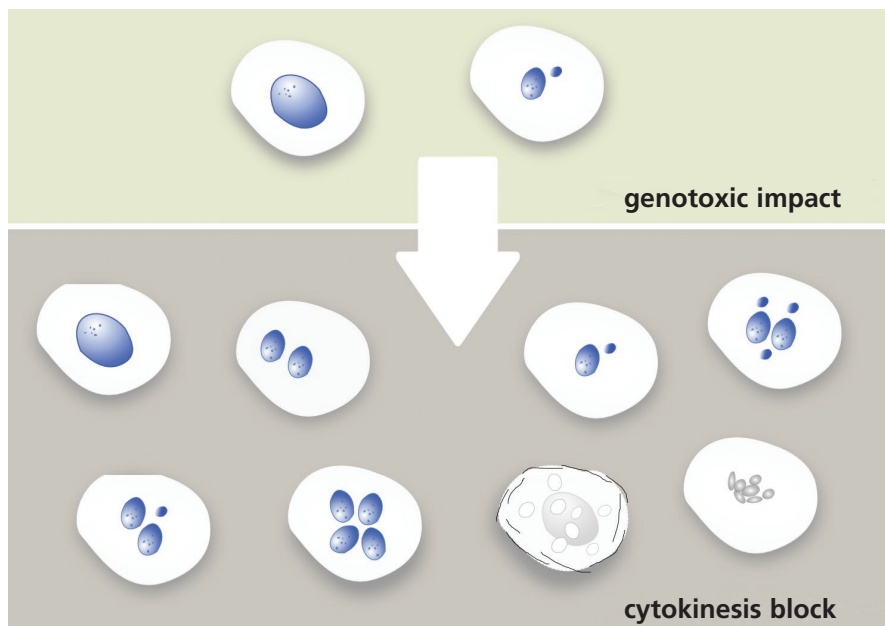


## Sophisticated Scoring Algorithm for Micronucleus Assays

### Key Features

- Novel Acapella® scoring algorithm for micronuclei frequency
- Identification of mono-, bi- and polynucleated cells
- Graphical feedback during parameter adaptation
- Automated image acquisition of fixed cells using the Opera® High Content Screening System



## Novel, Sophisticated Micronuclei Scoring Algorithm

### Background

Drug compounds with genotoxic side effects give rise to micronuclei (MN) formation in cell cultures. As a result, *in vitro* MN assays have become an important tool for assessing and pre-screening the mutagenic potential of drug candidates in the early stages of the drug discovery process. A micronucleus is a small DNA fragment in the cytoplasm of interphase cells – an erratic, small, extra nucleus. Micronuclei contain fragments or whole chromosomes that were not incorporated into the daughter cell nuclei during mitosis [Norppa and Falck, 2003].

Image-based methods for quantification of micronuclei formation rely on sophisticated methods for the identification of micronuclei occurrence in mononucleated and binucleated cells. Proper assignment of micronuclei to individual mono- and binucleated cells is a non-trivial task for image analysis and depends on algorithms with suitable scoring methods.

Here, we describe a new Acapella algorithm for micronuclei evaluation. We have used data from irradiated mammalian cells on the basis of the cytokinesis-block method [OECD guideline, 2009 and Fenech, 2007]. Addition of cytochalasin B to a cell population results in cells being trapped in their bi/multinucleated stage after one nuclear division, so that cytokinesis cannot be completed.

The algorithm allows for discrimination between mononucleated cells, which did not divide (either because they were damaged before treatment or because treatment was cytotoxic and caused cell cycle arrest), and binucleated cells which inaccurately completed the nuclear division after treatment.

## Application

One day before treatment, MCF-7 cells (breast cancer cell line) were seeded into a 384 CellCarrier™ microtiter plate (PerkinElmer) at a density of 6,000 cells per well and incubated overnight (37 °C, 5 % CO<sub>2</sub>). To induce chromosome damage which would result in micronuclei formation, cells were subjected to ionizing radiation from a Cs-137 source at doses of 0, 4 and 8 Gray (Gy). Following irradiation, 2 µg/ml cytochalasin B was added and the microplates were incubated for an additional 44 hr (37 °C, 5 % CO<sub>2</sub>). Cells were then fixed using 3.7 % formaldehyde and their nuclei/micronuclei stained using 16.23 µM Hoechst 33342. (Microplates kindly provided by Tony J. Collins, McMaster Biophotonics Facility, McMaster University, Hamilton, Ontario, Canada).

Confocal images were acquired using the Opera High Content Screening System equipped with the 20X water immersion objective, 405 nm laser excitation and a 450/50 nm emission filter. For each condition, twelve fields per well were measured in 179 wells so that approximately 50,000 cells were subject to MN evaluation.

Typical images are shown in Figure 1. Cells in both the control and irradiated wells (8 Gy) appear in their binucleated state after cytochalasin treatment. Irradiated cells also show micronuclear events.

The Acapella *Micronucleus Script* follows a sophisticated strategy involving multiple scoring parameters, which enable proper identification of mononucleated and bi/multinucleated cells and the micronuclei they contain. This is accomplished by combining and refining these main analysis steps:

- Nuclei and whole cell detection
- Identification of regularly shaped mono-, bi- or multinucleated cells
- Classification and assignment of micronuclei

In order to create a correct and reliable detection of micronuclei (MN) in mono- and binucleated cells and to avoid artifacts, several geometric and intensity properties of the nuclei and MN are identified, combined and weighed against each other:

- MN should be located in the cytoplasmic cellular area
- MN should have an intensity similar to that of the nuclei
- The MN diameter should be less than 33 % of the diameter of the main nuclei
- For binuclear cell identification: both nuclei are of a similar size, orientation and intensity, and are detached or marginally overlapping
- Irregular nuclei are excluded from the evaluation

The combination of all these aspects leads to an analysis strategy with four main elements (Figure 2).

The pivotal step of identification of binuclear cells (*Nuclear Pairing*) is further illustrated in Figure 3. Different cell types, cell cycle states, assay conditions and cytotoxic effects all contribute to a varied multitude of morphologically distinct nuclear features. Consequently, a sophisticated, flexible and adaptable multistep scoring system was created that meets these requirements and reflects the importance of this step.

To adapt the image analysis to different assay conditions, the last part of the script shown in Figure 2 (micronuclei detection) can also be controlled by adjusting a set of 10 parameters including threshold, contrast, minimum and maximum fractional area, minimum and maximum border distance.

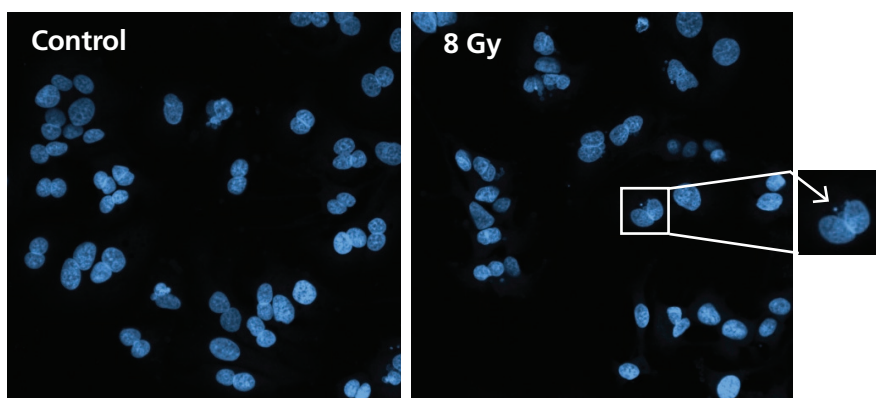
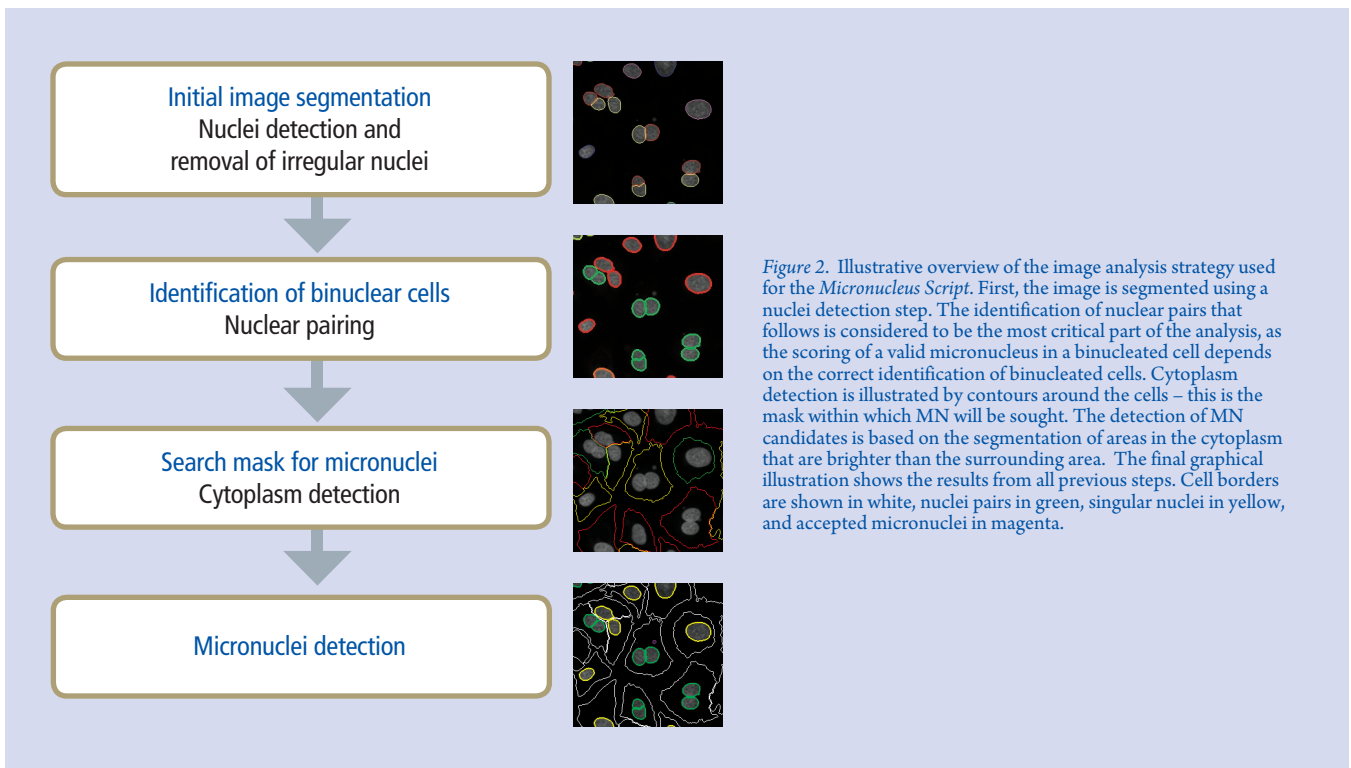


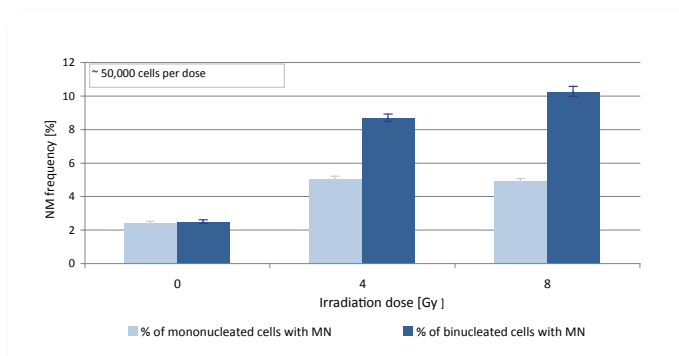
Figure 1. Images of MCF-7 control cells (left) and cells irradiated with 8 Gy (right). Hoechst 33342 stained nuclei and micronuclei are shown in blue. The confocal images were acquired on the Opera High Content Screening System using a 20X water immersion objective. A typical binucleated cell is shown enlarged, with a micronucleus resulting from the genotoxic impact of the irradiation.



*Figure 3. The Nuclear Pairing scoring system encompasses five measures (left), each of which contributes a factor in the assessment of all possible pairings of adjacent nuclei. These measures include assessment of the level of similarity between adjacent nuclei with respect to their size, brightness and roundness. The distance, which relates to the average diameter of the pair of nuclei being considered, and the orientation, which relates to the angle between the main axes of the two, is also assessed. Each measure is a contributing factor towards an overall score for each pairing. In the illustrations, the greener the line is, the higher the score, and the redder the line is, the lower the score. Yellow lines indicate score values where the pairing is just acceptable, as long as the average contribution from the other factors is not lower. The relative importance of the individual measures towards the overall score is determined by adjustable weights. For example, a weight of two would result in a factor being applied twice; whereas a zero weight would result in the measure being taken out of consideration. If the maximum score value is achieved, whereby the two nuclei touch each other and are equal in all examined properties, then this is considered to be a possible pairing of nuclei. The minimum score value that a pairing must reach in order to be accepted can be defined by the user as a fraction of this maximum. Yellow lines in the Overall score illustration indicate pairings with overall score values just above this limit. For each nucleus, the best possible and acceptable pairing is selected. If this is also the best pairing for the prospective partner then the pair is reconstructed into binuclear cells (Binuc score).*

By tuning all input parameters for the MCF-7 test assay, we were able to evaluate a dose-dependent increase in MN frequency in binucleated cells as the primary indicator of genotoxicity (Figure 4).

The numerical outputs generated by the script include the total number of cells, the number of mono-, bi-, polynucleated cells and the number of micronuclei in mono- and binucleated cells. These outputs provide the basis for simultaneously calculating the cytostatic/cytotoxic effects of tested compounds. Indices such as the Cytokinesis-Block Proliferation Index, the Nuclear Division Index or the Replicative Index can be derived from the data and provide additional useful information [OECD guideline, 2009 and Fenech, 2007].



**Figure 4.** MN frequency in mono- and binucleated MCF-7 cells after irradiation using 0, 4 and 8 Gy. The MN frequency in mononucleated cells is an indicator of the damage status of the cell population before treatment and for a negative influence on cell proliferation. The increasing frequency of binucleated cells with micronuclei reflects the genotoxic impact of the irradiation. Error bars denote standard deviations of the binomial distribution means.

## Conclusions

The analysis of micronuclei formation is challenging as it is a rare event and nuclei appear in different morphologies after geno/cytotoxic treatments. The Acapella *Micronucleus Script* gives the user maximum flexibility and reliability in detecting mono- and binucleated cells containing MN with its novel, sophisticated multistep scoring algorithm which correlates various relationship features of cellular nuclei. Visual feedback during the input parameter tuning greatly enhances the process. Additional numerical outputs can be derived to multiplex the MN frequency with readouts describing the proliferative response to the genotoxic event.

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