

Automatic detection of cellular necrosis in epithelial cell cultures

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ABSTRACT

Automatic discrimination and quantification of alive and dead cells in phase contrast microscopy images allows in vivo analysis of the viability of cultured cells without staining.

Unsupervised segmentation, based on texture analysis, classifies each image region into three groups: live cells, necrotic cells and background. The segmentation is based on three discriminant functions, built using a total of 12 parameters derived from the histogram and the co-occurrence matrix. These parameters were selected performing a discriminant analysis on a training set that included images from three different cultures. Once images are automatically segmented, the approximate number of live and dead cells is obtained by dividing each area by the average size of each cell type.

The number and percentage of live and necrotic cells have been obtained for primary cellular cultures in intervals of 48 hr. during two weeks. The results have been compared with the figures given by an experienced human observer, showing a very good correlation (Pearson's coefficient 0.95, kappa 0.87).

A reliable and easy-to-use tool has been developed. It provides quantitative results on phase contrast microscopy images of cell cultures, with preliminary results showing accuracy similar to that provided by an expert, allowing to count a higher number of fields.

Keywords: cell culture, texture, segmentation, cytometry, automated microscopy, co-occurrence matrix.

1. INTRODUCTION

Cell cultures are a common tool in biological research and assessments of cell viability are frequently required. Cells are cultured in a dish in specific conditions and the evolution of the population is studied under the microscope. With the use of a digital camera, images of the culture can be acquired, allowing computer analysis of the evolution.

Organ transplantation has experienced a great advance in the last decade due, among other factors, to the introduction of new immunosuppressors that allow to achieve a nearly rejection-free clinical evolution. One of these immunosuppressors, cyclosporine (CSA), has been shown to decrease the number of rejections but it may also be related to certain number of renal failures. Previous research has shown a direct effect of CSA on the proximal renal tubule¹.

In order to study the effect of two different immunosuppressors, an *in vitro* study is carried out. The experiment consists of adding two immunosuppressors (CSA and Tacrolimus, FK506) to different primary cultures of renal cells, measuring cell death kinetics. A third culture with no immunosuppressor is used as control. Cell death kinetics is measured by counting the number of cells and area occupied on each culture dish, analyzing images taken at different moments of their evolution. Starting on the fourth day, images are taken every two days and the number of alive and of dead cells is counted. To obtain significant statistics a large number of cells needs to be counted, so an automatic procedure would be desirable.

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The number of cells could be obtained accurately by identifying and classifying each cell on the image with some segmentation algorithm (border detection, followed by cell classification). However, this is a difficult task from the image processing point of view and no successful attempt is known by the authors. Instead, as alive and dead cells have different texture, a more feasible task is the identification of regions with each texture. This would allow to measure the area occupied by each class of cells, and the number of cells could be derived from their average size.

This paper presents an automatic method developed for cell counting. Images are captured with a camera mounted on the microscope, and transferred to a computer where regions corresponding to different type of cells are segmented. Texture parameters have been extracted from a set of training regions and discriminant functions were obtained based on the most discriminant of those parameters. Results and comparison with manual counting are presented.

2. MATERIALS AND METHODS

2.1. Sample preparation

All experiments were performed on primary cell cultures from swine. Proximal tubule suspensions were obtained from collagenase digestion of the renal cortex and isopicnic centrifugation on 45% Percoll gradient² and plated in plastic culture dishes (60 mm). Kinetics of culture with CSA were compared to cultures with no immunosuppressor and to cultures with FK506, as a positive control. Cultures were incubated at 37°C in a 95% air/5% CO₂ atmosphere. CSA was obtained from Sandimmun® and FK506 from Prograf®. In chronic toxicity experiments, cells were allowed to grow in the presence of CSA, FK506 or vehicle (control conditions) from zero time.

Images were acquired with an Olympus IX70 microscope with 100x magnification, and captured with a Sony DXC 151P color CCD camera. Monochrome (8 bits/pixel) 736 x 560 pixel images were transferred to a Pentium III computer for analysis.

Cultures are maintained for approximately fifteen days; during this period images are obtained every two days, starting from the fourth one. Three different dishes per treatment are chosen randomly. From each of them, seven images are usually obtained. Examples of images for two different days are shown in Figure 1. The aim of the method is to segment the regions of alive cells, dead cells and the areas with no cells.

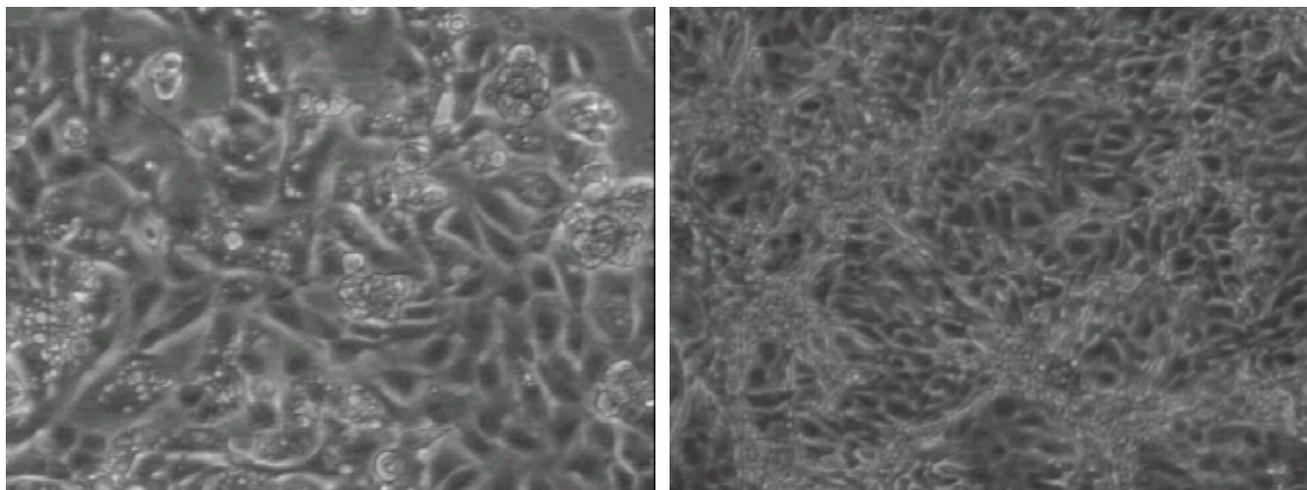


Figure 1. Images of the cell culture in day 4 (left) and day 11 (right)

2.2. Texture parameters

Texture parameters used in this application can be classified into first order statistics, computed from the histogram, and second order statistics, computed using the Co-occurrence matrix³. The normalized histogram is the probability density function for the gray levels of a specific region. If we denote by $p(z_i)$ the probability of each gray value z_i , the following parameters can be defined:

$$\begin{aligned} \text{Mean:} \quad \mu &= \sum_{z_i=0}^{N-1} z_i p(z_i) \\ \text{Variance:} \quad \sigma^2 &= \sum_{z_i=0}^{N-1} (z_i - \mu)^2 p(z_i) \\ \text{Skewness:} \quad \mu_3 &= \sigma^{-3} \sum_{z_i=0}^{N-1} (z_i - \mu)^3 p(z_i) \\ \text{Kurtosis:} \quad \mu_4 &= \sigma^{-4} \sum_{z_i=0}^{N-1} (z_i - \mu)^4 p(z_i) - 3 \end{aligned}$$

The histogram considers the gray level of each pixel separately and no spatial information is conveyed in these parameters. To analyze spatial distribution of the gray levels we make use of the gray-level co-occurrence matrix (GCM). Any GCM element $P_d(i, j)$ reflects the distribution of the probability of occurrence of a pair of gray levels (i, j) separated by a given distance d . The GCM is computed by mapping the gray-level co-occurrence probabilities based on spatial relations of pixels in different angular directions θ .

As with the histogram, a normalized version of the co-occurrence matrix can be computed, dividing each element by the total number of neighbors for each d and θ . These values depend on the texture (a rough texture should be better described with high values of d).

To improve computation speed, we can take benefit from the fact that the co-occurrence matrix is symmetric. The size of the matrix depends on the gray level resolution of the image. Texture parameters are invariant to gray level quantization. In this work, images were quantized to 4 bits/pixel before the matrix was calculated, to increase computational speed computation.

From the co-occurrence matrix, the following parameters were derived:

$$\text{Second order angular moment : } \mathit{AngScMom} = \sum_{z_i=0}^{N-1} \sum_{z_j=0}^{N-1} [p(z_i, z_j)]^2$$

$$\text{Contrast : } \mathit{Contrast} = \sum_{z_i=0}^{N-1} \sum_{z_j=0}^{N-1} (z_i - z_j)^2 p(z_i, z_j)$$

$$\text{Correlation : } \mathit{Correlat} = \frac{\sum_{z_i=0}^{N-1} \sum_{z_j=0}^{N-1} z_i z_j p(z_i, z_j) - \mu_x \mu_y}{\sigma_x \sigma_y}$$

$$\text{Sum of squares : } \mathit{SumOfSqs} = \sum_{z_i=0}^{N-1} \sum_{z_j=0}^{N-1} (z_i - \mu_x)^2 p(z_i, z_j)$$

$$\text{Inverse difference moment : } \mathit{InvDfMom} = \sum_{z_i=0}^{N-1} \sum_{z_j=0}^{N-1} \frac{p(z_i, z_j)}{1 + (z_i - z_j)^2}$$

$$\text{Entropy : Entropy} = - \sum_{z_i=0}^{N-1} \sum_{z_j=0}^{N-1} p(z_i, z_j) \log[p(z_i, z_j)]$$

$$\text{Sum Average : SumAverg} = \sum_{z_i=0}^{2(N-1)} z_i p_{x+y}(z_i) \quad p_{x+y}(k) = \sum_{z_i=0}^{N-1} \sum_{z_j=0}^{N-1} p(z_i, z_j) \text{ where } z_i+z_j=k=0,1,2,\dots,2(N-1)$$

$$\text{Sum variance : SumVariance} = \sum_{z_i=0}^{2(N-1)} (z_i - \text{SumAverg})^2 p_{x+y}(z_i)$$

$$\text{Sum Entropy : SumEntrp} = - \sum_{z_i=0}^{2(N-1)} p_{x+y}(z_i) \log(p_{x+y}(z_i))$$

$$\text{Difference variance : DifVarnc} = \sum_{z_i=0}^{2(N-1)} (z_i - \mu_{x-y})^2 p_{x-y}(z_i) \quad p_{x-y}(k) = \sum_{z_i=0}^{N-1} \sum_{z_j=0}^{N-1} p(z_i, z_j) \quad |z_i-z_j|=k=0,1,2,\dots,N-1$$

$$\text{Entropy of difference : DifEntrp} = - \sum_{z_i=0}^{2(N-1)} p_{x-y}(z_i) \log(p_{x-y}(z_i))$$

In these expressions, N is the number of gray levels, z_i are the different gray levels, $p(z_i, z_j)$ is the value of the GCM at point (i, j) , μ_x is the mean value of GCM values accumulated in the x direction and μ_{x-y} is the mean value of the distribution p_{x-y} .

2.3. Training: parameter selection and discriminant analysis

To select the most discriminant texture parameters⁴ and to establish the discriminant functions, a training set of 21 images (7 per type of culture) was obtained for each of the 5 days of study. From them, 222 regions of interest were extracted. Each region was manually classified as formed by alive cells, dead cells or background (dish). For each region, all histogram and co-occurrence matrix parameters were obtained using MaZda, a software developed at the Institute of Electronics, Technical University of Lodz, Poland⁵. Co-occurrence matrices for $d = 1, 2, 3, 4, 5$ and $\theta = 0^\circ, 45^\circ, 90^\circ, 135^\circ$ were computed.

In total, 229 texture parameters were computed for every region of the training set. The most discriminant variables were selected by means of a stepwise procedure, using an input and an output F-test to add and to eliminate variables. The F-test is based on Wilk's lambda, which measures the ratio of the variance in each group related to the total variance of all the variables. At each step the variable that minimizes lambda, considered together with previously selected variables, is chosen. Tolerance was computed to measure independence among variables. Tolerance of a variable X_j to variables $X_1, \dots, X_{j-1}, \dots, X_p$ is defined as $Tol_j = 1 - R_j^2$ where R_j is the multiple correlation coefficient of X_j and variables $X_1, \dots, X_{j-1}, \dots, X_p$. This procedure was applied using SPSS (SPSS Inc., Chicago, IL, USA). The 12 parameters selected are shown in Table 1.

Once the set of most discriminant parameters is obtained, discriminant functions⁶ are calculated to classify each sample into the three groups defined. We used linear discriminant or Fisher functions, of the following form:

$$D_s = B_{s1} X_1 + \dots + B_{sp} X_p + B_{s0}$$

where X_1, \dots, X_p are the quantitative independent parameters (texture parameters of the region) and $B_{s0}, B_{s1}, \dots, B_{sp}$ are the function coefficients.

Table 1 shows the coefficient of each parameter for each of the three discriminant functions obtained from the training set.

2.4. Implementation

Once the set of parameters and the discriminant functions were obtained, the segmentation process consists of computing all of them for a number of overlapping regions of the image. A running window of 32x32 pixels is moved along the image. Centered on that window, texture parameters are computed on a 60x60 pixel window. Based on these values, a

class is assigned to the pixels in the small window. The segmentation tool, shown in Fig. 2, was implemented using IDL (Research Systems Inc., Boulder, CO, USA). It allows the user to load a single image or a complete set of images that are automatically classified. The user can interactively correct the classification of any region. If desired, final results are saved to an ASCII file.

Table 1. Discriminant parameters and coefficients for each discriminant function.

	Alive Cells	Dead Cells	Dish
Percentile 99%	-0.964	-1.045	-0.691
InvDfMom(1,0)	30551	30603	31107
Entropy(1,0)	19650	19784	19777
AngScMom(1,-1)	-18022	-17895	-20953
InvDfMom(1,-1)	1120	863	1301
AngScMom(2,0)	18984	18904	22024
InvDfMom(2,0)	-1067	-940	-1737
InvDfMom(4,-4)	-4059	-4382	-4492
SumEntrp(5,0)	-12746	-12708	-12486
Contrast(5,5)	15	14	16
Entropy(5,5)	-2738	-2817	-3014
InvDfMom(5,-5)	5604	5993	6175
Constant	-15811	-15858	-16042

In order to estimate the number of cells in each class, the user introduces the mean size of each cell type either directly, or computing it by manually outlining a number of them. An interactive tool is also provided to manually segment several cells in order to compute the mean size. These values can be stored and used in subsequent sessions.

The program automatically computes the total area of alive and dead cells and their number as:

$$Number_i = \frac{Area_i}{Mean_size_i}$$

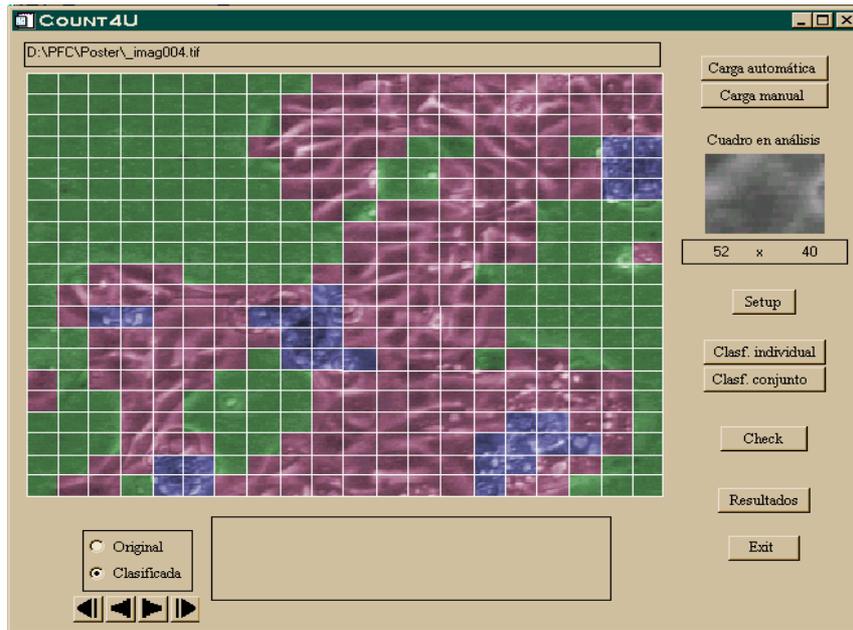


Figure 2. User interface of the application

3. RESULTS

Once the tool was deployed to end users, two different evaluations were carried out in order to assess the results of the automatic method. First, region classification was evaluated. In a second phase, cell number scoring was also evaluated.

The agreement in region classification has been evaluated by considering the number of rectangular windows that were correctly classified in a set of 4 images corresponding to different days. After the automatic classification, each image is divided into 400 regions (i.e., 1,600 regions in total) and the results are compared with the manual classification by a human expert (Table 2).

Table 2. Comparison of manual and automatic classification of regions

	Automatic classification			TOTAL
	Alive cells	Dead cells	Dish	
Alive cells	1069	29	26	1124
Dead cells	33	269	5	307
Dish	3	1	165	169
TOTAL	1105	299	196	1600

The diagonal correspond to perfect agreement among methods. A simple measurement is the rate of agreement, which corresponds to the observed agreement, computed as:

$$p_0 = \frac{\text{Number of agreements}}{\text{Total number of rectangles}} = \frac{1069 + 269 + 165}{1600} = 0.94$$

This rate of agreement between different image classification results can be considered as a reliability index in which a number of targets (image regions) are rated (classified) by different judges (methods). Another reliability measurement, known as the kappa coefficient (κ), has been proposed⁷ for this type of study. Kappa is a chance-corrected measurement of agreement, defined by:

$$\text{kappa} = \frac{p_0 - p_e}{1 - p_e}$$

where p_0 is the observed percentage of agreement between two methods and p_e is the agreement that would occur by chance. The expected number of randomly agreement is calculated as:

$$\text{Expected_cases} = \frac{\text{Row total} \times \text{Column total}}{\text{Total number of rectangles}}$$

In our case, p_e is 0.53, which gives a kappa of 0.87. The positive sign shows that the agreement is positive and it is close to the maximum value of 1. Therefore, there is a good agreement between methods in the measurement of areas.

To evaluate the results on the number of cells of each type, 15 images were selected randomly, from which the number of cells was computed both manually and automatically. The results are shown in table 3.

Table 3. Manual and automatic quantification of 15 images during 5 days.

	Day 4			Day 6			Day 8		
	Control	CSA	FK	Control	CSA	FK	Control	CSA	FK
Manual count	84	18	34	334	252	114	458	302	368
Automatic count	84	29	76	234	248	184	622	428	596

	Day 11			Day 13		
	Control	CSA	FK	Control	CSA	FK
Manual count	443	644	539	334	252	114
Automatic count	584	638	631	668	690	676

Altman⁸ proposed a simple method to compare classification techniques based on the analysis of individual differences. Differences between results are normalized with respect to the average of the results of the manual count, which are used as an estimate of the correct value. Figure 3 shows a graph of these differences.

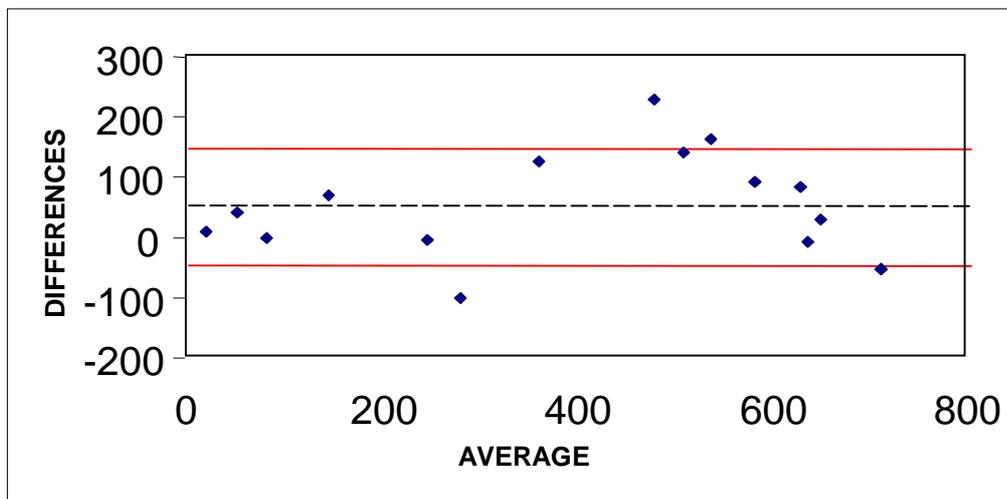


Figure 3. Normalized differences between manual and automatic scoring.

4. DISCUSSION

The proposed method provides quantitative figures (area and number of alive and dead cells) similar to those obtained by a human expert. Results of cell counting are worse than those for region classification. This may be due to the fact that the number of cells is calculated using a mean cell size. Cell sizes may differ significantly, specially in images taken after a few days of evolution. The final result of cell counting strongly depends on the particular selection of cells performed by the user for mean size calculation.

The number of ROIs of the training set used in the parameter selection step was small for the number of parameters used in the analysis⁹. A study with a larger number of ROIs could yield better and more reliable results.

In the present study, both windows used were of a fixed size. The size of the smaller window, pixels which are assigned to a certain class, determines the resolution of the classification. Using a smaller window increases the resolution but also the computational cost. The size of the window to calculate texture parameters must be chosen according to the expected textures on the image. We are currently exploring a multiresolution approach to obtain the optimal window size for each image.

The system developed could be used to segment cultures of other different cell types. The parameter selection and discriminant function calculation steps should have to be carried out for the new images.

5. CONCLUSIONS

An automatic system for scoring in vitro cultures of renal cells has been developed. From the clinical point of view, the system has two advantages. It allows to measure confluence, or degree of coverage of the dish with cells. On the other side, as segmentation is based only on a gray scale image, no staining is needed, thus not affecting cell evolution.

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