Automation of the cytokinesis-block micronucleus cytome assay by laser scanning cytometry and its potential application in radiation biodosimetry

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Here we describe the adaptation of laser scanning cytometry (LSC) to measure micronuclei (MN) automatically in lymphocytes. MN frequencies were determined in irradiated human lymphocytes using the cytokinesis-block technique, and the results from LSC were compared with visual scoring results obtained from slides of cells stained using Fast Green and 4´,6-diamidino-2-phenylindole (DAPI). This fluorescent approach allowed clear identification of binucleated cells and detection of MN. The dose responses measured visually and by LSC showed similar trends and correlated positively ($r = 0.9689$; $P < 0.0001$). High-content analysis was developed to further automatically score MN within mono-, tri- and tetra-nucleated cells and to determine the nuclear division index and nuclear circularity values. The high-throughput nature of LSC can provide unique advantages in future DNA damage diagnostics in experimental and epidemiological studies. Importantly, it allows for co-detection of other biomarkers of interest within a single lymphocyte, and further development of this capability is anticipated.

Micronuclei (MN) are biomarkers of genotoxic events and chromosomal instability that are measured in lymphocytes for biological dosimetry of radiation exposure with the use of the cytokinesis-block micronucleus cytome assay (CBMN) (1,2). This technique is one of the preferred methods to assess chromosomal damage induced by exposure to environmental and occupational factors in human populations (3). To address some of the limitations of this assay for large-scale studies, automated systems have previously been tested (i.e., Metafer Metasystem, PathFinder CellScan, imaging flow cytometry) (4–7). Such automation could nullify the variability in results between visual scorers and increase the throughput of the assay with a larger number of cells scored. However, an important limitation of the automated micronucleus assay protocol lies in its difficulty to be used in combination with detection of other established cytotoxicity and DNA damage markers (e.g., γH2AX, 8-OHdG).

Here we describe an automated scoring assay for MN in lymphocytes (within mononucleated and multinucleated cells with concomitant measurements of nuclear division index) that could offer a flexible capability for further DNA damage diagnostics if combined with immunodetection of other markers of interest within the same single cells. With this purpose in mind, we adapted the micronucleus assay to be used with the cutting-edge laser scanning cytometry (LSC) technology, an imaging cytometry-based approach previously shown to be particularly useful in scoring MN within buccal cells (8) as well as detecting multiple parameters within single cells by high-content imaging analysis (9). LSC uses four different excitation lasers, four multiplier tubes, and two photo detectors, allowing for the simultaneous use of chromatic stains or fluorescent antibodies (or both) in combination with a CBMN assay. Additionally, the LSC technology presented here has applicability in large-scale studies because human lymphocytes are relatively accessible and routinely collected in epidemiological studies.

Lymphocyte isolation using Ficoll–Paque (GE Healthcare, Silverwater, Australia) was performed as previously described (3). Lymphocytes isolated from collected blood were irradiated (0–4 Gy), cultured, and prepared on microscope slides before being analyzed by LSC (Supplementary Material). Micronucleus assessment

METHOD SUMMARY

Automated image analysis by means of laser scanning cytometry (LSC) was used with visual validation to determine micronuclei (MN) frequencies in irradiated human binucleated lymphocytes and was compared with visual scoring. The high-throughput and high-content nature of LSC can provide unique advantages in future DNA damage diagnostics in experimental and epidemiological studies.

was conducted on lymphocytes after X-irradiation by means of the CBMN method as described previously (3). The LSC automated procedure for MN slide analysis was performed as follows: (*i*) Regions defined around each cytospot (the location where the cells have been cytocentrifuged onto the microscope slide) were scanned with a previously designed fully automated LSC protocol (Supplementary Figure S1). (*ii*) When the scan was completed, the number of mononucleated, binucleated, and multinucleated (>2 nuclei) cells and the number of MN they contained was retrieved for each cytospot (a minimum of 1000 cells in total were scanned per microscope slide) using predefined settings. More precisely, these settings used specific contours around the cytoplasm, nuclei, and MN that allowed for identification of cells with associated MN. The cell segmentation process was conducted by plotting cells on a scattergram depending on the number of nuclei they contained. (*iii*) Saved images of measured events (i.e., mononucleated, binucleated, and multinucleated cells with or without MN) were displayed as a gallery (see Figure 1, A and B) that made it possible to distinguish true from false positives. After the data were collected by LSC, visual scoring was performed on the same microscope slides according to the criteria published previously (3). Scoring MN in a total number of 200 binucleated cells per spot was considered sufficient to detect exposure to ≥1 Gy of X-rays based on previous results using X-irradiation in human lymphocytes (10,11).

The automated LSC fluorescence approach allowed clear segmentation of cells using scattergrams (Supplementary Figure S1). A minimum of 500 binucleated cells were analyzed for detection of MN. A gallery of images is presented in Figure 1, A and B, showing a series of analyzed images from the LSC. The software used allowed the development of a protocol to automatically draw contours around the cytoplasm (green), nuclei (yellow), and MN (magenta), allowing separation of binucleated cells from the rest of the cell population and subsequent scoring of MN in this cell type. Visual scoring results were compared with the LSC scores of MN. Using LSC, it was noted that 5% of binucleated cells were false positives and that this percentage did not significantly differ between individuals or radiation doses. However, these false positives were easily disregarded from the analyses by visual examination of the image galleries generated by the software.

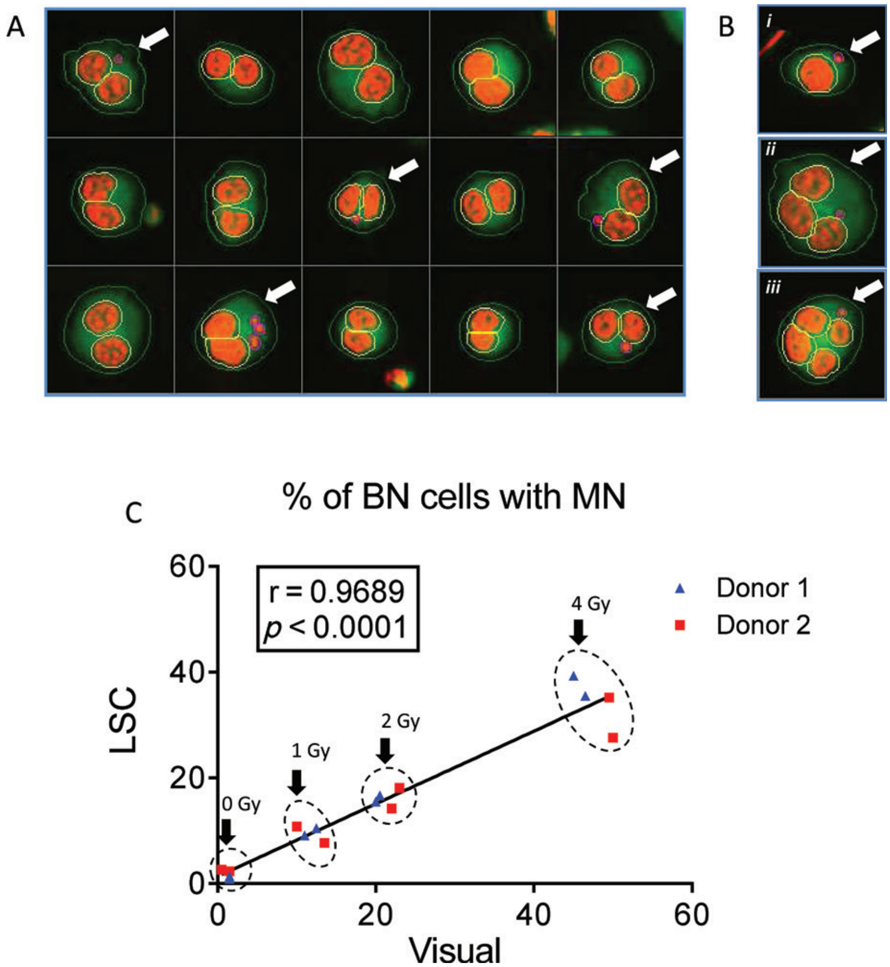

Figure 1. Gallery of images generated by laser scanning cytometry (LSC). A gallery of images collected by LSC and used for validation showing binucleated cells scored (A) and an example of a (*i*) mono-, (*ii*) tri- and (*iii*) tetra-nucleated cell (B). Cells containing micronuclei (MN) are indicated by white arrows. The contours automatically drawn by the software around cells, nuclei, and MN are represented in green, yellow, and magenta, respectively. (C) Example of correlation for the MN scores (percentage of binucleated cells with MN) measured for two donors' microscope slides by visual versus LSC. Two cytospots per individual per radiation dose were scored. The arrows indicate the radiation doses (Gy) to which the lymphocytes were exposed. BN: binucleated.

Table 1. Data generated by laser scanning cytometry (LSC) analysis of cytokinesis-block micronucleus cytome assay (CBMN) slides.

Radiation dose (Gy)	% of mononucleated cells with MN	% of binucleated cells with MN	% of tri- and tetra-nucleated cells with MN	NDI (a.u.)[a]	Circularity (a.u.)
0	0.67 ± 0.13	1.85 ± 0.42	6.44 ± 1.87	1.83 ± 0.06	16.91 ± 0.1
1	2.42 ± 0.98	10.03 ± 0.67**	10.33 ± 3.57	1.85 ± 0.06	16.89 ± 0.1
2	3.51 ± 0.86	16.90 ± 0.86****	18.15 ± 2.73	1.82 ± 0.08	16.9 ± 0.09
4	4.22 ± 0.90*	36.7 ± 2.55****	40.07 ± 4.82****	1.66 ± 0.05	16.81 ± 0.11

Data are presented as mean ± SEM. P values are in comparison to the 0 Gy control. *p<0.05, **p<0.01, ****p<0.0001.
[a]NDI was calculated using the formula: NDI = $(M_1 + 2M_2 + 3M_3 + 4M_4)/N$, where M_1–M_4 represents the number of cells with 1–4 nuclei, and N is the total number of viable cells scored (excluding necrotic and apoptotic cells).
a.u., arbitrary units; MN, micronuclei; NDI, nuclear division index

Results for visual and LSC scores from two donors are shown in Figure 1C. The dose responses for induction of MN measured visually and by LSC were well correlated (r = 0.9689; $P < 0.0001$). However, the LSC scores were slightly lower than the visual scores, likely due to small numbers of false negatives that would have been excluded from the analysis by the software protocol. Additionally, LSC allowed simultaneous scoring of mono-, tri- and tetra-nucleated cells, therefore enabling measurement of the nuclear division index (NDI) as well as MN scores specific to each of these cell populations. Data were also generated by the LSC software for an additional nuclear parameter: circularity (a measure of nuclear roundness). This parameter indicates how circular an object (e.g., a nucleus) is and was previously found to increase in buccal cells from individuals with Alzheimer disease, which is associated with abnormal nuclei and may be indicative of chromosomal instability (9). The percentage of each cell population containing MN, NDI, and circularity values recorded for combined cell populations exposed to different radiation doses is presented in Table 1. This combination of data shows the high-content potential of LSC when applied to the CBMN assay.

LSC provides a novel approach for automated scoring of MN in cytokinesis-blocked binucleated lymphocytes and was well correlated with visual scoring. Because LSC can also identify mononucleated cells, this technique offers the possibility to score MN within mononuclear lymphocytes, providing information on MN that were already induced in vivo before tissue culture (12,13). Moreover, instead of using only DNA fluorochromes, as conventionally done for detection of MN by image analysis or flow cytometry, we chose double-color differential staining of DNA with DAPI and protein with Fast Green. This led to a more reliable identification of MN compared with staining DNA alone because it distinguished nonspecific objects from MN based on their higher protein-to-DNA ratio. In terms of high-throughput as well as interscorer variation, this newly developed method for automated scoring of MN should be favorable when compared with visual scoring. In the same amount of time it took to visually score MN, the LSC method developed here resulted in a 20-fold increase in the number of cells analyzed, which makes this method more suitable for large population studies and lowers the detection limit in vitro to detect increases in MN induced by radiation exposure (14). Importantly, an additional advantage of this method for measuring DNA damage is that the staining protocol can be altered so that other targeted proteins within the same single cells can be quantified.

Compared with visual scoring, the LSC method developed here offers a convenient way to measure MN frequency in addition to other DNA damage markers such as γH2AX and 8-OHdG or centromere detection within MN, which further strengthens the use of the CBMN assay for radiation biodosimetry (1,2).

Author contributions

M.F. and K.H. carried out the LSC and visual scoring, respectively. W.L. carried out the radiation dosimetry of samples. M.F.F. and W.L. participated in the design of the study. All authors participated in the writing of the manuscript.

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Competing interests

The authors declare no competing interests.

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