Incorporating flow cytometry based automated scoring into the Reconstructed Skin Micronucleus Assay

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Introduction

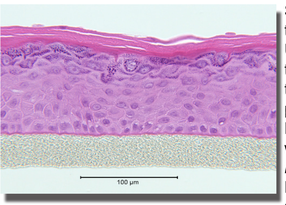


Figure 1: Cross section of MatTek EpiDerm tissue

Starting in March of 2009, *in vivo* genotoxicity testing for cosmetics sold in the European Union was banned by the 7th Amendment to the Cosmetics Directive. *In vivo* tests were used to confirm the results of *in vitro* tests, which produce a high number of false positives. The Reconstructed Skin Micronucleus Assay (RSMA) was developed as a possible replacement for *in vivo* tests in this tiered testing strategy. The RSMA uses a metabolically active 3-dimensional reconstructed human skin model with a functional stratum corneum (Figure 1). Test articles are applied topically, mimicking exposure of cosmetics. Cells from the basal layer are assessed for micronucleus induction by microscopy. Though this assay has performed well in prevalidation trials, scoring the micronuclei is labor intensive, which may limit widespread use of the assay. We have investigated the feasibility of automating the scoring of micronuclei from the basal cell layer using the In Vitro MicroFlow® system developed by Litron Laboratories.

Materials and Methods

Treatment of tissues

EpiDerm™ reconstructed skin constructs were obtained from MatTek Corporation and incubated at 37° C overnight. Mitomycin C (a clastogen) and Vinblastine (an aneugen) were administered topically in 10 µL of acetone, and the culture medium was replaced with fresh medium. Treatment and refeeding was repeated after 24 hours, and basal layer cells were harvested 24 hours later for a 48 hour treatment time. Two sets of tissues were treated identically in parallel; one set for analysis by In Vitro MicroFlow®, the other set for standard microscopic analysis. For tissues scored microscopically, Cytochalasin B (3 µg/ml) was included in the culture medium for the entire 48 hour treatment period to induce binucleation in dividing cells. Binucleation identified the relevant population of dividing cells, which served as an indicator of cytotoxicity.

After 48 hours, the basal cells were collected from the EpiDerm constructs by gentle trypsinization. Cells from tissues to be scored microscopically were fixed in methanol/acetic acid (3:1), affixed to slides, and stained with Acridine Orange (Figure 2). Cells from tissues to be analyzed by the In Vitro MicroFlow® system were washed in PBS and processed using the In Vitro MicroFlow® protocol for suspension cell cultures. Isolated nuclei were checked microscopically for staining and stored at 4°C before shipping to Litron Laboratories for analysis by flow cytometry.

Analysis by microscopy

500 cells were counted to determine % binucleation. Control cultures typically yield about 40% binucleated cells following 48h of treatment with cytochalasin B.

% Survival was calculated for each slide by dividing its % binucleation by that of the control.

To determine the **% Micronucleated cells**, 1000 binucleated cells per slide were scored for the presence of micronuclei. Cells containing multiple micronuclei were noted but scored as a single micronuclated cell.

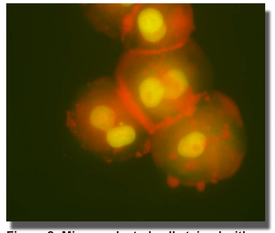


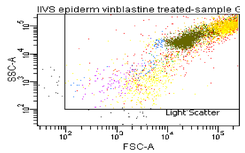
Figure 2: Micronucleated cell stained with Acridine Orange

Analysis by flow cytometry

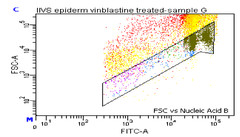
Processing cells for micronucleus analysis using the In Vitro MicroFlow® involves first staining whole cells in suspension with a DNA stain (EMA) that only penetrates necrotic and apoptotic cells. The cells are then lysed, and nuclei are stained with a second DNA stain (SYTOX) before analysis by flow cytometry.

Data acquisition was accomplished with a single-laser flow cytometer, 488 nm excitation. SYTOX-associated fluorescence emission was collected in the FL1 channel (530/30 band-pass filter), and EMA-associated fluorescence was collected in the FL3 channel (670 long-pass filter). Events were triggered on FL1 fluorescence. The FCM gating strategy developed for this MN scoring application required events to meet each of six criteria before they were scored as nuclei or MN. The incidence of MN was determined through the acquisition of at least 10,000 gated nuclei per culture.

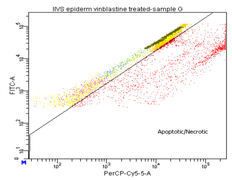
1. Total population of events detected by flow cytometry



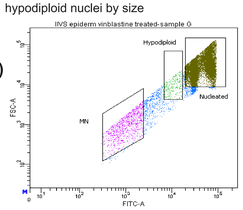
2. Identify nuclear material by size and fluorescence



3. Separate nuclear material from necrotic and apoptotic cells by differential staining



4. Differentiate nuclei, micronuclei, and hypodiploid nuclei by size



Correction for binucleation

Scoring cells by microscopy takes into consideration only micronuclei that are present in binucleated cells. Since the MicroFlow® system counts all micronclei and relates them to the total number of nuclei counted, a correction factor was needed to relate the number of micronuclei detected by flow cytometry to the number of actively dividing cells. The percent binucleation obtained from microscopic scoring was used in this correction.

% Micronuclei (MicroFlow) = m / N

% Micronuclei (MicroFlow; corrected for binucleation) = m / (NB/((1-B) + 2B))

m = micronuclei (by flow)
N = total nuclei (by flow)
B = binucleation (by microscopy)

Statistical analysis (one way ANOVA) was performed by GraphPad Prism version 4.03. Statistical significance ($p<0.05$) of the differences between each treatment group and controls was determined by Dunnett's Multiple Comparison Test.

Results

Mitomycin C

Scoring micronuclei from the tissues treated with Mitomycin C by either microscopy or MicroFlow® resulted in a statistically significant positive response at 10 µg/ml. However, the % of micronuclei per nucleus detected by MicroFlow was considerably lower than the % of binucleated cells containing micronuclei counted by microscopy. Introducing a correction factor to relate the number of micronuclei detected by flow cytometry to the number of actively dividing cells brought the % micronuclei detected by each method very close together (Figure 3).

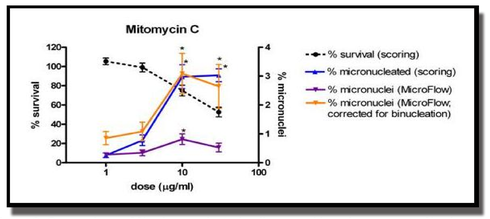


Figure 3: Comparison of % micronucleated cells scored by microscopy to % micronuclei obtained by MicroFlow® analysis following 48 h treatment with Mitomycin C (a clastogen). Means and standard errors are shown for each treatment group of three tissues. *Significantly different from controls by one way ANOVA

Vinblastine

Scoring micronuclei from the tissues treated with Vinblastine by MicroFlow® resulted in a much higher % micronuclei per nucleus than the % of binucleated cells with micronuclei detected by microscopy (Figure 4).

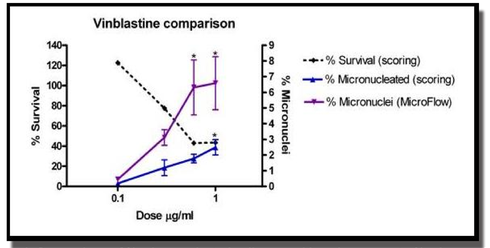


Figure 4: Comparison of % micronucleated cells scored by microscopy to % micronuclei obtained by MicroFlow® analysis following 48 h treatment with Vinblastine (an aneugen). Means and standard errors are shown for each treatment group of two tissues. *Significantly different from controls by one way ANOVA

Many of the binucleated cells contained multiple micronuclei, but were scored as a single micronucleated cell. In addition, vinblastine induced micronuclei in some *mono*nucleated cells, which were excluded from scoring. In the case of vinblastine, introducing a correction factor to the MicroFlow data would greatly exaggerate the % micronuclei.

Vinblastine treatment also caused a greater increase in hypodiploid nuclei and apoptosis/necrosis, which could be quantified by the MicroFlow® system. (Figure 5)

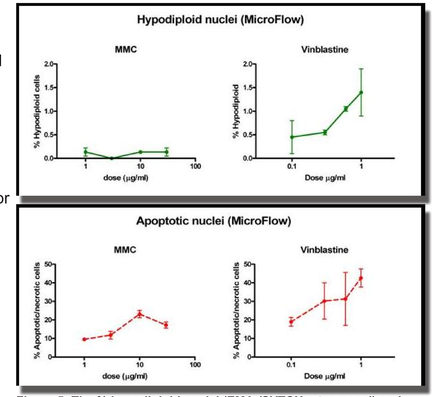


Figure 5: The % hypodiploid nuclei (EMA-/SYTOX+; top panel) and apoptotic/necrotic nuclei (EMA+/SYTOX+; bottom panel) identified by MicroFlow® from tissues treated as described in Figures 3 and 4.

Conclusions

The Litron MicroFlow® system worked well for identifying micronuclei induced by the clastogen Mitomycin C and the aneugen Vinblastine in the Reconstructed Skin Micronucleus Assay. Positive micronucleus induction was noted at similar doses when tissues were analyzed by either microscopy for flow cytometry. No major modifications were required to the RSMA protocol up to the preparation of the single cell suspensions of basal cells.

It was noted that the number of apoptotic/necrotic nuclei was higher in the cells from the EpiDerm tissues, compared to other cell lines that have been analyzed by *in vitro* MicroFlow®. In addition, a larger than usual proportion of cells was resistant to lysis. This likely reflects the presence of more highly differentiated keratinocytes in the stratified 3-dimensional tissue. The differentiation process involves both keratinization and a apoptotis-like program. The more highly differentiated cells are visually discernible and are normally excluded from scoring during microscopy.

One drawback of the MicroFlow protocol is that the current method for assessing cell proliferation in *vitro* cell lines, assessing the ratio of nuclei to fluorescent microspheres (i.e. counting beads), is not highly compatible with the EpiDerm™ constructs. One solution is to treat all tissues with cytochalasin B, reserve a portion of the basal cell suspension for scoring binucleation by microscopy, then proceed with the MicroFlow® protocol to quantify micronuclei. Cytochalasin B did not appear to affect the data obtained by flow cytometry (not shown). Automating only the scoring of micronuclei would dramatically reduce the amount of labor required to run this assay (Table 1).

Table 1: Comparison Between Microscopic Scoring (IIVS) and Automated scoring (Litron) of micronuclei

	Microscopic	Automated
Cells Counted per Sample:	1000	10,000-25,000
Time per Sample:	30-60 minutes	4 minutes

Future Goals

- **Eliminate the need to prepare a single cell suspension.** Collecting the basal cell layer in a single cell suspension is technically challenging and time consuming. We are evaluating methods to lyse cells directly in the tissue culture insert to release nuclei and micronuclei.
- **Automate proliferation.** Eliminating the need to prepare slides for evaluating survival by relative binucleation is highly desirable. Several markers for measuring proliferation by flow cytometry are commercially available and will be evaluated in the RSMA.
- **Test additional chemicals for genotoxicity.** Additional chemicals would include genotoxins and non-genotoxins that have been used during prevalidation of the Reconstructed Skin Micronucleus Assay.

Acknowledgements

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References:

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