Automated Image Analysis of Micronuclei in Binucleated Human Lymphocytes

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INTRODUCTION
The micronucleus assay is a well-known and recognized test to assess structural or numerical chromosome damage in cells following exposure to chemical compounds, radiation or environmental mutagens, or to investigate cellular DNA repair capacity after a mutagenic challenge. A micronucleus (MN) is formed during the metaphase/anaphase transition of mitosis (cell division). It may arise from a whole lagging chromosome (aneugenic event leading to chromosome loss) or an acentric chromosome fragment detaching from a chromosome after breakage (dissociation event) which do not integrate in the daughter nuclei.

The cytokinesis-block micronucleus (CBMN) assay is the preferred method for measuring MN in cultured (human) cells because scoring is specifically restricted to once-divided cells. These cells are recognized by their binucleated (BN) appearance after inhibition of cytokinesis by cytochalasin-B (an inhibitor of actin) [1]. Restricted scoring of MN in BN cells (Figure 1) prevents the confounding effects caused by suboptimal cell division kinetics which is a major variable in this assay. Although the scoring of micronuclei is relatively easy and certainly much less labour intensive or time consuming than, for example, scoring structural chromosome damage in metaphase, there is a growing need for an automated scoring procedure, especially if large amounts of cells (and individuals or samples) need to be scored. In the past, attempts to design automated scoring systems were more or less successful, e.g., [2-5] but it is only recently that automation of the micronucleus assay in binucleated human lymphocytes has been considerably improved.

We recently purchased an automated scanning and image analysis system which allows the automated detection of micronuclei in cytochalasin-B-blocked human white blood cells [6-7]. In order to validate the system we analyzed the frequency of micronuclei in human peripheral blood lymphocytes and recorded the results obtained with and without post-analysis corrections. We also investigated the same slides using a ‘classical’ microscopic analysis and compared the results obtained by both methods.

MATERIALS AND METHODS
Image analysis
The Metafer automated scanning platform from MetaSystems (Altusheim, Germany) was used (Figure 2). It consists of a Dell computer, a high-resolution JAI M1 CCD camera connected to a motorized Zeiss Axioplan microscope, and a Märzhäuser Wetzlar motorized scanning stage, enabling analysis of eight slides at a time. We also used the external slide feeder which loads up to ten 8-slide frames to the scanning stage automatically and unattended (see [7] for details). For analyses of micronuclei the MicroNude module for Metafer MSearch was used. It finds cells on a slide, and automatically counts the micronuclei. Each binucleated cell is captured and the image is displayed in the gallery. A real-time histogram lists the binucleated cells with the micronuclei counts. The co-ordinates of each binucleated cell are saved, so that their positions can be easily relocated.

Figure 1: Images of cytochalasin-B-blocked binucleated white blood cells. The number of detected micronuclei is shown in the lower right corner of each image. Courtesy of MetaSystems and Karger AG, Basel.
Cell cultures and measurements

For this comparative analysis we took advantage of an ongoing large-scale biomonitoring study for which 800 blood samples were obtained from individuals in the population at large. The blood was cultivated and prepared for micronucleus analysis using routine standardized methods [1]. Slides were stained by May-Grunwald (50%) followed by Giemsa (10%). Micronuclei were scored in all donors by the Metafer MSearch automated system. Of these, 50 donors were randomly chosen and also investigated by visual microscopical analysis. Scoring was always performed on coded slides. The slides were coded twice, before automated analysis and afterwards before visual inspection on the microscope. The code was broken only after all measurements were performed.

We also cultivated blood cells in the presence and absence of the chemical mutagen mitomycin C (MMC). The blood was obtained from two healthy donors recruited at our institute’s medical service. Two slides were prepared per concentration of MMC and each slide was measured five times with the Metafer MSearch automated system and once by visual microscopical inspection. The number of analyzed cells per subject or treatment was at least 1000 but if possible 2000 cells or more were scored.

All automated measurements were checked by the same operator who also performed the slide readings on the microscope. All BN cells with one or more micronuclei were inspected and corrected if necessary.

RESULTS

Figure 3 gives as an example the micronucleus frequencies found in the white blood cells of one of the donors after cultivation in the presence of MMC. The results are given for the classical microscopical scoring and for the automatic scoring with and without post-analysis corrections. A dose-dependent increase in micronucleus frequency was obtained, as expected, but it is obvious that the frequencies were considerably higher with automated analysis compared to the ‘normal’ microscopical inspection. Binucleated cells that were found to contain micronuclei were re-examined by inspecting the gallery and corrected if necessary. Following this rapid correction step, which took only a few minutes, micronucleus frequencies were obtained that were in perfect accordance with the results obtained by the classical microscopical analysis. This was found in the blood of both investigated donors.

Repeat measurements of the same slides also gave nearly the same results, demonstrating the reproducibility of the system. This is shown in Figure 4.

Results of the comparative analysis for the 50 donors (after correction) are given in Table 1 and Figures 5 and 6. It is clear that both methods gave the same results as the mean number of cells with micronuclei (per 1000 BN cells) was approximately identical. Figure 5 gives the correlation between the number of micronuclei per 1000 BN cells as measured by visual microscopical inspection and the Metafer MSearch automated system. This correlation was nearly 0.98. In Figure 6 the results are visualized using the Altman Bland method [8]. It shows only one deviation from the ±2SD interval. There were no systematic increases or decreases of the difference between both methods with increasing mean value. A t-test (paired two sample for means) did not reveal any difference between both methods (p = 0.51).

<table>
<thead>
<tr>
<th></th>
<th>Metafer MSearch Automated System</th>
<th>Microscopical Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of analyzed</td>
<td>Means: 1940</td>
<td>1595</td>
</tr>
<tr>
<td>individuals</td>
<td>Standard deviations: 148</td>
<td>439</td>
</tr>
<tr>
<td></td>
<td>Standard errors: 21</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Min - Max: 1500-2000</td>
<td>1010-2211</td>
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<tr>
<td>Number of binucleated</td>
<td>Means: 7.62</td>
<td>7.54</td>
</tr>
<tr>
<td>cells/individual</td>
<td>Standard deviations: 5.54</td>
<td>5.57</td>
</tr>
<tr>
<td></td>
<td>Standard errors: 0.78</td>
<td>0.79</td>
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<tr>
<td></td>
<td>Min - Max: 1-31.5</td>
<td>0.5-29.7</td>
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<tr>
<td>Number of cells with</td>
<td>Means: 8.23</td>
<td>8.14</td>
</tr>
<tr>
<td>micronuclei per 1000</td>
<td>Standard deviations: 6.07</td>
<td>6.08</td>
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<tr>
<td>BN cells</td>
<td>Standard errors: 0.86</td>
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<tr>
<td></td>
<td>Min - Max: 1-34</td>
<td>0.5-32.2</td>
</tr>
</tbody>
</table>

Table 1: Descriptive statistics of the micronucleus results obtained by Metafer automated system and microscopical analysis.

DISCUSSION

The data presented above are just a few examples of the results obtained. They clearly show that the MicroNuclei module for Metafer MSearch provides reliable data. As scoring is fully automated and up to 80 slides can be analyzed with the slide feeder, the investigation of 80 slides with, for example, 2000 cells per slide can be done overnight without any problem (scoring 2000 cells on a slide takes only a few minutes). Data are automatically saved and the system (including the microscope) can be automatically switched off if this is desired. With this system a few hundreds of slides can thus be scored within a week. It is clear however that a post-analysis correction should be made as the frequency of false-positive detections is considerable. It should also be stressed that only micronucleus positive cells need to be checked as we almost never found false-negative results. Corrections can be done with a simple mouse click and also take only a few minutes per slide at the most. It may also be important to indicate that the number of false-positive results can not only be influenced by the quality of the preparations but also by the staining method used. We used May-Grunwald-Giemsa but other staining dyes can be used such as...
acridine orange, Hoechst 33258 or propidium iodide. In particular, the use of fluorescent dyes may lower the number of false-positive counts.

CONCLUSIONS

There now exist a few commercial systems that allow micronucleus detection in binucleated cells. In our laboratory we chose the Metafer system because the MicroNuclei module for Metafer MSearch proved to be a reliable tool to determine the frequency of micronucleated binuclear cells in human lymphocytes as well as in other cell types (e.g. Chinese hamster cells, bone marrow cells and others). The system is fast and user friendly and requires only limited post-analysis corrections. In addition to the micronucleus detection the image analysis system allows metaphase finding and detection of chromosome aberrations (dicentrics), as well as the measurement of DNA damage in the comet assay, karyotyping, multicolor banding, cell signal analysis, FISH imaging, etc. Some of these applications are now also routinely used in our laboratory.

The micronucleus test should in most studies be accompanied by evaluation of the cytochalasin-B proliferation index (or the nuclear division index) by counting the frequency of mono-, bi-, tri- and tetra-nucleated cells also, extension to a cytome assay by additional scoring (e.g. of nucleoplasmic bridges between the main nuclei, budding, etc.) was recently proposed [9]. Such additional scorings are not yet automated but it is foreseen to be part of the system in the relatively near future.

REFERENCES


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