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Automation of Mouse Micronucleus Genotoxicity Assay by Laser Scanning Cytometry

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Background: The evaluation of the safety of drugs and other chemicals is an important aspect of toxicology work. The mouse micronucleus assay is a standard in vivo genotoxicity assay. Chromosomal damage is an indicator of genotoxicity, which manifests in the formation of micronuclei in polychromatic erythrocytes from bone marrow and in peripheral blood erythrocytes. The assay is laborious to perform by manual counting. The laser scanning cytometer allows automated and rapid quantitation of cellular and subcellular fluorescence in monodisperse cell samples on a microscope slide. The object of this study was to evaluate the application of this new technology in the mouse micronucleus genotoxicity assay.

Materials and Methods: One hundred forty-four mice of various strains were dosed with combinations of carcinogens and antioxidants. Duplicate blood films were prepared 3 days later. One set of slides was stained with acridine orange, and the proportion of micronucleated erythrocytes was counted in 5,000 cells per slide. The duplicates were stained with propidium iodide (40 μg/ml). Five thousand cells per sample were examined using a laser scanning cytometer. The proportion of micronucleated erythrocytes was measured.

Results: A coefficient of correlation of 0.96 was found between the data from the two assays. The automation of the assay on the LSC produced a considerable time saving and efficiency gain.

Conclusions: We conclude that with further development, laser scanning cytometry is likely to become the preferred modality for the performance of standard genotoxicity assays. Cytometry 44:153–155, 2001.

Key terms: laser scanning cytometry; mouse micronucleus assay; genotoxicity

The evaluation of the genotoxic safety of drugs and other chemicals is an important aspect of toxicology work in public health, academic research, and pharmaceutical industry laboratories. The mouse peripheral erythrocyte and bone marrow micronucleus assays are standard in vivo genotoxicity tests. Following in vivo administration of the agent under test, bone marrow or peripheral blood samples are harvested at specified times, and blood smears are prepared on microscope slides. Chromosomal damage manifests in the formation of micronuclei in polychromatic erythrocytes from bone marrow and in peripheral blood erythrocytes. Conventionally, smears are stained with acridine orange, and micronuclei are identified as intense spots of staining within cells. Trained technicians count samples for evidence of micronuclei. Normal samples of 5,000 counts typically contain 20 or so micronucleated cells, while a genotoxic agent may induce 60 or so micronucleated cells in a similar sample.

The conventional assay is laborious to perform by manual counting. Not only is it susceptible to observer fatigue and inaccuracy, but the margins for error are narrow. A large study may take weeks and considerable expense to perform. A machine which could automate the micronucleus assay, while allowing for direct observation and visual checking of results, would be a major advance in genotoxicity testing. Not only would it expedite current testing programmes, but it would make economic larger and more ambitious testing projects and research programmes.

The laser scanning cytometer (Compucyte, Cambridge, MA) allows automated and rapid quantitation of cellular and subcellular fluorescence in monodisperse samples of 5,000 or more cells on a microscope slide, and coarse discrimination of subcellular fluorescence such as would be attributable to fluorescence in situ hybridization probes or condensed, fluorochrome-tagged micronuclei (1,2). The object of this study was to evaluate the application of laser scanning cytometry technology to the automation of the mouse micronucleus genotoxicity assay.

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tion, and analysis were all under the control of Wincyte software (Compucyte) used in conventional mode. The scanning area was set to approximately that of the coverslip. Cells were identified by the programmed settings of threshold, background, and data contours using Wincyte, and the machine was preset to count 5,000 contoured cells. Threshold levels were set such that each cell was contoured above the background fluorescence. This was confirmed by viewing the image on the Scan Data Display window within Wincyte. The PI fluorescence from erythrocytes was detected, and the peak red signal was displayed against total red fluorescence, such that micronucleated cells could be clearly distinguished as a separate population on the (integral versus peak) dot plot. The data from each slide were stored as individual files in the computer memory.

Statistical Analysis

The correlation between manual and laser scanning cytometry (LSC) counts were estimated using the raw counts of micronuclei per 1,000 cells, using the MINITAB package (MINITAB, State College, PA). A general linear model analysis of variance following a logarithmic transformation of X1 was used to evaluate the effects of the carcinogen treatments for the manual and LSC counts.

RESULTS

Microscope/Manual Counting

Mature erythrocytes fluoresced a dull khaki/green, and micronuclei a bright yellow/green. No polychromatic erythrocytes were seen in the peripheral blood films.

Automated Imaging

A series of preliminary experiments was undertaken to standardize PI staining, machine settings, and Wincyte protocols. The laser scanning cytometer proved consistent and reliable in automated analysis of the sample population at standard settings for individual cells, but required resetting of threshold levels for each slide, owing to the low fluorescence from erythrocytes and variable background fluorescence.

Sample dot plots with gating on the micronucleated erythrocytes are shown in Figure 1. The results of the comparison between slides analyzed by visual microscopy and laser scanning cytometry are shown in Figure 2. A coefficient of correlation of 0.96 was found between the data from the two assays.

DISCUSSION

In this evaluation study, laser scanning cytometry is indicated to be a reliable and accurate quantitative tool for the automation of the mouse micronucleus genotoxicity assay. The automation of the assay, using laser scanning cytometry, produced a considerable time saving and efficiency gain. Each sample took on the order of 5 min to run on the machine and a further 5 min to analyze. While the mouse micronucleus assay is usually performed on polychromatic erythrocytes, our own studies deliberately made no discrimination between normo- and polychromatic erythrocytes.

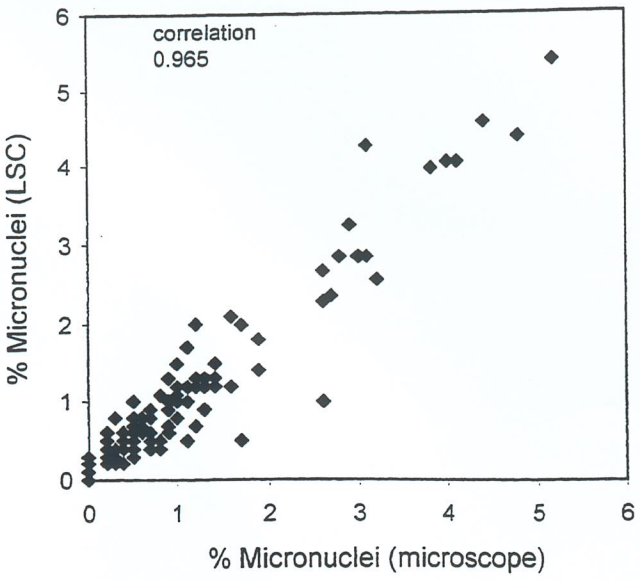

FIG. 2. Close correlation between percentage of micronuclei detected by manual and automated (LSC) techniques.

Our technique relies on the high intensity of bound propidium iodide in the micronuclei for gating and discrimination, and lower but sufficient red fluorescence in the erythrocyte to contour above background fluorescence. The relatively low levels of red fluorescence in the erythrocyte cytoplasm and variable levels of background fluorescence oblige the readjustment of the threshold level for contouring for each new slide. Alternative techniques may be developed to further refine this assay. For example, whole-cell contouring might be improved using green fluorochromes such as Oregon green to delineate the whole erythrocyte, with separate staining of the micronuclei with PI to improve discrimination.

We conclude that with further development, laser scanning cytometry is likely to become the preferred method for the performance of standard genotoxicity assays. It might also offer a suitable method for the fast or preliminary screening of samples prior to the more detailed analysis of micronuclei in polychromatic erythrocytes and similar toxicological models.

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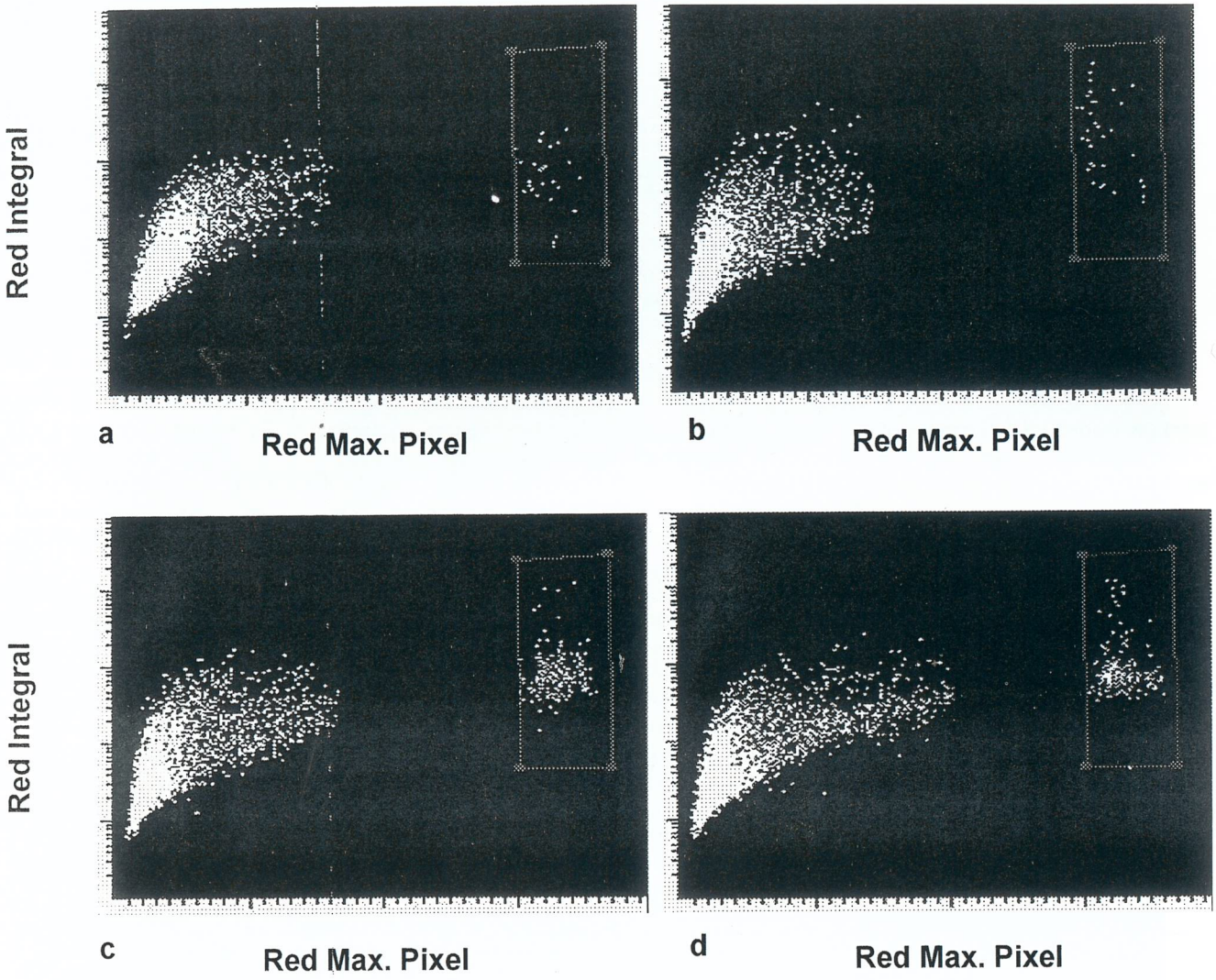

FIG. 1. Laser scanning cytograms of propidium iodide (40 μg/ml)-stained mouse erythrocytes. Gated area at right in each cytogram contains micronucleated erythrocytes. Other dots represent normal erythrocytes. **a, b:** Control slides. **c, d:** Slides from treated animals. Each cytogram is derived from approximately 5,000 cells.

MATERIALS AND METHODS

One hundred forty-four mice in four strains (A/J, Balb/C, 129/SvHsd, and NIH/01a), obtained from Harlan (Blackthorn UK), were dosed orally with 1 of 3 antioxidants; BHT, or BHA for 2 weeks, or diallyl sulfide for 3 days. A fourth control group was not treated with antioxidant. After a rest period, one third of the mice were each given a carcinogen (urethrane or 3-methyl cholanthrene), or saline by intraperitoneal injection. All fine chemicals used in this study were supplied by the Sigma-Aldrich Co., Ltd. (Poole, Dorset, UK). Altogether, there were four mouse strains, four antioxidant treatments including the untreated control, and three carcinogen treatments, including the saline control. These 48 treatment groups each comprised three mice in a factorial arrangement of treatments. Blood was taken 3 days after dosing by cardiac puncture from each mouse at postmortem. Blood smears were made in quadruplicate, according to the method described by McGregor et al. in 1980 (3). After air-drying, cells were fixed in methanol for 15 min, allowed to dry, and stored or stained with acridine orange (AO) or propidium iodide (PI).

One pair of slides was stained for conventional microscopy with acridine orange (40 μg/ml), according to the method described by Tinwell and Ashby in 1989 (4). The proportion of micronucleated erythrocytes was counted in 5,000 cells per slide at ×100 magnification, using an oil immersion objective and ×10 eyepiece.

The duplicate pair was stained with PI (40 μg/ml) for 15 min, washed with phosphate-buffered saline (PBS), and coverslipped for analysis by the Compucyte laser scanning cytometer (3). Five thousand cells per sample were examined, using the instrument with a ×40 objective, 488-nm argon laser at 5 mW output. Slide scanning, data collec-