Comparison of TK6 Micronucleus Data Generated by Flow Cytometry and Microscopy

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1 ABSTRACT

Good product stewardship requires assessment of potential genotoxicity using a battery of tests. The most recent addition to the genetic toxicity core battery of in vitro assays is the mammalian cell micronucleus assay. Historically, micronuclei (MN) are scored following chemical exposure by microscopy. However, flow cytometry also may be used to enhance the precision of the assay by increasing the number of cells scored per culture (e.g., 10,000 vs. 2,000 cells). Here we report the direct comparison of test results obtained from the same cultures using each method. Utilizing eight known genotoxic chemicals, including some promutagens requiring metabolic activation (S9), TK6 cells were exposed for 4 hours ±S9 and for 27 hours -S9 and dose-response curves were generated by both scoring methods. Cultures were harvested at the appropriate time and processed for each scoring method. Pooled data across the assays (N=198 cultures) indicated a good association between the scoring platforms (r^2 = 0.67). However, residuals indicated a bias towards higher MN frequencies in the flow cytometric data, which could be explained by the larger dynamic range of the assay. This inherent methodological difference did not adversely impact study outcome, as each chemical was either positive or negative across all exposure conditions and both scoring methods. The bias of flow cytometry to yield higher MN frequencies than microscopy was largely driven by results for the 27-hour -S9 treatment, as 5 of the 6 chemicals were positive in this treatment design at lower concentrations. This assessment was limited by the exclusion of weak genotoxicants, which would allow a more in-depth analysis of the sensitivity between the two methods. Cytotoxicity data also will be compared, as relative-nuclei counts are a built-in metric that may be appropriate regardless of the different generation times across exposure conditions. Overall, these data support the use of flow cytometry to score MN in TK6 cells.

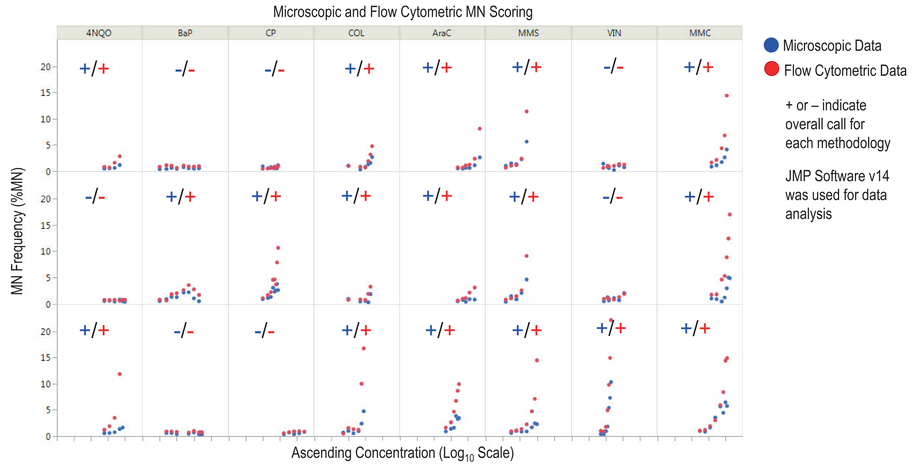
2 MICRONUCLEUS ASSAY

TK6 cultures were maintained in RPMI 1640 +L-glutamine (supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL penicillin-streptomycin [CCM]). On Day -1 (i.e., ~24 hours prior to treatment) TK6 cells were seeded in upright vented 75-cm^2 flasks at 1.3 to 1.8 × 10^5 cells/mL and grown in a humidified incubator set to 37 °C and 5% CO_2 in air. On Day 0, cells were adjusted to 3 x 10^5 cells/mL, seeded into upright vented 25-cm^2 flasks, and treated continuously for 27 hours -S9 or for 4 hours +/-S9 with a 40-hour recovery. The S9 mix contained ~17% (v/v) rat liver S9 homogenate (phenobarbital-5,6benzoflavone-induced) in a proprietary phosphate buffer containing NADP and G6P (Regenesys™, Moltox, Boone NC). The final concentration of S9 in culture was ~0.17% (v/v), and the vehicle (DMSO) was limited to 1% (v/v). The following chemicals were tested:

Mechanism of Action	OECD TG 487 Reference Chemicals	Acronym	CAS
Clastogen	Methyl methanesulfonate	MMS	66-27-3
	Mitomycin C	MMC	50-07-7
	4-Nitroquinoline-N-oxde	4NQO	56-57-5
	Cytosine arabinoside HCl	AraC	147-94-4
Pro-Mutagen	Benzo(a)pyrene	BaP	50-32-8
	Cylophosphamide monohydrate	CP	50-18-0
Aneugen	Colchicine	COL	64-86-8
	Vinblastine sulfate	VIN	143-67-9

At harvest, cultures were mixed, counted to determine cell density, and 1 mL was processed according to the instructions received with the In Vitro MicroFlow kit (Litron Laboatories, Rochester, NY). The remaining culture was processed to slides for microscopic scoring (centrifuged, resuspended in hypotonic solution, fixed in modified carnoy's fixative [9:1 ratio], and dropped onto slides). A total of 2,000 or 10,000 cells were scored for micronuclei by microscopy or flow cytometry, respectively. A positive response was defined as a statistically significant (1-tailed Fisher's Exact, $p < 0.05$, or $z' > 0.5$[1], for manual and flow scoring respectively), dose-dependent (Cochran-Armitage; $p < 0.05$) increase in MN frequency that exceeded the upper bounds of the 95% historical negative control interval (1.6% MN, microscopy only).

3 MICRONUCLEUS DATA



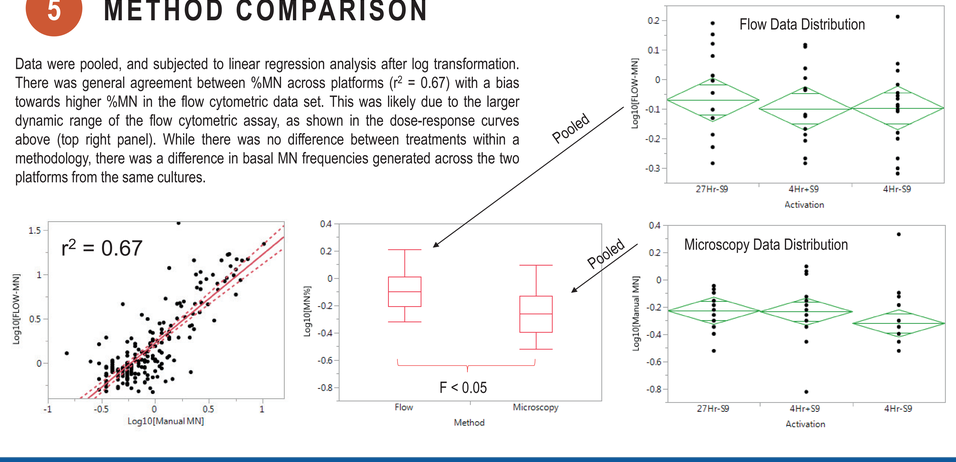
4 CYTOTOXICITY COMPARISON

Relative cytotoxicity can be computed using different techniques. Since MN should be evaluated only in cultures that exhibit <60% cytotoxicity, these different computations have been extensively studied using standard cell count data.[2] However, one advantage of the flow cytometric platform is inclusion of a cytotoxicity metric using inert fluorescent beads. Relative survival (RS), relative population doubling (RPD) and relative increase in cell counts (RICC) were all calculated using standard cell count data and were compared to the built in flow cytometric nuclei-to-bead ratio (NBR) cytotoxicity metric to evaluate differences in dose selction at ~50% relative cytotoxicity.

Metric	Score
RICC	6/8
NBR	4/8
RS	2/8
RPD	0/8

5 METHOD COMPARISON

Data were pooled, and subjected to linear regression analysis after log transformation. There was general agreement between %MN across platforms (r^2 = 0.67) with a bias towards higher %MN in the flow cytometric data set. This was likely due to the larger dynamic range of the flow cytometric assay, as shown in the dose-response curves above (top right panel). While there was no difference between treatments within a methodology, there was a difference in basal MN frequencies generated across the two platforms from the same cultures.



6 CONCLUSIONS

- Evaluating TK6 MN data by flow cytometry is a suitable alternative to microscopy (r^2 = 0.67)
  - There was 100% concordance of overall calls
  - The flow cytometric platform was more sensitive when evaluating dose-response of 27-hourr -S9 treatments (i.e., a lower concentration was considered positive)
  - There was a bias towards higher %MN results from the same cultures by flow, which was likely due to the higher dynamic range (and increased sensitivity) of the flow cytometric analysis
- The cytotoxicity metric of nuclei-to-bead ratio performed well in this limited data set
  - RICC was the most proficient metric when selecting concentrations that would not have the potential to be overtly cytotoxic, followed by NBR, RS, and RPD (which, was unexpected, as RPD is the *de facto* standard for cytotoxicity measurements in this assay)

7 REFERENCES

[1]Wojciechowski et al, 2016. Environ Mol Mutagen, 57(8):623-29. [2]Fowler et al, 2012. Mutat Res, 747(1):104-17