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Segmentation of Time-Lapse Images with focus on Microscopic Images of Cells

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Abstract. Phase contrast is a noninvasive microscopy imaging technique that is widely used in time-lapse imaging of cells. Resulting images however contain some optical artifacts, which makes automated processing by computer difficult.
We developed a novel algorithm for cell segmentation. It is based on processing of time differences between images and combination of sophisticated thresholding, blurring and morphological operations. We tested the algorithm on four different cell types acquired by two different microscopes. We evaluated the precision of segmentation against the manual segmentation by human operator and compared also with other methods. Our algorithm is simple, fast and shows accuracy that is comparable to manual segmentation. In addition it can correctly separate the dead from living cells.

Keywords: cell segmentation, phase-contrast microscopy

1 Introduction

The general experience that a picture is worth a thousand words also holds in the field of systems biology. The vast amount of image data which is generated by microscopy experiments of biological processes represents a firm data basis that contains important information on spatio-temporal aspects of these processes.

One issue that is studied is to determine the biocompatibility or biotoxicity of various materials. The motivation for this problem is the development of body implants. The body is very sensitive to foreign materials. Implants made of unsuitable materials may cause immune reactions of the body. Testing of various materials in clinical studies is very expensive and time consuming. Therefore, researchers are developing methods to test materials in vitro. Methods based on testing in the laboratory in vitro are currently used. Testing is performed with the cancer cells, which are more durable than normal body cells. If even cancer cells can not survive in the current environment, it is assumed that neither the

normal body cells can survive. The second reason for the use of cancer cells is that they are easy to grow in vitro. [1]

To achieve the highest fidelity, it is necessary to choose the least invasive capture method. A common choice is a microscope based on the phase contrast principle. Unlike fluorescent microscopes, phase contrast does not require any labeling of cells, uses a reasonable amount of light and provides high-contrast images of cells interior. Unfortunately, the resulting images contain artifacts like bright areas around the cell borders – halos.

Assessment of biocompatibility runs as follows. Cells are scanned at regular time intervals with a microscope. The acquired images are analyzed and the rate of cell growth is evaluated. Determining the rate of growth is a well-defined task and it can be automated using a computer. This can save a lot of time compared to manual processing. In the language of image processing, the task consists of segmentation of cells from the background. The area covered by cells during the experiment describes the rate of growth adequately.

Unlike humans, a computer can not automatically recognize what a cell is and what is background. All methods give only an approximate estimate of the correct solution. Segmentation by itself is a complicated task and in our case there are several factors that make it even harder, such as the presence of impurities in the solution, poorly focused specimen, shallow depth of focus of microscope images, presence of the halos, or texture-like background of images are all factors that make this task more difficult (see image examples in Fig. 1).

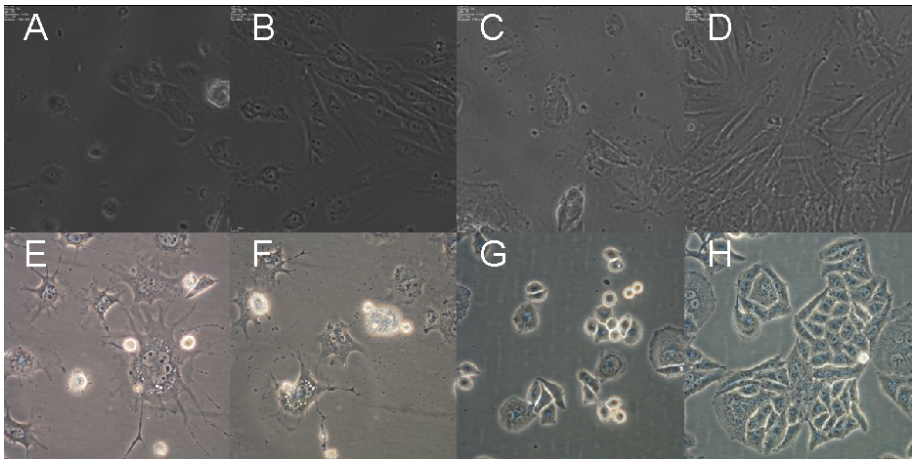

Fig. 1. Examples of data: A, B - MG63 cells, Nikon Biostation microscope (A - poorly focused); C, D - G10 cells, Nikon Biostation microscope (inpurities in solution - black dots outside the cells); E, F - HeLa cells, Olympus microscope; G, H - L929 cells (strange shapes of cells, dead cells), Olympus microscope (texture-like background, strong halos).

We cannot use classical segmentation method like thresholding or watershed method. In the literature there exist several approaches:

Active contours and levelset methods are relatively slow [2]. They must be initialized (manually or by some other method). Problems with connection of cells, halos around cells and change of topology of area covered by cells must be treated. The quality of segmentation depends on the initialization and in case of the manual initialization the results are poorly reproducible.

Methods based on machine learning [3, 4] depend on the cell type and microscope type. These methods can achieve satisfactory results but it is necessary to provide high quality and comprehensive learning data set.

Recently, a novel method [5] was developed based on removing of artifacts from images. The degradation model is adjusted for the phase-contrast microscope and it is in the form of convolution. Using a deconvolution algorithm we can obtain a modified image which looks similar to images from the fluorescent microscope. Segmentation is then performed by simple thresholding. Disadvantage of this method is the instability of the deconvolution. To obtain sufficiently good results we must use regularization and advanced optimization methods.

Besides these three approaches there exist several methods [6] based on transformation of images in order to be segmentable by thresholding. However, these methods often fail to provide accurate segmentation if images have either very small or very high coverage. We propose a novel method that belongs to this group and which solves problems with low and high coverage. Compared to the active contours our method is very fast. It is also versatile and highly accurate.

2 Methods

Before we start with the method description we must mention basic assumption on which our method stands. We process a series of time-lapse images and assume that (1) all images capture the same area (all images are registered), (2) there are no luminosity changes in images (such as automatic white balance and contrast correction), (3) background (area without cells) is still in time and changes only due to noise, (4) we can see movement of the cells and also movement of cell's interior and (5) cell coverage between consecutive images differs only slightly (our method works well only when frame rate of time-lapse capturing is sufficient – frequency of capturing must be equal or lower than one image per hour).

These five assumptions are necessary for a correct functionality of our method. Assumptions (1) and (3) are crucial and their violation will render the method useless. Violation of the other assumptions would make our method only less precise. Assumptions that we made are usually fulfilled or they can be guaranteed using proper experimental setting.

Our method consists of several steps that we group into three phases – preprocessing, thresholding and correction. In the preprocessing phase we change modality of images to improve contrast between areas with/without cells. The thresholding phase consists of dividing the image into areas with/without cells

(segmentation) and the correction phase improves segmentation using several heuristics (see Fig. 2).

2.1 Preprocessing

Images from the phase contrast microscope have poor contrast between cell and background regions and thus simple segmentation methods cannot be used. However, if we take the absolute value of differences of two consecutive images, contrast between these two regions is clearly visible. Here we use assumptions (3) and (4). When the background is still, differences between two consecutive images will be small (and depend only on the level of noise). On the other hand when we see movement inside the cells, it means that the brightness is changing and it is more probable to obtain higher differences between consecutive images in those areas.

The result of this step is a texture-like image that consists of regions with very low intensity values (background) and regions with both high and low intensity values (cells). Contrast between these two regions depend on the time interval between capturing these two images. When the frame rate is very high, cell's positions change very little. In this case we can not compare two consecutive images but use images more distant in time. Low frame rate is a more serious problem. When the time interval between two images is too long, cells might migrate to a completely new position. After the difference step we will see a signal of cell at both initial and final position. This kind of problem can be partially solved in the correction phase (see Sec. 2.3) but nevertheless makes the whole algorithm less precise.

The second step consists of blurring the difference image. Background regions stay unchanged and cells regions become more homogenous (see Fig. 2). We apply gaussian blurring with a very small kernel (standard deviation 1, kernel mask 3×3).

The last step in the preprocessing phase is thresholding. We assume that coverage in the current image will be about the same as in the previous image and we use this information to set a threshold value. When coverage in previous images was $C\,\%$, in this step we set the threshold value as the C-th quantile of image histogram. Thus $C\,\%$ of pixel with the highest values will become white and rest $(100 - C)\,\%$ black. Thus we increase contrast between regions that we want separate (see Fig. 2).

When the assumption about moderately changing coverage is not fulfilled, this step of the algorithm can lead to underestimated coverage increase/shrinkage. To our knowledge this assumption is fulfilled whenever frame rate is high enough. Movement inside the cells and migration of the cells is faster than the cells growth. To summarize this phase, we apply three operations (difference of consecutive images, blurring and thresholding) to change the modality of images. In this modality contrast between cells and background areas is grater compared to the original image. This allows us in the next phase of the algorithm to successfully apply a very simple segmentation method which makes the whole algorithm very fast.

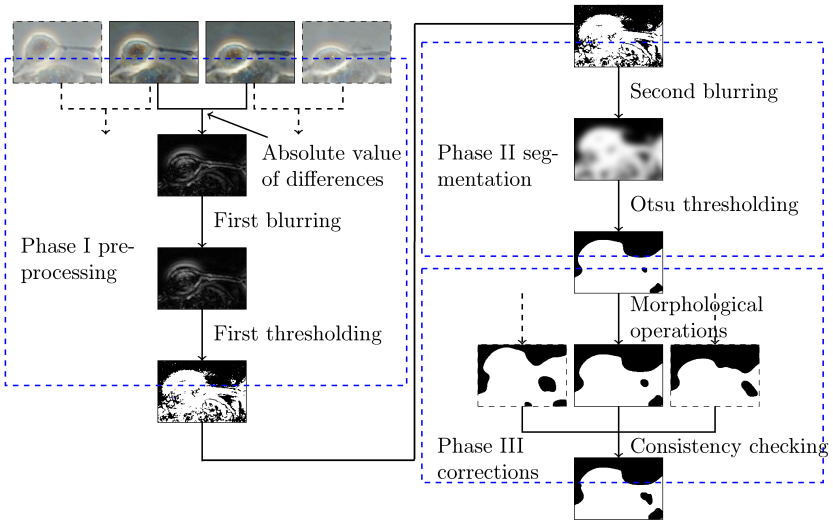

Fig. 2. Pipeline of our algorithm

2.2 Segmentation

Segmentation phase consists of two steps – blurring and thresholding. Blurring is here intended to fill small holes in otherwise uniform regions. Holes can correspond to some dirt moving in background or for example to a cell nucleus which did not change its position between the last two frames. Here blurring plays a different role than blurring in the preprocessing phase and also parameters of blurring differ. This time we use gaussian blur with larger standard deviation. It depends on microscope magnification and should be set from quarter to half of the smallest cell's diameter. This step can be omitted when necessary and the effect can be compensated in the correction phase. We omit this step for example when we want to detect thin tentacles, which connect some cells together (see Fig. 1). Excessive blurring can erase traces of tentacles, which may not be desired.

After blurring, we apply modified Otsu thresholding. The original Otsu thresholding automatically chooses a threshold value using only the image histogram. The threshold value is set to maximize the separability of resulting two (background/foreground) classes [7]. When two areas are about the same size, Otsu thresholding works very well. Problem occurs when one region is significantly smaller then the other. In these cases Otsu thresholding fails to find an optimal threshold near the edge of histogram and instead it chooses some point in the middle of histogram.

We modified Otsu thresholding to prevent this false behavior. When coverage in the previous image is too high or too low we apply Otsu thresholding only on a part of the histogram. Then the optimal value will be closer to the middle

of cropped histogram and the algorithm chooses the optimal thresholding value. (If coverage in the previous frame is between 0.25 and 0.75, we apply Otsu thresholding to the whole histogram. Outside this range we process only $100 \cdot 16 \cdot (1 - C)^2$ % resp. $100 \cdot 16 \cdot C^2$ % of the histogram.) This modification greatly improves segmentation precision in images with very small or very high coverage. After this step we obtain a very good approximation of real segmentation.

2.3 Correction Phase

This phase handles some irregularities in data and thus improves the segmentation quality. Possible sources of irregularities are debris in liquid, phantom images of out-of-focus objects or discrepancies caused by rapid movement of cells (for example shrinking/spreading before/after cell mitosis). First we apply a morphological operation erosion to move pixels affected by halo effect from the cells area to the background. We also remove objects smaller than minimum cells size by labeling the cells area and counting number of pixels in individual segments. Then we compare labeling of pixels in the neighboring images in time. Pixels labeled as cells only in the current image belong very probably to some irregularities such as phantom images of out-of-focus objects. In this case we classify such pixels as background despite their original classification.

2.4 Further Notes on the Algorithm

In the whole algorithm there are several moments where we need to tune some parameters. In the first phase we must decide whether to compare neighboring images or some more distant ones. The size of blurring seems to be a less critical parameter and in all experiments we used the same value given in Sec. 2.1. First thresholding is without any parameters.

Blurring in the second phase depends on the size of cells (magnification of microscope) and type of cells. The proposed algorithm is not much sensitive to changes of blurring size. Most of the parameters are present in the third correction phase. Influence of the third phase depends on the quality of original data. When movement of cells is moderate and no dirt and/or out-of-focus objects are present in data, there is almost nothing to modify in this phase. On the other hand when data contain lot of these irregularities, proper setting of correction parameters can improve segmentation.

We have experimentally evaluated that the algorithm is fairly robust to the choice of parameters and that only one set of parameters was necessary for all images captured under similar conditions (same type of microscope, same frame rate and similar type of cells).

3 Results

The main purpose of our method is to substitute the manual labeling of images by automated one. Therefore we adopt criteria of biologist to measure the quality

of segmentation. To evaluate the quality of the segmentation we use manually segmented images and we take them as ground truth.

When we determine the biotoxicity of materials we must correctly treat dead cells. Cells which didn't survive in the tested environment usually look similar to live ones but they didn't move, especially we don't see any movement inside the cells. In our evaluation we will consider the dead cells as the background.

Manual processing of images is a very tedious and time-consuming process. Results depend on the thoroughness of a human operator and could be biased due to their different subjective preferences and perception. For evaluation of our algorithm we chose 70 images of several types of cells captured by two different types of microscopes (details in Sect. 3.1). Each image was manually segmented by two experts. This work took them about 12 hours of work. Nevertheless the similarity of the segmentation was only 94.8 % (94.8 % of pixels was labeled to the same category). More detailed information about the precision of the manual labeling is in Tab. 1 together with the algorithm precision evaluation.

Relatively small accuracy of manual labeling is surprising. Part of the difference is due to a slight displacement of the cell borders. The segmentation sometimes differ also in what is regarded as a living cell and what is not. In the case of L929 cells where the accuracy decreases below 90 percent the biggest mistake arose when tracing tentacles of the cells (see Fig. 1).

3.1 Tested Images

To evaluate the quality of our algorithm we used 70 images in total. Microscopes used to capture the images were Nikon Biostation a Olympus X51S8F-3. We used images of four cell types: MG63 human osteosarcoma, G10 human gingiva, HeLa cerivix epitheloid carcino and L929 mouse fibroblasts. Magnification of the microscopes was 20×, time interval between images was 2 minutes, resolution of the images was 1280×960 (Biostation) and 2288×1712 (Olympus). For our evaluation we chose images evenly spaced in the whole time sequence, thus the images include the vast majority of potential cases (initial states where most of cells are dilated and circular, usual variants where most of cells are separated, situations where the cells take up most of the images and cells are arranged in large clusters).

To compare manual labeling with automated one we used precision (P), recall (R) and F1 statistics defined as: $P = |TP|/(|TP| + |FP|)$, $R = |TP|/(|TP| + |FN|)$, F1-measure is harmonic mean of precision and recall. Abbreviations TP, FN and FP denote true positive, false negative and false positive respectively number of pixels classified in this way.

For comparison of manual vs. automated labeling we calculated the statistics twice, once for each manually labeled set. Then we took only those with better correspondence (higher score) for each image. (We assumed that lower score was caused by random errors in manual labeling).

When comparing two manually labeled sets, we considered one as the ground truth and second as tested segmentation. If we swapped the sets we must exchange the precision and recall measures but the F1 statistic remain the same.

The results for each series are shown in Tab. 1, examples of segmentation are in Fig 3.

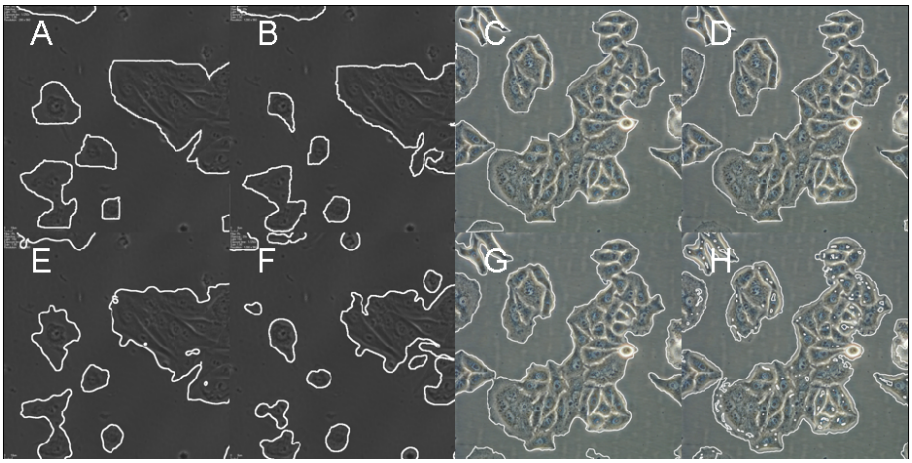

Fig. 3. Comparison of segmentation for MG63 (A, B, E, F) and HeLa (C, D, G, H) cell types: manual segmentations (A, B, C, D), our algorithm results (E, G) and TLA algorithm results (F, H).

The algorithm was implemented in Matlab using various built-in functions. The CPU timing (using Dual core 2.30 GHz) for segmentation of single frame was 1280×960 – 1.0 s, 2288×1712 – 4.0 s.

For comparison, we tested method from [2] based on active contours. Unfortunately this method failed to find all cells on the image. Although found cells were segmented well overall score was very bad. Therefore we didn't include score of this method in Tab. 1. The main problem lies in proper initializing the method. The manual initialization of active contours can improve precision but is very time consuming and thus of little difference from manual segmentation.

We also applied to all tested images the method present in the TimeLapseAnalyzer[8] – method woundhealing2. Although this method is meant for wound healing setting, this method is capable to be used also in regular time-lapse experiment.

Our method consistently scored better than the method from TimeLapseAnalyzer. The largest difference occurred for images with very high coverage ($>$ 95 %). Our algorithm also handled better the dead cells present in the images. In case of HeLa cells our method was about as accurate as manual labeling. This qualifies our method as a good candidate to substitute manual labeling.

The numerical values from our evaluation cannot be compared with the results in the literature. As can be seen, the accuracy depends heavily on the type of the cells and on the microscope type.

Table 1. Precision of the manual labeling and precision of the algorithm

Data	Statistic	MG63	G10	HeLa	L929
Manual labeling	P	0.90	0.96	0.95	0.88
	R	0.94	0.94	0.97	0.91
	F1	0.92	0.95	0.96	0.89
Our algorithm	P	0.88	0.91	0.97	0.61
	R	0.84	0.93	0.93	0.71
	F1	0.86	0.92	0.95	0.66
TimeLapseAnalyzer	P	0.64	0.79	0.90	0.54
	R	0.86	0.93	0.88	0.62
	F1	0.73	0.86	0.88	0.57

3.2 Universality of our Algorithm

Our algorithm can be used beyond system biology. It can be used to any time-lapse images. At Fig. 4 we can see its application to series of images showing a movement of caterpillars on a tree trunk[4].

Fig. 4. Segmentation of time-lapse images of caterpillars on a tree trunk, frames 6, 17, 44, 65, 86, 100

4 Conclusion

We developed a novel algorithm for segmentation of cells from time-lapse images acquired by phase contrast microscope. It is based on processing of time

[4] Could be found at http://bmumford.securewebsites.com/photo/creatures/

differences between images and combination of thresholding, blurring and morphological operations. Based on our preliminary results we can predict that our algorithm can be applied on wide range of cell types and various types of microscopes. Due to its speed it may be also suitable as a preprocessing step for some level-set methods.

We performed the analysis of the manual labeling and its precision. We compared the precision of our algorithm with manual one and realized that our algorithm can sometimes label cell similarly well as the human operator. Evaluation shows that our algorithm can be a good substitute for manual labeling.

The algorithm was implemented in Matlab and it is available on request from the authors. Program is currently being tested in the Tissue culture laboratory at Nové Hrady.

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