REVIEW

Laser scanning cytometry for automation of the micronucleus assay

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Laser scanning cytometry (LSC) provides a novel approach for automated scoring of micronuclei (MN) in different types of mammalian cells, serving as a biomarker of genotoxicity and mutagenicity. In this review, we discuss the advances to date in measuring MN in cell lines, buccal cells and erythrocytes, describe the advantages and outline potential challenges of this distinctive approach of analysis of nuclear anomalies. The use of multiple laser wavelengths in LSC and the high dynamic range of fluorescence and absorption detection allow simultaneous measurement of multiple cellular and nuclear features such as cytoplasmic area, nuclear area, DNA content and density of nuclei and MN, protein content and density of cytoplasm as well as other features using molecular probes. This high-content analysis approach allows the cells of interest to be identified (e.g. binucleated cells in cytokinesis-blocked cultures) and MN scored specifically in them. MN assays in cell lines (e.g. the CHO cell MN assay) using LSC are increasingly used in routine toxicology screening. More high-content MN assays and the expansion of MN analysis by LSC to other models (i.e. exfoliated cells, dermal cell models, etc.) hold great promise for robust and exciting developments in MN assay automation as a high-content high-throughput analysis procedure.

Attempts to automatise MN assay: flow cytometry—virtues and vices

Exposure of cells to ionising radiation or chemical agents that damage chromosomes or components of mitotic spindle leads to formation of micronuclei (MN; for reviews, see refs. 1–3). Either whole chromosomes or chromosome fragments that become separated from the rest of chromosomes during mitosis and at completion of telophase are not included into the daughter nuclei may form MN. Similar to whole nuclei, MN

are coated with a nuclear envelope, their DNA is often transcriptionally active and undergoes replication (4,5). The frequency of MN is considered to be a biological dosimeter of the *in vitro* or *in vivo* exposure to mutagens and carcinogens reporting the extent of chromosome damage. The MN assay, therefore, has become a preferred method to estimate mutagenic or carcinogenic properties of environmental factors and other agents.

The conventional approach for quantitative analysis of micronucleation by visual microscopy is tiresome and subjective leading to variability in results between scorers (6). Therefore, attempts have been made to use semi-automatic image analysis as the means for quantification of MN (7–15). Another approach to quantify MN semi-automatically involves the use of flow cytometry [FC; (16–26)]. By providing the means for rapid and unbiased quantitative analysis of MN based on DNA content measurement, FC offers certain advantages over the visual MN scoring or their enumeration by image analysis.

However, there are shortcomings of FC that limit its applications in the MN assay. The major limitation stems from the requirement to destroy integrity of the plasma membrane by lysing cells in order to release MN and measure them in suspension. Their identification is then based on characteristic distribution on DNA frequency histograms within a particular range of DNA content. Unfortunately, other particles that can be erroneously classified as MN may be present in such a suspension. Among them are (i) individual chromosomes or chromosome aggregates isolated from the lysed mitotic cells, (ii) fragments of nuclear chromatin from mechanically damaged cells, (iii) chromatin granules from the fragmented nuclei of apoptotic cells, (iv) individual apoptotic bodies and (v) contaminating microorganisms that can be present either in culture, in rinse buffers or in staining solutions. Since these objects may have similar DNA content as MN, they can be misidentified as MN ('false-positive MN').

Although strategies have been designed to discriminate between cell debris and MN (21,26,27), they may not always be effective. It is particularly difficult to differentiate between isolated chromosomes, fragments of chromatin or apoptotic bodies versus MN. This problem is amplified when among the cells subjected to the MN assay are numerous cells undergoing apoptosis. Then, the proportion of cellular fragments or apoptotic bodies versus MN is high. It should be noted that some apoptotic bodies are abundant in DNA, with DNA content close to that of MN (28). Likewise, lysis of cell suspensions containing a high percentage of mitotic cells (e.g. in cultures treated with mitotic poisons) releases a large number of individual chromosomes that masquerade as MN and can be misidentified by FC. Thus, unless the measured particles are sorted and examined by microscopy, their identity is uncertain and therefore the frequency of false-positive or 'false-negative' MN is unknown.

Still another limitation of FC is the inability to relate MN to individual cells and cell types. To give an extreme example, it is impossible to distinguish between the instances when (i) among 10 cells a single one contained 10 MN while 9 other had no MN versus and (ii) all 10 cells contained a single MN each. In both instances, 10 MN per 10 nuclei are detected by FC. Yet, the distinction is of relevance since in the first case only one cell in 10 (10%) while in the second all 10 cells (100%) demonstrated chromosomal damage. FC cannot be adapted to the cytokinesis-block micronucleus cytome (CBMNcyt) assay, which in addition to MN allows the measurement of other important biomarkers of chromosome damage such as nucleoplasmic bridges and nuclear buds (1,2,29). Still another shortcoming of FC is that the measured sample cannot be stored, e.g. for confirmation of the analysis, archival preservation or retrospective studies with other probes.

Laser scanning cytometry

Laser scanning cytometers (LSC; CompuCyte Corporation, Westwood, MA, USA) are the instruments offering unique analytical capabilities that combine those of flow and image cytometry (30–35) (Figure 1). 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 photo-multiplier tubes (PMTs). The dynamic range of fluorescence intensity measurement by LSC, therefore, 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. The latest version of the LSC software (iGeneration) offers powerful analytical tools for very accurate and multivariate data analysis.

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.

Scanning is done either with multiple lasers in a single pass or by one or more lasers in multiple passes. While spectral compensation may be employed to isolate signals from dyes whose fluorescence emission spectra overlap, multiple laser passes may eliminate or minimise the need for this spectral correction. LSC provides compensated images so that the isolated dye signals may be visualised and event segmentation may be based on these compensated images. LSC produces a full set of 14-bit image data from the fluorescence and light loss signals as well as the feature data derived from the processing and segmentation of these images.

LSC imaging and analysis are non-confocal by design. The resulting very high depth of focus allows collection of the total signal through the width of most samples. This in turn provides precise quantification of the measured signals (DNA content, for example), which is superior to many alternative technologies. Camera-based microscopy imaging systems (FIA) and (to a much greater extent) confocal imaging systems collect data from a very narrow plane through the sample and therefore do not provide the level of quantification available using LSC.

Fig. 1. iGeneration LSC technology schematic diagrams (CompuCyte Corporation). (A) Fluorescent measurement optical path; (B) Absorbance/scatter optical path and examples of corresponding images; (C) iCys Research Imaging Cytometer diagram.

Application of LSC for detection of MN was first reported in mice erythrocytes by Styles *et al.* (36) but the greater challenge was to develop methods for use with nucleated cells as described below.

LSC analysis of MN in nucleated cells growing *in vitro*

The cells analysed by LSC have to be deposited either on microscope slides or on multiwell culture plates. In the case of cells adapted to grow attached to culture flasks, the most convenient approach is to maintain them on mono- or multi-chamber microscope slide tissue culture vessels such as provided by the Lab-Tek, Nalge Nunc, Naperville, IL, USA (37–40). The subsequent steps of fixation, fluorochrome staining and fluorescence intensity measurement are then carried out with no need for cell detachment (trypsinization), on the same platform on which they were exposed to the agents expected to induce MN in cultures. In the case of cells that grow in suspension, the initial step, prior to fixation, is to deposit them on microscope slides by cytocentrifugation (38).

It has been observed that concurrent differential staining of 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. This is due to the fact that the ratiometric analysis of protein/DNA versus DNA content offers better means of MN identification than DNA content alone (38). 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 (41). 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. Alternatively, DNA can be stained with 4'-6-diamidino-2-phenylindole (DAPI), 7-aminoactinomycin D (7-AAD) or other DNA-specific fluorochromes with no need for RNase treatment (42).

Two strategies (methods) can be used to measure fluorescence intensity of the protein (e.g. FITC) and DNA-bound (e.g.

PI) fluorochromes in assessing frequency of MN by LSC. In the first method (Figure 2A), the 'threshold' contour is set based on the data computed from the photomultiplier measuring red fluorescence of PI. The 'integration' contour is then set within a range between zero and two pixels outside the threshold contour. In this way, the integral values of DNA (PI)- and protein (FITC)-associated fluorescence intensity of nuclei as well as MN are recorded in the same file. The distinction between nuclei and MN is then made based on difference in their DNA content. The data thus resemble those obtained by FC as the latter also rely on analysis of DNA content alone (20). As mentioned, however, the concurrent analysis of DNA and protein content of MN, in particular, the ratio of protein/DNA, which is similar in nuclei and MN, provides an additional parameter useful to distinguish MN from artifacts (Figure 3).

The second strategy makes use of the feature of LSC software that was designed for fluorescence *in situ* hybridisation (FISH) analysis (43). In this method, the threshold contour is set on the protein-associated (green-FITC) fluorescence (Figure 2B). Each cell is therefore identified, which allows one to obtain information about the number of nuclei and MN per individual cell (number of 'FISH spots'), as well as to measure intensity (integrated value) of red (DNA) and green (FITC) fluorescence per each spot as well as per whole cell (nucleus + MN). The value of DNA-associated fluorescence integrated over nucleus + MN provides information on the cell cycle position, discriminating between G₁, S and G₂M cells. A similar strategy of threshold contouring based on cellular protein-associated fluorescence has been used to analyse individual cells within cell colonies (44).

The capability of LSC to obtain and save images of the measured events allows their visual identification and thus makes it possible to accurately distinguish and separate MN from other objects, primarily cell fragments and debris. The image analysis revealed that >93% of the objects localised within the bivariate distribution window spanning the range between 0.1 and 5% of DNA content (PI fluorescence) of that of nuclei of G₁ (diploid) cells and having similar protein/DNA (FITC/PI) ratio as the nuclei (Figure 3) were MN (38). Thus, on

Fig. 2. Two different strategies for setting the threshold contour. (A) The mitomycin C-treated MCF-7 cells were fixed and then stained with FITC and PI. The threshold contour was set on red fluorescence of PI and the integrated values of green (FITC) and red fluorescence intensity were recorded for each nucleus and MN. (B) The mitomycin C-treated U-937 cells were cytocentrifuged on slides, fixed and then stained with FITC and PI. The threshold contour was set on green fluorescence and the LSC software dedicated for FISH analysis was used to contour the nucleus and MN within the cell. Thus, the number of nuclei plus MN and intensity of fluorescence emitted by each of them can be recorded for each measured cell. In panels A and B, MN are marked with arrows (38).

Fig. 3. Identification of MN based on analysis of DNA content (A) and protein/DNA ratio (B). To induce MN, HL-60 cells were treated with mitomycin C, then fixed and stained with FITC and PI. The threshold contour was set on the data from the photomultiplier measuring red fluorescence of PI. The events from different regions of these bivariate distributions were relocated and imaged to identify MN. The whole nuclei have the highest PI fluorescence and a pattern resembling G₁-S-G₂/M cell cycle distribution of DNA and because of treatment with mitomycin C, with a greater accumulation of cells on G₂/M. The highest proportion of MN can be found within the rectangular window marked on the PI versus FITC/PI bivariate distributions with dashed lines (B). This window had the top and bottom borders at the maximum and minimum range of FITC/PI fluorescence ratio of the whole nuclei, respectively. The lower and higher PI fluorescence borders of the window were within a range between 0.1 and 5% of the PI fluorescence of the G₁-phase nuclei. The events with the FITC/PI ratio higher than the upper limit of the whole nuclei (within the oval dashed line outline) were mostly cytoplasm fragments and other cell debris. The representative cell images from each region are shown as a 'cell gallery' above B. By the 'paint-a-gate' analysis, the events within the discussed regions in panel B reveal their distribution with respect to intensity of PI versus FITC fluorescence (A).

the bivariate PI versus FITC/PI fluorescence plots, three distinct clusters can be seen: (i) the cluster of the recorded events with the highest FITC/PI ratio and the lowest PI fluorescence which are the non-specific particles, mainly fragments of cells' cytoplasm; (ii) the cluster representing whole nuclei that had the highest PI fluorescence with the typical pattern reflecting the G₁-S-G₂/M cell cycle and (iii) the cluster representing predominantly MN.

Analysis of MN using the FISH approach is illustrated in Figure 4. Setting the threshold contour on FITC fluorescence makes it possible to record each individual cell and count the frequency of the objects emitting PI fluorescence such as MN ('FISH spots') and also to measure the integrated PI fluorescence over the whole cell (nucleus + MN). Thus, the cells with a single nucleus could be distinguished from the cells having a nucleus and one MN, from the cells having one nucleus and two MN, etc. It is evident from this data that in the cultures treated with increasing doses of mitomycin C (a cytotoxic and genotoxic drug), the percentage of cells without MN decreased concurrently with the increase in frequency of the cells with one, two and more MN.

To make the conditions of analysis of chromosome damage independent of the cell cycle kinetics, the MN assay has to be restricted to cells that made only a single division after

Fig. 4. Quantification of MN per cell using the FISH-dedicated software of LSC. The mitomycin C-treated HL-60 cells were fixed and stained with FITC and PI. The threshold contour was set on green fluorescence of FITC, as shown in Figure 2B and the data were collected using the FISH spot analysis software of LSC to count the number of nuclei plus MN per cell (30,43). The raw data plot is shown in panel (A). The bar plot (B) shows the frequency of cells with a single nucleus (1 'spot') and with the nucleus plus single MN (2 spots), plus two MN (3 spots) and plus three MN (4 spots) in cultures of HL-60 cells untreated (white bars) treated with 0.1 (grey bars) and 0.01 µg/ml of mitomycin C (black bars). This approach allows one also to present the integrated PI fluorescence (DNA content) of each detected nucleus and MN (data not shown).

exposure to the damaging agent. Towards this end, cytochalasin B is added into cultures to prevent cytokinesis in cells completing nuclear division after genotoxin exposure (1,2,29). In this CBMNcyt assay, only cells that have completed one nuclear division, identified as binucleated cells, are scored. Further nuclear division in the presence of cytochalasin leads to formation of multi-nucleated cells, which are not scored. 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 CBMNcyt assay to LSC (Figure 5). 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 tetra-nucleated 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 (38). The remaining objects were aggregates consisting of two or three cells in close proximity to each other; the contouring can mistakenly recognise such aggregates as single cells. Strategies that can be used to overcome the problem of close cell proximity or overlap in analysis of MN by LSC are discussed at the end of this chapter: 'Potential challenges'.

When cultured cells were treated with mitomycin C, the frequency of MN detected visually by microscopy at different mitomycin C concentrations correlated well with that assessed by LSC in both cases evaluated in binucleated cells (Figure 6). The highest MN frequency (12–14%) was seen at 0.1 µg/ml concentration. However, the MN frequency was diminished at both <0.1 µg/ml and >0.1 µg/ml concentrations of mitomycin C. At 10.0 µg/ml mitomycin C concentration, the cells were arrested in the G₂ phase (as was evident from the DNA content frequency histograms) which prevented cells from completing nuclear division and expressing the damage as MN. 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 (See legends to Figures 5 and 6).

Buccal cell MN analysis by LSC

The buccal mucosa (BM) is a stratified squamous epithelium consisting of four distinct layers. The 'stratum corneum' or keratinised layer lines the oral cavity comprising cells that are

constantly being lost as a result of everyday abrasive activities such as mastication. Below this layer lie the 'stratum granulosum' or granular cell layer and the 'stratum spinosum' or prickly cell layer containing populations of both differentiated and apoptotic cells. Integrated within these layers are convoluted structures known as rete pegs, containing the actively dividing basal cells known as the 'stratum germinativum' which produce cells that differentiate and maintain the profile and integrity of the buccal mucosa. The regenerative capacity of tissues and organs within the body is fundamental to growth, development as well as healthy ageing and is dependent on genomic stability and gene expression profile of the basal stem cells. The BM is an easily accessible epithelial tissue that can be sampled in a minimally invasive manner without causing pain to study participants and for this reason is

Fig. 5. Identification of cytochalasin B induced binucleated cells by gating analysis of the DNA content frequency histograms. U-937 cells were in the culture with cytochalasin B for 24 h, then fixed and stained with FITC and PI. The threshold contour was set on green fluorescence of FITC as shown in Figure 2, panel B, and PI fluorescence from all nuclei within the cell measured. The intensity of PI fluorescence integrated over the cell, thus, reflects total DNA content (DI) of either the mono-, bi- or multi-nucleated cell. The gating window can be set either on the bivariate DNA content versus green fluorescence scattergram (A) or on the DNA content frequency histogram (B). Within the gating window of DNA content between 2.2 and 3.8 DI, nearly all cells are binucleated and therefore they can be subjected to the CBMN analysis (38).

Fig. 6. Comparison of frequency of MN binucleated cells in relation to concentration of mitomycin C assessed visually by microscopy (white bars) and by LSC (black bars). MCF-7 cells were treated with different concentrations of mitomycin C for 6 h then transferred to fresh medium without mitomycin C and cultured for 24 h in the presence of cytochalasin B. The cells were stained with FITC and PI and the threshold contour on LSC was set on red fluorescence of PI. In double-blind test, the same slides were also subjected to scoring MN visually by fluorescence microscopy. The frequency is expressed as mean percentage of MN in relation to total events, nuclei plus MN, counted by LSC or by microscopy. Minimum 1200 binucleated cells were scored by LSC and 500 visually per sample. Standard deviation (five experiments) is shown atop the bars. The regression analysis has shown the correlation coefficient $r = 0.87$ between the visual versus LSC counts (38).

Fig. 7. LSC image of human buccal cells. Human buccal cells were stained with light green (cytoplasm) and Feulgen (nuclei). The Feulgen-stained nuclei are prominent and MN were indicated by an arrow. (A) High-resolution image of buccal cells showing a single MN (orange) within the (green) cytoplasm. (B) 'CompuColor' generated gallery images of two buccal cells showing distinct MN and (C) the same cells shown in (B) demonstrating the accuracy of the iCys-generated contour lines around the cytoplasmic periphery (red), nucleus (yellow) and MN (green).

an ideal tissue for *in vivo* MN diagnostics. This tissue provides a unique opportunity to study the regenerative capacity of epithelial tissue of ectodermal origin in humans and has been used successfully to study DNA damage by scoring MN using visual/microscopy techniques (45).

Micronucleated buccal cells are visually characterised by the presence of both a main nucleus and one or more smaller MN (Figure 7). The MN in buccal cells are usually round or oval in shape and their diameter may range between 1/3 and 1/16, the diameter of the main nucleus. Cells with MN usually contain a single MN, however, it is possible but rare to find cells with more than two MN. The nuclei in micronucleated cells may have the morphology of normal cells or that of dying cells (i.e. condensed chromatin). The MN must be located within the cytoplasm of the cells to be scored. The presence of MN is indicative of chromosome loss or fragmentation occurring during previous nuclear division. Indeed in our previous study, the frequency of MN was significantly elevated in the Down's syndrome cohort and were found to be roughly 10-fold higher than in the age-matched control group, confirming the observation of elevated genome damage in this syndrome (45).

LSC has been used at Commonwealth Scientific and Industrial Organization to score MN in fixed human buccal cells on microscope slides. Buccal cells that were stained initially for visual scoring of the buccal cytome MN assay using light green (cytoplasm) and Feulgen (nuclei) (45) 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 (Table I). 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.

The Feulgen absorbance of the nuclei can be used in conjunction with the cellular segmentation of the cytoplasm by incorporating an 'association' function in iCys. In this instance, the nuclei that are scored become associated with the 'cell event' and data obtained from nuclei can be correlated to the all set of data obtained from the different stages of cell differentiation in the buccal cell samples. The total amount of

Table I. Laser and detector selection for MN assay of buccal cells

Target	Dyes	Excitation lasers (nm)	Detectors
Nuclei	Feulgen	488	488LL PD (absorbance) + long red PMT (fluorescence)
MN	Feulgen	488	488LL PD (absorbance) + long red PMT (fluorescence)
Cytoplasm	Light green	633	Long red PMT (fluorescence)

signal detected in nuclei defines the 'DNA content' as mentioned earlier for cultured cells. MN segmentation was based on the same iCys-defined features of 'nuclei' segmentation but a smaller size restriction was used since MN are always much smaller than a typical '2N' nucleus. A filter 'FISH B' was added to the segmentation features to enhance the spatial resolution of the images, highlight small spots and therefore increase MN detection. In fact, by doing this, we noted that we could detect MN with the LSC that were far less apparent by visual scoring using a light microscope. To ensure that scored MN are only within the buccal cells, a 'peripheral contour' around the identified MN is applied to segregate the MN that are located within a cell from those that are not, by quantifying the light green fluorescence intensity of this contour. Using these approaches, it was possible to accurately score MN in human buccal cells using LSC, and indeed, there was a significantly higher score of MN in a Down's syndrome cohort (a model of premature ageing) compared with age-matched controls (Figure 8). These promising initial data suggest that it is possible to score MN frequency in buccal cells using LSC. Ongoing research is also exploring the possibility of scoring automatically by LSC other biomarkers and other buccal cell types including assessment of frequency of karyolytic cells, binucleated cells and basal cells. Such analysis could provide additional information on the regenerative potential of the buccal epithelium.

***In vivo* erythrocyte MN analysis by LSC**

The mouse erythrocyte MN assays are standard *in vivo* genotoxicity tests. After administration of the investigated agent at specified times, bone marrow or peripheral blood samples are collected and cellular smears are prepared on microscope slides. Genotoxic effects manifest in the formation of MN, which generally are scored in polychromatic erythrocytes (46). The conventional visual scoring assay of MN is laborious, susceptible to observer fatigue and bias. Attempts therefore have been made to assess MN in erythrocytes by FC (17,18,24,47–50). Styles *et al.* (36) have shown, however, that LSC can be effectively used to perform the erythrocyte MN assay automatically (36). The authors analysed 5000 cells per sample using the instrument with a ×40 objective, 488-nm argon laser at 5 mW output. No distinction was made between normocytes and polychromatic erythrocytes. The results of the comparison between slides analysed by visual microscopy and LSC showed a good

Fig. 8. Frequency of MN in human buccal cells. Human buccal cells on microscope slides were scanned by LSC. Using the features described in the text, MN were identified and scored in a Down's syndrome cohort (n = 10) and an age-matched control group (n = 10). Data are mean values ± SEM, P < 0.001.

correlation ($R = 0.96$) between the data from the two assays; the percent of MN in their samples varied between 0 and 6%. The authors conclude that 'LSC is likely to become the preferred method for the performance of standard genotoxicity assays' (36). New models of LSC having multiple laser excitation capability allow one to differentially stain DNA and RNA (e.g. DAPI versus thiazole orange) and thereby to restrict the MN analysis to polychromatic erythrocytes.

Advantages of MN assay by LSC

The presented data demonstrate that LSC can be easily adapted for the MN assay. The cell preparation is simple and the actual measurement is rapid and straightforward. In most specimens, the approximate time of analysis of MN in 1000 cells by LSC was 3–5 min. The LSC assay yielded a similar MN index as visual count under a light microscope. The assay can be carried on both types of cultured cells, i.e. the cells that grow attached to the slides such as MCF-7, as well as on cells that grow in suspension and then are deposited on slides by cytocentrifugation. Furthermore, we also demonstrated that the MN assay was particularly useful in scoring MN in buccal cells showing the expected higher level in Down's syndrome (45).

Instead of using only DNA fluorochromes, as it is conventionally done for detecting MN by image analysis or FC, we chose the double-colour differential staining of DNA with PI (or Feulgen for buccal cells) and protein with FITC (or 'light green' for buccal cells). This led to several advantages. The first advantage was that the non-specific objects could be distinguished from MN based on their higher protein/DNA ratio. The protein/DNA ratio measured by LSC, thus, was a useful parameter making identification of MN more reliable compared to staining DNA alone. The second advantage resulting from staining protein in addition to DNA was the possibility to use the protein-associated FITC fluorescence to set the threshold contour. Using the software of LSC developed for the determination of the FISH fluorochrome spots (30,43), the specimen was subjected to the analysis that revealed the frequency of MN and their DNA content in each individual cell. This approach offers a possibility to assess whether particular clastogenic agents generate cells with single or multiple MN. Thus, mechanistic studies can be carried out, for example to study the difference between the auegen- and clastogen-induced MN or to study whether different clastogen types preferentially induce a particular chromosome or set of chromosomes to separate and from a single or multiple MN per cell. In analogy to the present application, the FISH capabilities of LSC were extended before to analyse individual cell nuclei within cell colonies, in the clonogenicity assays (44).

The approach based on setting the threshold contour on FITC (or light green cytoplasmic stain) fluorescence offers still another advantage, namely the possibility of CBMNcyt assay. Indeed, using the specific range of cellular DNA content as a marker of binucleated cells, we were able to relocate them and visually confirm their identity. The MN count, thus, can be restricted to the cells that completed only one round of nuclear division, making it independent of differences in cell cycle kinetics.

Confirming the measurements by FC (19), we observed high variability in intensity of PI fluorescence, reflecting differences in DNA content, between individual MN (38). We were able, however, to detect MN with lower DNA content than that of MN detected by FC. Specifically, while our bottom limit of

DNA content of MN was $\sim 0.1\%$ of DNA content of the G_1 -phase nuclei, the lower limit measured by FC was reported to be between 0.5 and 0.75% (19). It is possible, thus, that the smallest MN measured by LSC may not be detectable by FC.

It is apparent that the more attributes of MN are measured the greater fidelity of their positive identification. With the capability of LSC for multi-laser excitation and multiparameter analysis, one may include additional features characterising MN, such as the presence of centromeres, telomeres, nuclear proteins (e.g. histones), chromosome identification markers, etc. Such multiparametric analysis of MN may not only be helpful for their identification but may provide new insight in mechanistic studies, e.g. aimed to correlate frequency, size and composition of MN with properties of the inducer, cell cycle position or other variables. Likewise, it is possible to detect DNA replication in MN by labelling cells with 5-bromo-2'-deoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU) followed by bivariate analysis of the incorporated precursor and DNA content (39).

Potential challenges in MN analysis by LSC

The analysis based on setting the threshold contour on protein-associated (e.g. FITC) fluorescence requires that the measured cells have to be separated from each other. Otherwise, with close cell proximity or partial overlap of their cytoplasm, the contouring encompasses cell doublets or larger aggregates. Thus, in order to analyse CBMNcyt by LSC, as well as by other automated imaging techniques, a caution should be exercised to have optimal cell density and relatively uniform spacing between the cells growing on slides. Similarly, in buccal cell, scoring the cells that are not completely separated may present a scoring challenge. We overcame this problem by using the 'seeded watershed' feature available in the LSC software, which very accurately defines the cytoplasmic boundaries of single cells in a clump of cells into well-defined single cells for scoring. The separation of the cellular boundaries can be assessed in either 'real-time' or post-acquisition to determine if this algorithm has adequately identified single cells in a clump. For any cell clumps remaining, a procedure can be used to 'gate out' events (cell clumps) that have a larger 'cytoplasmic area' and higher total cytoplasmic staining intensity ('integral') than the single cells. This is accomplished by plotting these twoparameters versus each other. 'Events' (cell clumps) that are obviously larger than a single cell can be identified using the 'create gallery' feature in iCyt/iCys and then removed from subsequent analyses.

Potential difficulty arises in analysis of cultured cells having low cytoplasm/nucleus ratio and are spherical in shape. MN in such cells may not be adequately spatially separated from the nucleus and thus they cannot be individually segmented. Cytocentrifugation at higher centrifugal force or treatment with mild hypotonic solution (e.g. 0.075 M KCl) also leads to more extensive cell spreading on slides. It would be expected, however, that because of the unfavourable geometry of spherical cells having low cytoplasm content, the MN index, assessed by any automated imaging including LSC, will be consistently lower compared to visual scoring. The extent of this bias, likely to be restricted to particular cell types, can be estimated by comparison of the same specimen scored visually versus by automated imaging.

As discussed, the strategy to use cellular DNA content (histograms) for gating binucleated cells in the CBMNcyt

assay does not allow one to distinguish between, e.g. G₂ versus binucleated cells having G₁ nuclei. However, by contouring on cytoplasm (e.g. protein—FITC fluorescence and using the FISH algorithm), it is possible to identify cells having two nuclei each with DI = 1.0 and one nucleus with DI = 2.0; the latter to be gated out. Alternatively, the contoured bi-nuclei can be distinguished from G₂ nuclei by use of the circularity parameter. This parameter is typically elevated in bi-nuclei as the ‘pinch-point’ at the interface of the nuclei pair creates an elongated perimeter in relation to the nuclear area. In doubtful cases, the imaging can be used to identify the binucleated cells.

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