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Automated Detection of Cytokinesis-blocked Micronuclei Using Fuzzy C-means Algorithm and Morphological Features

Jinggang An, Dandan Zhang, Datian Ye

***Abstract*—Increased frequency of micronuclei is positively correlated with the molecular dosimetry of genotoxic damages. The cytokinesis-block micronucleus test (CBMN test) is a well-established assay used in toxicological screening for potential genotoxic compounds. Since the method is simple and economical, CBMN assay can be employed on a large scale as a quantitative biological dosimeter. Automated detection of binucleated cells and micronuclei helps to increase the speed and reproducibility of CBMN method and is very beneficial for large-scale biomonitoring applications. Fuzzy c-means algorithm as well as mathematical morphology was employed in this preliminary study to identify the interested structures and helped to develop a robust comprehensive program to analyze the CBMN slides automatically. As a result, all the three kinds of binucleated cells with different nuclear morphologies were identified by our program. The structures that did not satisfied the criteria of binucleated cells and micronuclei were successfully excluded by the program.**

I. Introduction

THE development of industrialization, on the one hand, largely enhances the quality of life. Yet on the other hand, industrial activities increase the chance of exposure to chemical or physical genotoxins. The Japanese nuclear leakage happened in March this year is one of the examples that may seriously impair the health of human.

A micronucleus is the extranuclear chromosome fragments which are formed during the anaphase of mitosis or meiosis (Fig.1A-B). A micronucleus is morphologically similar to a normal nucleus but the diameter is between 1/16 and 1/3 of the latter [1]. Increased frequency of micronucleated cells and increased frequency of micronuclei in each cell have been proved to be positively correlated with the molecular dosimetry of genotoxic damages. The micronucleus test (*in vivo* or *ex vivo*) is a well-established assay used in toxicological screening for potential genotoxic compounds [2]. The most extensively used micronucleus assay, namely the cytokinesis-block micronucleus test (CBMN test), was developed and detailed described by Fenech and colleagues about 20 years ago [1, 3]. In CBMN test, the lymphocytes from human subjects exposed to genotoxins are cultured and stimulated by phytohaemagglutinin (PHA) to pass through one mitosis. Then the cytokinesis is blocked by cytochalasin-B thus allowing lymphocytes that have completed one nuclear division to be recognized by their binucleate appearance (Fig. 1C) [3]. Since the method is simple and economical, CBMN assay can be employed on a large scale as a quantitative biological dosimeter.

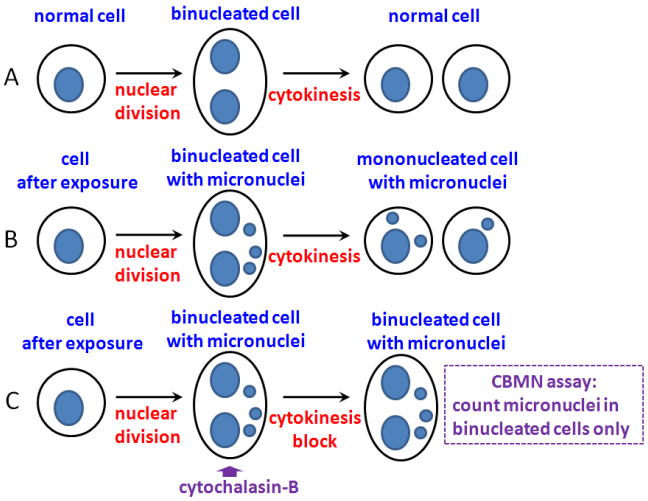

Fig. 1 Different types of nuclear division (schematic diagram). (A) normal mitosis: a mononucleated cell divides into two mononucleated cells; (B) after exposure: a mononucleated cell divides into two mononucleated cells with micronuclei; (C) after exposure and with cytochalasin-B added: cytokinesis was blocked so a mononucleated cell changes into a binucleated cell (or multinucleated cell) with micronuclei.

A detailed methodology of CBMN technique is described in the protocol paper by Fenech [4]. In general, two biomarkers of genotoxic exposure are essential in slide examination. *The micronucleus frequency* is defined as the number of micronuclei counted in at least 1000 binucleated cells divided by the number of total observed cells. *The micronucleated cell frequency* is defined as the number of binucleated cells containing micronuclei divided by the number of total observed cells. Although visual inspection of slides is easy to carry out, to search and discriminate thousands of cells is very time consuming. Automated detection of binucleated cells and micronuclei helps to increase the speed and reproducibility of CBMN method and is very beneficial for large-scale biomonitoring applications

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J. An is with the Department of Biomedical Engineering, Tsinghua University, Beijing, 100084 China (ankingkong@gmail.com). D. Ye is with the Biomedical Engineering Research Centre, Graduate School at Shenzhen, Tsinghua University, Shenzhen 518055 China (yedt6386@sz.tsinghua.edu.cn). D. Zhang is with the State Key Laboratory of Cognitive Neuroscience and Learning, Beijing Normal University, Beijing, 100875 China (zhangdd05@gmail.com).

For future correspondence contact D. Zhang (phone and fax number: 86-10-58802365).

[5]. Fuzzy c-means algorithm as well as mathematical morphology was employed in this preliminary study to identify the interested structures and helped to develop a robust comprehensive program to analyze the CBMN slides automatically.

II. Method

A. Acquiring of micronucleus images

According to the standard CBMN technique proposed by Fenech [1], fresh peripheral blood (0.3 ml) was collected by venepuncture and incubated for 72 hours in a 37℃ culture bottle with 4 ml culture medium. PHA was added at the beginning of incubation to stimulate nuclear division of lymphocytes. The cytochalasin-B was added to the cultures at 44th hour to stop cell dividing (i.e. cytokinesis blocking). After incubation, the cells were harvested and fixed on microscopic slides. Then Giemsa stain was performed to visualize chromosomes so nuclei and micronuclei could be identified by eyes or programs.

Slides were examined at 400 × magnification using a microscope (OLYMPUS CX21FS1, Japan) with a digital camera. The testing images (size: 640 × 480 pixels) used in this paper contained various cell morphologies, such as normally mononucleated cells, mononucleated cells with micronuclei, cytokinesis-blocked binucleated cells, binucleated cells with micronuclei, multinucleated cells, and naked nuclei (Fig. 2).

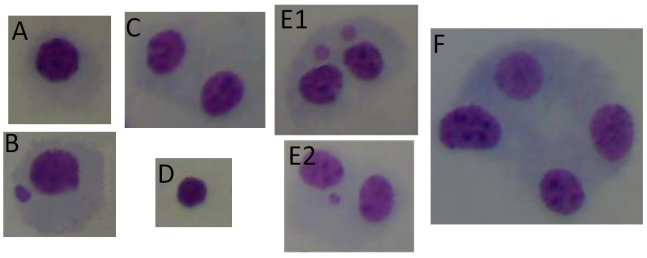

Fig. 2 Cell morphologies included in the testing images. (A) a mononucleated cell; (B) a mononucleated cell with a micronuclei; (C) a binucleated cell; (D) a naked nucleus; (E1-2) binucleated cells with one or two micronuclei; (F) a multinucleated cell. CBMN method requires to count micronuclei in binucleated cells only (i.e. E1 and E2).

B. Image preprocessing

In preprocessing, gray stretching (histogram equalization) and morphological operations were performed to increase the contrast level of images. Top-hat filtering computed the morphological opening of the image and then subtracted the result from the original image. In contrast, bottom-hat filtering subtracted the morphological closing from the original image. Combined use of top-hat and bottom-hat operators offered image enhancement to target objects. In particular, the original image was first added with the top-hat image and then subtracted by the bottom-hat image.

C. Pixel clustering by fuzzy c-means algorithm

The fuzzy c-means (FCM) was first developed by Dunn in 1973 [6]. It is a unsupervised clustering algorithm that calculates the degree of belonging to clusters of each point in a dataset.

Fuzzy segmentation of an image was carried out through an iterative minimization of the FCM objective function. The membership of each point and the cluster centers were updated in each iteration. [7].

Cluster number was defined as 3 before FCM iteration. At the end of computation, the mean of gray values in each cluster was calculated to automatically decide the cluster name. In particular, the cluster with the highest gray was labeled as image background; the cluster with the lowest gray was labeled as nuclei and micronuclei; and the cluster with a moderate gray was labeled as cytoplasm.

D. Target detection

After pixel classification, two more morphological operations, namely erosion and dilation, were employed to smooth the cell boundary and nuclear/micronuclear boundary. Then the cytoplasm area was region filled for nuclear searching. Simple morphological parameters, such as perimeter, area, and perimeter-to-area ratio (or curvature), were calculated to differentiate nuclei and micronuclei based on some predetermined thresholds [4].

III. Results

A. Detection of binucleated cells

In CBMN assay, binucleated cells were detected to limit the searching range for micronucleated cells and micronuclei. In an ideal situation, the two nuclei in a target cell had intact nuclear membranes and were situated apart within the cytoplasm boundary. This kind of normative binucleated cells could be easily detected by the program (Fig. 3A-B). There were sometimes binucleated cells with two nuclei touching each other at the edges. These binucleated cells were also accurately recognized by our method since the nuclear boundaries of each nucleus were distinguishable (Fig. 3C). In addition to the ideal binucleated cells and the cells with touched nuclei, CBMN technique also allows to count the binucleated cells with two nuclei connected by a narrow nucleoplasmic bridge (the bridge should be no wider than 1/4 of the nuclear diameter) [4]. In these cases (Fig. 3D), two pixels with the largest distance were first searched at the boundary of nuclear region. Then gray histogram was calculated on the direction given by these two pixels. The two peaks in the histogram were considered as two nuclear centers. Nucleoplasmic bridge was detected as with a large negative curvature.

In sum, all the three kinds of binucleated cells with different nuclear morphologies were identified by our program (11 ideal binucleated cells, 12 cells with touched nuclei, and 4 cells with bridged nuclei).

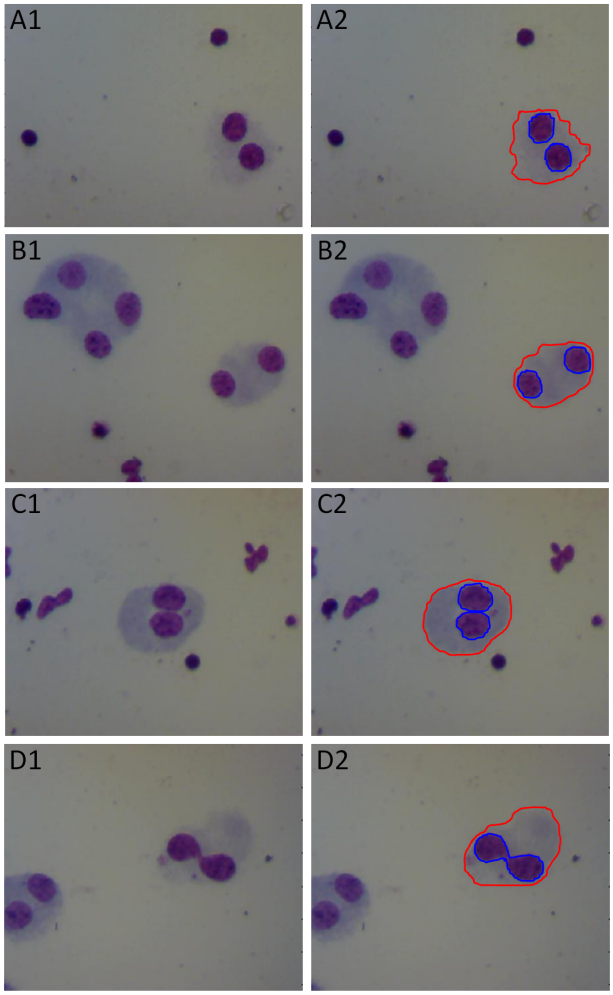

Fig. 3 Detected binucleated cells. (A) and (B) are ideal binucleated cells; (C) the two nuclei in the target cell touch each other at the edges; (D) the two nuclei in the target cell are attached by a narrow nucleoplasmic bridge. Left column shows the original images. Right column is the processed images, with red curves outlining the cell boundary and blue curves outlining the nuclear boundary.

The structures that did not satisfied the criteria of binucleated cells were successfully excluded by the program (Fig. 4). The principle of excluding illegal binucleated cells was that the cytoplasm boundary should be clearly distinguishable.

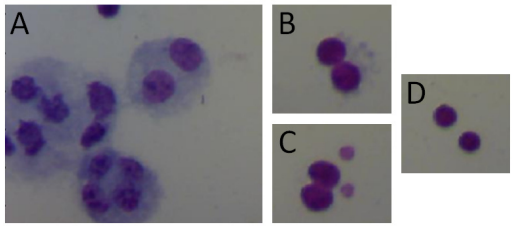

Fig. 4 Structures excluded by the program. (A) the membrane of binucleated cell (top right) did not separate from the membrane of adjacent cell so the binucleated cell was not suitable for micronucleus counting; (B) the membrane of the binucleated cell was not intact; (C) the membrane of the binucleated cell was not clearly distinguishable; (D) two naked nuclei with a close distance.

B. Detection of micronuclei

Micronuclei were detected inside the membrane boundary of the identified binucleated cells. The micronucleus area was limited to 1/256-1/9 of the mean area of the two nuclei. Eligible micronuclei should have clearly distinguishable nucleoplasm boundaries and were not connected to the nuclei. All 38 micronuclei in the testing images were detected successfully (examples shown in Fig. 5A-C). Chromosome fragments that did not satisfy the above criteria were excluded by the program (Fig. 5D).

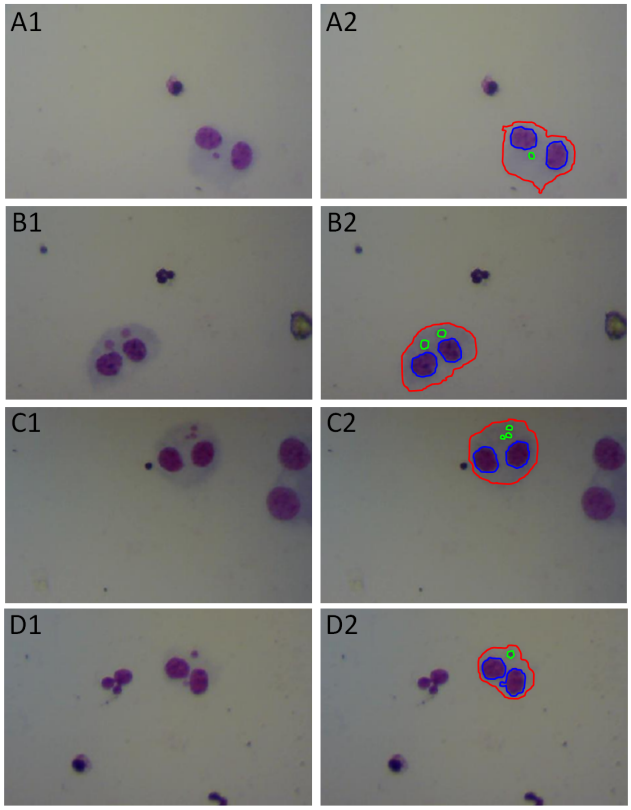

Fig. 5 Micronuclei detected by the program. (A-C) binucleated cells with one, two and three micronuclei, respectively; (D) only one micronucleus was identified in the binucleated cell. Another chromosome fragment was segmented into one of the main nucleus and did not recognized as a micronucleus. Left column shows the original images. Right column is the processed images, with red curves outlining the cell boundary, blue curves outlining the nuclear boundary and green curves outlining the micronuclear boundary.

C. Image processing GUI

We designed a graphical user interface (GUI) based on Matlab R2009a to integrate the image processing function in this study (Fig.6). After opening an original image, the main morphological parameters for binucleated cells and micronuclei could be manually set according to priori knowledge (or using default settings in the program). The detected targets were outlined and could be selectively displayed in the result image. The results of binucleated cell number and micronucleus number were shown on the screen and the processed images could be saved for further analysis.

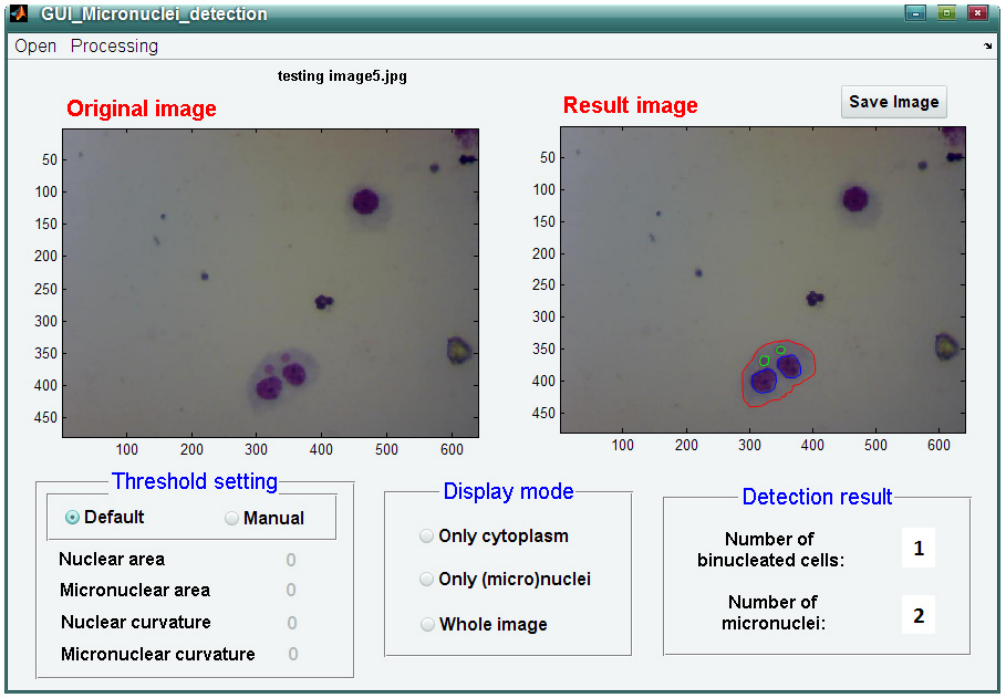

Fig. 6 The GUI developed for image processing.

IV. Discussion

The CBMN assay is the most extensively used technique for genotoxicity testing in cultured human lymphocytes. Many researchers and companies have been developing automatic image processing method for effective and accurate calculation of biomarkers such as the micronucleus frequency and the micronucleated cell frequency[5, 8]. In general, the detection algorithm employed by most of the existing system is mainly focused on processing of gray histogram (e.g. threshold segmentation of the histogram [9-10]). In this study, we employed the fuzzy c-means algorithm to classify pixels into three clusters, i.e. image background, cytoplasm and nuclei/micronuclei. Since the fuzzy c-means clustering is an unsupervised method, less priori knowledge was needed, making the image segmentation relatively more objective. Combining the fuzzy c-means classification and traditional empirical method based on mathematical morphology, the program correctly detected all the binucleated cells and micronuclei in the testing images.

The following efforts should be made in the near future to enhance the performance of the detection system: 1) a complete and large-sample image database should be acquired to rigorously test the result of the method; 2) the output of our system should be compared with other developed systems to investigate the advantages and disadvantages of our method.

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