fitted cell growth models. Another direction of the development is to fine-tune the cell contour detection module of the algorithm.

It is a lesson that it is not enough to target some preconceived precision, but the precision itself is the subject of serious examinations. Overall, the specification of a software requirement sometimes needs serious statistical considerations and the automatic use of professional standards is not sufficient.

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ДИНАМИКА НА СЛЪНЧЕВАТА АКТИВНОСТ И ТЕМПЕРАТУРНИТЕ АНОМАЛИИ НА ПОВЪРХНОСТТА НА ЧЕРНО МОРЕ

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DYNAMICS OF SOLAR ACTIVITY AND TEMPERATURE ANOMALIES ON THE SURFACE OF THE BLACK SEA

Angel Manev, Veselin Tashev, Lubomira Raykova, Miroslav Kostov

Alstract: *Peculiarities of the dependence of the temperature anomalies on the surface of the Black Sea and the dynamics of the solar activity at the same time have been studied. The causal relationship between the increase in solar activity and the increase in the temperature of the Black Sea surface is demonstrated. Meteorological data to explain the asynchrony in the time series are also included in the analysis process.*

Ke words: *Black Sea , eolar activity, temperature , anomalies.*

Енергийните процеси в ядрото на Слънцето водят до изхвърлянето на енергия в космическото пространство във вид на електромагнитно лъчение. Върху динамиката на това лъчение най-силно влияние оказват промените в собствената му атмосфера и динамиката във времето на магнитното му поле. Вариациите на енергийното излъчване на Слънцето и последвалите ефекти в атмосферата на Слънцето определят понятието Слънчева активност. Изменчивостта на Слънчевата активност може да се анализира в различни времеви диапазони чрез различни измерени характеристики.

Най-отдалечените във времето данни за Слънчевата активност са от порядъка на преди около 700 милиона години. За това време се датират промените в дебелината на след-декамбрийския слой елатин в Австралия. Макар данните да са спорни все още остава открит въпросът с датирането на слънчевите събития в най-далечните времена. По-късните във времето данни са в пределите на отпреди десетки хиляди години. Данните от този период са важни за изследването на продължителността та Холоцена.