Application Note

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

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Abstract

Double-strand DNA breaks represent a critical genotoxic effect. Associated with cell death and tumorigenesis, they are well defined by the phosphorylation of histone 2AX (H2AX) to γH2AX as part of the DNA repair process. In the procedure described, following immunostaining, automated fluorescent imaging and dual-mask spot counting is performed to quantify labeled foci per nuclei after drug treatment.

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 γH2AX. Here, a positive feedback loop is created between γH2AX 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 γH2AX levels can be visualized and quantified via immunofluorescence microscopy.

The conventional, manual method of evaluating foci via a γH2AX 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 overall population assessment captured within an image or set of images, they are not conducive to analysis of individual foci within the population.

This application note demonstrates an automated γH2AX assay workflow using a novel cell imaging multimode reader with advanced data analysis capabilities to process and image multiple samples simultaneously, including dual-mask spot counting capabilities to determine real-time population-level data as well as foci numbers per individual nuclei, 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 and media

U251 (part number 09063001) and U373 (part number 08061901) (human glial) cells were purchased from Sigma-Aldrich (St. Louis, MO).

Assay and experimental components

Topotecan (part number 1672257) and neocarzinostatin (NCS) (part number N9162) were purchased from Sigma-Aldrich (St. Louis, MO). The Alexa Fluor 647 anti-H2A.X-Phosphorylated (Ser139) antibody (part number 613407) was purchased from BioLegend (San Diego, CA). RAD51 (D4B10) rabbit monoclonal antibody (part number 8875S) was purchased from Cell Signaling Technology (Danvers, MA). Donkey anti-rabbit IgG secondary antibody, Alexa Fluor 488 (part number A-21206) was purchased from Thermo Fisher Scientific (Waltham, MA).

Agilent BioTek Cytation 5 cell imaging multimode reader

Agilent BioTek Cytation 5 is a modular multimode 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 nuclei using the DAPI imaging channel, and fluorescently labeled foci using the CY5 or GFP imaging channels. Integrated Agilent BioTek Gen5 microplate reader and imager software controls Cytation 5, and also automates image capture, processing, and analysis.

Methods

γH2AX and RAD51 assay performance

For the γH2AX assay alone, U373 cells were treated with concentrations of topotecan ranging from 0 to 10 µM. For the γH2AX/RAD51 assay, U251 and U251 knockout cells were treated with 0 or 150 ng/mL NCZ. Immunostaining was then performed using the parameters listed in Table 1.

Table 1. Immunostaining procedure using Alexa Fluor 647 anti-H2AX-phosphorylated (Ser139) antibody and 1° and 2° antibodies for RAD51. With some cell models, a nonPBS-buffer (e.g., Tris-buffered saline) is recommended.

γH2AX and RAD51 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-X100 in PBS, pH 7.4	10 minutes
4	Wash with PBS, pH 7.4	2x
5	Dispense 50 µL/well of 1º antibody (1:1,000 γH2AX or 1:1,000 γH2AX and RAD51) in 3% BSA plus PBS	Overnight at 4 °C
6	Wash with 0.1% Triton-X100 in PBS, pH 7.4	1x
7	Wash with PBS, pH 7.4	2x
8 (Only When Adding RAD51 Antibody)	Dispense 50 µL/well of 2° antibody (1:1,000 donkey anti-rabbit Alexa Fluor 488 antibody) in 3% BSA plus PBS	1 hour at 4 °C
9 (Only When Adding RAD51 Antibody)	Wash with 0.1% Triton-X100 in PBS, pH 7.4	1x
10 (Only When Adding RAD51 Antibody)	Wash with PBS, pH 7.4	2x
11	Dispense 150 µL/well of Hoechst 33342 (0.2 µg/mL) in PBS, pH 7.4	1 hour at 4 °C

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.

Table 2. Automated γH2AX and RAD51 imaging parameters.

Imaging Parameters	
Nuclei Imaging Channel	DAPI
γH2AX Imaging Channel	CY5
RAD51 Imaging Channel	GFP
Objective	20x
Montage	2 × 2
Montage Overlap	Auto for stitching
Exposure	Based upon positive control wells

Image preprocessing

Image preprocessing 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. Rolling ball diameter was reduced to 10 µm, which provided the most optimal final images for subsequent analysis.

Table 3. Image preprocessing parameters.

Imaging Preprocessing 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	10 µm
Image Smoothing Strength	0
Third Image Channel	GFP
Background	Dark
Rolling Ball Diameter	10 µm
Image Smoothing Strength	0

Image stitching

Individual preprocessed image tiles from the 2 × 2 montage were stitched together to create a final complete image for analysis using the parameters in Table 4.

Table 4. Image stitching parameters.

Image Stitching Parameters	
Registration Channel	DAPI
Fusion Method	Linear blend
Crop Stitched Image to Remove Black Rectangles on the Borders	Checked
Downsize Final Image	Checked

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. As the foci are located within the nucleus, "Measure within a Primary mask" and "Count Spots" are selected.

Table 5. Primary mask nuclei analysis parameters.

Primary Cellular Analysis Parameters	
Channel	Tsf[DAPI]
Threshold	Auto (8)
Background	Dark
Split Touching Objects	Checked
Fill Holes in Masks	Checked
Minimum Object Size	10 µm
Maximum Object Size	50 µm
Include Primary Edge Objects	Unchecked
Analyze Entire Image	Checked
Advanced Detection Options	
Rolling Ball Diameter	Auto
Image Smoothing Strength	4
Analysis Metric	
Metric of Interest	Cell count

Table 6. Secondary mask spot counting analysis parameters.

Secondary Spot Counting Analysis Parameters		
	γH2AX	RAD51
Channel	Tsf[CY5]	Tsf[GFP]
Minimum Object Size	0	0
Maximum Object Size	5	5
Advanced Detection Options		
Rolling Ball Size	0.2x Max spot size	0.3x Max spot size
Threshold	2,000	2,000
Analysis Metric		
Metric of Interest	Object spot count [Tsf[Stitched [CY5]]]	Object spot count [Tsf [Stitched[GFP]]]

Results and discussion

Automated 96-well γH2AX assay imaging and preprocessing

Automated γH2AX assay imaging, performed in 96-well format, was validated using U373 cells exposed to various topotecan concentrations. Cytation 5 automatically imaged the samples using a 2 × 2 image montage to ensure that a statistically relevant number of nuclei were analyzed per image. Exposure settings (Table 2) were optimized using positive 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 preprocessed and stitched the image tiles 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 cell, 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 (10 µM) concentration of topotecan. While individual labeled foci can be seen in certain nuclei, others containing higher foci numbers appear where the nuclei is covered with large areas of fluorescent signal from the labeled secondary antibody. This phenomenon precludes the possibility of performing accurate analysis of DNA damage within these nuclei.

By performing the preprocessing step in Gen5, with the incorporation of a rolling ball diameter of 10 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.

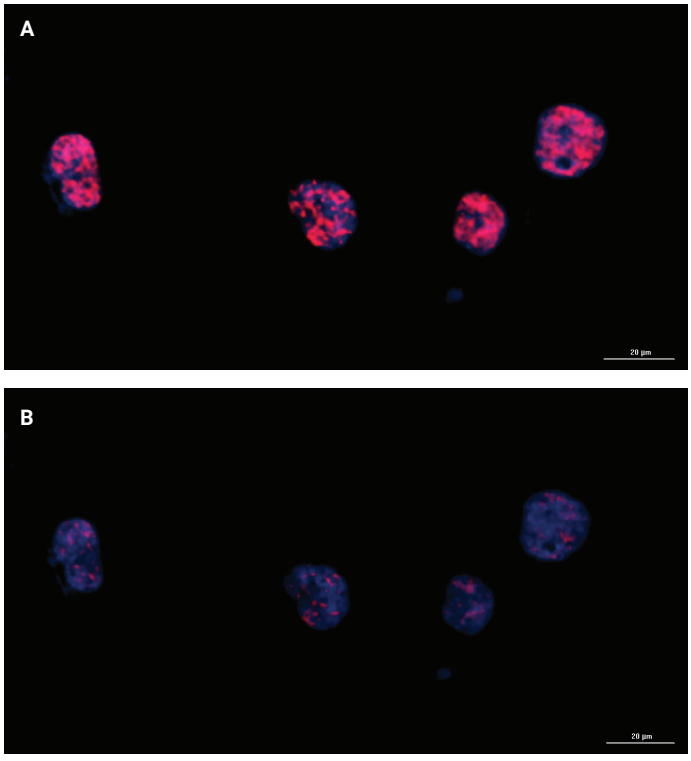

Figure 1. Image background signal removal via preprocessing. Zoomed 20x images (A) before, and (B) after preprocessing criteria (Table 3) applied using Agilent BioTek Gen5 software.

Following image capture, Gen5 automatically stitched together the four individual image tiles in the original configuration of the 2 × 2 montage. This allows analysis of the entire imaged area of the well (Figure 2).

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 topotecan showed increasing numbers of nuclei affected as well as an increasing number of labeled foci per nuclei (Figures 3B to 3F).

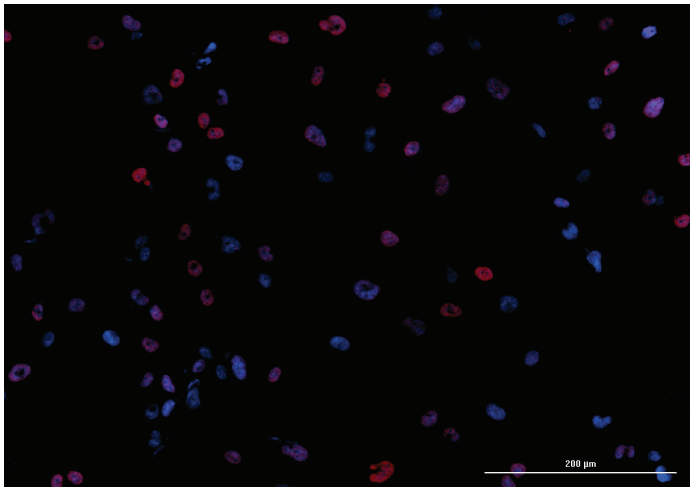

Figure 2. Image stitching. Final stitched image of the four tiles included in the 2 × 2 montage.

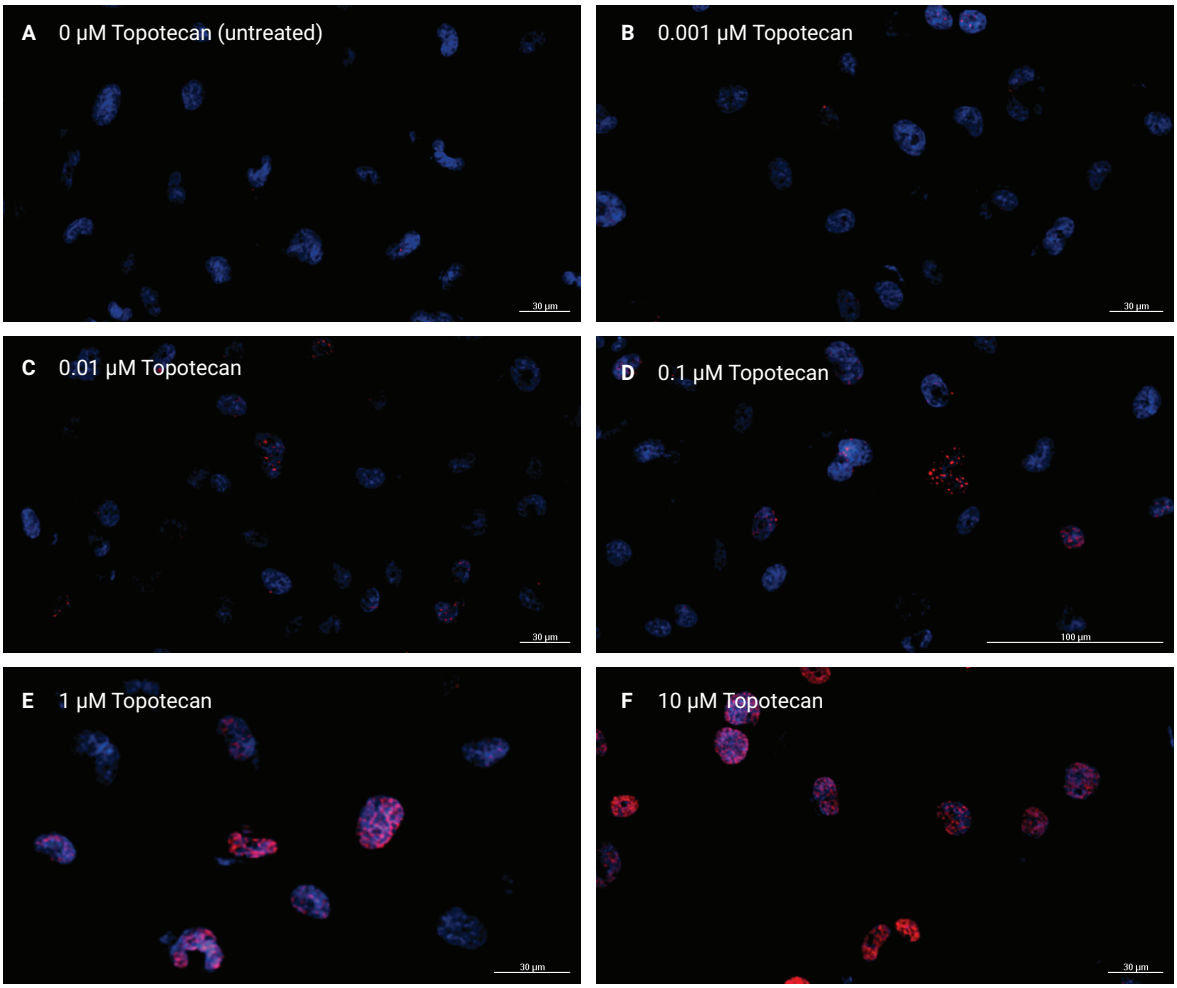

Figure 3. Final processed images following topotecan treatment and immunofluorescent staining. (A) Zoomed topotecan; (B) 0.001 µM topotecan; (C) 0.01 µM topotecan; (D) 0.1 µM topotecan; (E) 1 µM topotecan; or (F) 10 µM topotecan. Blue: Hoechst 33342 stained nuclei; Red: CY5 signal from labeled foci.

Automated γH2AX assay analysis

Primary and secondary cellular analysis criteria (Tables 5 and 6) were applied to all images captured using Gen5. When only the DAPI channel is selected in the analysis step, and the CY5 channel is deselected, it can be seen that primary analysis places object masks automatically around each nuclei (Figure 4A). By keeping the object masks applied within the image, and selecting/deselecting the opposite channels, the labeled spots per nuclei are then visible (Figure 4B).

Finally, when applying the secondary analysis spot counting parameters, Figure 4C demonstrates how each spot is accurately identified.

By using the dual-mask analysis to initially mask nuclei, the foci identified were properly linked to each nuclei, allowing analysis on a single-nucleus level (Figure 5).

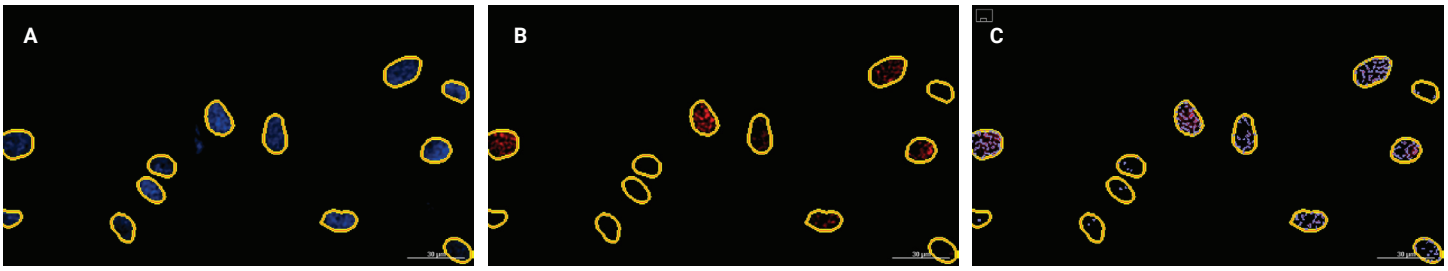

Figure 4. Automated γH2AX dual-mask analysis. (A) Object masks placed around nuclei. (B) Labeled foci imaged via CY5 imaging channel. (C) Spot object masks placed around labeled foci. Images captured using a 20x objective, 2 × 2 image montage, in addition to DAPI and CY5 imaging channels.

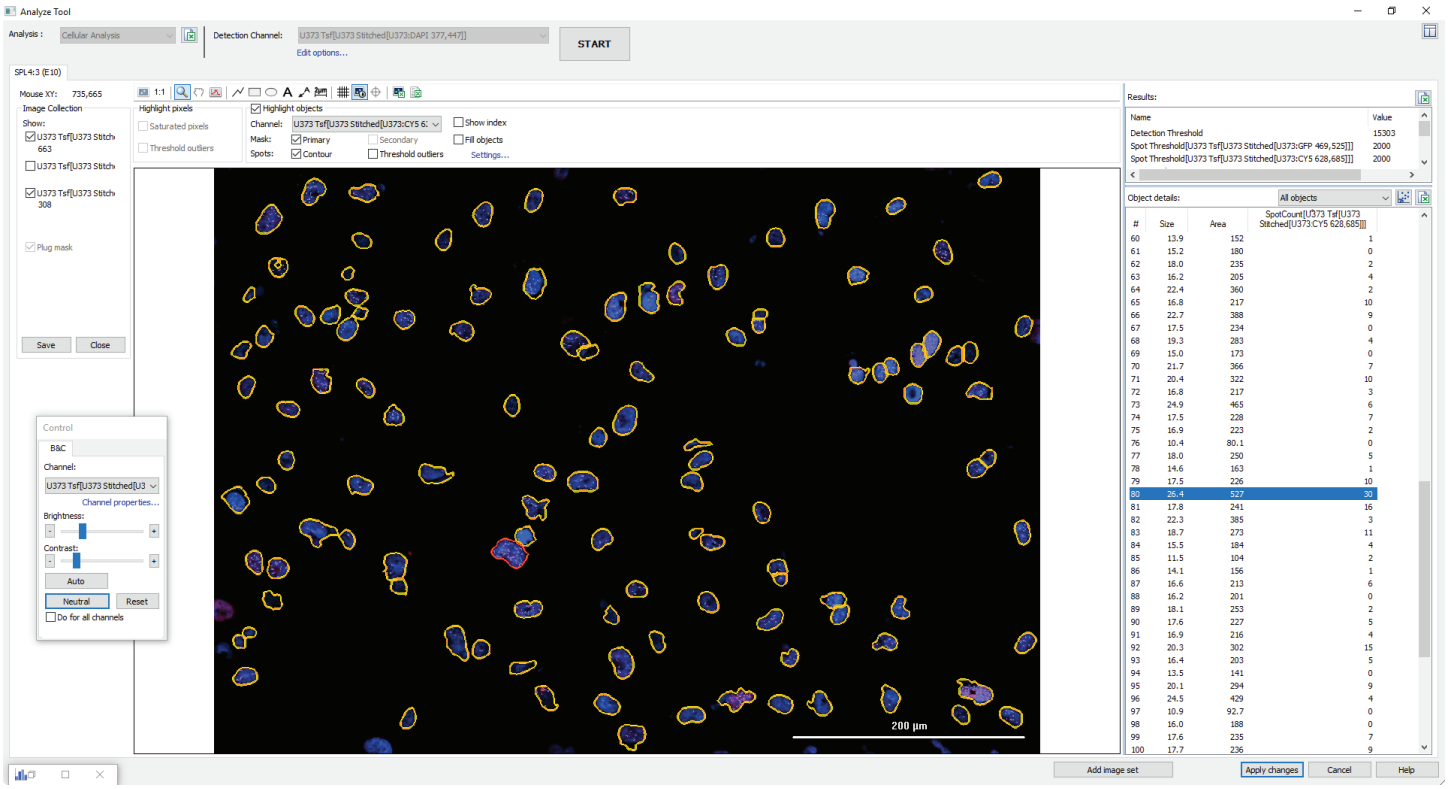

Figure 5. Individual nuclei spot count results following automated γH2AX dual-mask analysis.

The average number of spots counted per nucleus from replicate wells was then plotted in terms of the U373 cell treatment concentration (Figure 6). The results confirm what was observed visually, that the topoisomerase I inhibitor topotecan stabilizes cleavable DNA complexes, leading to the formation of increasing numbers of DNA double strand breaks[1] in a concentration-dependent manner.

Subpopulation analysis was then applied to identify nuclei in each image exhibiting positive DNA damage. As the number of double strand breaks increases, the number of labeled foci also increases per nuclei. Using a scatter plot within Gen5, a minimum number of spots within a nucleus can be established that is statistically greater than that seen within untreated cells (Figure 7), which can then be used as a cutoff spot count value to use for positive DNA damaged nuclei identification.

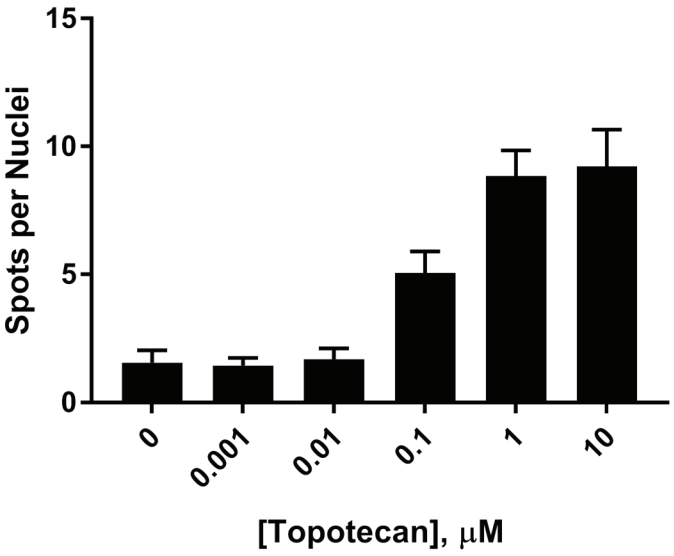

Figure 6. Labeled foci analysis of U373 cells exposed to various topotecan concentrations.

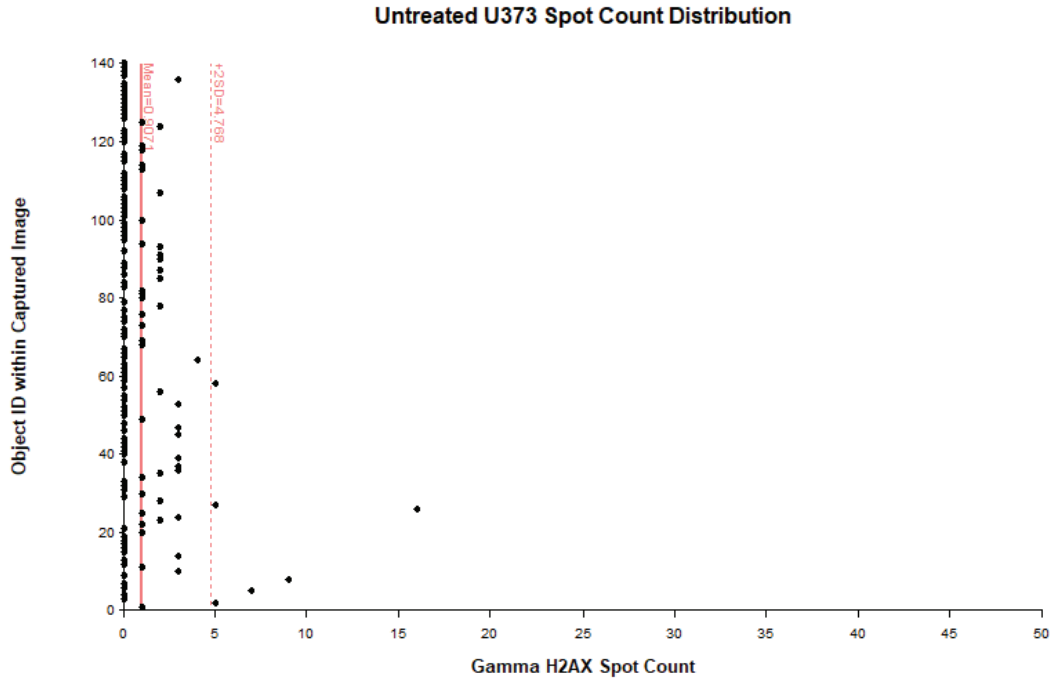

Figure 7. Scatter plot of spot count distribution for untreated U373 cells.

By dividing the number of positive DNA damaged nuclei by the total number of identified nuclei per image, a percentage of DNA damaged cells per treatment concentration was then determined. A similar trend was again seen as with the number of labeled foci per nucleus, where a positive correlation was observed between the percentage of DNA damaged cells per image and the concentration of topotecan added to replicate wells (Figure 8).

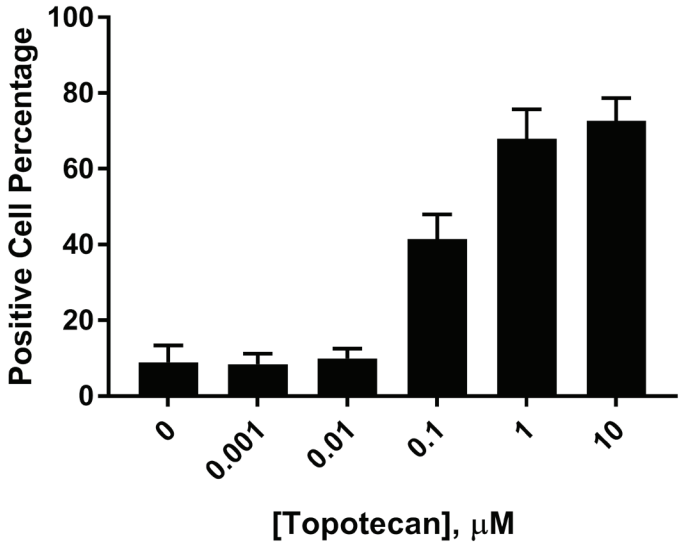

Figure 8. Positive DNA damaged cell percentage from U373 cells exposed to various topotecan concentrations.

γH2AX and RAD51 dual spot assay analysis

By taking advantage of the multicolor imaging capabilities of the Cytation 5, spot counting can be performed in two fluorescent channels to monitor two individual events involved in DNA double strand repair. In the experiments performed, immunofluorescent staining was performed to once again detect histone H2AX phosphorylation, in addition to the presence of RAD51, a protein known to assist in double-strand DNA repair (Figure 9).

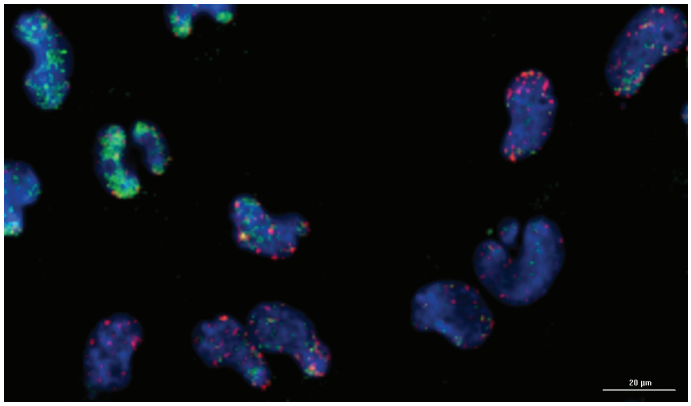

Figure 9. Final stitched, processed image following NCS treatment and immunofluorescent staining. Zoomed 20x processed, stitched image demonstrating staining to detect multiple DNA repair events. Blue: Hoechst 33342 stained nuclei; Red: CY5 signal from γH2AX labeled foci; Green: GFP signal from RAD51 protein labeled foci.

The dual-mask spot counting procedure was then tested using U251 and U251 RAD51 knockout cells, untreated or treated with neocarzinostatin (NCS) at 150 ng/mL. Untreated U251 knockout cells are expected to exhibit low RAD51 foci numbers as protein expression of the gene has been disrupted. In addition, when DNA damage is uninduced, little to no γH2AX foci should also be present. The opposite is then true when normal U251 cells, treated with the DNA damaging agent NCS, are stained and imaged. Following primary object mask placement around nuclei (Figure 10A), secondary spot analysis was carried out independently in the CY5 and GFP channels to identify γH2AX and RAD51-labeled foci, respectively (Figures 10B and 10C). Positive responding cells were also identified using histogram results from double negative control wells and subpopulation analyses (Figure 10D).

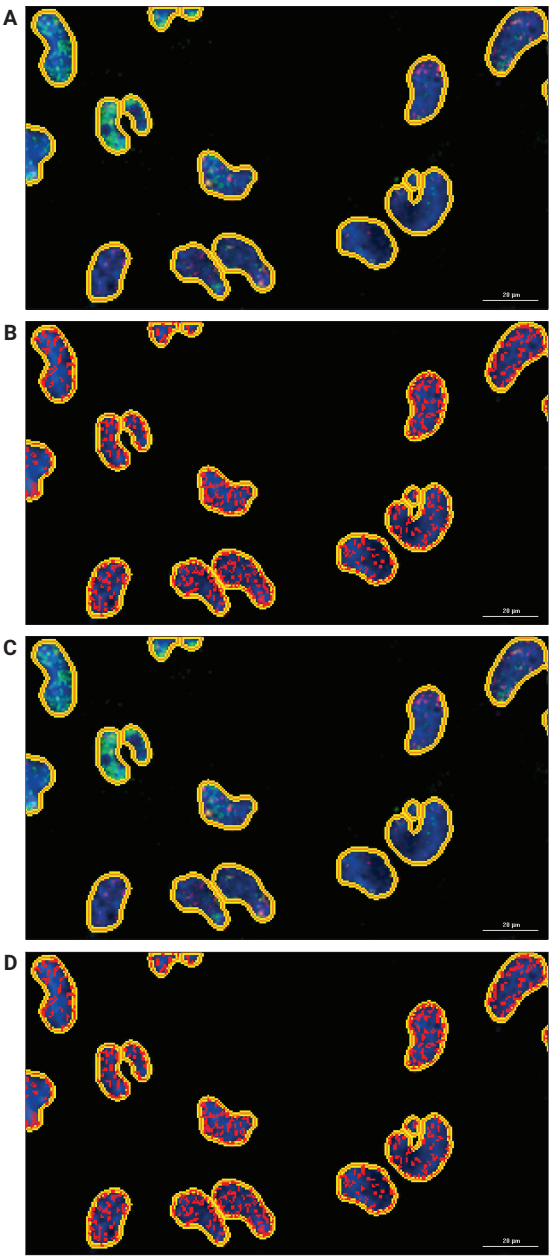

Figure 10. Automated γH2AX and RAD51 dual-mask analysis. (A) Object masks placed around nuclei. (B) Spot object masks placed around γH2AX foci via CY5 imaging channel. (C) Spot object masks placed around RAD51 foci via GFP imaging channel. (D) γH2AX and RAD51 positive nuclei identified via subpopulation analysis. Images captured using a 20x objective, 2x2 image montage, in addition to DAPI, GFP, and CY5 imaging channels.

Upon visualization of spots per nucleus and positive responding cell percentage graphs (Figure 11) from double negative, double positive, and combination wells, it was apparent that results agreed with previously described expected findings, validating the combined multichannel imaging and spot analysis process.

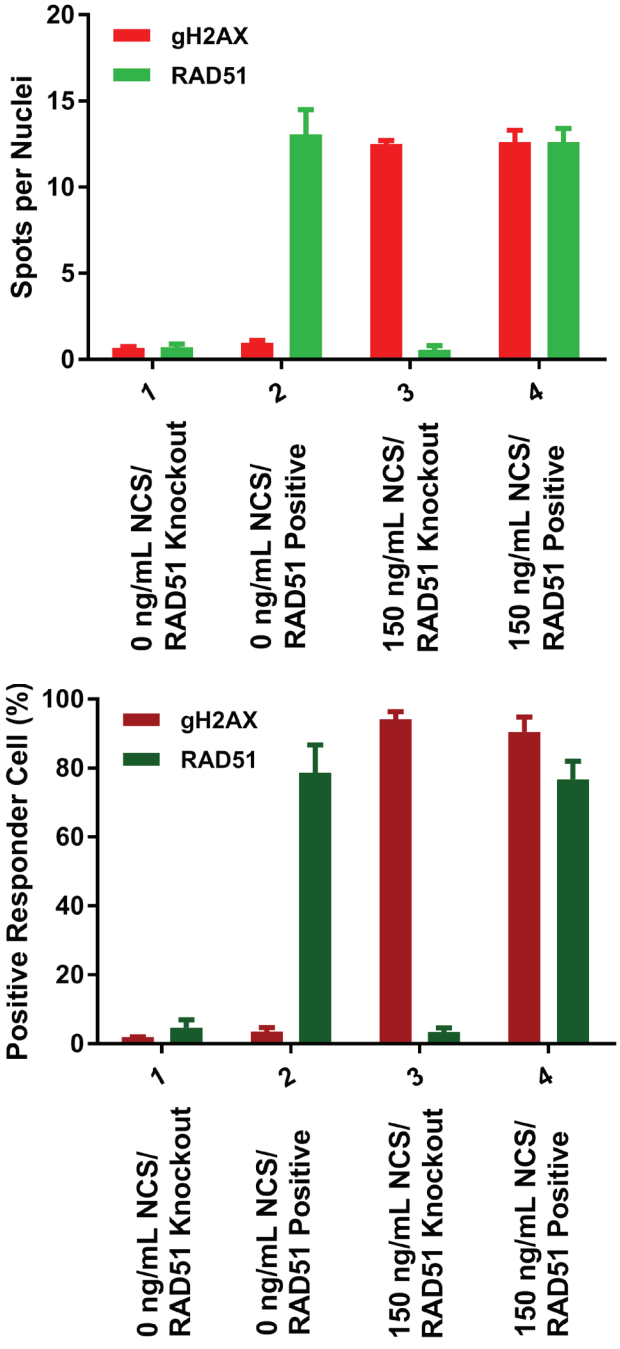

Figure 11. γH2AX and RAD51 dual spot counting results. (A) Labeled foci numbers; and (B) positive responder cell percentages for U251 and U251 knockout cells exposed to 0 and 150 ng/mL NCS.

Conclusion

The Agilent BioTek Cytation 5 cell imaging multimode reader provided the ability to automate and simplify the γH2AX assay image capture procedure. Incorporation of dual-mask cellular analysis, including spot counting, then enables accurate object mask placement around labeled foci within the nucleus and linking of counts to individual nuclei. This feature allows population-level analysis, as well as scrutiny and identification of individual cells positively responding to experimental test conditions. The combination creates a robust, user-friendly, and highly accurate method to detect DNA damage within mammalian cells.

Reference

1. Zhao, H. *et al.* Relationship of DNA Damage Signaling to DNA Replication Following Treatment with DNA Topoisomerase Inhibitors Camptothecin/Topotecan, Mitoxantrone, or Etoposide. *Cytometry A* **2012**, *81(1)*, 45–51.

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