REVIEW

Automated image analysis of micronuclei by IMSTAR for biomonitoring

Ilse Decordier*, Alexandre Papine[1], Kim Vande Loock, Gina Plas, Françoise Soussaline[1] and Micheline Kirsch-Volders

Laboratorium voor Cellulaire Genetica, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium, [1]IMSTAR, 60, rue Notre Dame des Champs, 75006 Paris, France

*To whom correspondence should be addressed. Tel: +32 2 629 34 28; Fax: +32 2 629 27 59; Email: idecordi@vub.ac.be

For many years, the analysis of micronuclei (MN) has been successfully applied to human biomonitoring of *in vivo* genotoxin exposure and provides a sensitive and relatively easy methodology to assess genomic instability. However, there is a need for automation of MN analysis for rapid, more reliable and non-subjective MN detection. In this review, we evaluate the application of automated image analysis of the *in vitro* cytokinesis-block MN assay on human lymphocytes for human biomonitoring, starting with the requirements that should be fulfilled by a valid and efficient image analysis system. Considering these prerequisites, we compare the automated facility developed in the framework of the European Union-project NewGeneris with other already published systems for automated scoring of MN. Although the automated scoring of MN is now put into place, extension to other cytome assay end points such as apoptosis, necrosis, nuclear buds and nucleoplasmic bridges would greatly enhance the specificity and sensitivity of future biomonitoring studies. Inclusion of these end points would also allow an automated approach to *in vitro* genotoxicity testing. In addition, automated scoring of the MN assay in exfoliated buccal cells would be very beneficial for large-scale biomonitoring studies, as cells can be collected in a minimally invasive manner.

Introduction

Human biomonitoring has become a central tool in environmental and occupational medicine and research in the identification, control and prevention of population exposures to potentially harmful compounds. Many approaches and techniques have been developed for the monitoring of human populations exposed to environmental mutagens. Human biomonitoring of early genetic effects requires accurate, sensitive, simple and not too time-consuming methodologies to assess mutations.

The analysis of micronuclei (MN) has become a standard approach for the assessment of chromosomal damage in human populations exposed to different environmental, occupational or lifestyle factors and for *in vitro* genotoxicity testing (1).

Scoring of MN is usually performed in peripheral blood lymphocytes (PBL) but MN can also be relatively easily scored in other cell types relevant for human biomonitoring, such as fibroblasts, exfoliated epithelial cells (from buccal, nasal mucosa or bladder cells in urine) and in erythrocytes [for review see ref. (2)].

The International Collaborative Project on Micronucleus Frequency in Human Populations (the HUMN project, http://www.humn.org) provided a detailed description of the scoring criteria for MN in PBL and used combined databases to assess intra- and interlaboratory variation in MN scoring, background MN frequencies and the influence of culture conditions, age, gender and smoking on MN frequencies (3–6). Furthermore, evidence was provided that baseline frequency of MN in PBLs is a predictive biomarker of cancer risk (7,8).

The cytokinesis-block micronucleus (CBMN) assay (9) is the most extensively used method for measuring MN in cultured human lymphocytes. This methodology allows distinction between a mononucleated cell, that did not divide, and a binucleated cell that has divided once. It was recommended by Kirsch-Volders and Fenech (10) to include mononucleated cells with micronuclei (MNMONO) for a more comprehensive CBMN assay for biomonitoring purposes. This parameter reflects genome instability accumulated *in vivo*, while micronuclei in binucleated cells (MNBN) indicate the chromosome/genome mutations accumulated before cultivation plus lesions expressed during *in vitro* culture (Figure 1).

As for many methodologies used in biomonitoring, the MN assay can have high inter-scorer variation, even under optimised laboratory conditions (3). Therefore, large cell numbers should be scored to generate statistically relevant data, especially for biomonitoring of populations characterised by low baseline MN frequencies as occurs in children (11). However, although the visual scoring of MN is relatively easy, scoring of thousands of cells is very time consuming. Therefore, although the MN assay is a well-validated biomarker of early genetic effects and easy to realise and score, there is need for automation of MN analysis for quicker and more reliable and objective detection of MN, allowing applicability on a large scale for environmental biomonitoring. This issue is still more critical when cohorts from different countries need to be compared, as is the case with our European Union-Integrated Project NewGeneris (Newborns and Genotoxic exposure risks, http://www.newgeneris.org), which investigates the role of prenatal and early-life exposure to genotoxic chemicals present in food and environment in the development of childhood cancer and immune disorders. Nevertheless, one cannot exclude differences even with the same instrument across laboratories. This could be due to modifications that different laboratories might make to the cell and MN classifiers that may be modifiable in the software or programme used.

Two different types of automated MN analysis are presently used, flow cytometry (12–14) and image analysis
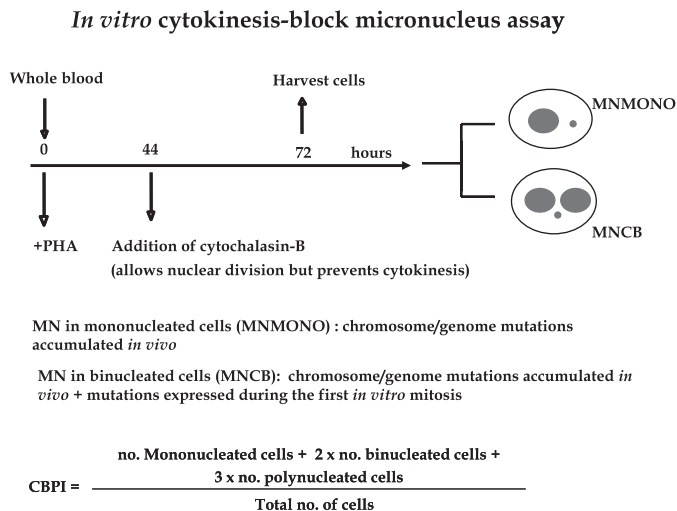
In vitro cytokinesis-block micronucleus assay

MN in mononucleated cells (MNMONO) : chromosome/genome mutations accumulated *in vivo*

MN in binucleated cells (MNCB): chromosome/genome mutations accumulated *in vivo* + mutations expressed during the first *in vitro* mitosis

$$CBPI = \frac{\text{no. Mononucleated cells} + 2 \times \text{no. binucleated cells} + 3 \times \text{no. polynucleated cells}}{\text{Total no. of cells}}$$

Fig. 1. Scheme depicting the protocol of the *in vitro* CBMN assay with the explanations of MNMONO, MNBN and CBPI.

(15–22,23,24). In this review, we consider the application of automated image analysis of the *in vitro* CBMN assay on human lymphocytes for human biomonitoring. We compare the efficacy and validation level of the automated facility we developed in the framework of NewGeneris (21), with other already published systems for automated scoring of MN (Table I). In addition, we discuss the knowledge gaps and future perspectives of automated MN scoring.

General concepts behind a valid image analysis system for the CBMN assay for biomonitoring

Owing to the fact that visual scoring in the CBMN assay is time consuming, several efforts to develop automated image analysis system have been made (15–18). The development of new computer software allowed the use of more advanced automated image analysis systems for MN scoring. To the best of our knowledge, besides the automated facility we have developed recently in collaboration with IMSTAR (21), only one other system has recently been introduced on the market, that by MetaSystems (19).

To develop a valid image analysis system for the CBMN assay for biomonitoring, one should start from a set of essential prerequisites.

(i) The detection algorithm should be well defined (a binucleated entity or the whole cell based on a cytoplasmic detection unit).

(ii) The automated image analysis system should be applicable to PBL. Since automation of MN scoring is particularly beneficial for human biomonitoring, PBL are the preferred cell types since they can easily be obtained in a minimal invasive manner, in large numbers and are synchronised. In addition, they can be seen as reflecting the overall body environment to which they are exposed since they are circulating throughout the body and have a relatively long lifespan. Furthermore, PBL can be used for both *in vitro* test and *in vivo* biomonitoring.

(iii) Both MNMONO and MNBN should be scored allowing the identification of genome instability events accumulated *in vivo* (MNMONO) and chromosome/genome

mutations accumulated before cultivation plus lesions expressed during *ex vivo* culture (MNBN) (10).

(iv) The MN scoring criteria should follow the HUMN project criteria (6).

(v) The automated image analysis system should be thoroughly validated, comparing automated versus visual scoring, either in a random sample survey of cases versus control or in PBL cultures from one or a few donors treated *in vitro* with mutagens. In the latter case, mutagens inducing MN by different mechanism (e.g. clastogens versus aneugens) should preferentially be used.

(vi) The false-positive (FP) and false-negative (FN) rates should be as low as possible.

(vii) Determination of cell proliferation should be included, by measuring mono-, bi- and polynucleated cells to obtain a measure of mitogen response and nuclear division. Moreover, one can calculate the cytokinesis-block proliferation index, which indicates the number of cell cycles per cell during the period of exposure to cytochalasin-B (26).

(viii) It should allow analysis of a large number of cells in less time when compared to visual scoring to detect small differences in MN frequency, thereby increasing the statistical power.

We aimed at developing the automated image analysis fulfilling these criteria in order to obtain robust, reliable and reproducible results that can be used for effective for biomonitoring. Comparison of the automated facility developed by Schunck *et al.* (19) with the system that we introduced (21) reveals that the main difference lies in the detection algorithms. We developed specific algorithms starting from the cell as a detection unit. The whole detection and scoring process was separated into two distinct steps: in the first step, the cells and nuclei are detected and then in the second step, the MN are searched in the detected cells. The specific algorithms developed for the detection of the cells and nuclei consisted of (i) cytoplasm detection for which the threshold is set automatically, based on the optical density (OD) histogram analysis. The thresholding result is filtered by size and shape to remove artefacts; (ii) detection of the nuclei regions for which threshold is set using the global analysis of the OD distribution, individually in every cytoplasm region detected previously; (iii) separation of the touching cells, avoiding cuts through the nuclear regions, using the watershed algorithm; (iv) enhancing the nuclei–cytoplasm and weakening the intranuclei contrasts; (v) detection of the nuclei candidates within the cells using the enhanced image; (vi) filtering of nuclei by shape, relative size and texture and (vii) filtering of cells by shape, size and number of nuclei. The specific algorithms developed for the detection of MN were (i) subtraction of the large background variations; (ii) detect the MN candidates by automatic thresholding. The threshold is set using analysis of global OD distribution and filtering by the relative size; (iii) filter the MN candidates by the relative (to the nuclei in the same cell) size, shape, colour, relative contrast and circular symmetry (21). A detailed description of the algorithms can be found in the supplementary material in Decordier *et al.* (21). MN are detected in both mono- and binucleated cells.

A similar approach starting from the cell as detection unit was used by Tates *et al.* (15), Castelain *et al.* (16) and Böcker *et al.* (18). The algorithms applied by Schunck *et al.* only detected binucleated entities, consisting of two similar nuclei

Table I. Overview of the automated image analysis systems for MN scoring

	Principle of detection algorithm	Protocol adaptation for slide preparations	Staining	HUMN scoring criteria followed	Scored parameters	Information on cell proliferation	Validation			Name and company of the system
							In vitro tested compound for validation	*In vitro* studies/ case–control studies	Comparison with visual MN scoring	
Tales *et al.* (15)	Cell (cytoplasm) → BN → MN	No	Gallocyanin (nuclei and MN)— naphthol yellow-S (cytoplasm)	Not yet available	BN, MNBN	No	Dose range of X-rays	*In vitro* study	Detection rate of ∼57%, FP: 10%; FN: not mentioned	Matrox Machine Vision Processor-Microscan B (The Netherlands)
Castelain *et al.* (16)	Cell (cytoplasm) → nucleus → MN	Yes	Feulgen-Congo-Red and Giemsa	Not yet available	MONO, BN, POLY, MNBN	Yes, but not discussed	1 dose of X-rays and MMS	*In vitro* study	Detection rate of ∼51%, FP: not mentioned; FN: 49%	Magiscan MD image processor— Applied Imaging (UK)
Verhaegen *et al.* (17)	Binucleated entity (2 touching nuclei) → MN	Yes (Vral et al. (25)	Giemsa	Not yet available	BN, MNBN	No	1 dose of X-rays	*In vitro* study	Detection rate of ∼89%, FP: not mentioned; FN: 12%	Discovery—Becton Dickinson Image Cytometry Systems (The Netherlands)
Böcker *et al.* (18)	Cell (cytoplasm) → nucleus → MN	No	Giemsa	Not yet available	BN, MNBN	No	Dose range of X-rays	*In vitro* study	Detection rate of ∼75%, FP: 6.8%; FN: 26.2%	MIAS hardware platform—Leitz (Germany)
Schunck *et al.* (19); Varga *et al.* (20)	Binucleated entity (→ MN)	No	DAPI	Yes	BN, MNBN	No	1 dose γ-rays		Detection rate of ∼34.5%, FP: not mentioned; FN: not mentioned	Metafer system— Metasystems (Germany)
Decordier *et al.* (21)	Cell (cytoplasm) → nucleus → MN	Yes	Giemsa	Yes	MONO, BN, POLY, MNBN	Yes: CBPI	Dose range of MMS; X-rays; NOC; Car and STP	*In vitro* study	Detection rate of ∼68.5%, FP: 1%, therefore manual validation step; FN: <10%	PathFinder™ platform— IMSTAR (France)
Willems *et al.* (24)	Binucleated entity (→ MN)	Yes	DAPI	Yes	BN, MNBN	No	Dose range of γ-rays	*In vitro* study for radiation biodosimetry	Detection rate of 87%, FP: 1%; FN: 1%	Metafer system— Metasystems (Germany)

BN, binucleated cells; CBPI, cytokinesis-block proliferation index; MMS, methyl methanesulphonate; NOC, nocodazole; STP, staurosporine, CAR, carbedanzim; DAPI, 4′,6-diamidino-2-phenylindole.

close to each other but completely separated, with or without MN, a methodology that was also used by Verhaegen *et al.* (17).

The fact that our designed software protocol started from the cell as a detection unit, and hence the identification of mono-, bi- and polynucleated cells, and MN in these different sub-populations of cells, allows the assessment of cell proliferation through nuclearity index, which is important for an efficient assessment of mitogen response and cytostasis in human biomonitoring as these are indicative of immune function and cytotoxicity (1,2,10,27). Moreover, scoring of mononucleated cells also allows the detection of MN in these cells (MNMONO) and allows a more comprehensive CBMN assay for biomonitoring purposes (10). This parameter does not only provide information on genetic damage but is also a measure for mitotic slippage (tetraploid mononucleated cells identifiable by DNA content measurement and nuclear size) in presence of microtubule inhibitors, which is important when investigating the aneugenic potential of chemicals [reviewed in ref. (28)].

For most biomonitoring studies, large numbers of samples need to be prepared in parallel and often by different laboratories. Therefore, it is crucial to reach high concordance between laboratories by applying the same cultivation, harvesting and slide preparation procedures. Therefore, we first aimed at implementing a well-standardised slide preparation protocol ensuring a high reproducibility and in order to perform an unambiguous and consistent image analysis. To meet this requirement, we first adapted the slide preparation protocol by mainly focussing on adjusting the hypotonic treatment to obtain uniformity in cell size. Besides this change in the hypotonic treatment, a few other steps in the slide preparation protocol were slightly adapted: cells were resuspended in a higher volume of fixative as compared to that used for the standard protocol before spreading onto slides. This was performed to obtain optimal spreading of the cells, without too much overlap, prior to slides being stained with 5% Giemsa (16) instead of 10% (29), in Sörensen buffer, to obtain an optimal contrast between the nuclei and cellular cytoplasm. Giemsa solution was freshly prepared and filtered twice to avoid artefacts in the detection due to debris present in the Giemsa solution (21).

Since the automated image analysis system for MN analysis was developed in the framework of NewGeneris for which the aim is to analyse MN frequencies in newborns, we also established an adapted protocol for slide preparation of whole blood cultures from cord blood.

Differences with cultures from adult peripheral blood are as follows: 1:3 dilution of the cord blood in phosphate buffer solution before cultivation and hypotonic solution of 90 mM potassium chloride (KCl) instead of 110 mM.

Vral *et al.* (25) also presented a modified slide preparation procedure for the *in vitro* CBMN assay. A fixation with a methanol/acetic acid ratio of 25:1 instead of 3:1 and a culture time of 64 h instead of the classic 72 h was proposed. This protocol was suggested since the slightly overlapping nuclei were essential for the detection algorithm of the automated image analysis system used by the same group (17). When using a 3:1 ratio, large numbers of binucleated cells have non-overlapping nuclei. In addition, a culture time of 64 h reduces the number of cells that have undergone a second or third mitosis yielding polynucleated cells, that were a source of artefacts for the automated system described (25). For our

automated MN detection system, we did not change the methanol/acetic acid ratio or the cultivation time of the PBL since we aimed at detecting mono-, bi- and polynucleated cells and the developed algorithms started from the cells as detection unit. Castelain *et al.* (16) also compared different fixation methods to fix cells, thereby varying the way to put the cells onto slides. Nevertheless, no robust standardised protocol was established at that time.

As automation of MN scoring is of particular benefit for human biomonitoring, whole blood cultures are preferred since only a small amount of blood is required. Most of the automated image analysis systems discussed (Table I) used slides prepared from whole blood cultures. Castelain *et al.* (16) suggested that purification of PBL after cultivation may improve the quality of the slides. The modified slide preparation protocol we established is applicable for both whole blood and isolated PBL.

To validate the image analysis system for the automated scoring of MN, we compared the results of automated and visual scoring of MN induced by different mutagens *in vitro* in whole blood cultures from a healthy donor. Two clastogens: ionizing radiation and methyl methanesulphonate (without threshold in dose–response); two aneugens: nocodazole and carbendazim (with threshold in dose-response) and a non-genotoxic apoptogen, staurosporine. For each compound, three concentrations and corresponding controls were analysed. Including both aneugens and clastogens allowed us to verify whether our system was able to detect small MN, which are usually produced following clastogen exposure. In addition, by including an apoptogen, we were able to verify whether our automated analysis system was able to discriminate between MN and apoptotic bodies. The other studies described used only clastogens to evaluate their automated image analysis systems. Comparison of automated versus visual scoring revealed that the IMSTAR system is able to produce biologically relevant and reliable results (21).

Similarly to our study, Castelain *et al.* (16) and Verhaegen *et al.* (17) used PBL cultures from one or a few donors treated *in vitro* with mutagens. Varga *et al.* (20) on the other hand evaluated the Metafer system developed by Schunck *et al.* (19) by comparing γ-rays induced MN in more donors. For a first evaluation, they used 76 persons: 27 with breast cancer and 26 female and 20 male controls. A further improvement of their system was confirmed in a validation sample of an additional 21 controls and 20 cases performed as a small prospective study. Later on, the system was applied to a larger population of breast cancer patients and controls (23), where a significant difference in MN frequencies was observed between cases and controls after *in vitro* challenging with γ-rays. The same system was recently used for the first time in a biomonitoring study focused on the measurement of MN for the assessment of chromosomal damage as a result of environmental mutagen exposure (22). In addition, the same software was used by Willems *et al.* (24) to demonstrate the suitability of automated MN scoring for population triage in case of large-scale radiation accidents, for which it is important to differentiate severely exposed individuals from those less exposed, in order to provide an efficient medical treatment and follow-up. In the very near future, biomonitoring data obtained with our automated image analysis system will be available: comparison of genotoxic responses in newborns versus their mothers (NewGeneris project) and the genotoxicity surveillance of workers exposed to cytostatics (I. Decordier, K. Vande Loock, P. Boogaerts, M. Hotat, M. Kirsch-Volders, unpublished data).

As far as FP results are concerned, our FP rate was not always below the required 1%. Therefore, we introduced an interactive visual validation step, in which is performed visually on screen by a scorer. However, this human interaction is not time consuming, provides a good quality control and allows re-analysis of the same sample. Moreover, we verified the inter-scorer variability of this manual interaction and did not find a significant difference. Verhaegen *et al.* (17) also introduced a manual operation step in which FP could be removed. Most of the other mentioned systems (Table I) did not report on FP rates (16,20).

As far as the FNs are concerned, the dose–effect curves produced with the automated system were consistently lower than the ones obtained by the visual scoring (22). This means some MN visible under the microscope are missed by the system. This phenomenon can be partly explained by the stricter scoring criteria imposed by the automated detection system. When scoring under the microscope, the user may slightly change focus in case of a doubtful object and thus resolve the MN. Whereas the automated system deals with the static images, so in the case of the slightly out-of-focus MN or doubtful object, it is systematically rejected. Although it is not possible to compare directly the different systems discussed (Table I) with the IMSTAR system, in all cases, lower MN frequencies were detected compared to visual scoring. Nevertheless, comparison shows that the IMSTAR system has a relatively high detection rate of 68.5% (21).

Further improvements and knowledge gaps relating to automated scoring of the CBMN assay for biomonitoring

Although the automated scoring of MN is starting to find its way in human biomonitoring, some further adaptations would improve its usability. First of all, a more efficient detection of MN should be reached. Secondly, since the CBMN assay can be considered as a 'cytome' assay (for review see ref. (30)), not only providing information on the chromosome breakage and loss but also on additional measures of genotoxicity and cytotoxicity, such as apoptosis, necrosis, cell division inhibition (by estimation of the proliferation index), nuclear buds (NBUDs) and nucleoplasmic bridges (NPBs), the automated detection of these different parameters would also contribute to the high specificity, sensitivity and completeness of biomonitoring studies. Therefore, specific algorithms for the detection of these different end points should be developed, following the HUMN scoring criteria, and a similar validation process as we performed for the classic CBMN assay should be performed in order to obtain a robust detection system for end points such as apoptosis, necrosis, proliferation, NBUDS and NPBs.

In addition, since our pathfinder system can be equipped with a filter for fluorescent light, the automated analysis of MN could be combined with FISH (fluorescence *in situ* hybridization) with pancentromeric probes to identify centromere negative or positive MN and with centromeric chromosome-specific probes to detect chromosome non-disjunction.

Future perspectives

Recently, the MN assay in exfoliated buccal cells is increasingly been used in epidemiological studies (for review see ref. (31)). Moreover, the HUMN project is currently focussed on harmonising and standardising the buccal MN assay. Since this methodology holds great promise as a minimally invasive method for human biomonitoring, automated scoring would be very valuable for large biomonitoring studies.

In addition to biomonitoring, automated image analysis of MN could contribute considerably to *in vitro* genotoxicity testing. The *in vitro* MN test has been recognised as a scientifically valid alternative for the *in vitro* chromosome aberration assay for genotoxicity testing by the European Centre for the Validation of Alternative Methods and several steps during the last 20 years have led to the approval by the Organisation for Economic Co-operation and Development of the *in vitro* MN test guideline 487 (for review, see Kirsch-Volders *et al.*, in this special issue (32)). Therefore, the automated image analysis system should be evaluated for *in vitro* genotoxicity testing. This implies that the system should be able to read slides prepared from different types of cell lines. Since several cell lines do not have a typical circular shape, specific algorithms will need to be developed.

Since the assessment of toxicity is essential in determining the appropriate dose range for investigating the genotoxic potential of a compound. Cytotoxicity has different aspects that include inhibition of nuclear division (i.e. cytostasis) as well as cell death by apoptosis or necrosis. Information of cell proliferation and on apoptotic and necrosis is essential. In our view, special attention should be paid to the development of robust algorithms for the detection of apoptotic and necrotic cells. In addition, an automated image analyser should not be restricted to the detection of only binucleated cells. Therefore, our IMSTAR system could be a very promising tool for *in vitro* genotoxicity testing.

Another issue that should be considered in the future is the application of automated MN scoring in nano-(geno)toxicology. Proposals are formulated in this special issue by Gonzalez *et al.* (33).

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