Imaging and Evaluation of DNA Damage Employing the γH2AX-Assay with CELLAVISTA® and the YT-software®

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Application Note
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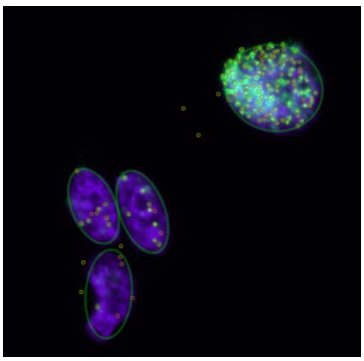
Abstract



Although gDNA is protected inside the nucleus, there are multiple possibilities to damage DNA. For instance oxidative stress, caused by reactive oxygen species (ROS), mutations caused by ionizing radiation or compound treatment. Therefore some of these factors have proven valuable to a multitude of research interests. Particularly in cancer research and environmental research the specific damaging of DNA can be a valuable tool if used efficiently and specifically, e.g. inducing DNA damage in specific tumor cells in therapy to hinder tumor growth. Biodosimetry and environmental research also partly rely on the monitoring of DNA damage. Thus a quick, precise and efficient method of monitoring the introduction of double strand breaks (DSBs) in a single cell based manner is key to identify factors or treatments that induce DSBs. In this Application Note we treated the breast cancer cell line MDA-MB-231 with various concentrations of the DSB-inducing Camptothecin. To monitor occurrence of DNA damage we used immunostaining with a primary antibody against phosphorylated serine 139 of γH2AX and a secondary antibody conjugated with Alexa 488. Nuclear counterstaining was performed with Hoechst. For all measurements we used SYNENTEC's CELLAVISTA® and NYONE® cell imager and the associated image analysis YT®-software with the automated high throughput foci counting operator.

Keywords: Cancer Research, Histone, Cell Line Development, γH2AX, Double-Strand-Breaks (DSBs), Biodosimetry, DNA Damage, Apoptosis, Automated Interpretation, Irinotecan, Topotecan

Introduction

Humans are increasingly exposed to DNA-damaging factors like ionizing radiation, toxic substances or environmental toxins.

Acute exposure to high doses of radiation leads to major injuries, especially to the immune system and effectively can cause cancer. Therefore having a reliable tool for radiation biodosimetry is absolutely necessary.

On the other hand, DNA damage can be used to specifically eliminate cancer cells. On a molecular level, the exposure of cells to ionizing radiation or radiometric agents leads inter alia to the formation of double strand breaks (DSB).

Whereas high DSB levels lead to cell death, low levels may induce cellular senescence or genomic rearrangements that lead to cancer.[1]

One of the earliest events detected in cells after exposure to DNA damaging agents or exposure to radiation is the phosphorylation of serine 139 in a SQ-motif of the H2AX by a PI-3 kinase.

H2AX is a member of the histone H2A family, which is one of the four core histones constizuting up the nucleosom core particle. Two molecules of each of the four core histones (H2A, H2B, H3 and H4) form an octamer, which assciates with approximately 146 bp of DNA to ultimately condense the DNA into chromosomes. The C-terminal phosphorylation of the H2AX, the γH2AX first occurs around the DSB site, then forms a focus around it, which seems to have recruiting function for DNA repair factors, like the 53BP1-protein or p53, which plays a keyrole in multiple cancer studies. [2]

In addition to its role in DNA-damage repair, H2AX is also required for DNA fragmentation during apoptosis and is phosphorylated by various kinases in response to apoptotic signals, leading to a formation of large quantities of γH2AX during apoptosis.[3]

On the other hand, H2AX undergoes constitutive phosphorylation in healthy cells which is considered to be in large part a reflection of oxidative DNA damage, caused by the metabolically generated oxidants (ROS).

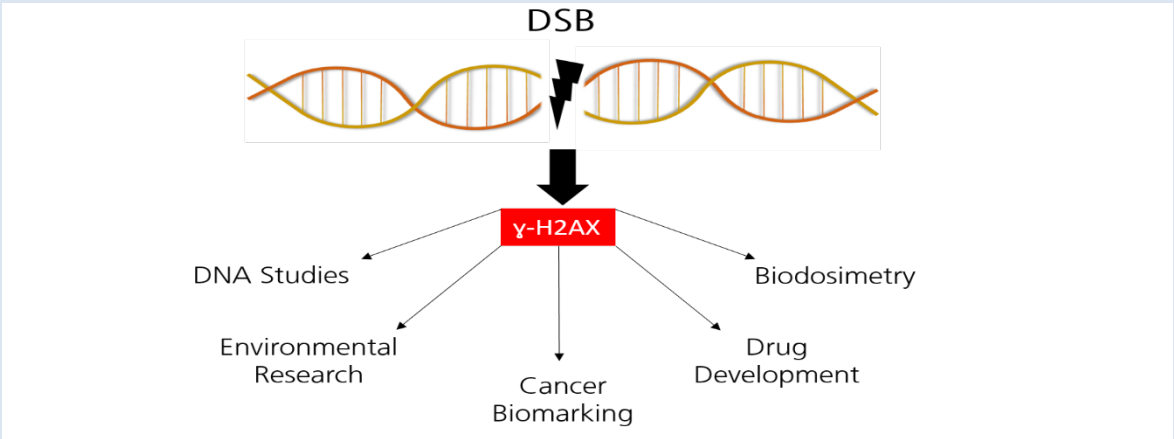


Fig. 1: Overview of the benefits of γH2AX-level analysis
Numerous applications emerge from analysing the amount of γH2AX. From basic DNA research to drug development up to individual biodosimetry studies.

Analyzing the formation of γH2AX foci therefore can yield information about individual cell's or cell lines' radiosensitivity, DNA-repair kinetics, the oxidative stress level and about the apoptosis state as well (Fig.1).
The amount of γH2AX can either be measured in cell lysates with ELISA or directly in cells or tissues with high resolution fluorescence microscopy using imagers like CELLAVISTA® or NYONE®.

The use of imaging enables researchers to monitor formation of bright foci and thus the detection and quantification of individual DSBs. Counting foci can gather valuable information, especially since cells will be read out individually. Other methodologies are less stringent, especially if the DNA damage is heterogenous in the homogenized sample.

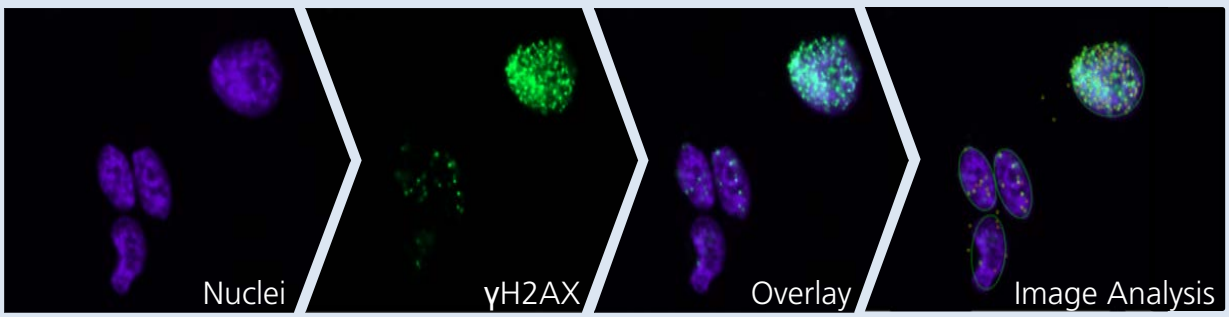


Fig. 2: Workflow of SYNENTEC's nuclei real dot count operator
Hoechst-stained nuclei are imaged via UV-excitation (377 nm), immuno-stained γH2AX foci by exciting the Alexa 488 fluorophore with blue LED (475 nm). These images are overlayed and analyzed by the image processor.

The most common approach is to manually count the foci either directly bye eye using a mircoscope or within images previously captured. Both are time consuming and subject to human bias. Therefore we have developed a high throughput foci counting image processing operator for SYNENTEC's YT-Software® (Fig. 2).

To induce DSBs, we used camptothecin, an alkaloid inhibiting the function of DNA topoisomerase I (TOP1) effectively inducing DSBs. TOP1 is an essential human enzyme, whose physiological function is to reduce shear stress of DNA via supercoiling during DNA replication. It is the only known target of camptothecin, from which the potent anticancer agents irinotecan and topotecan are derived.[4] These topoisomerase inhibitors are able to bind topoisomerase-DNA-complexes. This interaction effectively prevents a re-ligation of DNA resulting in double strand breaks in dividing cells due to stabilizing the complex, ultimately leading to apoptosis.

Following the occurence of DNA double strand breaks, rapid phosphorylation of serine 129 on H2AX is detectable within minutes (Fig. 3).[4] In order to visualize the phosphorylated H2AX, a monoclonal primary anti-γH2AX-mouse antibody in combination with a goat-anti-mouse Alexa Fluor 488 labeled antibody was used for immunofluorescence microcopy.

SYNENTEC's CELLAVISTA® or NYONE® cell imager and the associated YT®-software with the automated high throughput foci counting operator, allows the user to detect even low-abundant events like γH2AX in a high content measurement.

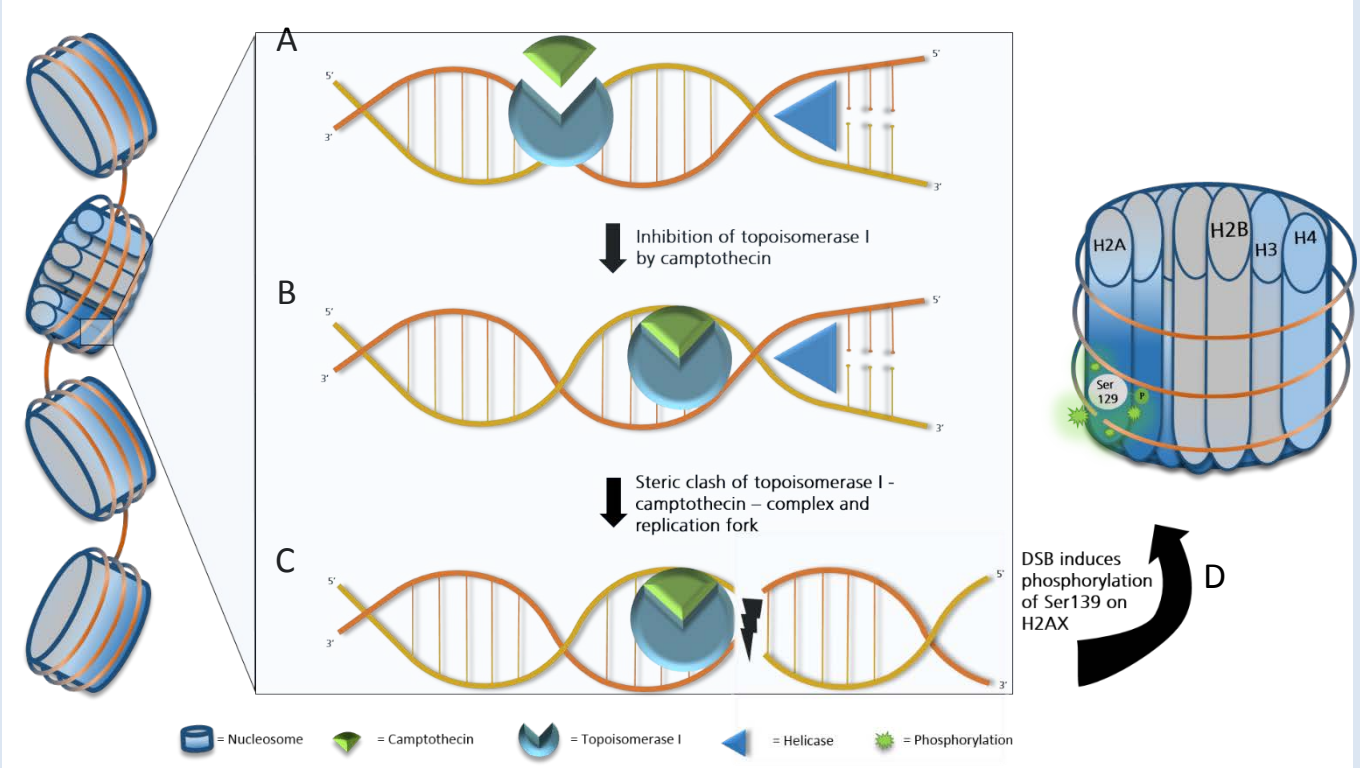


Fig. 3: Schematic process of phosphorylation of H2AX after DSB induction with camptothecin
Camptothecin binds to TOP1 (A) and stabilizes the TOP1-DNA-cleavage complex, inhibiting religation (B). Clashing with the replication fork leads to a double strand break (DSB) (C), whereupon Ser139 on histone H2AX is phosphorylated (D).

Material and Methods

Materials:

- Adherent MDA-MB-231 cells
- DMEM/Ham's F12 1:1 with 10 % fetal bovine serum and 1 % Penicillin/Streptomycin (PAN-Biotech)
- Phosphate Buffered Saline (with Ca^{2+} & Mg^{2+}) (PAN-Biotech)
- Trypsin 0.05 %/EDTA 0.02 % (PAN-Biotech)
- 96 well microplate, black, glass bottom (Greiner)
- Camptothecin (Santa Cruz Biotechnology)
- BSA (EMD Millipore)
- Poly-L-lysine 0.1 mg/mL (Biochrom)
- Triton X-100 (Fluka BioChemika)
- Paraformaldehyde (Alfa Aesar)
- Anti-phospho-Histone H2A.X antibody JBW301 (Merck Millipore)
- Alexa Fluor™ 488 goat anti-mouse antibody (Invitrogen)
- Hoechst 33342 (Santa Cruz Biotechnology)
- One of SYNENTEC's imaging systems, e.g. CELLAVISTA®

Method:

MDA-MB-231 cells were cultivated in T-flasks until confluence was reached, subsequently the cells were washed with PBS^{++}. Afterwards cells were detached with trypsin and resuspended in warm growth medium. A diluted cell suspension of 7500 cells/well was seeded into a 96-well microplate and incubated for 24h. Glass bottom plates were used in this experiment. These are highly recommended when conducting high content measurements with 40x magnification. The surface should be coated with poly-L-lysine before seeding the cells, for better cell-glass-adhesion. The cells where incubated until they reached 90% confluency. Medium was aspirated after incubation and 150 µL ´camptothecin added in a dilution range between 1nM and 20 nM for 1 h at room temperature (RT) to induce DSBs.

After aspirating Camptothecin and carefully washing three times with PBS^{++} the cells were fixed by protein-crosslinking, adding 150 µL 2% ice cold PFA and cooling the plate at 4°C for 15 min. Following another washing step, cells were permeabilized and blocked using 100 µL 0,5% Triton and 3% BSA in PBS^{++}, incubating for 30 min at RT. Afterwards the permeabilization/ blocking solution was aspirated and 200 µL of anti-γH2AX antibody (1:800 diluted in 1% BSA) added, incubating for 1h at RT. Subsequently, 200 µL of the secondary antibody (follow the stated dilution instructions of the manufacturer) and 1:3000 Hoechst (5 mg/mL stock) were added, incubating for 1h in the dark. Following a last washing step, the measurement was started with 100 µL PBS^{++} per well.

Tab. 1: Optical settings for Nuclei Real Dot Count wizard

Channel	Nuclei	Dots
Objective	40x	
LED	UV	Blue
Emission Filter	Blue	Green
Intensity	100 %	100 %
Exposure Time	23 ms	300 ms
Gain	0 %	20 %

Results and Conclusion

Results:

Imaging of cells showed clear foci formation in a concentration range between 0 and 50 nM camptothecin. Nuclei Real Dot Count image processing identifies nuclei based on Hoechst staining and counts foci per nucleus (Fig. 4).

Four foci/cell was set as base threshold for image processing to label cells as γH2AX dot-positive.

The control group without camptothecin treatment had 7% cells identified as positive. This is due to the fact that H2AX is constitutively phosphorylated in healthy cells. This is considered to be in large part a reflection of oxidative DNA damage caused by metabolically generated oxidants. Therefore, we could reproduce commonly available data regarding γH2AX in non-treated cells.[5]

Focus formation within camptothecin-treated cells was evaluated and showed a positive correlation to drug concentration (Fig. 5, 6).

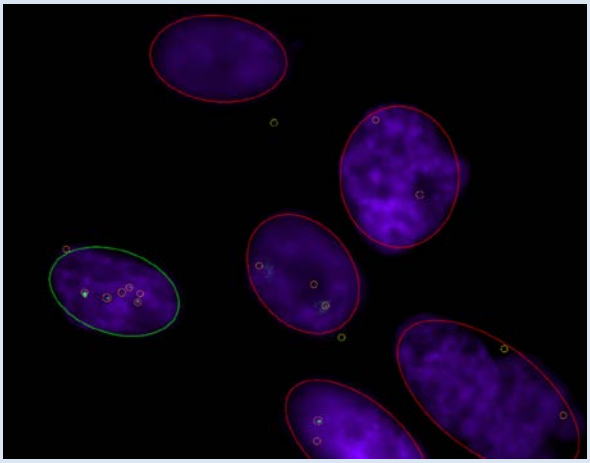


Fig. 4: γH2AX foci formation in Hoechst-stained MDA-MB-231 cells without camptothecin treatment

Constitutive H2AX phosphorylation caused by oxidative stress occurs in all cells. Therefore, the dots/cell threshold to count a cell as γH2AX-positive was set to min. 4 dots per cell. As the nucleus to the left containes more than four foci, it is counted as positive (greend elipse), while the others are counted as negative (red).

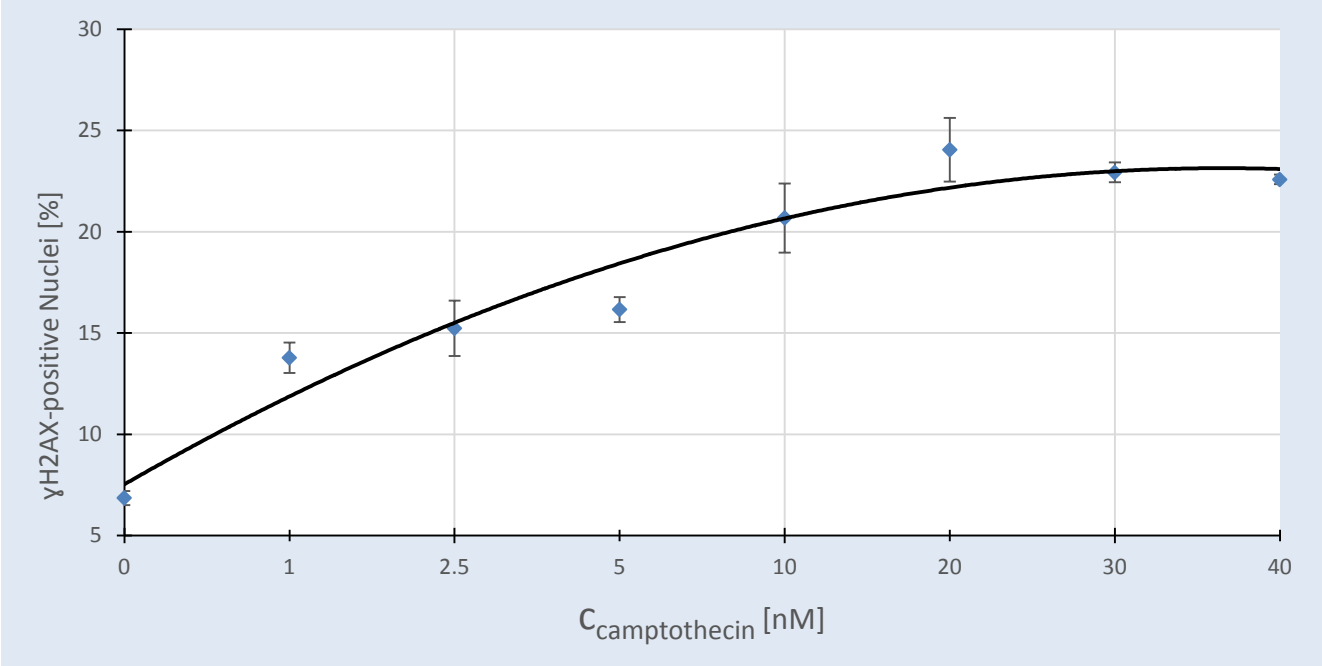


Fig. 5: Percentage of γH2AX-positive cells after treatment with different concentrations of camptothecin

MDA-MB-231 cells were treated with camptothecin for 1 hour and the mean percentage of γH2AX-positive cells determined as described above. Each concentration was measured in triplicate.

Conclusion:

In this Application Note, we demonstrate that SYNENTEC's high throughput Nuclei Real Dot Count application allows the user to count foci formation in a cell-based manner, using large sample sizes in high throughput format. The assay is taking advantage of the highly sensitive imaging capabilities, expecially in terms of high dynamic range of signal analysis. As it is shown below, very dim foci can be counted (Fig. 6A) as well as very bright foci after treatment with high doses of DNA-damaging agents (Fig. 6C). With higher doses of camptothecin used, more DSBs are formed, leading to increasing γH2AX foci formation. In fact, the amount of γH2AX-positive cells nearly tripled within the range of 1 nM to 20 nM. When treated with doses above 20 nM, signal abundance in the nuclei inhibits detection and quantification of focus formation, due to local overexposure.

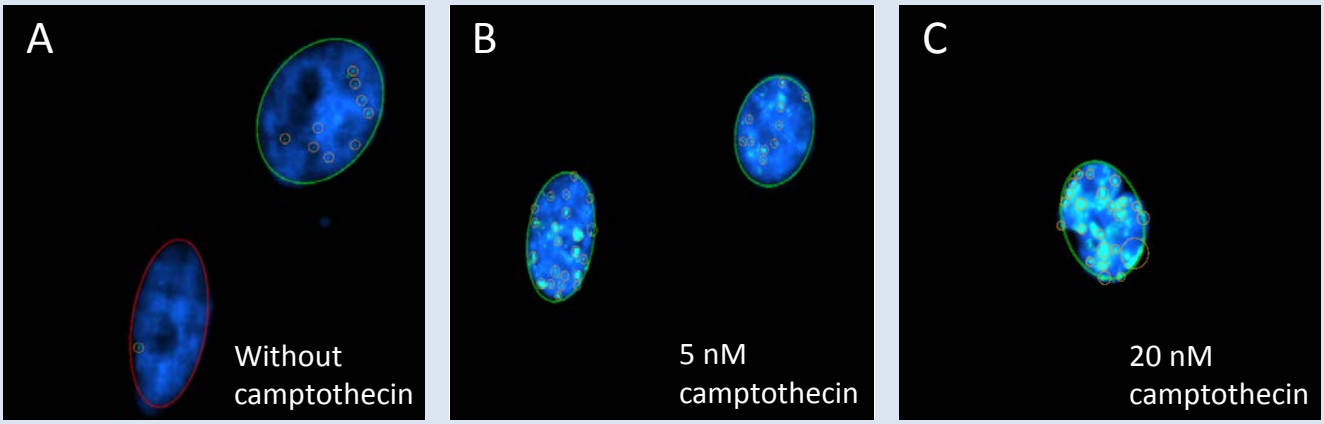


Fig. 6: Foci formation in Hoechst- and γH2AX-stained MDA-MB-231 cells without camptothecin (A), with 5 nM camptothecin (B) and with 20 nM camptothecin treatment for 1h (C)

Outlook

Wider ranges of signal intensities can be measured by using one of SYNENTEC's soon to be launched Scientific line of NYONE® and CELLAVISTA®. These will use highly sensitive 16 bit sCMOS cameras, combined with high numeric aperture lenses. The Scientific instruments will reach better resolution in a higher dynamic range, using a quantum efficiency of over 70%. This allows the user to perform assays with higher dynamic range, due to a better signal to background ratio.

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