Increase Sensitivity with Confocal Acquisition in a High-Throughput Imaging Assay for Detecting DNA Damage and Genotoxicity

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OVERVIEW

- Purpose - 1) evaluate differences between running a nuclear puncta assay using widefield or confocal optics for imaging and 2) demonstrate ability to detect genotoxicity with automated high throughput imaging
- Methods – 1) use immunofluorescent assays to detect nuclear puncta positive for the markers phospho-H2AX and 53BP1, indicating the presence of DNA damage and 2) analyze nuclear morphology using the common stain Hoechst 33342 for signs of genotoxicity such as an increased incidence of micronuclei
- Results – 1) the markers phospho-H2AX and 53BP1 can be reliably measured and dose-dependent responses seen when using compounds known to cause DNA damage. The co-localization of the markers can be shown more clearly using confocal imaging with high magnification and 2) genotoxicity can be quantified in a high throughput manner using automated imaging and pre-configured software analysis modules

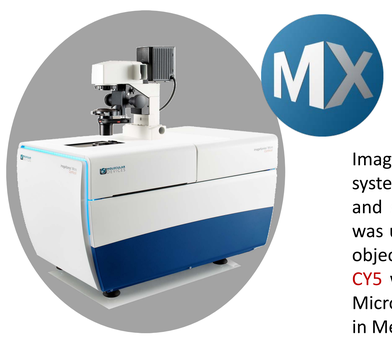
INTRODUCTION

The phosphorylation of histone H2AX on serine139 has been shown to be an early event and sensitive indicator of DNA double-strand breaks. It is also accepted that the tumor suppressor protein 53BP1, which coordinates DNA repair, becomes phosphorylated and forms nuclear foci in response to DNA damage. The presence of morphologically abnormal nuclei also signifies accumulated DNA damage. Micronuclei are small extra-nuclear bodies that result when mitosis progresses improperly, leaving behind fragmented or whole chromosomes that are encapsulated in a nuclear envelope. The evaluation of the ratio of cells with micronuclei is a standard test for genotoxicity during drug development.

This study reports our development of a three-color, rapid, and automated high-content imaging assay for assessing the ability of compounds to stimulate genotoxicity or DNA damage based on the immunofluorescent detection of phosphorylated histone H2AX and 53BP1 or the formation of abnormal nuclei, including micronuclei, upon cell division. The assay was conducted in 384-well microplates amenable for high-throughput screening. Widefield acquisition was compared to confocal imaging to demonstrate trade-offs in speed and assay robustness between the two processes.

MATERIALS & METHODS

- Seed and culture cells at 5000 - 7500 cells/well in 384 well thin-bottom polystyrene microplate
- Treat 24 hours with compounds and treat 120 minutes with hydrogen peroxide (H2O2) serially diluted 1:2 or UV irradiate 10 minutes
- Replace media and allow cells to recover for 30 minutes before fixing with 4% formaldehyde for 20 minutes at room temperature
- Wash cells with PBS then block and permeabilize cells for 1 hour at room temperature
- Add primary antibodies, anti-H2AX and anti-53BP1, and incubate overnight at 4° C
- Wash three times and add fluorescently labeled secondary antibodies. Incubate 1 hour at room temperature
- Add 10 uL Hoechst at 16 µM (final) and incubate an additional 15 minutes at room temperature
- Wash cells three times with PBS and acquire images



ImageXpress® Micro Confocal high content imaging system (Molecular Devices) equipped with widefield and spinning disk confocal optics (60 um pinhole) was used to acquire images with 20X, 40X or 60X air objectives using filter cubes for DAPI, FITC, TRITC, & CY5 wavelengths. The images were analyzed with a Micronuclei Application Module or a Custom Module in MetaXpress® software.

ANALYSIS OF NUCLEAR PUNCTAE

Multiple parameters reflecting DNA damage or genotoxicity can be measured at once with a user-designed software analysis module as shown below.

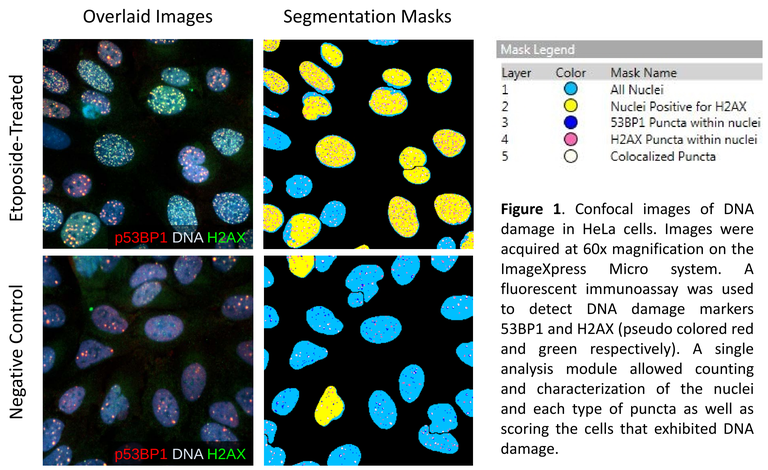


Figure 1. Confocal images of DNA damage in HeLa cells. Images were acquired at 60x magnification on the ImageXpress Micro system. A fluorescent immunoassay was used to detect DNA damage markers 53BP1 and H2AX (pseudo colored red and green respectively). A single analysis module allowed counting and characterization of the nuclei and each type of puncta as well as scoring the cells that exhibited DNA damage.

The segmentation masks allows one to correlate H2AX and p53BP1 puncta counts to severity of DNA damage. The counted puncta can be graphed to show a dose response curve.

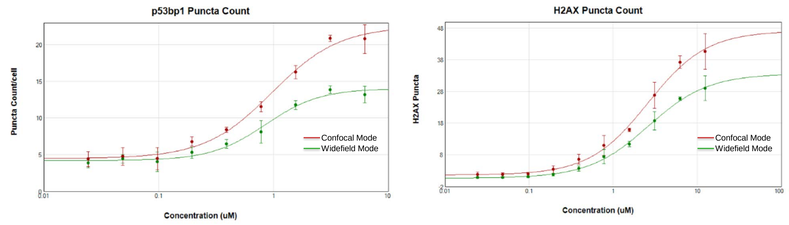
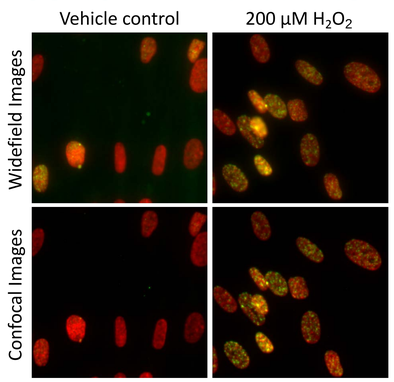


Figure 2. Widefield and confocal comparison of DNA damage in response to etoposide treatment. Puncta counts of each marker were measured using the confocal (red) or widefield (green) modes in ImageXpress Micro Confocal System. Images acquired with confocal mode showed a higher dynamic range and better sensitivity for both proteins.

CONFOCAL IMAGES YIELD MORE ROBUST RESULTS IN DNA DAMAGE ASSAY



WI-38 human lung fibroblast cells were treated for 2 hours with hydrogen peroxide to stimulate double-stranded DNA breaks. Multiple sites in each well were acquired for 8 replicate wells each of untreated and treated cells to evaluate assay dynamic range and Z´ factor of confocal vs. widefield images.

Figure 3. Images acquired with a 40X PA (0.95NA) objective in confocal mode illustrate improved puncta visibility and reduced background fluorescence compared to widefield images.

Table 1. Assay robustness was improved when images were acquired in confocal mode.

Parameter measured	Signal/Background		Z Factor	
	Widefield	Confocal	Widefield	Confocal
Puncta count (H2AX)	2.6	8.3	0.17	0.57
Puncta area/cell (H2AX)	10.2	19.6	0.42	0.54
Puncta Integrated Intensity/cell (H2AX)	22.6	27.8	0.29	0.35

NUCLEAR MORPHOLOGY REFLECTS GENOTOXICITY

Drug candidates are routinely tested for potential genotoxicity using an *in vitro* micronucleus assay. To avoid false positive results when steep toxicity curves are observed, the cell count of a compound dilution is plotted so an IC_{50} may be calculated. The micronucleus induction is evaluated at a concentration near each compound's IC_{50}. The percent of cells exhibiting micronuclei is compared to the vehicle control cells and results are reported as a fold-induction. In these experiments HeLa or U2OS cells were treated 24-27 hours with compounds serially diluted 1:2 so both overall cell toxicity and genotoxicity could be measured.

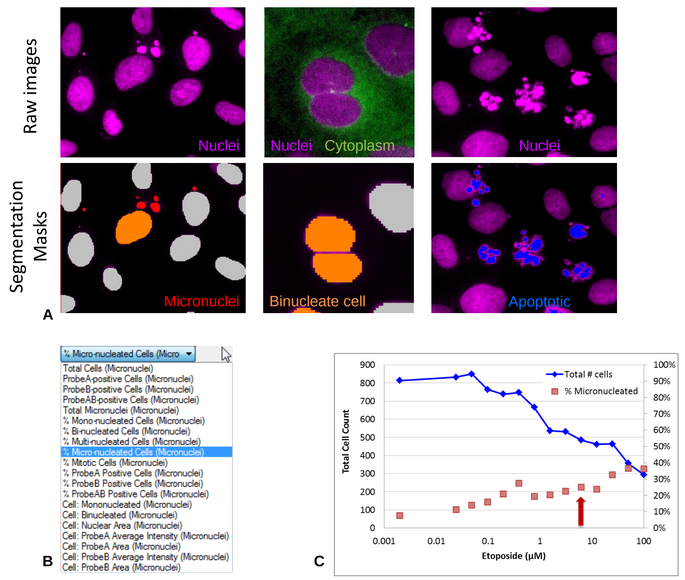


Figure 4. **A**. Genotoxic effects such as abnormal formation of micronuclei and bi-nucleated cells can be discriminated from apoptosis as seen in the zoomed 60X images of U2OS nuclei treated with compound (Hoechst staining is pseudo colored magenta) **B.** The MetaXpress Micronuclei software module reports out multiple parameters using only a nuclear stain or adding an additional 1-2 probes for markers indicating DNA damage or apoptosis. **C.** An example response for etoposide imaged at 40X magnification. The micronuclei results were reported from wells near the 50% toxic dose of each compound (red arrow for this compound).

Compound	Compound Concen. nearest IC_{50} (uM)	Number of nuclei analyzed	% Micronuclei	Micronuclei Fold-induction	% Apoptotic cells
Etoposide	6.25	485	25.0	2.8	2.7
Mitomycin C	6.25	490	36.0	4.0	7.6
Camptothecin	50	721	16.8	1.9	1.2
Hydroxyurea	12.5	455	44.2	4.9	22.2
Doxorubicin	1.56	512	31.9	3.5	2.9
EGCG	50	514	15.3	1.7	3.7
Control		786	9.0		1.1

Table 2. Results are from the dose of compound that caused no more than a 50% decrease in cell number. Total number of cells per well (over multiple fields of view), micronuclei fold-induction compared to control, and % apoptotic (based on a Caspase 3/7 marker) are shown.

SUMMARY

- Assays with low Z factors due to insufficient image quality can be improved by automated confocal acquisition.
- The ImageXpress Micro Confocal System acquires the high quality images required for accurately segmenting nuclear puncta indicating DNA damage so that co-localization can be measured.
- Screening assays that require confocal imaging can be run in 96, 384 or 1536 well plates and analyzed in less than or equal to the amount of time it takes to acquire.

MOLECULAR DEVICES