Assessing DNA damage by quantification of punctate nuclear markers

Introduction
Damage to DNA or chromosomes is an increasingly popular area of interest in biology because of its implications in genetic mutation, cancer, and aging. DNA damage may occur spontaneously or as a result of exposure to ionizing radiation, environmental toxins, or chemical toxins. There are several in vitro assays that can be used to identify and characterize the mechanism of action of a compound’s genotoxicity. The phosphorylation of histone H2AX on serine139 has been shown to be an early 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.1

This study describes our development of a three-color automated high-content imaging assay for assessing a compound’s ability to stimulate DNA damage, based on the immunofluorescent detection of phosphorylated histone H2AX and 53BP1. Levels of DNA damage were compared between a control group and cells treated with Mitomycin C (MMC), Hydrogen Peroxide (H₂O₂) or Etoposide.

Visualize nuclear puncta indicating DNA damage
DNA damage can be visualized and characterized even in heterogeneous cell populations using commercially available reagents and the ImageXpress Micro High Content Imaging Systems.

Primary antibodies against H2AX and 53BP1 (EMD Millipore) were detected with fluorescently labeled secondary anti-species antibodies (Life Technologies) with fluorescence excitation/emission profiles of 495/519 and 640/680 nm respectively. To accurately detect puncta within these cells’ nuclei, images are typically acquired using a high magnification (at least 40X) and are detected using standard FITC and Cy5 filter sets.

Assay workflow

Fix cells following treatment
Add DNA damaging compounds
Add primary antibody
Add fluorescently labeled secondary antibody and nuclear stain
Overnight
1 hr.
Read on ImageXpress Micro

Benefits
• Use a pre-configured software module for simple quantitation of DNA damage markers
• Count individual puncta accurately using confocal imaging
• Measure co-localization of two markers to determine the mechanism of DNA damage
The multi-parameter DNA Damage assay workflow was as follows:

- Seed cells at 5000-7500 cells/well in 384 well black-walled, clear thin-bottom polystyrene microplates
- Culture U2OS or CHO cells overnight at 37°C
- Treat cells with a serial dilution of MMC or Etoposide for 24 hours or treat cells with a serial dilution of hydrogen peroxide (H$_2$O$_2$) for 2 hours
- Replace media and allow cells to recover for 30 minutes at 37°C 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 with 1x PBS
- Add fluorescently labeled secondary antibodies and incubate for 1 hour at room temperature
- Add 16 µM Hoechst 33342 nuclear stain (final concentration) and incubate for an additional 15 minutes at room temperature
- Wash cells three times with 1x PBS
- Acquire images at 3 wavelengths
- Perform image analysis

DNA damage was quantified using three different methods which are described, from least to most complex, in the next section.

**Use simple Cell Scoring analysis for identifying damaged nuclei**

A pre-configured MetaXpress® software application module, Cell Scoring, allows the nuclei to be classified as positive or negative for a DNA damage marker, and the percentage of cells exhibiting DNA damage is calculated. The acquisition and analysis can be performed rapidly since imaging a single site with a 10X objective in widefield mode is often sufficient (Figure 1). A single field-of-view yielded >500 cells in the highest concentration of the compound and >2800 cells in wells below the toxic concentrations of Mitomycin C (MMC).

![Figure 1. DNA Damage response in U2OS cells after 24 hours treatment with MMC (an inhibitor of DNA replication).](image)

**A**. Top: all stained nuclei are pseudo-colored purple and nuclei positive for H2AX are marked with green. **Bottom**: segmentation masks showing healthy nuclei as red and DNA damaged-nuclei identified as green.

**B.** A plot of the dose response, showing the percentage of H2AX positive cells against MMC concentration.
Improve individual nuclear puncta count accuracy using confocal microscopy

The punctate staining of individual markers can be characterized by several parameters such as number, average intensity, total area, or total sum intensity on a per-cell or per-well basis. In this experiment, multiple images in each well were acquired on the ImageXpress Micro Confocal System using either widefield mode or 60 µm pinhole confocal mode. Both modes used a 40X Plan Apo objective for imaging. Images were analyzed with a simple user-configured MetaXpress software module that specifically counts puncta within the nuclei. Since confocal imaging rejects fluorescence from out-of-plane objects, puncta appear sharply distinct (Figure 2B) and are more easily segmented. Our results showed that images generated using confocal optics allow more accurate counting of individual puncta, resulting in a more robust assay as defined by a larger dynamic range (Signal/Background) and higher z’ factor (Figure 2).

Measure co-localization of two markers

Assessing the co-localization of two distinct DNA damage markers can provide important insights into the biological pathways involved in a compound’s mechanism of nuclear toxicity. The presence of the repair protein, 53BP1 can be calculated along with the measurement of the marker for double-stranded DNA breaks presented previously (% of cells positive for H2AX marker, and puncta count/intensity/area present per-cell) by combining a user-configured software analysis module with high magnification confocal images (Figure 3) to generate a co-localization value for the cells and their corresponding wells (Figure 4).

![Figure 2. A. Graph comparing DNA damage responses to Etoposide treatment using confocal (red) vs. widefield (green) imaging. The number of H2AX stained puncta per-cell were plotted. B. Images acquired with a 40X PA (0.95NA) objective in confocal mode (right) illustrate improved nuclear puncta visibility and reduced background fluorescence compared to widefield images (left). C. The confocal images yielded a more robust assay, as reflected in the higher signal/background ratios and higher z’ factors presented in the table.](image-url)
Summary
We have shown a sensitive multiplexed assay for detecting H2AX and 53BP1 using the ImageXpress Micro Confocal or widefield systems to enable high-throughput screening for DNA damage quantification. Traditionally, confocal imaging is utilized for more accurate identification of individual punctae in assays where measuring co-localization of nuclear markers is desired. This was reflected in our experiment, where the z’ factor resulting from analysis of widefield images was <0.5 whereas images acquired with confocal mode provided acceptable assay robustness. In addition, the ability to multiplex fluorescently labeled markers to study the various mechanisms of DNA damage allows the most efficient use of cells, reagents, and time.

Reference

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