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Three-Dimensional Cell Counting based on Two Dimensional Cross-Section Images

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Introduction

Arabidopsis thaliana are short flowering plants related to cabbage and mustard. The importance of this plant has become more significant since it was the first plant genome to be sequenced. Many genetics and molecular biology studies have been developed in the last few years. In this research, the plant reaction to the nitric oxide (NO) exposure needs to be measured. It is currently unknown how the NO controls the root cell proliferation [1]. In order to measure the effect of this monoxide, the number of cells in the apical root tip of these plants needs to be obtained. A images processing method is presented here for carrying out the cell counting and the tracking operations.

This paper is organized in three different sections. The section 1 explains the methodology of the procedure. The second part discusses the techniques used to make the cell detections. The section 3 presents the cell tracking, and finally some conclusions and experimental results are presented in the last paragraphs.

Method procedures

The techniques of fluorescence cell detection have become a very useful solution for many bio-medical applications [2]. The process begins with the insertion of a fluorescence lipid which allows the detection of the cells distribution by the means of an optical microscope. The size and the cells translucency make the plant very well suitable for light microscopy analysis. The instrument also allows taking dissection images of each of these root tips at the same time. Each cross-section image varies only in the z-axis position along the root tips (Fig. 1). The gap between consecutive images is 1 micrometer and the number of frames or cross-section images taken for each root tip is 36. The resolution of these input images is 768x1024 pixels and their output type is Unsigned 8-bit integer with an Output Range from 0 until 255. The application also provides a tool to select the region of inters (ROI), so that only the wanted portion of the image is performed and the rest is filtered out.
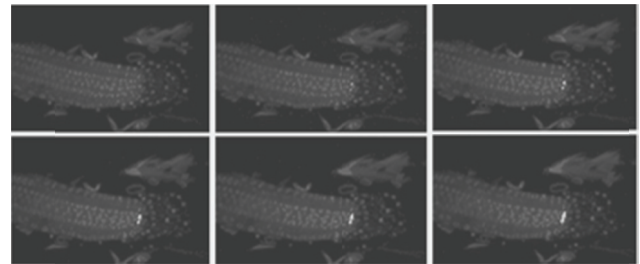

Fig. 1. Input images to be processed

Cell detection in microscopic images is still a complicated task due to the often blurred images, high noise and the difficulties of adapting and improving available image segmentation approaches. The proposed method is able to automatically detect and track the cells over all frames and to provide the number of found cells.

Some previous works [3,4] use semi-automatic tracking procedures to follow the cells over the frames; the selection of the cells has to be made in a previous step and afterwards the system starts to track the sequence of frames. Nevertheless, when the number of cells to track is very big, the task becomes a tedious work and an automatization is strongly demanded. This is the case of our method, in which the detection provides the initialization for the tracking procedure.

Other previous studies described in [5] implements correlation functions to accomplish the same aim of our project. However, these techniques cannot be used in this case because the size and the shape of the cells are very different depending on their age and position. Active contour methods can handle detections of different forms but they fail when the degree of overlap is high. The approach uses watershed algorithm for a similar cell segmentation purpose. They can separate a cluster if the seeds are well chosen, but excessive seeding results in over segmentation.

This paper proposes a cell tracking process that uses a combination of methods in order to reliably track individual cells which are overlapped between frames. An overall of all methods implemented can be seen in Fig. 2. Each and every single of them are described in the corresponding section.
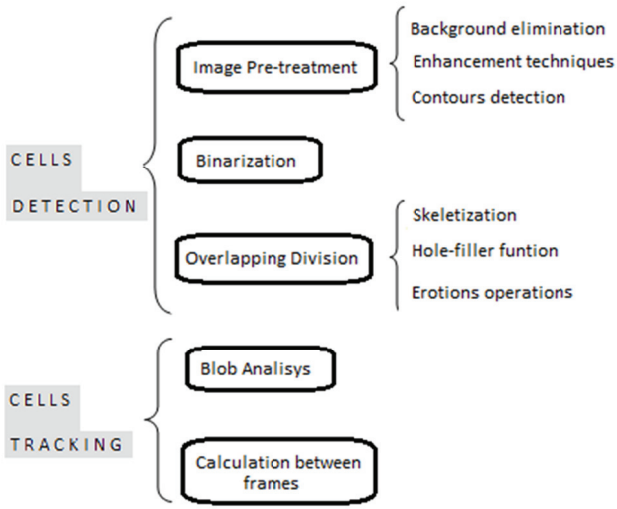

Fig. 2. Diagram of the methods implemented

Cell Detection

The cell detections process involves all the calculations related to the search of each and every one of the cells in all frames. This process brings with it many problems that need to be solved. The cell detection system must be able to detect all cells, regardless of the shape, size and position (overlapping). This is the main reason why the method proposed does not depend of a single one but a combination of different active methods. The test of diverse combinations of methods has been accomplished until obtaining the best result for our application.

Some image pre-treatment were performed in first place. The initial issue to be estimated is the value of the background pixels. In the input images, the background illumination is brighter in the center of the image than the other sides. This is the reason why some segmentation method applied failed. The segmentation techniques might delimit the background from the image clustering the images in different class. Nevertheless, the gray level similarity between the intensity of the background and the cells in many cases makes more difficult to obtain a good result, even when the number of class is very high. The alternative method used to estimate the background illumination is the morphological opening operation. This technique consists in an erosion followed by a dilation, using the same structuring element for both operations. The erosion process computes the minimum of each pixel's neighborhood, whereas the dilation computes the maximum. The formula used for performing the dilation is represented in (1)

$$G(j,k) = \left[F(j,k) \ominus H(j,k) \right] \oplus H(j,k), \quad (1)$$

The pixels in the neighborhood are defined by the structured element $H(j,k)$. The effect of the opening operation is to remove the objects that cannot completely contain the structuring element. The structuring element chosen is a circumference with a higher ratio than the bigger cell (15 pixels), so that it cannot fit entirely inside any cell (Fig. 3b).

Further on, some Image enhancement techniques were examined. The goal of image enhancement is to improve the perception of the image through modification of intensity function or image spectral content. For that purpose, histogram equalization method was initially tested. This technique did not provided good results because the noise of the image was highly increased. Better results were obtained utilizing contrast stretching. This approach maps the intensity of the image to a new range using thresholding operations as follows:

$$T(u) = \begin{cases} v_{min}, & if \ u < u_{min}, \\ v_{min} + \dfrac{(v_{max} - v_{min})(u - u_{min})}{u_{max} - u_{min}}, & if \ u_{min} < u < u_{max} \\ v_{max}, & if \ u > u_{max}. \end{cases} \quad 2)$$

In our particular case $u_{min} = 40$ and $v_{min} = 0$, therefore the values inferior to 40 are thresholded to 0, the rest are linearly mapped. No clipping is required, thus $u_{max} = 1$ and $v_{max} = 1$. This equation removes the pixels that have been tested that are not part of the image and spreads out the gray level intensity of image (Fig. 3c).

The cell density variation over the frames in all microscopy images makes the cell detection more difficult. Since there is not a single method that can detect cells perfectly without missing match or having false detections, a contour based segmentation algorithm is proposed and used in parallel in order to improve the final results. The approached used to find edges in the grayscale input images is the Canny edge detection algorithm. The low error rate and the better detection, localization and response are the reasons to use this technique.
Firstly, the algorithm attempts to filter out any noise in the original image by smoothing it. The mask of the Gaussian filter used has a standard deviation of σ= 1.4 and it is shown in (3)

$$K(k,l) = \frac{1}{159} \begin{bmatrix} 2 & 4 & 5 & 4 & 2 \\ 4 & 9 & 12 & 9 & 4 \\ 5 & 12 & 15 & 12 & 5 \\ 4 & 9 & 12 & 9 & 4 \\ 2 & 4 & 5 & 4 & 2 \end{bmatrix}. \quad 3)$$

The images are convoluted with the mask filtering out any noise before trying to detect any edge

$$O(i,j) = \sum_{k=1}^{m} \sum_{l=1}^{n} I(i+k-1, j+l-1) K(k,l). \quad (4)$$

Secondly, the Canny algorithm finds edges by looking for local maxima of the gradient of the input image. The gradient is calculated using the derivative of a Gaussian filter. Two mask are used, one estimates the gradient in the x-direction and the other estimates the gradient in the y-direction.

(5)

The magnitude or the edge strength of the process is calculated by computing the module of the two numbers obtained. The following approximation is also made.

$$|G| = \sqrt{G_x^2 + G_y^2} \approx |G_x| + |G_y|. \qquad (6)$$

The algorithm finally tracks along these regions to reduce the error rate by the means of using two thresholds. If the magnitude is below the first threshold, it is set to zero (non edge detection), if the magnitude is above the high threshold, it is set to 1(edge detection). Finally, if the magnitude is between the 2 thresholds, then it is set to zero unless there is a path from this pixel to a pixel with a gradient above second threshold.
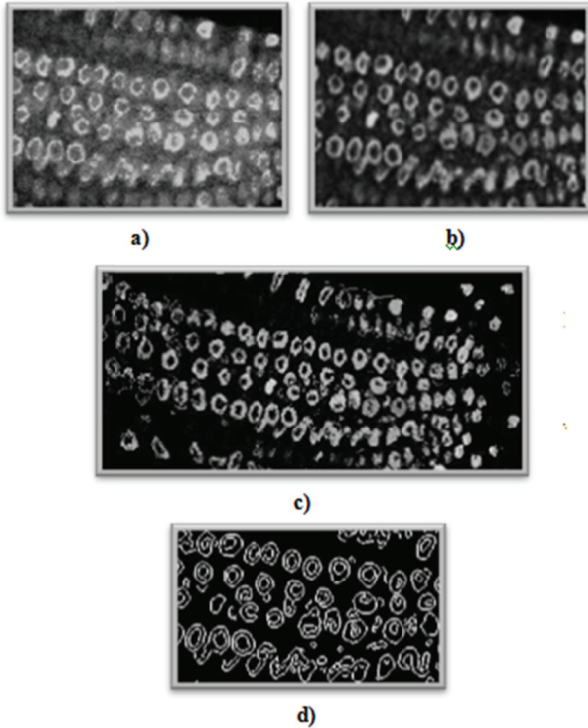

Fig. 3. Input grayscale image (a), background elimination (b), image enhanced (c), contours detection (d)

The image with the cell edges detected (Fig. 3d) is added to the previous image obtained (Fig. 3c) achieving new pictures with the contours enhanced. These images are the input for the binarization algorithm. However, before proceeding to the binarization part, a median filter is also utilized to perform noise reduction in the obtained images. Each output pixel contains the median value in the 2-by-2 neighborhood around the corresponding pixel in the input image. The window mask to implement the process is small to preserve edges while removing noise.

The binarization aim is to separate the objects of interest (foreground) from the background. The gray scale image is reduced to a binary picture where the background is represented by zeros and the foreground by ones. A global threshold did not generate good results since the intensity of the background is uneven. Adaptive thresholding provides more robustness to variations in illumination. An algorithm for adaptive thresholding of the image histogram is proposed. The following steps describe the way this approach works:

1) *Subregions selection.* Divide the input images into equal subregions (NxN pixels). A different threshold will be calculated for each of them. The size of these subregions can be defined by the user when the application is running.

2) *Overlapping subregions assignment.* Since the images illumination variations are smooth, a better result is obtained when the thresholds are calculated over subregions that are overlapped a certain number of pixels. The size of subregions size will be the same but overlapped between each other. The number of overlapping pixels can also be selected by the user.

3) *Threshold calculation.* After all pixels in the region have been assigned, the threshold is calculated using the Otsu method. The algorithm assumes that the image to be thresholded contains two classes of pixels (foreground and background). The algorithm tries to find the threshold value (T) where the sum of foreground and background spreads is minimal

$$\sigma^2_{Intra-Class} = n_B(T)\sigma_B^2(T) + n_F(T)\sigma_F^2(T), \qquad)$$

here, $n_B(T)$ and $n_F(T)$ are the the number of pixels in background class and forecast respectively, and $\sigma_B^2(T), \sigma_F^2(T)$ are the variance of intensities of each class.

The Otsu method proves that minimizing the intra-class variance is the same as maximizing inter-class variance

$$\sigma^2_{Inter-Class}(T) = \sigma^2 - \sigma^2_{Intra-Class}(T) =$$
$$= n_B(T) \cdot n_F(T)\left[\mu_B(T) - \mu_F(T)\right]^2. \qquad (8)$$

4) *Binarization.* Once the threshold value for each subregions is calculated the grayscale image is reduced to a binary image.

$$f_{kl} = \begin{cases} 0, & if \quad b_{kl}(i,j) \le T_{kl}, \\ 1, & if \quad b_{kl}(i,j) > T_{kl}. \end{cases} \qquad 9)$$

The binarization result (Fig. 4a) visually shows recognizable cells, but there are still overlapped cells and false detections that need to be removed. To improve these results morphological operations are performed.

Based on our experience, it was proved that objects smaller than 30 pixels were noise elements that did not form part of cells. A function was implemented to remove the small objects which have a smaller area than 30 pixels.

In order to separate the overlapped cells, two different functions are performed, the first one is a skeleton function and the second consisted of erosion operations. The fundamental idea of using the skeleton function is to reduce the image shapes to the minimal set of points. It removes pixels on the object boundaries but it does not allow objects to break apart. Therefore, the overlapped cells remained together but with the littlest number of pixels, what means that it will be easier to separate by means of erosion functions.

Because of the erosion function need to be very aggressive, a hole-filler function is previously implemented. This function sets to 1 all the area of dark pixels surrounded by lighter pixels. Although not all the cells has their boundaries completely closed and cannot get filled, the majority of overlapped cells are filled. This is the reason why the erosion process has to be very selective. Not all the cells can be erased as it would result in the loss of a lot of data. In order to do that, a blob analysis has to be made. Blob analysis is the identification and the study of the regions in an image that have all the pixels connected. Two cell properties need to be considered to make the selection process. One is the number of pixels in the region or area, and the other is the solidity. The solidity is the number of pixels in the area that has 8 connected neighborhoods. It was tested that the minimum area of the filler cells were 250 pixels and the solidity was higher than 40%. Once the cells selection is done, an erosion followed by a closing operation is performed to them.
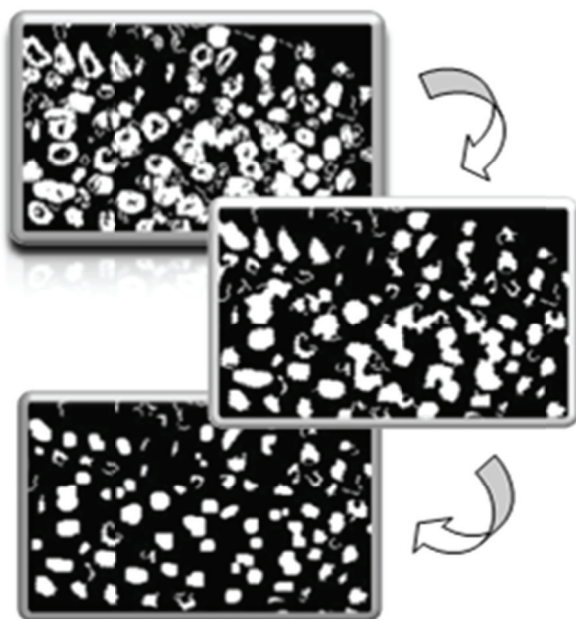

Fig. 4. Binarization result, holes filler function and result after the erosion computations

Cell tracking

The tracking system works with the processed images through the whole frame sequence. It is performed after all cell detection calculations are made.

A bob analysis is calculated in a very first place to determinate the number of cells detected in each frame.

Each detected cell is associated to its centroid in order to initialize the tracking procedure to quantify the total number of cells. It was observed that the cells size along the z-axis varied between 4 and 9 frames. The bigger the cell is, the more spread it is along the z-axis. It was also noticed that the centroid variation between consecutive frames was around 2-3 pixels and 7 pixels was never raised in any case.

The modus operandi of the tracking algorithm is very simple. It processes all the cell centroids along the 36 frames. For every centroid detected, the algorithm tries to find another centroid in the same position in the consecutives frames with a deviation inferior to the maximum observed. If at least a sequence of 4 centroids has been detected, then this is count as a cell. The cell is labeled with the same number in all frames in which it appears. The minimum number of frames is 4 for small cells and the maximum is 9 for the big ones. A noise false detection is rarely labeled because in the majority of cases it is not extended more than the minimum number of frames. An example of the tracking process is shown in the Fig. 5.
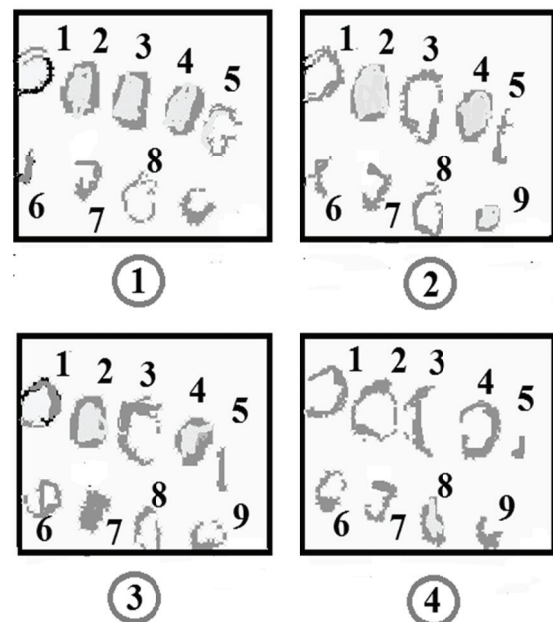

Fig. 5. Tracking process along the first 4 frames

Experimental Results

Using some image pre-treatment techniques, the input images were processes in order to prepare the images for a more successful binarization and removing the unwanted noise from them. The functions parameters and the functions order were changed many times until the observed results were as good as possible. If the contrast stretching threshold was raised more than 40, several poor illuminated cells at the left size of the images were eliminated.

If the Canny threshold were raised more than 60, a lot of noise was added to the images because many false cell boundaries were also detected. If this threshold was decreased, the edges of the poor illuminated cells were ignored.
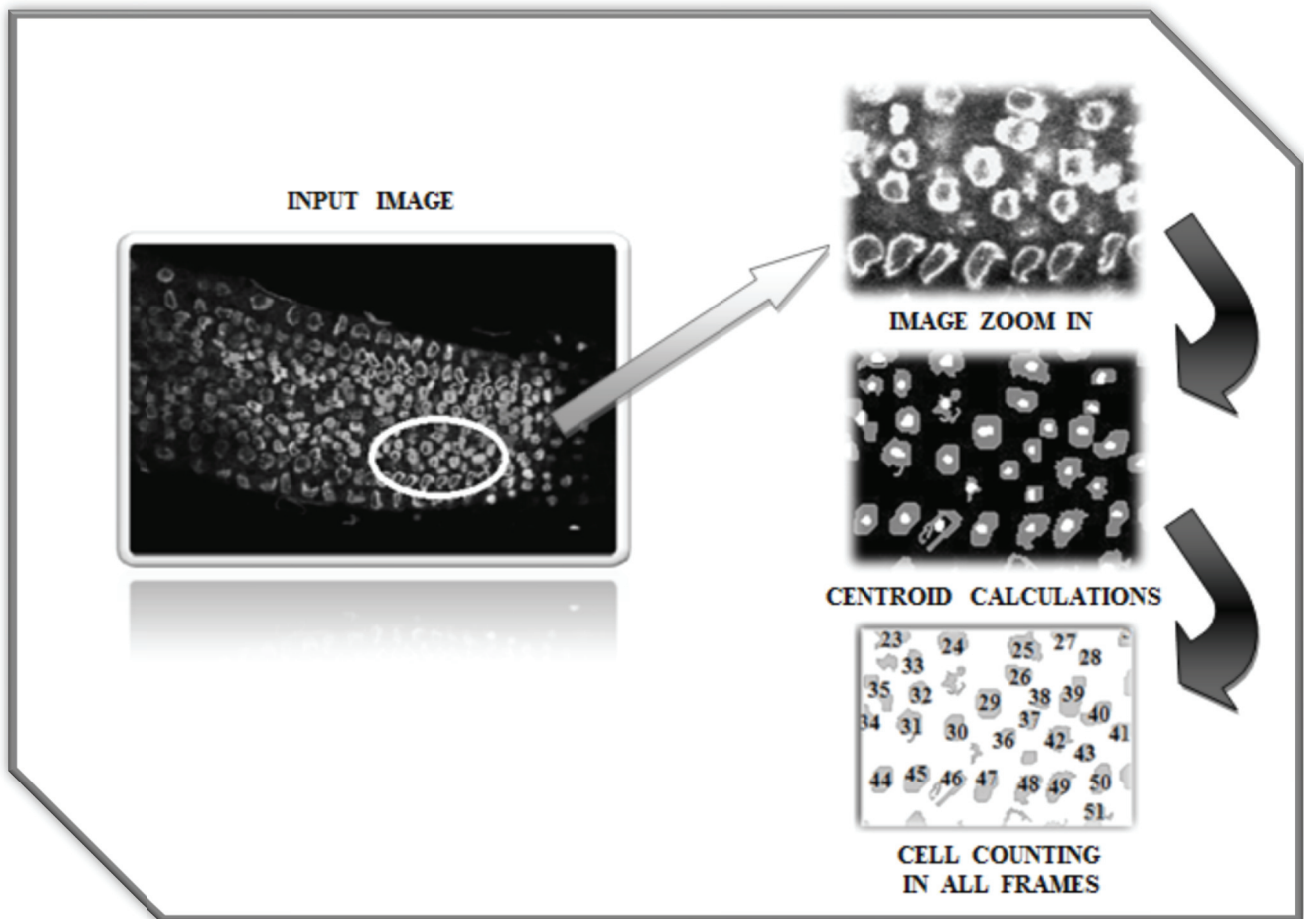
Fig. 6. Cells Tracking processes

Some other experiments were made to convert the gray level morphological image into binary images. It was observed that the best results were obtained with a subregion size of 50 pixels and an overlapping size of 25. Those are the options the program set by default. If the subregion size is very small, all cells are correctly detected with a very small overlapping, nevertheless, the processed images have a lot of noise around making very difficult to determinate which objects are cells and which are not. When the subregion size is settled to a high number, the overlapping cells increase gradually. Moreover, if the overlapping size increase more than 25 pixels, the computational time increase quite a lot and the result is practically the same since the illumination variation of the images are not bigger than that.

The final morphological operations generated in order to separate the overlapped cells cannot be more aggressive because it will remove the smallest cells. The program achieves to separate the 90% of the overlapped cells. It was also proved that 94% of the cells are tracked correctly along all frames. The cells that cannot be separated are normally tailed as a single one in the majority of cases. Moreover, if the Region of interest (ROI) is not carefully selected by the user at the beginning of the application, some surrounding root cap proteins can also be false detected as cells.

Conclusions

Boundary segmentation of adjacent cells is a very difficult and challenging task. In this work, we have developed an automated method for tracking the cells along cross-section images of different Arabidopsis root tips. This is a generic method, capable of segmenting different cell regardless of the size and shape.

The quantification results obtained for a set of test images exceed the result previously acquired using manual methods.

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This paper presents an automated method for effective quantitative measurement of cells in different Arabidopsis root tips. The plant reaction to the nitric oxide needs to be measured, therefore the root cell proliferation has to be calculated. Cross section images of these root tips are the input of the application implemented. It consists of a two parts methods which are presented here: 1) A combination of several image processing algorithms is proposed in order to overcome images defects (burry, overlapping) and correctly detect the cell distribution. 2) Using the information from the processed images, a three-dimensional tracking process over all frames is accomplished in order to calculate the total number of cells in each root tip. Practical simulations and experimental results are presented to show the performance and efficiency of this method for cell tracking. Ill. 6, bibl. 5 (in English; abstracts in English and Lithuanian).

J. Anton, T. Saar. Trimačių ląstelių apskaičiavimas taikant dvimačių vaizdų skersinius pjūvius // Elektronika ir elektrotechnika. – Kaunas: Technologija, 2011. – Nr. 6(112). – P. 89–94.

Pristatomas automatizuotas kiekybinis ląstelių matavimo metodas. Būtina įvertinti šaknies ląstelių proliferaciją tiriant azoto oksido įtaką augalams. Pasiūlytas algoritmas, gebantis apdoroti keletą vaizdų ir taip padedantis išvengti defektų. Pateikti modeliavimo ir eksperimentiniai rezultatai. Il. 6, bibl. 5 (anglų kalba; santraukos anglų ir lietuvių k.).