Flow Cytometry for In vitro Assessment of Genotoxic Substances

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Rationale:

Flow cytometric analysis offers new possibilities in genetic toxicology by providing an easy way to increase both the number of samples and the number of cells per sample, therefore increasing the statistical power of the results. Here we detail the implementation of the micronucleus assay by flow cytometry for the purpose of *in vitro* genotoxicity assessment.

Micronuclei are created whenever a fragment of DNA, either acentric chromosomes or even complete chromosomes, that won't migrate to the pole during cell division. The method also allows a mechanistic interpretation of DNA damages with the detection of damages caused by aneugens and clastogens. Since micronuclei represent damages transmitted to daughter cells the demonstration of mitosis is required unless some mitosis blocker is used (hence the population doubling calculation for each experiment).

Aneugens
Substance that interacts with components of the mitotic and meiotic cell division cycle apparatus leading to a deviation from the normal diploid/haploid number of chromosomes.[1]

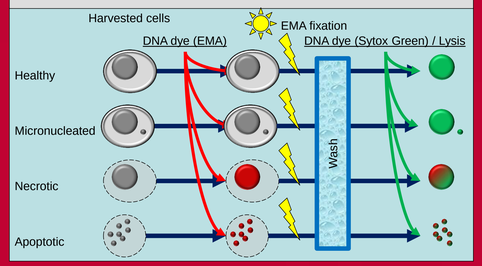
Clastogens
Substance that causes structural chromosomal aberrations in populations of cells.[1]

Experimental Procedures:

CHO-K1 cells were exposed to either vehicle or known genotoxins (Cyclophosphamide, CPA; Colchicine, CLC; and Mitomycin C, MMC) in two modes of exposure, short (approx. 4 hours with additional 24 hours in normal media) and extended (approx. 26 hours). Dead cells were tagged with Ethidium Monoazide (EMA), a fluorescent DNA dye (red) that can be covalently fixed by a simple exposure to visible light wavelengths . Cells were then lysed and labeled again with a fluorescent DNA dye (green). Fragmented DNA from apoptotic or necrotic cells can thus be excluded from analysis by selecting out bi-colored events.

DNA fluorescence intensity was acquired on FacsCanto II flow cytometer for the quantitation of haploid/diploid nuclei, hypodiploid nuclei and micronuclei. The relative population doubling of each sample was calculated with the help of fluorescent beads.

Labelling for flow cytometry



Results:

Exposure with CPA (1, 2, and 3 µg/mL) induced measurable dose-dependent in the short protocol with addition of metabolic activator (S9 liver fraction). The MN frequency reached 12.0%. Exposure with MMC (0.05, 0.10, and 0.25 µg/mL) induced measurable dose-dependent increases in micronuclei in the short and extended protocols. The MN frequency reached 9.5% and 12.2% in the short and extended exposure protocols respectively. Significant toxicity was present in the highest dose at the extended exposure. Exposure with CLC (0.1, 0.2, and 0.3 µg/mL in the short exposure and 0.05, 0.10, 0.15 µg/mL in the extended exposure protocol) induced measurable dose-dependent increases in both hypodiploids and micronuclei. The MN frequency reached 6.5% and 14.3% in the short and extended exposure protocols respectively. Significant toxicity was present in the highest dose at both the short and extended exposure. The range of valid genetic effects and cytotoxicity is more limited. The Hypodiploid frequency reached 3.0% and 9.8% in the short and extended exposure protocols respectively.

Cell Culture Acceptance criteria

Run population Doubling (from microscopy counts): 1.5-2.0

$$\text{Doubling} = \frac{\log_{10}(\text{Cells}^{\text{Post dosing}} \div \text{Cells}^{\text{Pre-treatment}})}{\log_{10}(2)}$$

Relative Population Doubling (RPD): ≥ 45% (cytotoxicity < 55%)

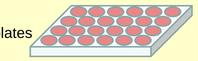
$$\text{RPD} = \frac{\text{Population doublings in treated cultures}}{\text{Population doublings in control cultures}}$$

where individual well population doubling is deduced from the cell-to-bead ratio

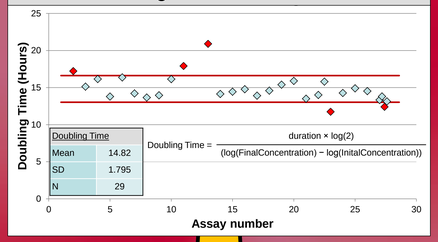
$$\text{PD} = \log_{10}\left(\frac{\text{Post-treatment cell-to-bead ratio}}{\text{Pre-treatment cell-to-bead ratio}}\right) \times 100$$

Plates preparation:

- Seeding of 24-well cell culture plates
- Incubation >16 hours
- Collection of cells for Pre-treatment cell counts
- Dosing of cell cultures
- Incubation for 26 hours
- Collection of cells for Post-treatment cell counts



CHO-K1 Doubling Times



Genetic Aberrations Acceptance Criteria (Positivity)

Power: recommendation of 10,000-20,000 events acquired per well, in replicates. Values of replicate wells pooled unless there was a technical error, or a failed relative population doubling value (RPD outside 1.5-2.0).

Significance level: 0.001 (χ^2)

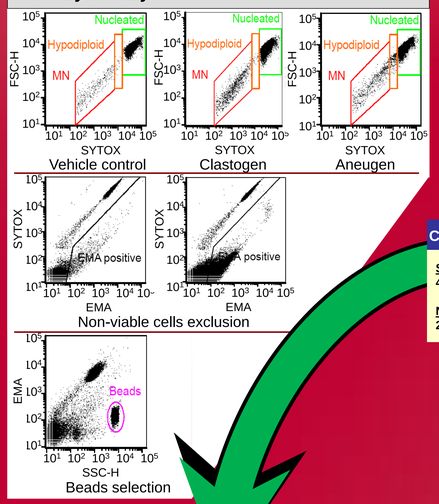
	Aberrations	
	-	+
Treatment	a	c
Vehicle	b	d

Biological relevance: Odds ratio (OR) with ±95%CI ≥ 2

$$\text{OR} = \frac{a/b}{c/d} \qquad \text{SE}\{\ln(\text{OR})\} = \sqrt{\frac{1}{a}+\frac{1}{b}+\frac{1}{c}+\frac{1}{d}}$$

95%CI = from exp (ln(OR) - 1.96 × SE{ln(OR)}) to exp (ln(OR) + 1.96 × SE{ln(OR)})

Flow Cytometry



Colchicine / Mitomycin C

Short exposure
4 hours

No exposure
22 hours

Cyclophosphamide

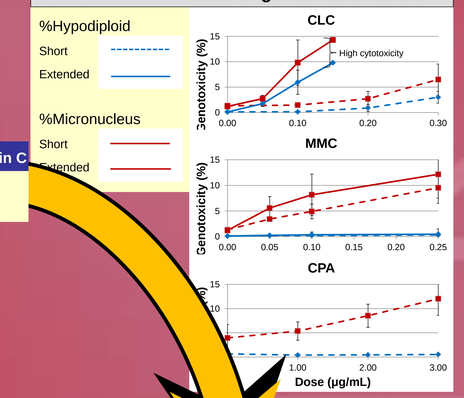
Short exposure
4 hours
Metabolic activation (S9)

No exposure
22 hours

Colchicine / Mitomycin C

Extended exposure
26 hours

Dose-related increases in genetic aberrations



Labelling Protocol and Flow Cytometry Acquisition

Short exposure, without metabolic activation

Treatment	Dose µg/mL	Hypodiploid Min / Max	Hypodiploid Mean	Hypodiploid SD	Hypodiploid n	Micronuclei Min / Max	Micronuclei Mean	Micronuclei SD	Micronuclei n
Vehicle	0	0.0 / 0.2	0.1	0.05	37	0.7 / 2.0	1.3	0.36	37
CLC	0.10	0.1 / 0.3	0.1	0.06	32	0.7 / 2.5	1.5	0.48	32
	0.20	0.2 / 2.4	0.9	0.69	23	0.8 / 5.5	2.7	1.44	23
	0.30	0.9 / 4.4	3.0	1.18	14	1.4 / 11.3	6.5	3.06	14
MMC	0.05	0.1 / 0.3	0.2	0.05	34	1.4 / 5.7	3.4	1.23	34
	0.10	0.1 / 0.3	0.2	0.05	33	1.8 / 8.3	4.9	2.01	33
	0.25	0.1 / 0.5	0.3	0.09	27	3.4 / 14.8	9.5	3.47	27

Short exposure, with metabolic activation

Treatment	Dose µg/mL	Hypodiploid Min/Max	Hypodiploid Mean	Hypodiploid SD	Hypodiploid n	Micronuclei Min/Max	Micronuclei Mean	Micronuclei SD	Micronuclei n
Vehicle	0	0.1 / 2.7	0.6	0.64	37	1.5 / 14.4	3.9	2.78	37
CPA	1.00	0.2 / 1.8	0.4	0.30	26	2.8 / 12.3	5.4	1.89	26
	2.00	0.3 / 0.6	0.4	0.09	22	4.9 / 12.5	8.5	2.39	22
	3.00	0.3 / 0.6	0.5	0.10	21	6.5 / 16.6	12.0	3.44	21

Extended exposure, without metabolic activation

Treatment	Dose µg/mL	Hypodiploid Min/Max	Hypodiploid Mean	Hypodiploid SD	Hypodiploid n	Micronuclei Min/Max	Micronuclei Mean	Micronuclei SD	Micronuclei n
Vehicle	0	0 / 0.2	0.1	0.04	37	0.6 / 2.0	1.2	0.33	37
CLC	0.05	0.9 / 2.8	1.7	0.57	23	1.7 / 3.9	2.7	0.66	23
	0.10	1.0 / 8.7	6.0	2.39	21	1.3 / 15.4	9.8	4.47	21
	0.15	NA	9.8	NA	1	NA	14.3	NA	1
MMC	0.05	0.1 / 0.7	0.2	0.10	35	1.6 / 10.8	5.6	2.25	35
	0.10	0.2 / 0.8	0.4	0.15	28	2.6 / 16.1	8.2	4.03	28
	0.25	0.2 / 0.8	0.5	0.16	22	5.1 / 20.6	12.2	4.65	22

Discussion:

The assessment of micronucleus formation in cell cultures requires that the cell division machinery be induced. Unlike the microscopy-based method which can rely on visual confirmation of the chromatin duplication after cell division block, the flow cytometry method necessitates the release of the genetic material through cell lysis. Therefore, an indirect method is used to demonstrate active cellular division, through cell population doubling.

CHO-K1 doubling times in culture has been calculated to be 14.82 ± 1.795 hours. In order to achieve more than 1.5 but less than 2.0 population doublings it was determined to proceed with a total cell culture time of 26 to 28 hours.

Of a total of 27 experiments, 3 had failed the population doubling requirement (including one for an obvious technical error).

Micronuclei are usually rare events and a large number of events are required to obtain analyzable values (10K in duplicate wells or 20K in single wells). Flow cytometry is a high throughput technology which provide great statistical power. It is not abnormal in such type of experiments to obtain statistical significance from small variations between samples. Such statistical analysis must therefore be accompanied by criteria for biological/clinical significance. For this method each condition replicate from cultures passing the population doubling requirement were evaluated individually. The relative population doubling (using counting beads in the labeling solution) was first used to exclude any well with excessive cytotoxicity (<45%RPD). Then the number of nuclei with or without aberration were compared to the vehicle control with a Chi-square statistical method (p<0.001). The biological relevance for genetic toxicology was set using the odds ratio and its confidence interval (95%), specific wells being accepted as positive when the OR ± 95%CI fell above the pre-set value of 2.

Using a two-pronged analysis approach, statistical significance with the χ2 method and biological significance with the OR ± 95%CI ≥ 2, a dose-response relation was obtained from the contact of CHO-K1 cells with CLC (both Hypodiploid and MN), CPA (with metabolic activation), and MMC.

Conclusions:

The analysis of in vitro micronuclei by flow cytometry provide an alternative to microscopic slides reading for detecting both aneugens and clastogens, with capacity for high throughput.

Bibliography:

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