Assessment of Nuclei-Based Cytotoxicity Measurement as an Alternative to Bead-Based Methods in Flow Cytometry.

[1]Sean Oh, [1]Dong Ma, [1]Landen Power, [1]Katarina Aleksa.

[1]Labstat International, Kitchener, ON, Canada.

Background and Purpose

Cytotoxicity is a key factor to consider in in vitro mammalian cell genotoxicity tests, as it can sometimes lead to chromosome aberrations or micronuclei formation through mechanisms that are not relevant to genotoxic risk. The percentage cytotoxicity is calculated based on the number of cell counts, and an incorrect cell calculation can lead to inaccurate cytotoxicity. Therefore, it is important to ensure that cell counting methods are precise and consistent.

Flow cytometry is a commonly used platform for this purpose. In the MN assay using flow cytometry, beads are often employed to estimate cell numbers for calculating cytotoxicity. However, lot-to-lot variation in beads, even from the same vendor or batch, has been observed, which can introduce variability into assay results.

The OECD 487 guideline permits substituting cell count with the number of nuclei in the cytotoxicity calculation formula. In this study, we compared cytotoxicity percentages using two methods with 1R6F reference cigarettes: (1) estimating cell numbers using beads and (2) using nuclei counts instead. The goal was to determine whether these methods produce significantly different results or are comparable.

Using nuclei counts for cytotoxicity calculations offers several advantages. It eliminates the need for beads, improving assay sensitivity, reliability, and overall performance. Importantly, the method used for cytotoxicity calculation does not affect the %MN results, ensuring consistency regardless of the approach. Additionally, nuclei count-based calculations provide a more direct measure of toxicity, as a reduction in the number of nucleated events can be observed with increasing test item concentration, directly reflecting cytotoxic effects. This approach is more reliable than using beads, which are susceptible to variability due to lot-to-lot differences and fluctuations in flow cytometer conditions.

Materials and Methods

- **Cell Lines**: CHO-WBL (Chinese hamster ovary).
- **Test Articles**: 1R6F reference cigarettes.
- **Flow cytometry: BD FACSLyric**
  Volumetric collection: Flow rate = 2 µL/sec
  - If the sample is collected for the same length of time, the same volume will be obtained because the flow rate is fixed at 2 µL/mL per minute (i.e. Collection for 10 sec = 200 µL sample).

Materials and Methods

- **Cell Number Estimation Formula using beads:**

  Estimated cell #/µL = (A/C) x (B/D)

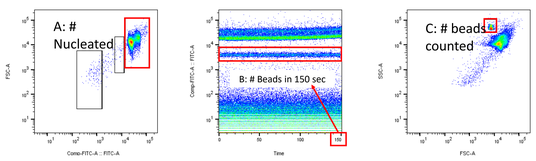
  Where:

  A = # of Nucleated events

  B = Beads concentration (Total # of beads/200 µL)

  C = # of Beads counted

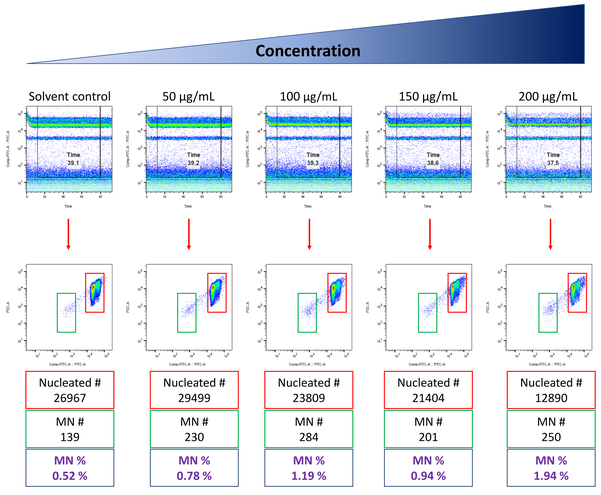
  D = Final sample volume (200 µL)



Results

Figure 1. The number of nucleated cells decreased as the concentration increased. The percentage of cytotoxicity can be calculated based on the difference from the baseline (solvent control).

Sample: Schedule 1 - 1R6F TPM, 3 hrs treatment, 27 hrs recovery, without S9. % MN and % cytotoxicity graphs are displayed in Figure 3.



Results

Figure 2. Representative raw data from Schedule 1, The same well was analyzed using two different cytotoxicity calculation methods, showing comparable %cytotoxicity and consistent %MN results.

Note: % MN values remain unchanged and are equivalent, irrespective of the cytotoxicity calculation method applied.

% Cytotoxicity calculation #1: using Beads.
Beads method = (A/C) X (B/D). The final sample volume is 200 µL.

TPM (µg/mL)	Nucleated (A)	# MN	# Beads total (B)	# Beads counted (C)	Est. cell #	% Cytotoxicity (100 - RPD)	% MN
T0	8608	N/A	4361	3575	5.25E+04	N/A	N/A
0	26967	139	4825	3508	1.85E+05	0	0.5
50	29499	230	5021	3639	2.04E+05	-11	0.8
100	23809	284	4988	3595	1.65E+05	6	1.2
150	21404	201	5159	3692	1.50E+05	17	0.9
200	12890	250	5184	3736	8.94E+04	56	1.9

i.e.) % Cytotoxicity of TPM 150 µg/mL

- PD (Control) = 1/Log(2) X Log(Final # Cells)/(Starting # Cells)
  = 1/Log(2) X Log(185,000/52,500) = **1.8**
- PD (TPM 150 µg/mL) = 1/Log(2) X Log(150,000/52,500) = **1.5**
- RPD = (PD of Treatment) / (PD of Control) X 100 = 0.8/1.8 x 100 = **83**
- % Cytotoxicity = 100 - RPD = 100 - 83 = **17%**

% Cytotoxicity calculation #2: using Nucleated events.
Number of cells can be substituted with Number of nuclei (nucleated) for calculating % cytotoxicity - *OECD 487*.

TPM (µg/mL)	Nucleated (A)	# MN	# Beads total (B)	# Beads counted (C)	Est. cell #	% Cytotoxicity (100 - RPD)	% MN
T0	8608	N/A	4361	3575	5.25E+04	N/A	N/A
0	26967	139	4825	3508	1.85E+05	0	0.5
50	29499	230	5021	3639	2.04E+05	-9	0.8
100	23809	284	4988	3595	1.65E+05	6	1.2
150	21404	201	5159	3692	1.50E+05	19	0.9
200	12890	250	5184	3736	8.94E+04	62	1.9

No need to use

i.e.) % Cytotoxicity of TPM 150 µg/mL

- PD (Control) = 1/Log(2) X Log(Final # Nucleated)/(Starting # Nucleated)
  = 1/Log(2) X Log(26967/8608) = **1.6**
- PD (TPM 150 µg/mL) = 1/Log(2) X Log(21404/8608) = **1.3**
- RPD = (PD of Treatment) / (PD of Control) X 100 = 1.3/1.6 x 100 = **81**
- % Cytotoxicity = 100 - RPD = 100 - 81 = **19%**

Results

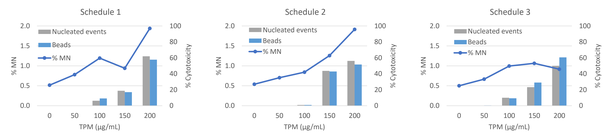
Figure 3. The response of 1R6F - % MN and % Cytotoxicity.

Schedule 1: 3 hrs treatment without S9.

Schedule 2: 3 hrs treatment with S9.

Schedule 3: 30 hrs treatment without S9.

1R6F response across all three schedules. Schedule 1 data from this set was used as a representative example in Figures 1 and 2 to demonstrate the new cytotoxicity calculation method using nucleated events and its comparability to the bead-based approach.



Conclusion

Our findings suggest that using the number of nuclei instead of beads to calculate the percentage cytotoxicity offers a more consistent and reliable method. This approach reduces the variability, streamlines the analysis process, and provides a more accurate assessment of cytotoxicity.

Reference

1. Bryce et al., 2007. "*In vitro* micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity", Mutation Research. 630(1-2): 78–91.

2. Bryce et al., 2010. "Miniaturized Flow Cytometry-Based CHO-K1 Micronucleus Assay Discriminates Aneugenic and Clastogenic Modes of Action", Environmental and Molecular Mutagenesis. 52:280-286.

3. Avlasevich et al., 2011. "Flow cytometric analysis of micronuclei in mammalian cell cultures: past, present and future", Mutagenesis. 26(1), 147-152.

4. OECD Guideline for the Testing of Chemicals No. 487, 2023. "*In Vitro* Mammalian Cell Micronucleus Test".