Mutat Res Fund Mol Mech Mutagen 806 (2017) 9–18

Contents lists available at ScienceDirect

Mutat Res Fund Mol Mech Mutagen

journal homepage: www.elsevier.com/locate/mut

Automatic detection of micronuclei by cell microscopic image processing

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ARTICLE INFO

Keywords:
Micronucleus test
Cytogenetic analysis
Ionizing radiation
Image processing
Image segmentation
Computer-aided-design system

ABSTRACT

With the development and applications of ionizing radiation in medicine, the radiation effects on human health get more and more attention. Ionizing radiation can lead to various forms of cytogenetic damage, including increased frequencies of micronuclei (MNi) and chromosome abnormalities. The cytokinesis block micronucleus (CBMN) assay is widely used method for measuring MNi to determine chromosome mutations or genome instability in cultured human lymphocytes. The visual scoring of MNi is time-consuming and scorer fatigue can lead to inconsistency. In this work, we designed software for the scoring of in vitro CBMN assay for biomonitoring on Giemsa-stained slides that overcome many previous limitations. Automatic scoring proceeds in four stages as follows. First, overall segmentation of nuclei is done. Then, binucleated (BN) cells are detected. Next, the entire cell is estimated for each BN as it is assumed that there is no detectable cytoplasm. Finally, MNi are detected within each BN cell. The designed Software is even able to detect BN cells with vague cytoplasm and MNi in peripheral blood smear. Our system is tested on a self-provided dataset and is achieved high sensitivities of about 98% and 82% in recognizing BN cells and MNi, respectively. Moreover, in our study less than 1% false positives were observed that makes our system reliable for practical MNi scoring.

1. Introduction

The micronucleus (MN) assay is a common technique to estimate chromosomal damage in human populations exposed to different environmental, occupational or lifestyle factors and for in vitro genotoxicity testing [1]. MNi are small fragments that form whenever a chromosome or a fragment of a chromosome is not embedded into one of the daughter nuclei after cell division [2]. Their usage for biomonitoring is first introduced in 1973 [3–5]. The assay has been applied successfully for biomonitoring of in vivo and in vitro testing and also as a predictor for radiosensitivity and cancer risk of normal tissue and tumor [6–10]. MN induction in humans has been reported for erythrocytes, fibroblasts, hair root cells, buccal cells and human peripheral blood lymphocytes (PBLs). As such, lymphocytes remain the gold standard in toxicological screening [11]. The CBMN is a widely used method for measuring MNi to determine damage in cultured human lymphocytes. The scoring of MN reflects the chromosome mutations or genome instability accumulated before cultivation together with other lesions expressed during in vitro culture.

Although the visual scoring of MN is relatively easy, the scoring of the large number of cells is difficult due to the following reasons [12,13]:

1) The visual scoring of MN is time-consuming and a tedious task,
2) The accuracy of manually MN counting of MN depends on the technician's skill,
3) Technicians may have different understanding of the criteria for MN identification, so various counts will be reported by different technicians for a single slide. This matter weakens the reproducibility of the manual MNi counting.
4) The repeatability of manual MN counting is not reliable for inexperienced users meaning that a specific technician may reports various counts for a single slide in different time.

Designing and developing automated/semi-automated techniques for the radiation biology are widely spread in the biodosimetry and radiobiology laboratories. The CBMN assay is frequently used in radiation biodosimetry to estimate unknown doses by correlating the rate of MNi per BNC in PBLs to dose [14–17]. Automated detection and scoring of MNi in PBLs is proposed by using various methods for practical approaches to biological dosimetry [18–21]. In addition, automation of MN analysis provides faster and more reliable results than

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http://dx.doi.org/10.1016/j.mrfmmm.2017.07.012
Received 14 January 2017; Received in revised form 15 June 2017; Accepted 28 July 2017
Available online 12 August 2017

traditional procedure with minimizing subjective MN identification. Some effort is also done in this regard by researchers. Szirmai et al. [19] have presented automatic scoring of MNi in which they identify peripheral lymphocytes and their MNi by Cyt-B. The decision of the software is based on the contour lines of the cells, nuclei and MNi. The Giemsa color is used for staining and gray-level images are obtained. Objects are extracted by thresholding method which needs nuclei, cytoplasm and background be significantly different in intensity. Bocker et al. [20] proposed an algorithm for automatic MN assay in BN PBLs in two steps using empirical methods based on mathematical morphology. First, BNs with preserved cytoplasm are detected in a low magnification (x100) and their positions are saved. Then, MNi are detected in a high magnification (x630). Decordier et al. [22] designed an automatic system for the scoring of the in vitro CBMN assay for biomonitoring on Giemsa-stained slides. They first achieved optimum cell density by controlling the way of slide preparation. The scoring procedure is comprised of two steps: first, cells and nuclei are detected and second, MNi are sought in the detected cells. These objects are easily detected using some optic disk thresholding methods followed by some post-processing due to optimum slide preparation. Lyulko et al. [23] have developed software to score the frequency of MNi in BN lymphocytes automatically. Dual staining of the samples is done and cytoplasmic and nuclear images are processed separately to find MNi. They mentioned that Mono-nucleated to BN cells ratio might be useful to detect high doses as an extra symptom of cell proliferation. There are also some commercial systems designed for automatic MN scoring which are summarized afterwards. A priority system in this regard is Metafer MNScore (MetaSystems) [24] which has been widely used by researchers [21,24–26] for different studies such as technique validation, cancer research, radiation biodosimetry, etc. Metafer provides automatic detection of BN cells based on the adjacent nuclei and morphology. Then, an area of interest is defined around each BN cell and MNi are automatically detected in this area. The parameters used for BN cells and MNi are based on morphometric criteria such as size, aspect ratio, concavity. The system introduced by Decordier et al. [22] for MN scoring is well-known as IMSTAR Pathfinder™Screentox Auto-MN automated imaging system in which a standard protocol for slide preparation has been utilized and as mentioned beforehand successive steps are performed to detect cytoplasm, nuclei and MNi within cells. A different system for automatic MN scoring is iCyte® (CompuCyte), a laser scanning cytometry (LSC) system [27,28] that unites the abilities of flow and image cytometry. The application of LSC and its development systems has been investigated by researches [29–31]. A recent review is done by Fenech et al. [32] for automated micronucleus assays using image cytometry systems.

Some research [12,21,33] are also done that confirm automatic scoring of MNi outperforms conventional procedures, but there is still some limitation for these systems. In this work, we designed software for automatic scoring of in vitro CBMN assay for biomonitoring on Giemsa-stained slides which is able to detect BN cells even with various cytoplasm color intensity and vague MNi in peripheral blood smear. The paper is organized as follows. In Section two details of the proposed algorithm are explained as well as results of each step for more clarity and keeping the cohesion of the context. Final results and discussion are presented in Section three and four, respectively. Eventually, the paper concludes in Section five.

2. Materials and methods

In this section, first the protocol and method for preparing the dataset are described and the algorithm for image processing is explained afterward.

2.1. Dataset

Whole blood sample was taken from veins and was collected in heparinized vacutainer tubes. 0.5 ml of whole blood was added to 4.5 ml of culture medium including RPMI 1640 (Gibco) containing 15% FBS (Biosera), 1% of 200 mM L-glutamine (Biosera), 100 units/mL penicillin, 0.1 µg/ml Streptomycin. Cells were transferred to four 25 cm^3 flasks (Spl). Peripheral blood cultures for the CBMN assay were incubated according to standard protocol as described by Fenech et al. [1].

2.1.1. Gamma irradiation

The capacity of ionizing radiation to induce micronuclei formation in cells has been discussed previously [34]. Flasks were placed on a water phantom (10 × 30 × 30 cm) in a 15 × 15 cm field at 80 cm from the Co-60 (Phoenix Theratron) source with an average output of 60/79 cGy/min at dose of 2 Gy challenge dose. Dosimetry study was performed with Farmer-type 0.6 cm3 ionization chamber with a Farmer 2581 electrometer.

2.1.2. Slide preparation

After irradiation, 100 µl PhytoHaemAgglutinin (PHA) (Gibco) was added to the culture medium and cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2 in air. Cytochalasin B (Sigma Aldrich, USA) at the recommended final concentration of 6 mg ml^{-1} was added at 44 h after PHA stimulation. PBLs were harvested after another 28 h (1.5 doubling time) following to addition of Cytochalasin B and cells were fixed [35]. The cells were centrifuged at 300*g* for 10 min. The supernatant culture medium was discarded and the cells were hypotonically treated with 75 mM KCl. After adding saline, cells were fixed with methanol: acetic acid (3:1) (Merck, Germany) for three times. The cells were resuspended gently in a small volume of fixative solution and dropped onto clean glass slides. Finally, slides were stained using 10% aqueous solution of Giemsa dye (Merck, Darmstadt, Germany) for 20 min.

2.1.3. Imaging system

A Sony high resolution digital camera (model: DSC-H9) coupled to an Olympus CH40RF200 microscope is used for imaging. With an objective power of 40X, this microscope will yield a total magnification of 400. Images are stored in JPEG format with a res olution of 3872 × 2592 pixels, the maximum resolution of the camera. Totally, 200 images are captured for implementing the proposed algorithm.

2.2. Image processing algorithms

In this work, we are faced a problem in which various cytoplasm color intensity because of non-uniform staining, what is common in everyday staining procedures in laboratories. In order not to lose these group of cells, and since the cytoplasm of cells might be ambiguous, we assume that there is no cytoplasm for cells and the cytoplasm will be estimated from nuclei. This assumption is very useful for cases in which cytoplasm is not easily detectable. The block diagram of general system proposed in this paper for the detection of MNi is shown in Fig. 1 Each part of the block diagram is described in the following.

2.2.1. Overall nuclei segmentation

Fig. 2 shows the block diagram of proposed system for initial detection of nuclei within the input image. Since, resolution of captured

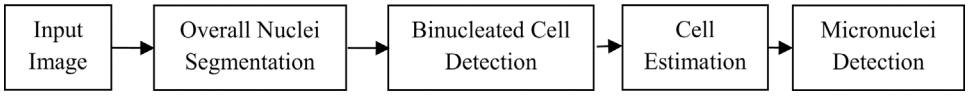

Fig. 1. The block diagram of general system for the detection of MNi while acquiring an input image.