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HUMN project initiative and review of validation, quality control and prospects for further development of automated micronucleus assays using image cytometry systems

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ABSTRACT

The use of micronucleus (MN) assays in *in vitro* genetic toxicology testing, radiation biodosimetry and population biomonitoring to study the genotoxic impacts of environment gene-interactions has steadily increased over the past two decades. As a consequence there has been a strong interest in developing automated systems to score micronuclei, a biomarker of chromosome breakage or loss, in mammalian and human cells. This paper summarises the outcomes of a workshop on this topic, organised by the HUMN project, at the 6th International Conference on Environmental Mutagenesis in Human Populations at Doha, Qatar, 2012. The aim of this paper is to summarise the outcomes of the workshop with respect to the set objectives which were: (i) Review current developments in automation of micronucleus assays by image cytometry; (ii) define the performance characteristics of automated MN scoring using image cytometry and methods of assessment for instrument validation and quality control and (iii) discuss the design of inter-laboratory comparisons and standardisation of micronucleus assays using automated image cytometry systems. It is evident that automated scoring of micronuclei by automated image cytometry using different commercially available platforms [*e.g.* Metafer (MetaSystems), Pathfinder™ (IMSTAR), iCyte® (Compucyte)], particularly for lymphocytes, is at a mature stage of development with good agreement between visual and automated scoring across systems (correlation factors ranging from 0.58 to 0.99). However, a standardised system of validation and calibration is required to enable more reliable comparison of data across laboratories and across platforms. This review identifies recent progress, important limitations and steps that need to be taken into account to enable the successful universal implementation of automated micronucleus assays by image cytometry.

Introduction

Micronuclei (MN) are a chromosomal damage or mitotic abnormalities because they originate from chromosome fragments or whole chromosomes that fail to be incorporated into daughter nuclei during mitosis (Fenech et al., 2011a; Kirsch-Volders et al., 2011). MN are typically scored in cells that have completed nuclear division and the best validated method to do this, is to score MN immediately after nuclear division in telophase in binucleated cells which can be accumulated by blocking cytokinesis using cytochalsin-B (Fenech, 2007). The use of micronucleus (MN) assays in *in vitro* genetic toxicology testing, radiation biodosimetry and population biomonitoring to study the genotoxic impacts of gene-environment interactions has increased steadily over the

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past two decades. The lymphocyte cytokinesis-block micronucleus (CBMN) test is recommended by the International Atomic Energy Agency as a biodosimeter for the exposure to ionising radiations (International Atomic Energy Agency, 2011) and by the OECD for *in vitro* genetic toxicology testing (OECD, 2010). Moreover, scoring MN frequencies in cytokinesis-blocked human lymphocytes is a predictive biomarker for pregnancy complications, cancer risk and cardiovascular disease mortality (Furness et al., 2010; Bonassi et al., 2007; Federici et al., 2008). As a consequence there has been a strong interest in developing automated systems to score micronuclei, a biomarker of chromosome breakage or loss, in mammalian and human cells. Automation of MN analysis is needed for more reliable, faster detection and improved scoring accuracy, while reducing the need for highly trained personnel. Automated MN scoring also allows large scale analysis for multicentre cohort studies which are particularly important for population studies both in developed and in developing countries, which are suffering from different adverse environmental exposures that can harm the genome. Moreover it is almost impossible to do multicentre cytogenetic studies without a standardised system for slide preparation and an automated system for MN scoring which are required to eliminate the confounding effects of inter-laboratory and scorer variation. Automated procedures can play an important role in accelerating the process of unbiased data acquisition which can contribute effectively in risk assessment and help in understanding the association between environmentally induced chromosome damage and disease risks. Furthermore automated image analyses and automated cytogenetics, will play a pivotal role in triage and supportive medical management following a nuclear radiation mass-casualty event. This paper summarises the outcomes of a workshop on this topic, organised by the HUMN project (www.humn.org), at the 6th International Conference on Environmental Mutagenesis in Human Populations in Doha, Qatar, 2012. The aim of this paper is to summarise the outcomes of the workshop with respect to the set objectives which were to: (i) review current developments in automation of micronucleus assays by image cytometry; (ii) define parameters and processes for instrument validation and quality control and (iii) discuss the design of inter-laboratory comparisons and standardisation of micronucleus assays, using automated image cytometry platforms. Some additional information regarding automated MN scoring systems that was not discussed at the workshop is also included for completeness.

Why do we need automation in scoring micronuclei?

Analysis of micronuclei (MN) is a commonly used method with wide applications as evident from more than 7300 references found using the keywords “micronucleus” or “micronuclei” in PubMed database as of January 2013. However, both slide preparation and visual scoring of micronuclei are time consuming and laborious particularly when hundreds of samples need to be analysed rapidly, reproducibly and accurately. In comparison with other laboratory methods (*e.g.* ELISA, quantitative PCR or gene expression analysis arrays which allow analysis of up to thousands of samples per day), a highly trained microscopist or cytogeneticist can analyse micronuclei in approximately 8–12 samples per day reliably by visual scoring of at least 1000 binucleated cells per slide. Moreover, visual scoring usually does not involve keeping the full image galleries for future reanalysis and quality control and there is an increased risk, regardless of level of training, of within and/or between person variability in scoring due to scorer fatigue and/or differences in vision capacity and/or interpretation of scoring criteria (Fenech et al., 2003; Patino-Garcia et al., 2006). The two main methods of automated MN scoring are based on flow cytometry or image cytometry. Flow cytometry has important limitations relative to image cytometry: (i) the cell has to be lysed to release MN which can result in the release of other DNA containing bodies such as nuclear fragments from apoptotic cells or chromosomes from cells in metaphase/anaphase which confound the result as they appear as false positives; (ii) other nuclear anomalies such as nucleoplasmic bridges and nuclear buds cannot be scored which are otherwise scoreable in slide preparations; (iii) the sample cannot be preserved or archived in the event that re-analysis is required. For these reasons automated image cytometry (AIC) is the preferred option.

AIC for MN in binucleated lymphocytes was first attempted in the late 1980s (Fenech et al., 1988) and the early 1990s (Tates et al., 1990; Castelain et al., 1993), only a few years after the crucial description of the processing of samples based on the cytochalasin-B blocking of cytokinesis (Fenech and Morley, 1985). Later on, the development of improved computer algorithms allowed the application of more advanced image analysis systems for use in the CBMN assay *e.g.* Metasystems Metafer MNScore – preferably used for fluorescence microscopy of DAPI-stained samples (Schunck et al., 2004; Varga et al., 2004), IMSTAR Pathfinder™ Screentox Auto-MN preferably used for non-fluorescent (Giemsa) stained samples (Decordier et al., 2009) and Compucyte iCyte® Laser Scanning cytometer (Darzynkiewicz et al., 2011).

Methods were also reported on automated image analysis scoring of MN in bone-marrow or peripheral blood erythrocytes (Romagna and Staniforth, 1989) which however, is an assay rarely used in human studies relative to the lymphocyte CBMN assay. Exfoliated cells collected in a minimally invasive manner, such as from buccal mucosa, can also help as a screening test that can be used on large scale. Use of AIC technology needs standard operating procedures (SOPs) to guide training of researchers about the proper preparation and staining of cells on slides, the need for constant quality control and awareness of limitations and performance dependencies of the automated systems. International cooperation is a key approach to exchange the experience and transfer the knowledge from more experienced to recently established laboratories. Also, networking between laboratories and inter-laboratory comparison studies (Wilkins et al., 2008) can encourage the implementation of comparative research and validate the techniques for more meaningful and reliable results.

The significant achievements, current state of application, restrictions and recommendations for future research for all the above-mentioned AIC CBMN assay systems were recently addressed in a special issue of Mutagenesis (Rossnerova et al., 2011a; Decordier et al., 2011; Darzynkiewicz et al., 2011). Advances in molecular cytogenetics (*e.g.* use of centromere and telomere probes to distinguish MN resulting from chromosome breakage or loss) and use of recent automated scoring techniques in cytogenetic studies can play an important role in accelerating the process of scoring and improving diagnosis which is essential in large population studies.

Points to consider for an automated image cytometry system for micronucleus assays:

For an accurate assessment of genotoxicity using the MN test the optimal automated system based on image analysis of microscopy slide preparations should fulfil a set of technical criteria. Some of the critical criteria are detailed below but, these should not be considered as a definitive list:

- Ideally the system should be able to detect cytoplasm as well as nuclei and MN to allow mono-, bi- and multi-nucleated cells to be identified and quantified. This will enable MN to be scored

in all cell types along with the nuclear division index, an important biomarker of cytostatic effects commonly associated with genotoxicity, to be measured.
- The system should have high detection efficiency (detection of small MN frequency differences) and be able to detect MN in mononucleated and binucleated cells in the CBMN assay.
- Moreover the system should allow an assessment of MN from cultures at varying degrees of cytotoxicity where often the quality of cytogenetic preparation are not optimal at relatively high cytotoxic culture conditions.
- Another important aspect is the use of sufficiently high magnification to obtain the maximum confidence on the quality of the object to be accepted or rejected as MN.
- Besides the development of an automated system, protocol optimisation for slide preparation should be a prerequisite to allow a reproducible cell density/spacing for cytogenetic preparation, and reproducible homogeneous nuclear and cytoplasm staining.
- The system should be able to be used in different testing modes either for environmental *in vivo* biomonitoring where differences in MN frequencies may be small or for *in vitro* genotoxicity testing (chemicals or radiations) where cytotoxicity can be a challenging aspect for MN analysis.
- The system should allow some degree of flexibility and adaptability for inter-laboratory differences in cytogenetic preparations under different laboratory conditions although a protocol harmonization is recommended.
- One crucial aspect for chemical genotoxicity testing that should be considered during the development of an image analysis system, is the relocation of detected objects for artefact rejection. This is very important, especially at elevated cytotoxicity where a high level of artefacts related to cytotoxic culture conditions can compromise an accurate detection of micronuclei. Therefore storage of the stage coordinates of each object classified as MN during the automatic scanning should be essential. Moreover, the ability to characterise quantitatively the morphology and the position relative to the nuclei within the cytoplasm of the object classified as MN, provides an efficient tool for artefact rejection and, eventually, the assessment of accuracy using an internal standard for inter-laboratory comparisons and standardisation of micronucleus assays.
- Quantification of other challenging but interesting cytogenetic events such as nucleoplasmic bridges, nuclear buds, necrotic or apoptotic cells, when possible, can lead to a very powerful automated "cytome" system. Therefore, ideally, the system should also be able to recognise and quantify these events. Software that recognises and quantifies nuclear buds and nucleoplasmic bridges has recently been described (Sun et al., 2010; Zhang et al., 2010).
- The system should be built on a scalar architecture, allowing adaptability and freedom for inclusion or exchange of components (*e.g.* microscope, camera, slide feeder) depending on local requirements.
- The individual system components, including slide preparation and staining, should be standardised and Standard Operating Procedures (SOP) be developed and adapted for each platform.

Current developments in automation of micronucleus assays by image cytometry – a brief review of current systems and instruments

Metafer MNScore (MetaSystems)

The first commercially available system – Metafer MNScore(MetaSystems) became a widely used platform in many laboratories during a short period of time, which is documented by various publications of users focused on the validation of technique (Varga et al., 2004; Maes et al., 2007; Willems et al., 2010; Bolognesi et al., 2011), cancer research (Varga et al., 2005, 2006), biomonitoring studies in the field of air pollution (Rossnerova et al., 2009, 2011a,b,c), radiation biodosimetry (Thierens and Vral, 2009; Willems et al., 2010) and in cell lines in *in vitro* studies (Cariou et al., 2010; Doherty et al., 2011). This system, first introduced in 2004 (Schunck et al., 2004), allows the automatic detection of mononucleated and binucleated (BN) cells based on proximity of nuclei and morphology, and the consequent automated detection of MN, identified by their morphology, in a defined area of interest around the nuclei of the selected BN cells. The parameter set (classifier) used to identify BN cells and micronuclei in the automated scoring process is based on morphometric criteria (size, aspect ratio, concavity) for nuclei and micronuclei. Mononucleated and BN cells scored, with or without MN, are displayed in an image gallery, and the number of MN is shown in the corners of the respective display. Data are also shown as a histogram, allowing for fast selection of sub-populations (*e.g.* MN-positive cells). Different protocols for sample processing and slide preparation can be applied which may, however, vary in terms of accuracy of genotoxin dose estimation, reproducibility of the analysis and correlation with visual scoring. Besides these achievements, MetaSystems now also offers new improvements including the possibility of identification of mononucleated, binucleated, and multinucleated cells, automated detection of MN in binucleated cells with centromere FISH identification in MN and automated detection of MN in the erythrocyte MN assay (http://www.metasystems-international.com/).

Use of the Metafer automated MN assay platform in biomonitoring studies in the Czech Republic allowed obtaining probably the largest monothematic data-set of results with this system in a single laboratory in a relatively short time (Rossnerova et al., 2009, 2011a,b,c). The total published data-set includes 885 subjects (aged 0–65 years) investigated in connection with exposure to various concentrations of carcinogenic polycyclic aromatic hydrocarbons (c-PAHs), mostly to benzo[a]pyrene (B[a]P) from ambient air. These complex studies focused mainly on the model groups (city policemen, bus drivers, administrative workers, children, mothers and their newborns and laboratory workers). The participants in these studies lived prevalently in the capital city of Prague (a place with medium levels of pollution by B[a]P), some from Ostrava region (the most polluted region of the Czech Republic) and some from Ceske Budejovice (a regional city in a rural area). This valuable information allows a better understanding of the connection between the dose of pollutants and biological effect. These studies demonstrated the capacity of the system to easily evaluate high numbers of BN cells per subject from 1000 per subject (Rossnerova et al., 2009) to more than 6000 in the case of evaluation of a small group of laboratory workers (Rossnerova et al., 2011a).

Pooled data from above-mentioned studies allowed evaluation of the effect of age, gender and smoking on the frequency of MN measured by Metafer MNScore. The effect of age was analysed in a subgroup of 744 participants of our studies (repeatedly analysed subjects and smokers were excluded). The effect of age on the frequency of MN was previously analysed in a group of 445 subjects (Rossnerova et al., 2011a). The update of these results ($N = 744$, $y = 0.103x + 2.992$, $R = 0.63$, $p < 0.001$; where y is the MN frequency and x is age in years) confirms previously published data and their similarity with the data obtained by visual scoring in the Human MicroNucleus (HUMN) project which also reported a steady increase in MN frequency in lymphocytes with age (Bonassi et al., 2001) (http://www.humn.org). Gender is another well-known factor described in many studies which has an impact on the frequency of MN. The broad analysis from visual scoring data found females to have a 19% higher MN frequency than males (Bonassi et al., 2001).

Similar results were obtained by Metafer MNScore: 16% higher frequency of MN in girls in the control group compared to control boys (Rossnerova et al., 2011b), or a higher frequency of MN in a group of mothers from Prague (Rossnerova et al., 2011c) in comparison with a group of city policemen (Rossnerova et al., 2009) (both groups of similar age).

In the event of a large scale radiological emergency biological dosimetry is an essential tool that can provide timely assessment of radiation exposure to the general population and enable the identification of those people exposed, who need immediate medical treatment. A number of biodosimetric tools are potentially available, but they must be adapted, improved and validated for a large-scale emergency scenario. In the EU project Multibiodose (Multi-disciplinary biodosimetric tools to manage high scale radiological casualties, http://www.multibiodose.eu/) the validation of automation of the lymphocyte CBMN assay using Metafer MNScore for radiation exposure biodosimetry makes good progress. Calibration curves were established and blind samples after exposure under simulation of different exposure conditions (acute whole body and partial body exposure or protracted exposure) were analysed. Furthermore a control group was established for CBMN assay and scoring in triage mode was investigated (Willems et al., 2010). In 2011 a NATO biological dosimetry exercise was performed. In total, 10 blind samples of whole blood were irradiated (X-ray, 240 kVp) with doses between 0.0 and 6.4 Gy and shipped to the partners. Dose estimates were performed based on dicentric assay, the gold standard for radiation biodosimetry, and the CBMN assay. It was the first time that the dose estimations were performed using automated dicentric assay and CBMN assay in parallel with conventional manual scoring. The results simultaneously obtained with the automated assays in different laboratories looked very promising and gave reliable information for clinical triage decisions by allowing reliable estimates of exposure to 1 Gy or greater doses of low linear energy transfer ionising radiation (Beinke et al., 2012; Romm et al., 2012).

A recent validation exercise of MNScore for radiation biodosimetry focused on the frequency of binucleated cells with micronuclei (BNMN cells), considering the low efficiency of the system in detecting more than one MN in a single BN cell. Dose-response curves at different doses of gamma radiation obtained by fully and semi-automated scoring of BNMN cells were compared with visual analysis performed by an experienced scorer. A good correlation ($R = 0.705$) between visual and automated scoring with visual correction was observed over the dose range. A lower correlation ($R = 0.584$) was detected between the frequencies of BNMN detected by visual scoring and by automated system directly without visual correction. The results showed that a semi-automated approach involving visual checking of the gallery of automatically detected MN positive binucleated (BN) cells has to be preferred at the present time (Bolognesi et al., 2011). The analysis of the overall results obtained with the Metafer MNscore system allowed identification of the critical factors impairing the automated scoring. The presence of apoptotic nuclei and a wide cell size range decreases the efficiency of the system in detecting BN cells. In addition the system may fail to identify all MN if they are close or attached to the main nuclei or if there are more than one MN in the same BN cell. Two key steps in the protocol for sample processing and slide preparation can be optimised to improve the BN cell detection. A carefully controlled hypotonic treatment during the sample processing allows obtaining BN cells of the same size and the use of diluted cell suspension (4–5 drops/slide) in slide preparation leads to a monolayer of well separated cells. Cyto-centrifugation of samples can further help in producing highly reproducible slides with improved quality. With this method it is also relatively easy to adjust cell density for optimal automated scoring to enhance speed and accuracy of automated cell detection.

In conclusion, automated detection and counting of MN with the Metafer MNScore system is a reliable tool for the assessment of chromosomal damage. The main advantages of the system are as follows: (1) saves time (scanning of one slide lasts ~3 to 5 min assuming optimal density of cells), (2) minimises interpersonal variability, (3) keeps full image galleries as a virtual slide for future re-analysis and quality control, (4) flexible classifier settings (set of parameters for identification of BN cells and MN) which adjust to laboratory protocol, (5) the possibility to analyse large numbers of BN cells which is particularly important for improvement of statistical power of results, (6) determination of the ratio of mono- and binucleated cells, which provides important information about the proliferation kinetics of the investigated samples. Moreover, the effects of age and gender are comparable with the data obtained in the HUMN project.

IMSTAR Pathfinder™ Screentox Auto-MN

The application of the IMSTAR Pathfinder™ Screentox Auto-MN for automated scoring of MN in the CBMN assay was developed as part of the European Union Framework Programme NewGeneris ((Newborns and Genetic exposure risks, http://www.newgeneris.org/) aimed at exploring the possible role of *in utero* and maternal exposure to genotoxic compounds from diet and environment as a possible risk factor for the development of cancer during childhood. For this purpose, an automated image analysis system was developed, validated and implemented in three steps.

In the first step, the IMSTAR Pathfinder™ Screentox Auto-MNautomated Image Cytometry System (ICS) dedicated to MN scoring was developed in close collaboration between IMSTAR and VUB Cellular Genetic Laboratory. Particular attention was given to the crucial prerequisite for successful automated MN scoring which is a standardised slide preparation protocol as shown previously (Decordier et al., 2009, 2011). The IMSTAR Pathfinder™ Screentox Auto-MN automated imaging system for the CBMN assay is based on advanced unique algorithms, in successive steps, starting from the detection of the cytoplasm of each individual cell within the whole sample, then, accurate recognition of the number and morphology characteristics of nuclei, and micronuclei within every cell, thus enabling the MN frequency scoring in mono-, bi- or polynucleated-cells, the measurement of the cytokinesis-block proliferation index (CBPI), as well as the validation by an expert/technician. For this purpose, an extensive set of digital filters were applied in order to correct for intra-cytoplasm staining variations, non-homogeneous background, to take into account heterogeneous staining, to separate touching or overlapping cells, to avoid cuts through the nuclear regions, to detect the nuclei and micronuclei "candidates" within the cells, using enhanced contrast image, and finally, to eliminate the large majority of artefacts based on morphology criteria (shape, relative size and texture).

In a second step, validation of the automated scoring procedure was undertaken. Intercapture and interscorer variability was analysed for two clastogens (ionising radiation and methyl methane-sulphonate), two aneugens (nocodazole and carbendazim) and one apoptogen (staurosporine). Very similar results were obtained between different capturing sessions combined with the visual validation step. For each capturing and analysis session combined with the visual validation by two independent scorers, a clear dose-response in MN frequencies was observed. In addition, frequencies of binucleated cells with MN (MNBN) found for the five capturing sessions and the visual validation step performed by the two different scorers were highly correlated with those of the visual scoring, indicating the ability of the automated scoring system to detect biological differences as observed by visual scoring. In addition, comparison of the results of visual and automated scoring

of micronucleated mono- (MNMONO) and micronucleated binucleated cells (MNBN) was performed. Although the absolute MN frequencies obtained with automated scoring were lower as compared to those detected by visual scoring, a clear dose response for MNBN frequencies was observed with the automated scoring system, indicating the ability to produce biologically relevant and reliable results. These observations, together with its ability to detect cells, nuclei and MN in accordance with the HUMN scoring criteria, confirm the usability of the automated MN analysis system for biomonitoring (Decordier et al., 2009).

In the context of the NewGeneris project, the MN scoring data obtained with the **IMSTAR Pathfinder**™ Screentox Auto-MN system, were reported for single cohorts, in Crete (Vande Loock et al., 2011) and Norway (Hochstenbach et al., 2012). Cytologists of the different cohorts were trained at the VUB laboratory, to harmonize the slide preparation protocols. All CBMN assay preparations were analysed at the VUB laboratory by the same researcher to ensure that the data capture is standardised which is essential for multi-centre studies.

In the Crete cohort, statistically significant higher MNBN and CBPI levels were observed in peripheral blood lymphocytes of mothers compared to lymphocytes from cord blood of newborns. In newborns, MNMONO and MNBN were correlated ($r = 0.35$, $p < 0.001$) and a moderate correlation was found between MNMONO in mother and newborns ($r = 0.26$, $p < 0.001$). MNMONO frequencies in newborns increased with mother's BMI and decreased with gestational age while no predictors were found for MNBN or CBPI. In conclusion, the results indicate the importance of taking into account both mono- and bi-nucleated T-lymphocytes (Kirsch-Volders and Fenech, 2001) for biomonitoring of newborns, since the first one reflects the sensitivity of newborns to MN induction caused by *in utero* exposure and the latter is additional accumulated damage that is expressed *in vitro* in BN cells (Vande Loock et al., 2012).

In the Norway cohort, the link between MN frequencies and global gene expression was investigated in umbilical cord blood samples and compared with the CALUX®-assay for measuring dioxin(-like), androgen(-like) and oestrogen(-like) internal exposure, and acrylamide-haemoglobin adduct levels determined by mass spectrometry. Male newborns showed a higher number of genes significantly correlating with MN frequencies, when compared to female newborns (1397 *vs* 95) and, only in males, acrylamide and glycidamide haemoglobin adducts correlated significantly with MN frequencies (Hochstenbach et al., 2012).

In parallel, the design, development and validation of automated image cytometry scoring systems were implemented for a variety of preparation techniques, staining, for different testing modes, environmental *in vivo* biomonitoring and *in vitro* genotoxicity testing (chemical compounds, radiations), all involving visual review and validation of the gallery of MN positive automatically detected mono or binucleated cells. The detection of cytogenetic events such as nucleoplasmic bridges and nuclear buds is in progress, using an extended set of algorithms. In this context, the experience accumulated over the last decade could greatly contribute to the identification of the processes necessary for standardisation, evaluation and inter-comparison methods of such comprehensive cytometry systems.

In conclusion, the performance characteristics that have been or are being addressed by the IMSTAR system (http://www.imstarsa.com), which are relevant to all automated image cytometry systems for MN scoring, include:

- Detection of all MN in mono- and bi-nucleated cells and cell proliferation index evaluated by measuring the ratio of mono-, bi- and multi-nucleated cells.
- Functional parameters are automatically calculated during each slide scanning, following the HUMN scoring criteria (Fenech et al., 2003) as described in validation studies.
- IMSTAR image cytometry system provides for each cell type, all nuclei and MN characteristics and numbering; additional requirements for cytome analysis (8) are also included as it recognises and counts nuclear buds, nucleoplasmic bridges, metaphases, apoptotic and necrotic cells.
- IMSTAR has developed automated MN assays with lymphocytes, L5178Y mouse, HepG2, V79, CHO, TK6 cells using a variety of stains (Giemsa, Feulgen, DAPI, Acridine orange) with or without FISH centromeric probes.
- An accuracy measurement process enabling sensitivity and specificity (false negative, false positive) by comparative inter-scorer visual review validation (1–2 min/slide for review) and assessment of repeatability by results from comparative inter-capture/analysis.
- A scalable workflow is achievable, namely 12 slides/hour to 200–400 slides/day (with automated slide loader) depending on the hardware configuration, using a parallelised, multi-processor image processing server, for a minimum of 2500 cells to be detected.

iCyte® automated imaging cytometer, CompuCyte

Laser scanning cytometry (LSC) using iCyte® (CompuCyte Corporation, Westwood, MA, USA) is an alternative method suitable for automated micronuclei scoring which offers unique analytical capabilities that combine those of flow and image cytometry (Kamentsky, 2001; Darzynkiewicz et al., 1999; Henriksen, 2010; Henriksen et al., 2011; Luther et al., 2004; Peterson et al., 2008; Pozarowski et al., 2005). Unlike the fluorescence imaging analysis (FIA) instrumentation in which the fluorescence intensity of individual cells is recorded by charge-coupled device (CCD) cameras, in LSC it is measured by photomultiplier tubes (PMTs). The dynamic range of fluorescence intensity measurement by LSC is greater, offering higher sensitivity and accuracy in fluorescence measurement than FIA instruments. The dynamic range of PMTs is adjusted by altering the voltage applied to the device, whereas output signal levels from CCDs are a function of time: low-light samples require extended CCD exposure times. New generation (iGeneration: iCyte®-, iCys®- and iColor®-) LSCs provide fluorescence excitation with up to four laser wavelengths (selected from 405, 488, 532, 561, 594 and 633 nm) and four PMTs allowing fluorescence measurements in wavelength bands appropriate for the respective excitation lasers. Forward laser light scatter and/or laser light loss can be measured simultaneously with the fluorescence measurements, using photodiode detectors. Forward scatter measurement yields images similar to differential interference contrast (DIC; Nomarski illumination) while laser light loss measurements allow imaging and quantification of chromatic dyes. Combining three concurrent measurement modes namely fluorescence, scatter and absorption enables simultaneous utilisation of both fluorescent and chromatic dyes in the analysis.

Application of LSC for detection of MN was first reported in mouse erythrocytes (Styles et al., 2001). The greater challenge was to develop methods for use with nucleated cells as described below. DNA and protein of the cells subjected to MN analysis by LSC with fluorochromes of different emission colour is more advantageous than staining DNA alone, because ratiometric analysis of protein/DNA *versus* DNA content offers better means of MN identification than DNA content alone (Smolewski et al., 2001). A variety of fluorochromes can be used to differentially stain cellular DNA and protein within a given sample. A simple approach, in which fluorescence is excited with a single 488 nm laser, utilises propidium iodide (PI) and fluorescein isothiocyanate (FITC) as DNA and

protein fluorochromes, respectively (Crissman et al., 1985). The use of PI to selectively stain DNA requires removal of RNA which is accomplished by incubation of the fixed and permeabilized cells with RNase A.

The strategy of setting the threshold contour on green (FITC) fluorescence combined with selection of cells within a specific range of cellular DNA content can be used to adapt the CBMN cytome assay to LSC. Specifically, the cytochalasin-arrested binucleated cells are expected to contain DNA content between 2.0 (both nuclei in G1) and 4.0 DNA index (DI) (both nuclei in G2). However, the binucleated cells with 2.0 DI overlap on DNA content frequency histograms with single-nucleated G2-phase cells. Furthermore, the tetranucleated cells containing G1-phase nuclei may have 4.0–8.0 DI DNA content and overlap in DNA content with binucleated cells containing G2-phase nuclei. Therefore, the range of cellular DNA content between 2.2 and 3.8 DI is the most reliable to represent the binucleated cells. Indeed, imaging of cells whose PI fluorescence (DNA content) was within this range confirmed that 80% of these cells were binucleated (Smolewski et al., 2001). The remaining objects were aggregates consisting of two or three cells in close proximity to each other. The frequency of MN in binucleated cells detected visually by microscopy at different mitomycin-C concentrations, correlated well with that assessed by LSC. When the frequency of MN in the same specimens was analysed visually by microscopy and compared with that assayed by LSC, in double-blind tests, rather good correlation ($r = 0.87$) was observed between both assays (Smolewski et al., 2001).

Previously mentioned studies (Styles et al., 2001; Smolewski et al., 2001) were done very early in the development of LSC technology. With the advent of new iGeneration® LSC technology high resolution imaging, new generation image analysis and the ability to add more relevant markers significantly enhanced MN analysis. For example, more recently, LSC has also been used successfully at the Commonwealth Scientific and Industrial Research Organisation (CSIRO, Australia) to score MN in fixed human buccal cells on microscope slides. Buccal cells that were stained initially for visual scoring of the buccal MN cytome assay using light green (cytoplasm) and Feulgen (nuclei) (Thomas et al., 2009) were subsequently scanned with the LSC. We noted strong red fluorescence from the 'light green' stain (cytoplasmic contents) when the 633 nm excitation laser was used. The LSC iCys® software enabled us to use a feature termed 'CompuColor' to force the colour of the cytoplasm to appear as the pseudo colour green (the closest possible as it is observed when visualised under light microscopy). Additionally, in the same scan, we set-up the protocol to also quantify the nuclear (Feulgen) staining of buccal cells; however, some 'compensation' was required, and it should be noted that the LSC software is well equipped to manage overlapping or background signals. Feulgen fluorescence was also detected with a red filter following 488-nm excitation. Interestingly, when the Feulgen 'absorbance' was used to determine the chromatic light loss at 488 nm, we found that the DNA histograms yield more reproducible data upon analysis compared with Feulgen fluorescence, and for this reason, we chose to use the Feulgen chromatic light loss (absorbance) at 488 nm to quantify nuclei and MN in human buccal cells. Using this automated LSC system we showed a significant increase in MN in buccal cells from cases with Down syndrome relative to age-matched controls (Darzynkiewicz et al., 2011). An LSC protocol was successfully developed using this approach that allowed automated scoring of the buccal cytome by identifying and measuring successfully the proportion of basal, transitional, differentiated, pyknotic and karyolitic cells as well as determining the frequency of cells with aneuploid nuclei; the ratios of these cells differed significantly with age (Leifert et al., 2011).

Cellomics

Another commercially available system is the Cellomics Automated scoring system for measuring MN in cytokinesis-blocked mammalian cells. The system was tested for use in the *in vitro* micronucleus assay using CHO-K1 cells in 96-well plates (Diaz et al., 2007). CHO-K1 cells were pre-loaded with a cell dye that stains the cytoplasm, after which the cells were treated with the test compounds for either 3 h (for the +S9 condition) or 24 h (for the −S9 condition). At the end of the incubation period the cells were fixed and their DNA was stained with Hoechst. The visualisation and scoring of the cells was done using an automated fluorescent microscope coupled with proprietary automated image analysis software provided by Cellomics (Pittsburg, PA). A total of 46 compounds were used in this evaluation, including 8 aneugens and 25 clastogens with varied mechanisms of action. Thirteen non-genotoxic compounds were also included. The automated scoring had a sensitivity of 88% and a specificity of 100%, with a predictive positive value of 100% and a predictive negative value of 76%, compared to data from the literature that was obtained with visual scoring.

INCell Analyser 1000, GE Healthcare

Shibai-Ogata et al. (2011) reported for the first time on the use of the IN Cell Analyser 1000 for automation of high-throughput genotoxicity screening using *in vitro* MN assay with CHL/IU cells grown in 96 well microplates without cytochalasin-B. Cells were stained with Hoechst 33342 for nuclei and Cell MaskRed for cytoplasm. For automated analysis cytoplasmic, nuclear and micronuclear boundaries were contoured, nuclei removed from images using pre-set nuclear area cut-off thresholds leaving the micronuclei to be scored within each cell automatically. Results for 30 chemicals tested *in vitro* were in high concordance with previous *in vitro* MN data obtained with visual scoring. In a separate analysis within the same study the authors performed both visual and automated MN scoring and found that the correlation factor was 0.94.

Other systems for automated scoring for MN that are not yet commercially available

Other systems for automated micronucleus assay measurement have been developed but are not commercially available. These include:

The ROBIAS (Robotic Image Analysis System) system developed at Novartis Institute for Biomedical Research consists of a PC with a MATROX Meteor II frame grabber, a Leica DM RXA/2 fully automatic microscope with SONY DXC-390P colour camera, an EPSON robotic slide feeder (130 slides), an in-house developed software for automatic pattern recognition, based on Visual Studio and Matrox MIL library. This system is being used routinely for measuring MN in mono-nucleated and bi-nucleated cells using Giemsa staining. From an image analysis point of view, the advantage of using a Giemsa-based slide preparation and staining protocol is the segmentation of valid cells, nuclei and MN, without having to worry about fading fluorescence dyes. A ×20 objective for good visualisation of cells, nuclei and micronuclei at the same time proved to be sufficient for scanning. For the visual rejection of "artifacts", a 40x objective with subsequent ×1.6 magnification was chosen for ROBIAS to obtain the maximum confidence on the quality of the object to be accepted or rejected as MN. Here it is emphasised that a "Relocation" program to control and adjust for false positives is considered to be an important and integral part of the MN application. All the requirements listed in the earlier section "Points to consider for an automated image cytometry system for micronucleus assays" had been appropriately addressed for the design and development of the ROBIAS lymphocyte automated MN scoring system

except the detection of nucleoplasmic bridges and nuclear buds. Hence, the software was developed to cope with effects of strong cytotoxicity (discrimination of cellular debris), to detect mono-, bi- and polynucleated cells as well as mitotic figures (metaphases), and determine artefacts from true micronuclei and link them with the different cell types. A series of tests for comparing visual scoring with automatic image analysis showed that there was a very good concordance between the results obtained with both methods. Using identical criteria for a positive effect in the assessment of the data obtained by visual or automatic slide reading, the conclusions were the same in 24 out of 25 experiments, irrespective of the method used. The correct detection of >90% of the micronuclei is probably the highest achieved so far for automated scoring of MN by image analysis. The throughput per technician could be doubled using ROBIAS when compared to visual scoring. Details on the image analysis steps and validation data can be found in (Frieauff et al., 2012).

The Cytogenetic Laboratory Automated Scoring Platform (**CLASP**) system for automated scoring of dicentric chromosomes and micronuclei developed by Adarsh Ramakumar at the Armed Forces Radiobiology Research Institute (AFRRI, USA) using artificial intelligence, contextual image mapping and neural network systems classifies chromosome aberration in high-throughput. CLASP operates as a two stage process, (1) Automation of sample preparation and (2) Automation of sample analysis. Automation of sample analysis involves image acquisition, image analytics and dose estimation. CLASP provides a robust and high-throughput platform for various cytogenetic assays. Automated high-throughput platform with automated feature extraction, storage, cross-platform data linkage, cross-platform validation and inclusion of multiparametric biomarker approaches will provide the first generation high-throughput platform systems for effective medical management, particularly during radiation mass casualty events where very high volumes of samples have to be analysed for dose estimation. The SOP's for each of the robotic instruments involved in CLASP, for the various tissue culture processes, slide preparation and scoring have been developed and cross validated against human manual assay preparation and visual scoring by microscope. The overall SOP to switch between various protocols such as dicentric chromosome analysis for simulated medical casualty (triage) *vs* the use of CBMN assay to score MN's have also been validated. The SOP's developed for CBMN assay and associated slide preparation are based on the latest IAEA protocol (International Atomic Energy Agency, 2011). This process of using an internationally accepted protocol such as the IAEA-endorsed protocol, will further help in harmonisation of the automation process and guide development of industry standards for automated CBMN assay systems. The entire system of CLASP incorporates state of art automation technology, built on the backbone of strong information management system supported by sophisticated IT hardware.

The Rapid Automated Biodosimetry Tool (**RABiT**), developed by the Center for Minimally Invasive Radiation Biodosimetry at Columbia University, is a completely automated, ultra-high throughput biodosimetry system for the the lymphocyte CBMN and gammaH2AX assays (Garty et al., 2010, 2011). The RABiT analyses lymphocytes from fingerstick-derived blood samples to estimate past radiation exposure or to identify individuals exposed above or below a cut-off dose. Through automated robotics, lymphocytes are extracted from fingerstick blood samples into filter-bottomed multi-well plates. Depending on the time since exposure, the RABiT scores either micronuclei or phosphorylation of the histone H2AX. Following lymphocyte culturing, fixation and staining, the filter bottoms are removed from the multi-well plates and sealed prior to automated high-speed imaging. Image analysis is performed online using dedicated image processing hardware. Both the sealed filters and the images are archived. Parallel handling of multiple samples through the use of dedicated, purpose-built, robotics and high speed imaging has the potential to allow analysis of up to 30,000 samples per day.

Finally, a summary and further information on both the commercially available and those not yet in the market is provided in Table 1. In this Table the relative advantages and disadvantages, the number of studies published with each system and information on results of comparisons of automated and visual scoring are concisely described.

Statistical Issues for instrument validation and inter-laboratory comparison of automated systems

Large collaborative studies published in the last decades have revealed that inter-laboratory, and especially inter-scorer variation are the most important source of variability, setting this heterogeneity as a priority field to address. The recent development of automated systems for chromosome damage scoring should dramatically change the level of reliability of these biomarkers. Before introducing these methods, robust standardisation studies have to be started, aimed at comparing automated systems in different settings and different software and hardware platforms. The two critical properties of a biomarker are its validity and reliability. While the main purpose of validity studies is to determine whether the test measures what it was intended to measure, reliability addresses the question of whether the test is measuring accurately and consistently. This second quality is more specific in standardisation studies, where only reliable and consistent measures can be processed.

A critical feature to evaluate in these studies is the extent of inter-scorer agreement. Several approaches are available, such as the Test-Retest reliability, the Inter-Rater reliability (Birkimer and Brown, 1979), or the more common and robust Cohen's Kappa (Cohen, 1968), which avoids the problems described above by adjusting the observed proportional agreement for the amount of agreement which would be expected by chance (Rosner, 1986). In particular cases, such as for continuous data or when the number of scorers is bigger than two, the intra-class correlation coefficient (ICC) can be used to assess consistency and reproducibility of quantitative measurements made by different observers measuring the same quantity (Rosner, 1986). When more laboratories are involved in the validation study the parameter of interest is the inter laboratory agreement. Comparative tests conducted between several laboratories using the same method and the same type of equipment on one or several test samples circulated between them is the key approach in evaluating the heterogeneity among laboratories. This is a critical issue in collaborative studies such as the HUMN project (Fenech et al., 2003, 2011b) which gathers data from several laboratories. The most commonly used methodology is the round robin test, an inter-laboratory test performed independently several times.

The methodology for assessing the extent and the sources of inter-scorer and inter-laboratory variability is well established. However, a critical role for reaching a good degree of standardisation is played by international collaborative studies, which brings together several laboratories in the same field including key laboratories and opinion leaders. Agreement reached on protocols and standard techniques proposed by these consortia may remarkably increase the reliability of a biomarker (Taioli and Bonassi, 2002). When a novel technique is introduced in a new field, the use of well established methods of standardisation is the obvious first choice. However, the experience of "omics" biomarkers, where reducing technical heterogeneity was a "standardise or die" condition, should be taken into consideration, and the identification of a set of minimal criteria, *e.g.*, MIAME criteria for transcriptomics

Table 1
Comparison of image cytometry systems reported in the literature for automated scoring of micronuclei in mammalian and human cells.

System	Advantages	Disadvantages	Number of studies published	Outcomes of comparison with visual scoring
Metasystems Metafer MNScore	Validated for use with DAPI-stained slides using a standardised slide preparation procedure.[a] MN in BN cells, MN in MONO cells and NDI can be scored in lymphocyte CBMN assay. This system can also be used to measure other DNA damage biomarkers using antibody or PNA probes (*e.g.* γ-H2AX, centromere respectively) in nuclei and micronuclei. Performance: Conditions: Slide scanning zone 1 cm^2; 1000–2000 BN cells detected in <2 min. With 800 slide SlideFeeder ×80 device, allowing unattended processing during scanning, 500–700 slides per day can be analysed.	NDI can be scored but requires a separate scan	*N* = 16 Beinke et al. (2012) Bolognesi et al. (2011) Caryou et al. (2010) Doherty et al. (2011) Maes et al. (2007) Romm et al. (2012) Rossnerova et al. (2009, 2011a,b,c) Schunck et al. (2004) Thierens and Vral (2009) Varga et al. (2004, 2005, 2006) Willems et al. (2010)	The correlation coefficient (*R*) between visual and automated scoring of MN in BN lymphocytes for base-line values was 0.84 and for γ-irradiated samples at different doses it was 0.91; CV for repeat counts ranged from 5.4 to 9.3% depending on classifiers used (Varga et al., 2004). Subsequent studies by Willems et al. (2010) using Metafer 4 software showed a correlation coefficient (*R*) for MN in BN lymphocytes between visual and automated scoring of 0.96; the false positive and false negative rates were 6.3% and 1% respectively. In contrast the study of Bolognesi et al. (2011) using a similar approach reported a lower correlation coefficient for visual and automated scoring (*R* = 0.58).
IMSTAR Pathfinder™ Screentox Auto-MN	Validated for use with Giemsa stained slides using a standardised slide preparation procedure.[b] MN in BN cells, MN in MONO cells and NDI can be scored in lymphocyte CBMN assay. This system can also be used to measure other DNA damage biomarkers using antibody or PNA probes (*e.g.* γ-H2AX, centromere respectively) in nuclei and micronuclei. Performance: Conditions: Slide scanning zone 1 cm^2; 1000–2000 BN cells detected in <4 min; with 200 slide loader device allowing unattended processing during scanning: 200–400 slides per day can be analysed depending on hardware configuration.		*N* = 4 Decordier et al. (2009, 2011) Vande Loock et al. (2011) Hochstenbach et al. (2012)	In the conditions described in the articles, the correlation coefficient (*R*) between visual and automated scoring of BN lymphocytes with MN is 0.96. Sensitivity: 75% of cells detected; >90% of MNs in BN and MN detected, *i.e.* the false negative rate is <10% specificity: the false positive MN rate is <1%. After visual validation the false positive rate is 0%. Decordier et al. (2009).
CompuCyte iCyte®	Validated for use with PI-stained slides MN in BN cells can be scored in CBMN assay using MCF-7 and HL-60 cells.\ Methods for measuring MN in peripheral blood or bone marrow erythrocytes and buccal cells have also been reported. This system can also be used to measure other DNA damage biomarkers using antibody or PNA probes (*e.g.* γ-H2AX, centromere respectively) in nuclei and micronuclei.	Not yet validated for use with Giemsa stained slides. NDI can be scored but not yet validated. Number of slides that can be analysed per day is relatively low in the range of 10–20 per day.	*N* = 4 Styles et al. (2001) Smolewski et al. (2001) Darzynkiewicz et al. (2011) Leifert et al. (2011)	The correlation coefficient (*R*) between visual and automated scoring of MN in erythrocytes was 0.95 (Styles et al., 2001) and for scoring MN in BN cells it was 0.93 for MCF-7 cells and 0.87 for HL-60 cells (Smolewski et al., 2001).

Table 1 (*Continued*)

System	Advantages	Disadvantages	Number of studies published	Outcomes of comparison with visual scoring
Cellomics	Validated for use with Hoechst-stained CHO-K1 cells. This system can also be used to measure other DNA damage biomarkers using antibody or PNA probes (*e.g.* γ-H2AX, centromere respectively) in nuclei and micronuclei MN in BN cells can be scored in CBMN assay.	Not yet validated for use with Giemsa stained slides or lymphocytes. Number of slides analysed per day not reported.	*N* = 1 Diaz et al. (2007)	Comparison with visual scoring not done or not reported.
GE Healthcare IN Cell Analyser 1000	Validated for use with Hoechst-stained CHL/IU cells for MN assay. This system can also be used to measure other DNA damage biomarkers using antibody or PNA probes (*e.g.* γ-H2AX, centromere respectively) in nuclei and micronuclei.	Not yet validated for use in CBMN assay. Only validated for MN assay in mononucleated cells. Number of slides analysed per day not reported.	*N* = 1 Shibai-Ogata et al. (2011)	The correlation coefficient (*R*) between visual and automated scoring of MN in mononucleated cells was 0.93.
ROBIAS	Validated for use with Giemsa stained slides	System not yet available commercially but hardware components and software characteristics available.	*N* = 1	Coefficient of determination (*R*) for automated and visual scoring ranged between 0.98 and 0.99.
	MN in BN cells, MN in MONO cells and NDI can be scored in lymphocyte CBMN assay	Not yet validated for use with staining using DNA specific dyes. Number of slides that can be analysed per day is relatively low in the range of 10–20 per day.	Frieauff et al. (2012)	False negative and false positive rates were 10% and 5% respectively (Frieauff et al., 2012)
RABiT	Fully automated system using microcultures of buffy coat cells collected in fingerstick capillary tubes. Developed for use with Hoechst stained cells on membranes. MN in BN cells can be scored in lymphocyte CBMN assay. This system can also be used to measure fluorescent spots using antibody or PNA probes (*e.g.* γ-H2AX, centromere respectively) in nuclei and micronuclei. The system is designed for scoring thousands of samples per day.	Not validated for use with Giemsa stained slides. System not yet available commercially.	*N* = 2 Garty et al. (2010, 2011)	Comparison with visual scoring not reported.
CLASP	Fully automated system using whole blood cultures and same culture/staining method for classical metaphase analysis.	Validation studies on use with CBMN assay or other MN assays not reported.	Nil	Not reported.

MN, micronucleus; BN, binucleated cell.

[a] There are ongoing efforts to develop a validated automated system for Giemsa stained slides.

[b] There are ongoing efforts to develop a validated automated system for slides stained with Feulgen, DAPI and Acridine orange stained slides.

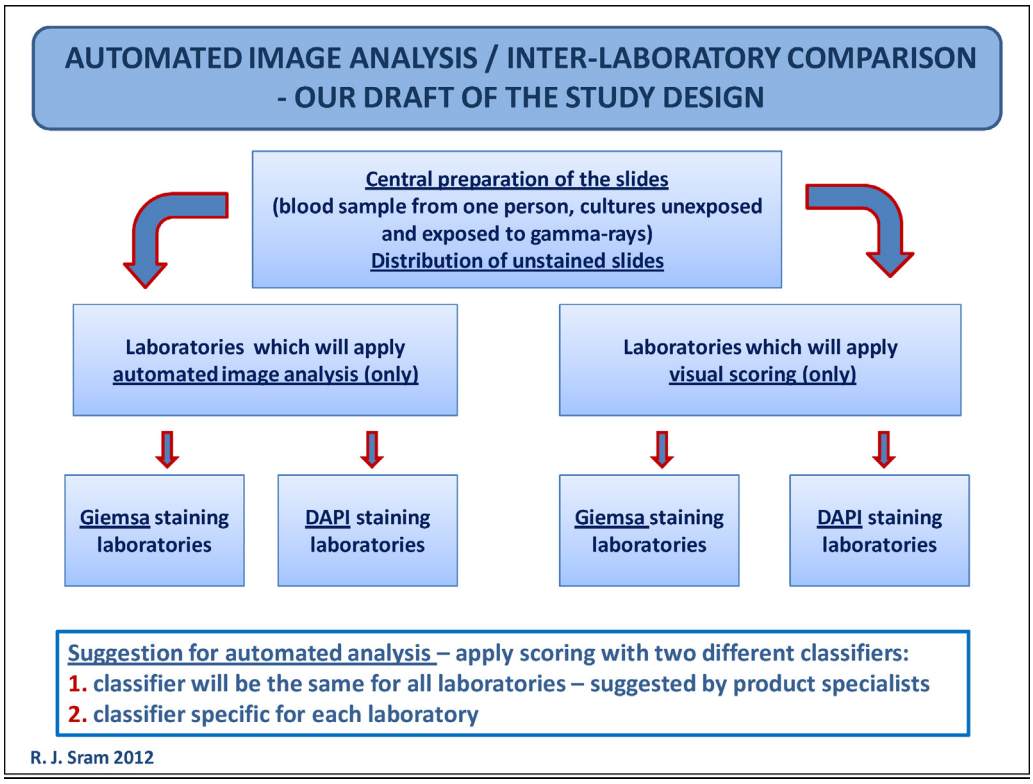

Fig. 1. An example of an inter-laboratory comparison scheme for evaluating the performance of automated image cytometry systems relative to visual scoring of MN in the lymphocyte CBMN assay. Lymphocyte slides will be prepared from whole blood cultures using either hypotonic treatment to remove erythrocytes or by density gradient to isolate lymphocytes post-culture.

(Brazma et al., 2001), may be considered interventions that may improve the quality of publications in the field.

Design of inter-laboratory comparisons and standardisation of micronucleus assays using automated image cytometry systems

The ongoing Multi-Biodose project using the Metafer MNScore (MetaSystems) instrument is the first attempt to test the inter-laboratory variability in MN frequency measurement in lymphocytes exposed to varying doses of ionising radiation. However, the biggest challenge will be to improve automated systems to an extent that base-line frequencies of MN can be measured reliably and reproducibly within a single laboratory and across laboratories using the same or different automated system platforms. Standardised procedures will be required regarding slide preparation, slide staining and software settings (classifiers) to reliably distinguish cells based on the number of nuclei and the presence of micronuclei within them. It is important to emphasise that ultimately the performance of any automated image cytometry system depends on the quality of slide preparation with regards to cleanliness, cell density and cellular staining.

For this reason the HUMN project is planning an inter-laboratory automated slide-scoring exercise to determine the extent of variability between laboratories using the same or different automation platforms and the performance characteristics of these platforms relative to visual scoring by experienced laboratories. A possible plan that is currently being considered (Fig. 1) could involve the preparation of unstained slides by a central laboratory and distribution of these slides to participating laboratories with expertise in automated image cytometry of MN in lymphocytes. These laboratories may then stain the slides according the appropriate method for their automated system (*e.g.* Giemsa for Pathfinder™ Screentox Auto-MN IMSTAR, DAPI for Metafer MNScore MetaSystems, Acridine orange for iCyte® Compucyte) and measure the MN using two classifiers, one of which will be the set recommended by the manufacturer of the instrument and the other selected by the participating laboratory to meet specific criteria that will require justification (*e.g.* minimising false positive and false negative rates; MN size and shape criteria to meet visual scoring criteria).

When agreement is achieved regarding the final design of the inter-lab comparison an announcement will be made for participation. Selection of participating laboratories is likely to be subject to a set of criteria defining a minimum level of expertise both for visual and automated scoring of MN using the lymphocyte CBMN assay.

Conclusion

Substantial progress has occurred in the development and commercialisation of instruments that are capable of automated scoring of MN in mammalian and human cells since the first Automated Micronucleus Workshop that was held in Milpitas California [(Automated Micronucleus Scoring Workshop November 17–18, 1988 Mutagenesis (1988) 3(6): 532, http://dx.doi.org/10.1093/mutage/3.6.532)]. The increasing use of automated image cytometry for automated scoring of MN in human lymphocytes for *in vivo* biomonitoring and *in vitro* genetic toxicology testing is a critically important development that necessitates international collaboration to standardise criteria and evaluation processes for acceptable performance of automated image cytometry systems. These advances should allow the possibility of reliable mass screening at lower cost and help to realise the implementation of MN assays in public health strategies aimed at DNA damage prevention and improving genome integrity in human populations and ultimately in all ecosystems which humans inhabit. This will further help both developed and developing countries with

high genotoxin exposure and/or poor nutrition/life style conditions to test whether implementation of new public health policies are actually effective in reducing DNA damage burden. Ultimately international cooperation is essential to implement standardisation of procedures across laboratories that would allow MN data to be obtained accurately and efficiently in multi-centre studies.

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