Automated Imaging and Dual-Mask Analysis of γH2AX Foci to Determine DNA Damage on an Individual Cell Basis

Brad Larson, BioTek Instruments, Inc., Winooski, VT USA
Asha Sinha and Sachin Katyal, University of Manitoba, Winnipeg, Manitoba, Canada

Key Words:

Gamma H2AX
Gamma H2A.X
Histone H2AX
Serine 139
Double Strand DNA Break
DNA Damage
DNA Repair
Genotoxicity

Introduction

In mammalian cells, one of the most serious types of DNA damage is a double-stranded break (DSB), where the DNA double helix is completely severed. DSBs are associated with cell death and tumorigenesis, so they are of keen interest in understanding cancerous mechanisms as well as developing therapeutic treatments. One of the most characterized early markers of DNA DSBs is the phosphorylation of the histone 2AX (H2AX) to γ-H2AX. Here, a positive feedback loop is created between γ-H2AX and phosphatidyl inositol 3' kinase-related kinases, where repair systems are recruited to the damage, creating nucleation sites, or foci, that correspond to the level of DSB. When the foci are bound to fluorescently labeled antibodies, overall γ-H2AX levels can be visualized and quantified via immunofluorescence microscopy.

The conventional, manual method of evaluating foci via a γ-H2AX assay offers limited throughput and is subject to operator variability. Incorporating automated imaging and analysis into the assay workflow can help to enhance throughput, assay robustness and accuracy, while also increasing overall laboratory efficiency. Additionally, while manual methods allow for overall population assessment captured within an image or set of images, they are not conducive to analysis of individual foci within the population.

Here, we demonstrate an automated γ-H2AX assay workflow using a novel cell imaging multi-mode reader with advanced data analysis capabilities to process and image multiple samples simultaneously, including dual-mask capabilities to assess real-time population-level data as well as individual foci data, for a complete data set. This automated assay format provides an accurate, robust method to assess DNA damage in mammalian cells.

Materials and Methods

Materials

Cells

U251 (human glial) cells (Catalog No. 09063001) were purchased from Sigma Aldrich (Saint Louis, MO).

Assay and Experimental Components

The known cytotoxin, camptothecin (Catalog No. 208925) was purchased from EMD Millipore (Billerica, MA). The Alexa Fluor® 647 anti-H2A.X-Phosphorylated (Ser139) antibody (Catalog No. 613407) was purchased from BioLegend (San Diego, CA).

Cytation™ 5 Cell Imaging Multi-Mode Reader

Cytation 5 is a modular multi-mode microplate reader combined with automated digital microscopy. Filter- and monochromator-based microplate reading are available, along with laser-based excitation for Alpha assays. The microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield and phase contrast. With special emphasis on live-cell assays, Cytation 5 features shaking, temperature control to 65 °C, CO_2/O_2 gas control and dual injectors for kinetic assays. The instrument was used to image the stained DNA using the GFP imaging channel. Integrated Gen5™ Microplate Reader and Imager Software controls Cytation 5, and also automates image capture, analysis and processing.

Methods

Gamma H2AX Assay Performance

Cultured U251 cells were treated with concentrations of camptothecin ranging from 1-0 µM. Immunostaining was then performed using the parameters listed in Table 1.

BioTek Instruments, Inc.
P.O. Box 998, Highland Park,
Winooski, Vermont 05404-0998 USA
Phone: 888-451-5171
Outside the USA: 802-655-4740
Email: **customercare@biotek.com**
www.biotek.com

Gamma H2AX Immunostaining Procedure		
Step Number	Description	Time/Iteration
1	Fix cells with 4% paraformaldehyde	10 minutes
2	Wash with PBS, pH 7.4	2x
3	Permeabilize with 0.5% Triton-X 100 in PBS, pH 7.4	10 minutes
4	Wash in PBS, pH 7.4	2x
5	Dispense 50 µL/well of 1° antibody (1:1000; γH2AX) in 3% BSA plus PBS	Overnight @ 4 °C
6	Wash with 0.1% Triton-X 100 in PBS, pH 7.4	1x
7	Wash with PBS, pH 7.4	2x
8	Dispense 150 µL/well of Hoechst 33342 (0.2 µg/mL) in PBS, pH 7.4	1 hour @ 4 °C

Table 1. Immunostaining procedure using Alexa Fluor® 647 anti-H2AX-Phosphorylated (Ser139) antibody.

Following completion of the staining procedure, the plate was then imaged by the Cytation 5. Table 2 lists the settings used to perform automated image capture of each sample well.

Imaging Parameters	
First Imaging Channel	DAPI
Second Imaging Channel	CY5
Objective	10x
Montage	3 Row by 3 Column
Montage Overlap	Auto for Stitching
Exposure	Auto Exposure based upon Positive (1 µM Camptothecin) and Negative (0 µM Camptothecin) Control Wells

Table 2. Automated gamma H2AX imaging parameters.

Image Pre-Processing

Image pre-processing was applied to remove excessive background signal from the images using the criteria listed in Table 3. As the individual foci are small in diameter, and may be in close proximity within the nucleus, an optimized subset, or area, of the image that is analyzed to distinguish between background and true signal was required. A greatly reduced value of 2 µm provided the most optimal final images for subsequent analysis.

Imaging Pre-Processing Parameters	
First Imaging Channel	DAPI
Background	Dark
Rolling Ball Diameter	Auto (136 µm)
Image Smoothing Strength	0
Second Image Channel	CY5
Background	Dark
Rolling Ball Diameter	2 µm
Image Smoothing Strength	0

Table 3. Image pre-processing parameters.

Primary Foci Analysis

Primary mask cellular analysis criteria (Table 4) were applied to automatically place object masks around individual foci. The average area of an individual foci was then used in conjunction with results from the dual-mask analysis to calculate the number of foci per nuclei on an individual foci basis.

Primary Cellular Analysis Parameters	
Imaging Channel	CY5
Threshold	5000
Background	Dark
Split touching objects	Checked
Fill holes in masks	Checked
Min. Object Size	0 µm
Max. Object Size	8 µm
Include primary edge objects	Unchecked
Analyze entire image	Checked
Advanced Detection Options	
Background Flattening Size	2 µm (Rolling Ball diameter)
Image Smoothing Strength	0 Cycles of 3x3 average filter
Evaluate Background On	5% of Lowest Pixels
Primary Mask	Use Threshold Mask

Table 4. Foci analysis parameters.

Dual Mask Individual Nuclei Foci Analysis

Primary mask cellular analysis criteria (Table 5) were applied to automatically place object masks around nuclei in each captured image. Secondary mask cellular analysis criteria (Table 6) were then also applied to place linked additional masks around individual foci. The area of the primary mask was minimally expanded such that areas within the primary mask meeting the criteria to identify foci would still fall within the nucleus. "Threshold in Mask" was also selected to allow individual secondary masks to be placed anywhere within the initial primary mask meeting the included criteria.

Primary Cellular Analysis Parameters	
Imaging Channel	DAPI
Threshold	Auto (35)
Background	Dark
Split touching objects	Checked
Fill holes in masks	Checked
Min. Object Size	10 µm
Max. Object Size	100 µm
Include primary edge objects	Unchecked
Analyze entire image	Checked
Advanced Detection Options	
Background Flattening Size	Auto 300 µm (Rolling Ball diameter)
Image Smoothing Strength	2 Cycles of 3x3 average filter
Evaluate Background On	5% of Lowest Pixels
Primary Mask	Use Threshold Mask

Table 5. Primary mask nuclei analysis parameters.

Secondary Cellular Analysis Parameters	
Imaging Channel	CY5
Measure Within a Secondary Mask	Include Primary and Secondary Area in Analysis
Expand Primary Mask	1 µm
Threshold	5000
Background	Dark
Method	Threshold in Mask
Fill Holes in the Mask	Unchecked

Table 6. Secondary mask foci analysis parameters.

Results and Discussion

Automated 96-Well γH2AX Assay Imaging and Pre-Processing

Automated γ-H2AX assay imaging, performed in 96-well format, was validated using U251 cells exposed to various camptothecin concentrations. Cytation 5 automatically imaged the samples using a 3x3 image montage to ensure that a statistically relevant number of nuclei were analyzed per image. Auto exposure was used with each imaging channel (Table 2) and optimized using positive and negative control wells to ensure that the integrated Gen5 Microplate Reader and Imager Software settings accurately imaged the nuclei and labeled foci in each image.

Following image capture, Gen5 automatically pre-processed the samples to remove excess background signal and properly prepare each image for analysis. In the case of assays where the signal of interest emanates from small punctate areas within the well, this step is critical, particularly when large numbers of puncta fall within small areas of the image. This is observed in Figure 1A where cells were exposed to a high (1 µM) concentration of camptothecin. While individual labeled foci can be seen in certain nuclei, others containing higher foci numbers appear where the nuclei is completely covered with the fluorescent signal. This phenomenon precludes the possibility of performing accurate analysis of the DNA damage within these nuclei.

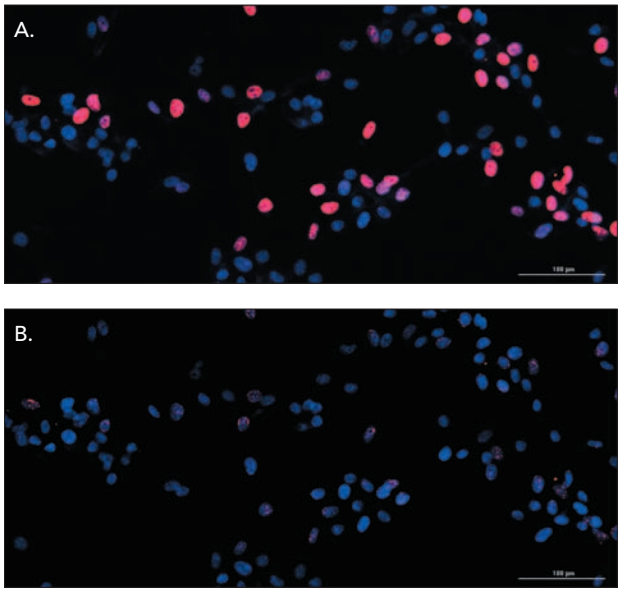

Figure 1. Image background signal removal via pre-processing. Zoomed 10x images (**A**) before; and (**B**) after pre-processing criteria (Table 3) applied using Gen5 Software.

By performing the pre-processing step in Gen5, with the incorporation of a 2 µm diameter in which to analyze each portion of the image, superfluous fluorescent signal not associated with the labeled foci is eliminated from the image (Figure 1B). Individual labeled foci are then visualized in each nuclei and accurately analyzed.

Following image capture Gen5 automatically stitched together the nine individual image tiles in the original configuration of the 3x3 montage. This allows for analysis of the entire imaged area of the well.

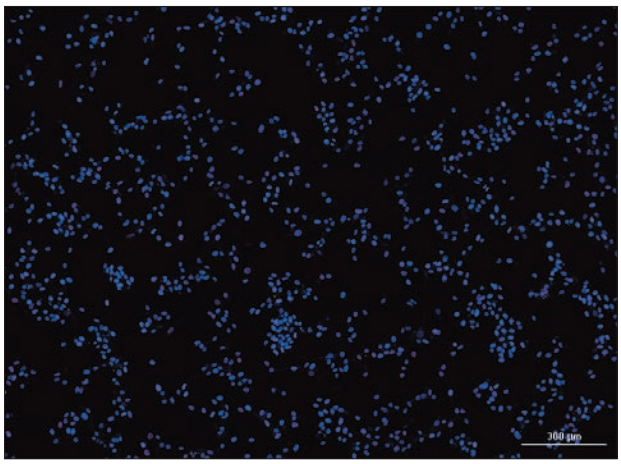

Figure 2. **Image stitching.** Final stitched image of the nine tiles included in the 3x3 montage.

The final images accurately portrayed the extent of DNA damage from each test condition, where low numbers of labeled foci per nuclei were seen in untreated cells (Figure 3A), while those treated with camptothecin showed increasing numbers of nuclei affected as well as an increasing number of labeled foci per nuclei (Figures 3B-D).

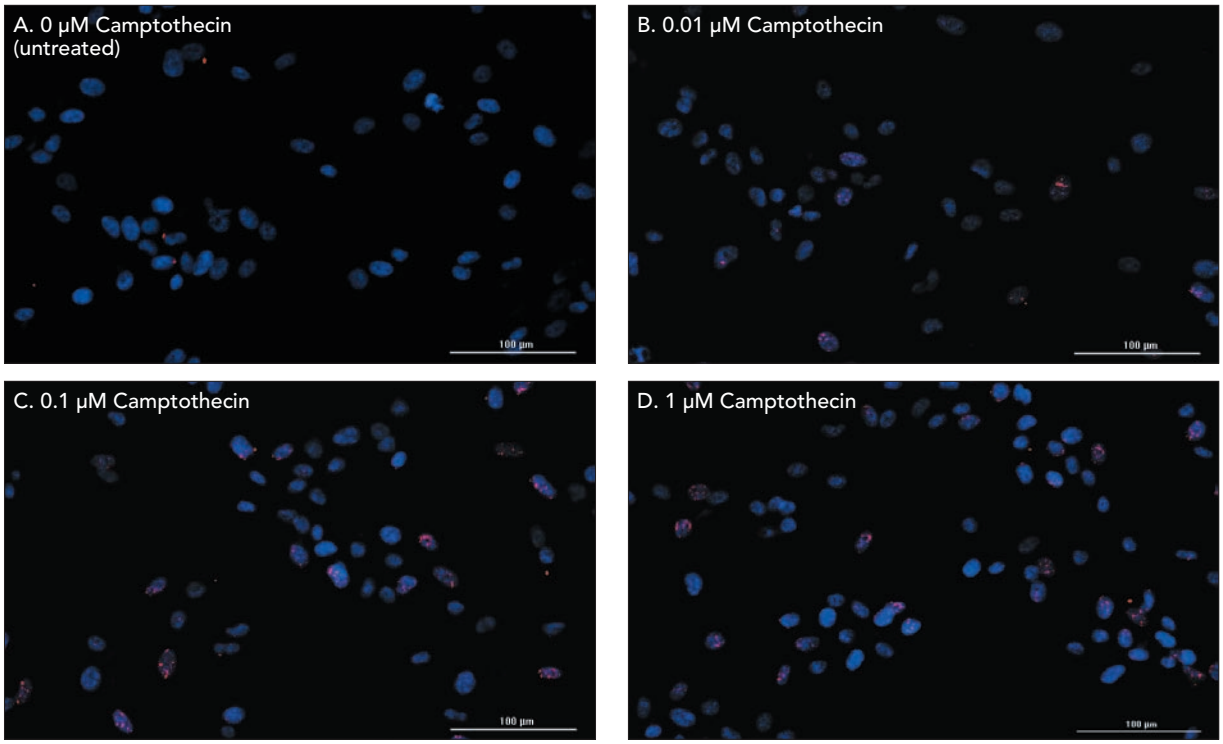

Figure 3. **Final processed images following camptothecin treatment and immunofluorescent staining.** Zoomed images captured using a 10x objective and 3x3 montage of U251 cells exposed to (**A**) 0 µM (untreated) camptothecin; (**B**) 0.01 µM camptothecin; (**C**) 0.1 µM camptothecin; or (**D**) 1 µM camptothecin. Blue: Hoechst 33342 stained nuclei; Red: CY5 signal from labeled foci.

Automated γH2AX Assay Analysis

Individual Foci Identification

Primary cellular analysis criteria (Table 4) were applied to all images captured with Gen5 Software using the fluorescent signal from the CY5 channel to place object masks around individual labeled foci (Figure 4). Minimum and maximum object sizes were set low due to the small size of each labeled foci. The background flattening size, or rolling ball diameter, was also set to 2 µm as was seen in the image pre-processing step, to aid in accurate identification of each foci. The area of each individual foci was reported and averaged per image. A value of 1.95 µm was determined as the average area of a labeled foci. This value was then used in the second cellular analysis step to calculate the number of labeled foci within each individual nuclei.

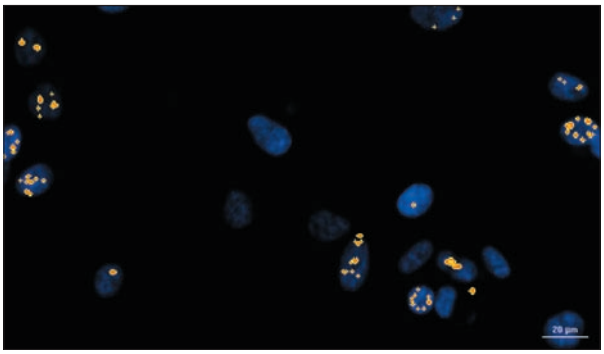

Figure 4. **Automated cellular analysis for specific identification of labeled foci.** Object masks placed using signal from antibody labeled foci. Images captured using a 10x objective, 3x3 image montage, and DAPI and CY5 imaging channels.

Dual-Mask Identification of Labeled Foci per Nuclei

Primary and secondary cellular analysis criteria (Tables 5 and 6) were applied to all images captured using Gen5. Primary cellular analysis automatically masked each nuclei (Figure 5A), while secondary analysis automatically placed individual masks around nuclear foci specifically within the original primary masks (Figure 5B).

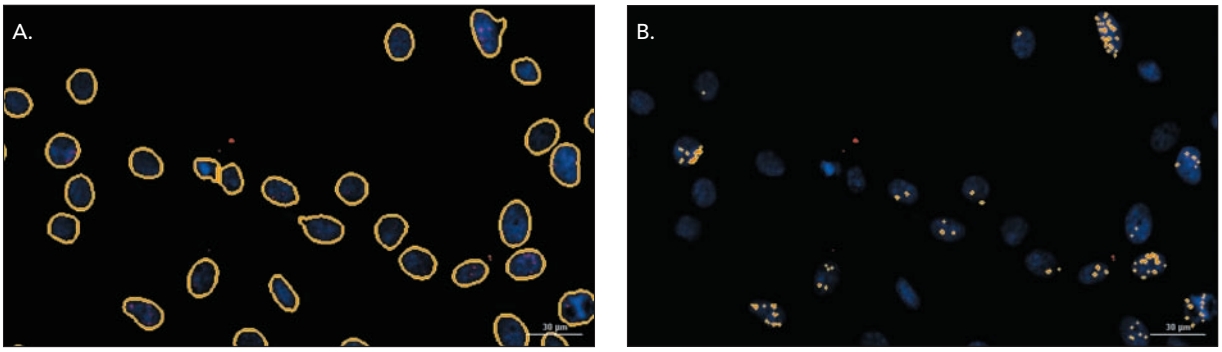

Figure 5. **Automated γH2AX dual-mask analysis based on user-programmed parameters.** Primary cellular analysis object masks showing (**A**) nuclei and secondary masks showing (**B**) labeled foci, respectively. Images captured using a 10x objective, 3x3 image montage, in addition to DAPI and CY5 imaging channels.

By using primary cellular analysis to initially mask nuclei, the foci identified were properly linked to each nuclei; allowing for analysis on a single nuclei level.

Subpopulation analysis was then applied to identify nuclei in each image exhibiting positive DNA damage. The total signal from the CY5 channel within objects identified using secondary analysis was used to apply threshold criteria. As foci appear and increase in number per nuclei the total fluorescence in the CY5 channel within the object masks placed around the foci also increases. Therefore, a value greater than 100,000 RFU was set to eliminate nuclei exhibiting low levels of fluorescence in the CY5 channel not emanating from true labeled foci. Using this criteria, nuclei containing distinct labeled foci meet the set threshold parameter for being γH2AX positive cells (Figure 6).

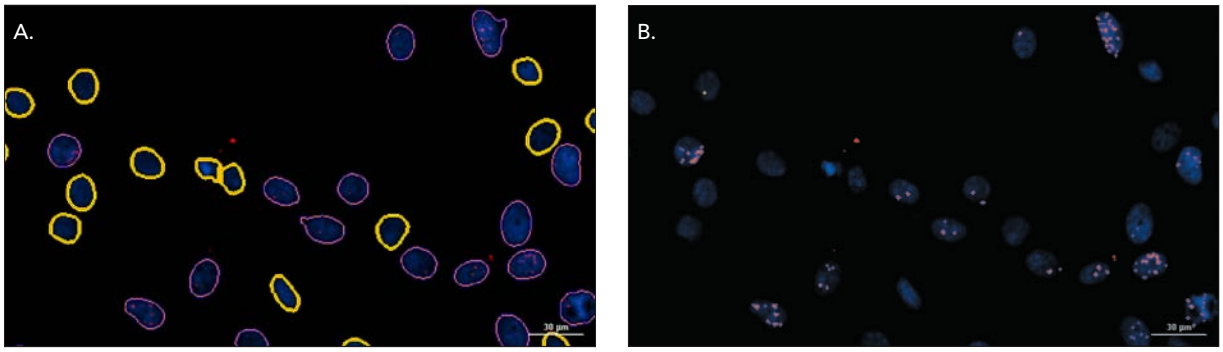

Figure 6. γH2AX positive nuclei. (A) Nuclei meeting γH2AX positive subpopulation criteria; and (B) labeled foci within positive nuclei. Positive γH2AX nuclei in purple and negative nuclei in yellow.

Labeled Foci per Individual Nuclei Calculation

Upon completion of the γH2AX assay procedure and image preparation, Gen5 was used to calculate the commonly used assay metric "labeled foci number per nuclei" on an individual nuclei basis. This was generated using the following formula and object metric of interest (Table 7), in addition to the average foci area value from the initial foci analysis (1.95 μm^2), to automatically calculate the number of foci per nuclei:

$$M_1/1.95$$

Secondary Mask		
Calculated Metric	Description	Data Reduction Designation
Area_2[Tsf[Cy5]	Total foci area value within the secondary masks of an identified nuclei	M_1

Table 7. Number of foci per nuclei metrics.

When the total area is divided by the average area of a labeled foci, the final foci count within nuclei is yielded.

Dual-Mask γH2AX Assay Validation Data

The results in Figure 7A illustrate that the total coverage area per nuclei of labeled foci increases in direct correlation to the camptothecin treatment level. Then by applying the formula explained above, the number of labeled foci per nuclei is calculated (Figure 7B). This value agrees with expected results as the number of foci in an individual nucleus starts at an insignificant level in wells containing untreated cells, indicating little to no DNA damage, and increases directly with camptothecin treatment.

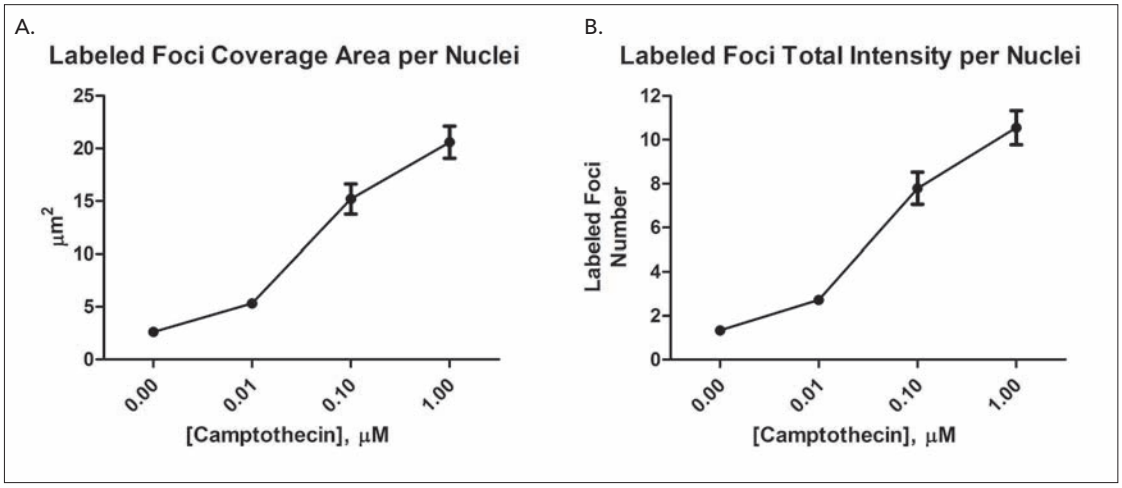

Figure 7. Labeled foci analyses using U251 cells exposed to various camptothecin concentrations. (A) Total foci coverage area per nuclei, and (B) calculated foci number in individual nuclei. Twelve replicates tested at each compound concentration.

Because the foci count is calculated from the total foci coverage area for each nuclei counted in the image, using the dual-mask analysis, it is also possible to quickly assess results for an individual nuclei by clicking on that object. The linked data is then highlighted (Figure 8). Therefore, if an interesting phenomenon is observed in a particular object, all relevant data can be immediately scrutinized.

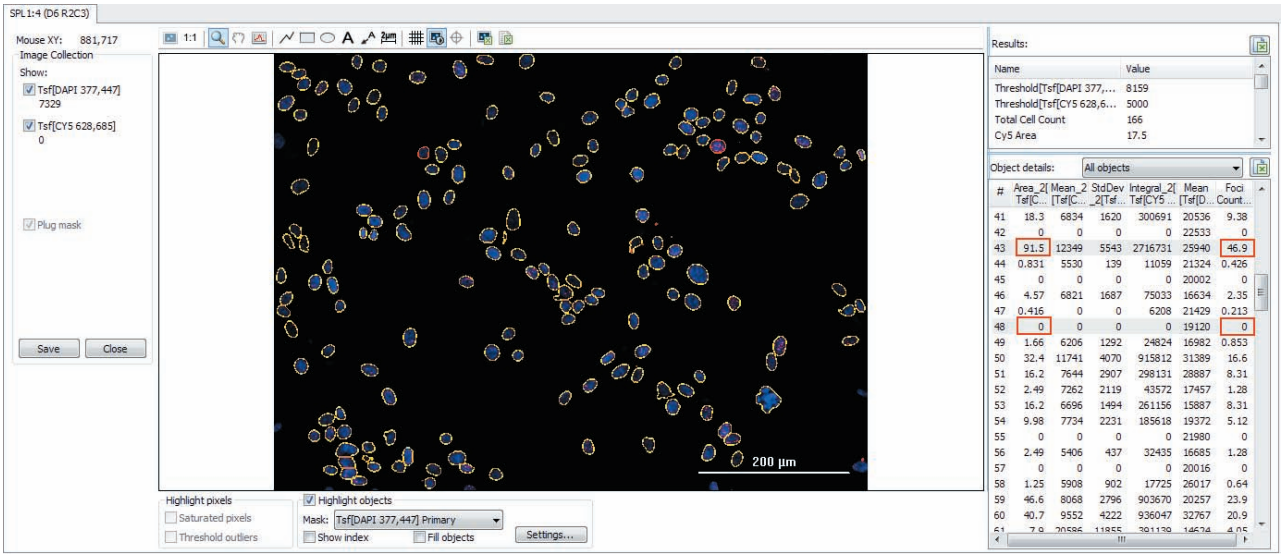

Figure 8. **Observation of single object data. Objects of interest highlighted in red.** Relevant data such as labeled foci area coverage and calculated foci count highlighted in the data table to the right.

Finally, using the data from the dual mask individual nuclei foci analysis, subpopulation parameters were applied to the primary objects, representing txd in each image using the criteria listed in Table 8. The criteria used was the combined total signal within object masks placed around foci interior to previously identified nuclei. The established condition separates the nuclei exhibiting DNA damage, as witnessed by a minimal total signal from identified labeled foci, from undamaged nuclei.

Subpopulation Parameters		
Criteria	Description	Condition
Integral_2 (Secondary Mask CY5 Integral Signal)	Total CY5 fluorescence from identified foci within an individual nuclei	>100000

Table 8. Gamma H2AX positive cell criteria.

Using the subpopulation parameter listed in Table 8, the number of γH2AX positive nuclei per test condition was also automatically determined (Figure 9A). By comparing this number to the total nuclei in the image, a normalized γH2AX positive nuclei percentage was then calculated (Figure 9B).

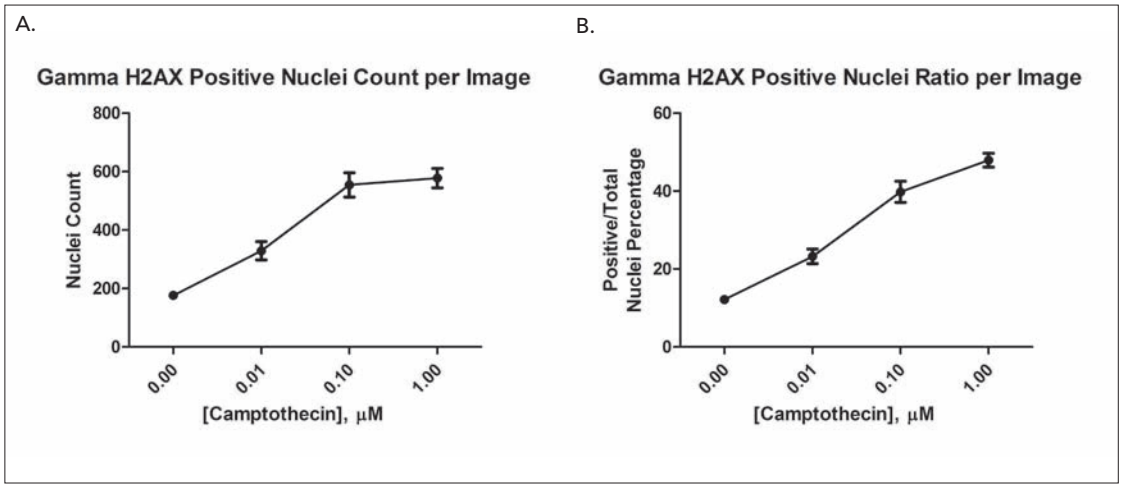

Figure 9. **γH2AX positive (A) nuclei count and (B) ratio to total nuclei counted.** U251 cells exposed to various concentrations of camptothecin. Twelve replicates tested at each compound concentration.

From the graphs in Figure 9 it is also evident that the number and ratio of nuclei meeting the established subpopulation criteria increase when cells are exposed to higher concentrations of camptothecin.

Results for both foci count per nuclei and percent γH2AX positive nuclei exhibit similar trends across all camptothecin concentrations tested, confirming the ability of the automated imaging procedure and dual-mask analysis method to deliver accurate, robust data on an individual nuclei and population basis.

Conclusions

Incorporating a cell imaging multi-mode reader to automate the γ-H2AX assay provides increased throughput to aid in laboratory efficiency. At the same time, dual-mask capabilities allow population level analyses as well as single nuclei scrutiny, and all analyses are automatically performed without the need for separate software. Automated imaging and analyses also eliminates human subjectivity and analysis errors that could potentially skew results. This creates a robust, user-friendly and accurate method to detect DNA damage in mammalian cells.