Supervised Mitotic Index Scoring as a Tool in Cytotoxicity Studies

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Abstract

The supervised classifier to be contained in an automatic PC based mitotic index scoring system is presented. An interactive statistical training based on size, shape and distance between objects measurements is combined with fast cluster identification techniques to produce an accurate count of cells and mitotic clusters. Results analysing lymphocytes culture preparations are presented.

Keywords: Image Analysis, Mitotic Index, Supervised Classification.

Introduction

Mitotic index is the percentage of cells in a culture which are in the process of division. It has proven to be a good an easy marker to evaluate and compare cell proliferation rate [1]. Normal cell proliferation can be affected by exposure to physical, chemical, and biological agents. This can lead to cytotoxicity, which is expressed as a decrease in cell proliferation, lowering the mitotic rate among other parameters, like DNA synthesis and cell viability. Cell toxicity analysis is used in chemical safety evaluation of new substances, mainly those involved in food and drug products. It is included as an in vitro test for every batch of vaccines. Cell proliferation evaluation is also used in the screening of new anticancer drugs, due to the fact that their action is to stop cell growth [2]. Conversely, in clinic, an increase in mitotic index in certain tissues is used as a diagnostic marker for some types of cancer.

The determination of mitotic index is usually made by light microscope analysis of slide preparations. The analyst identifies and counts thousands of cells, and reports the percentage of mitotic counts found among the cells. It is a long and tedious process, and the problem of subjective and biased identification is unavoidable. Several semiautomatic solutions have been proposed. Some commercial equipment is limited to the finding of potential mitotic clusters, which are analyzed visually by the expert. Some other solutions identify cells automatically, but require the visual identification of mitotic clusters to extrapolate to the whole preparation [3]. In many cases, an oil immersion objective (100x) is needed, as well as illumination adjustment in every field of view.

In the current study we introduce a new methodology which includes a supervised personal computer classifier to be contained in an automatic mitotic index scoring system. The classifier is initially trained to identify objects using a 20x objective. It classifies them based on cell size, cell shape, distance between chromosomes, and mitotic cluster size. Due to the nature of the objects to be classified, propagation and shrinking in pyramids of images was chosen as the most adequate technique for cluster identification. Results with lymphocyte cultures are presented.

Material and Methods

Culture preparation

0.5 ml of heparinized blood are added to 6.0 ml RPMI-1640 culture medium, supplemented with glutamine (2 mM) and non essential aminoacids (0.1 mM), in the presence of bromodeoxiuridine 32 μM. 2.7 % fitohemaglutinin is used to stimulate lymphocytes growth.

Cultures are incubated at 37° C for 72 hrs. 2 hrs before fixation of cells, Colcemid (2μg per culture) is added in order to stop cycling cells in mitosis. Fixation is done with Carnoy solution (methanol-acetic acid 3:1) after incubating cells in KCl 0.075M for 30 minutes.

Slide preparation

Quality of slides is a critical point for a good automated analysis of mitotic index. Therefore, fresh solutions ought to be used in fixation: cell pellets must be thoroughly washed as many times as needed, in order to eliminate cell debris. Pellets are resuspended in 0.5 ml fresh Carnoy and dropped onto cold alcohol moistened slides. Staining is done as described in [4].

Hardware and software specifications

The program was written in TURBO PASCAL 4.0 using an IBM AT PC computer operating with MS-DOS version 3.30. It is 20 Kbytes long, which corresponds to 800 lines of PASCAL code. The PC was employed as the core of the image acquisition and processing system. A frame grabber (Matrox PIP 1024B) plug-in card was used, together with a dedicated analog RGB monitor (NEC Mutisync) and a RS-170 monochrome TV camera (Panasonic), attached to an Olympus BH-2 microscope. 512 x 512 pixel resolution was used, with 256 grey levels. Halo 88 drivers and software library were used for displaying through a PGD high resolution monitor. The training process was performed using IMAL System (BIOCOM, France).

Digitization

The microscope's objective setup, field selection and image digitization through a standard vidicon RS-170 B/W camera are performed interactively. Before a single frame of the desired field is taken, adjustment of the input video signal's offset and gain is performed in a continuous frame grabbing mode. This is done by manipulating the digitizer's input Look-up Table (LUT) together with the camera's offset and gain, and the microscope's illumination adjustments. In this way, saturation is achieved in areas that are to be ignored and the full dynamic range of the digitizer's A/D converter is employed on the objects to be analyzed. Once these parameters are established, a whole set of images size 512 x 512 pixels is digitized under similar illumination conditions.

Training process

The system requires an initial training process in order to compute the mean and standard deviation of cell size, the mean and standard deviation of cell circularity, mean distance between chromosomes, mean area of mitotic clusters, as well as the mean ratio between background area and objects area. This is done interactively using the IMAL System. The expert segments manually the image, points to cells representative of a class, in order to obtain statistics. In another step, he (she) points to pairs of chromosomes, in order to compute distance between them. In a last step, he (she) surrounds mitotic clusters and points to them in order to compute mitotic mean area. It is possible to generate several classes of cells, as well as several types of mitotic clusters. Normal distribution is assumed in all classes. Statistics are kept in a disk file accesible to any spreadsheet type software.

Automatic segmentation

Images are segmented automatically, due to the fact that cells and chromosomes are dark spots, and background is much lighter and has a modal distribution. The histogram is analyzed automatically, using a priori knowledge obtained during the training process, of the percentage of the image corresponding to background. A local minimum in that area of the histogram is found, and this value is used to threshold the whole image and produce a binary image.

Cell identification

After automatic segmentation is done, and the image is binarized, it is scanned and all 4-connected components are identified using classical algorithms [5]. Their area and perimeter are computed, and together with their extreme coordinates are kept in a table. Components in the borders of the image are eliminated because of the impossibility of classification. All objects are processed by the minimum distance classifier, which assigns to each object its coordinates in a two dimensional space, where x represents its area, and y represents its circularity. The Mahalanobis distance [6] of each object to the mean values of the cells classes is computed, and only those objects with the probability (parameter given by the user) of belonging to a cell class are classified as cells, are counted, and are eliminated from the image.

The remaining objects are classified either large or small, regardless of their circularity, in order to eliminate large artifacts. This is done by computing their density using the cell area density function. Again, those objects with the probability (given by the user) of being large are eliminated from the image.

The rest of the objects are assumed to be either isolated chromosomes, clusters of chromosomes, or small isolated artifacts. This information is reduced in size using pyramids techniques, in order to accelerate CPU time, and afterwards is processed by the Mitosis Identifier.

Pyramids

The usage of pyramids technique [7] was chosen in order to accelerate processing time during mitosis identification. It is considered that cluster characteristics are preserved in a smaller resolution image, and its processing is less time consuming. Once cells and large artifacts are identified (see Cell identification), the 512 x 512 binary image assuming to contain only chromosomes, clusters of chromosomes, and small isolated artifacts is used as input at the bottom of a pyramid. The next level of the pyramid is computed averaging 4 x 4 windows, and a 256 x 256 image is obtained. A 128 x 128 third level is obtained in the same way. Again a binary image is generated and processed by the mitosis identifier.

Mitosis identification

Classification of clusters is done in resemblance to what the human eye identifies as objects close together. The procedure of propagation and shrinking is used to identify clusters of chromosomes in the low resolution image [8]. Propagation and shrinking is done as many times (divided by 4) as the mean distance between chromosomes computed in the training process, in order to connect components that distance apart. During propagation, pixels with value 0 are changed into 1's if they have any 1's as neighbors. During shrinking, pixels with value 1 are changed into 0's if they have any 0's as neighbors. As a result, large connected components appear over the clusters of chromosomes in the original image.

The image produced by the propagation and shrinking procedure is again analyzed by the connected components identifier. For all objects their density is computed using the mitosis area density function, and only those with probability (given by the user) of belonging to the class are assumed to be a cluster of chromosomes, which are counted, and together with the number of cells found, are stored as a final result in a disk file.

Results

The system we developed has been tested with preparations containing lymphocytes cultures. A typical image, as the one shown in Figure 1.a, is processed and analysed in aproximately 1 minute, with one class of cells and one class of mitotic clusters. The result is shown in Figure 1.b, where the count of nuclei of lymphocytes and mitotic clusters appears on the right hand side of the screen. With these few statistics, inhomogeneous illumination conditions, and great variability in cell and clusters size, a set of 15 images was processed. The mitotic index obtained automatically was compared to the visual result, leading to a difference of 1% (see Table 1). Although this result is highly encouraging, there is confusion between different types of objects.

Depending on the type of cells analyzed, it could be desirable to minimize this error. Although in this present study, the result obtained is within the accepted limits, there are several possibilities of improving the classification process. Round cell-like artifacts are likely to be counted as cells. As they are differentiated visually only by color, this confusion can be avoided using color information, besides shape and size of cells. Non-identification of very sparse mitosis, or confusion of compact mitosis with either cells or artifacts can be avoided by increasing the number of mitotic classes. The system allows the usage of as many classes as needed, which can help identify different types of mitosis, provided the classification time does not increase significantly.

Conclusions

The software we developed for obtaining the mitotic index of a culture has been proven to be reliable within the conditions of the present study. As in all supervised classification problems, its success rests on the quality of the training process. The classifier will become the core of an automatic mitotic index scoring system, integrated to a microscope with automatic slide device. Although several problems with illumination and segmentation are expected, results obtained this far are greatly encouraging. Validation with a larger data sample still remains, which will undoubtedly lead to an improved version of the system.

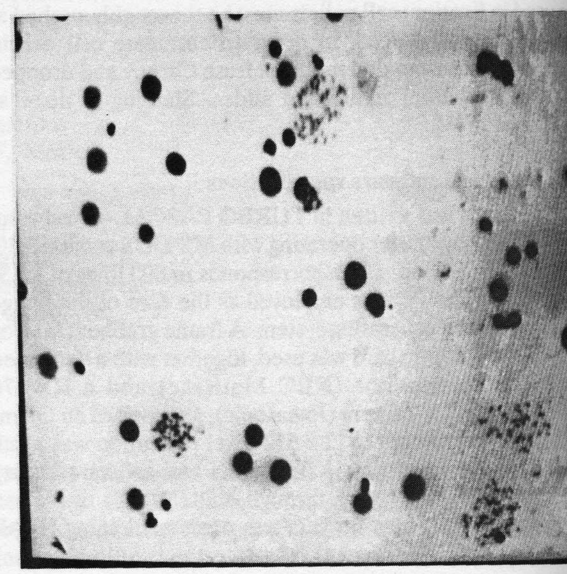

Figure 1.a. Original digital image using 20x objective, showing nuclei of lymphocytes and mitotic clusters.

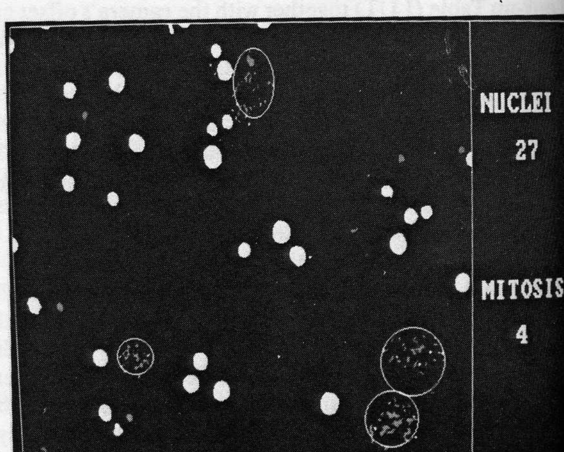

Figure 1.b. Clasification of image 1.a. showing the nuclei of lymphocytes and the mitotic clusters found.

Table 1. Comparison of visual and automatic scoring of mitotic index in a set of images. A difference of 1% was obtained.

VISUAL		AUTOMATIC		VISUAL	AUTO-MATIC
Cells	Mitosis	Cells	Mitosis	Mitotic Intex	Mitotic Index
331	16	308	18	0.048	0.058

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