A Comparison of HCA ImagAmp and Conventional Indirect Immunofluorescence for High Content Analysis (HCA) Applications

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Introduction

HCA ImagAmp™ is an amplification system designed to provide significant enhancement of target-specific fluorescence signals and to improve sensitivity in immunofluorescence-based assays. Amplification is achieved through enzyme-mediated deposition of multiple fluorophores in close proximity to a given target. The technique utilizes the ability of horseradish peroxidase (HRP) to convert certain fluorophore-labeled substrates into highly reactive molecules that will covalently bind to tyrosine residues in or very close to the enzyme (Hunyady *et al.,* 1996).

We evaluated the HCA ImagAmp kit in two typical HCA assays for cytotoxicity: apoptosis and DNA damage. Apoptosis can be induced with staurosporine and Cytochrome C staining used as a marker. The mitochondrial protein Cytochrome C loses its specific localization during apoptosis, and becomes distributed more evenly in the cell. Detection of apoptotic cells is therefore based on a redistribution of the marker between cellular compartments. DNA damage is induced by neocarzinostatin (NCS), causing DNA double strand breaks. As a result, phosphorylation of Histone H2A.X occurs, which can be detected with an antibody (Ab) against the phosphorylated protein (Rogakou *et al.,* 1999). To measure the degree of signal enhancement by HCA ImagAmp in either assay, the kit was compared to a common indirect immunofluorescence approach using fluorophore-labeled secondary antibodies (secAb).

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Application

To perform immunofluorescence staining in the two cytotoxicity assays, a standard protocol was used. HeLa cells were seeded at a density of 6000 cells per well in CellCarrier™-384 plates. After overnight incubation, the appropriate molecule was introduced to induce apoptosis (1 µM staurosporine for 4 hours) or DNA damage (1 µg/mL NCS for 1 hour). The cells were then fixed in 3.7% formaldehyde and permeabilized with a 0.1% Triton/PBS solution. Simultaneously, the nuclei were counterstained by adding 2 µg/mL Hoechst®33342 to the solution. Primary Ab and HRP-labeled secAb incubation steps were done in PerkinElmer blocking solution, followed by a 10 min incubation step with HCA ImagAmp reagent. To compare the performance of the HCA ImagAmp with a commonly used indirect immunofluorescence approach, cells were stained in parallel with a secAb labeled with an Alexa Fluor® fluorophore of the corresponding wavelength, under otherwise identical conditions. Cells were imaged on the Operetta® High Content Imaging System, using a 20X WD objective.

Apoptosis Assay

In the apoptosis assay, Cytochrome C is bound to mitochondria in untreated control cells, while in apoptotic cells it distributes more evenly in the cell and can also be found in the nucleus (Figure 1).

After initial optimization of the anti-Cytochrome C antibody concentration, robust staining was obtained with HCA ImagAmp in both untreated and staurosporine-treated cells (Figure 1, A and B).

In contrast, Alexa Fluor®-labeled secAb stained cells, when imaged under exactly the same conditions, did not yield a significant staining (Figure 1, C and D). To determine the percentage of apoptotic cells in the images obtained with HCA ImagAmp, the average intensity of the Cytochrome C staining was determined within the nucleus and within a perinuclear ring outside of the nucleus. A cell was considered as apoptotic when the nuclear/perinuclear ratio increased over a threshold. The results are summarized in Table 1 and show that the ratio almost doubled after staurosporine treatment. Accordingly, 72.5% of the cells were found apoptotic, in contrast to only 3.9% in untreated controls. To quantify the extent of amplification by HCA ImagAmp compared to standard immunofluorescence, the concentration of the primary Ab and the exposure time were adjusted in each application to produce equivalent intensity levels with HCA ImagAmp and an Alexa Fluor®-labeled secAb. The results are summarized in Figure 2. Judged by the average intensity of the perinuclear Cytochrome C signal, HCA ImagAmp allowed a 20-fold greater dilution of the anti-Cytochrome C Ab, as well as a 10-fold reduction in exposure time as compared to the Alexa Fluor®-labeled secAb.
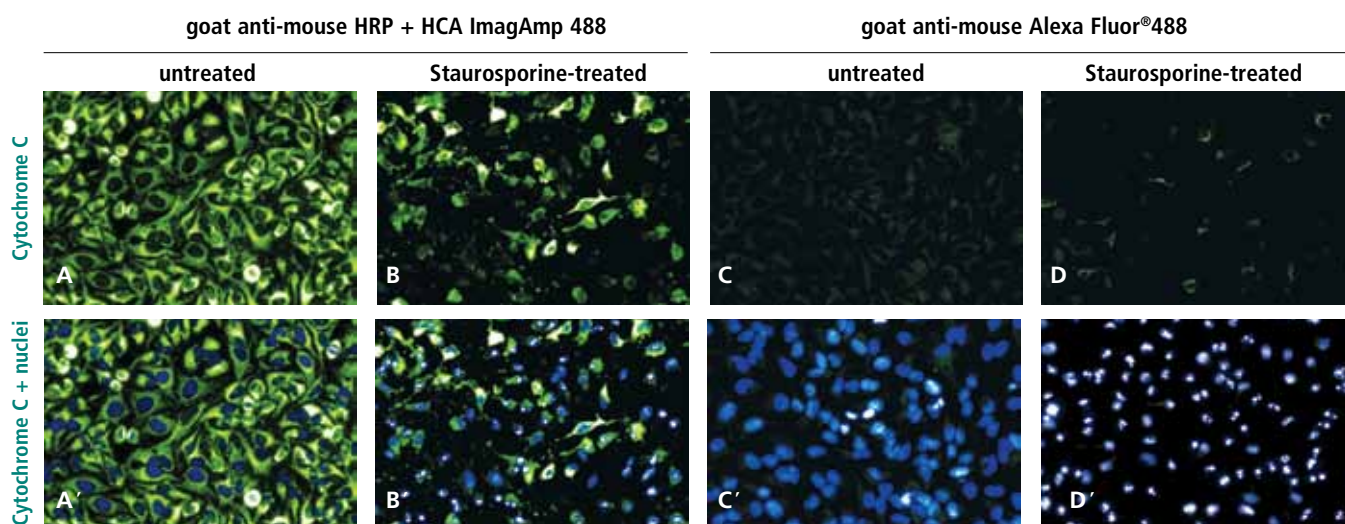

Figure 1. Apoptosis induction imaged with HCA ImagAmp 488. HeLa cells were grown overnight in CellCarrier-384 plates, then incubated with 1 µM Staurosporine for 4 hours and fixed. All Cytochrome C images (green) were taken at 35 msec exposure time. A-D) Anti-Cytochrome C Ab (0.5 µg/mL). A, B) secAb goat anti-mouse HRP (1 µg/mL) + HCA ImagAmp 488. C, D) secAb goat anti-mouse Alexa Fluor®488 (5 µg/mL). A′-D′) Overlay of Cytochrome C staining with nuclear counterstain Hoechst®33342 (blue).

Table 1. **Apoptotic cells were identified by an increase in the ratio of nuclear/perinuclear (cytoplasmic) Cytochrome C ImagAmp 488 staining. ± represents standard deviation. Z′ was calculated comparing average ratios of treated and untreated wells (4 wells per condition).**

	Avg. ratio nuclear/ perinuclear Cytochrome C	% apoptotic cells	Z′ untreated/ Staurosporine-treated
untreated	0.47 ± 0.03	3.9% ± 0.8	0.72
Staurosporine-treated	0.91 ± 0.01	72.5% ± 6.0	

HCA ImagAmp 546   **Alexa Fluor®546**
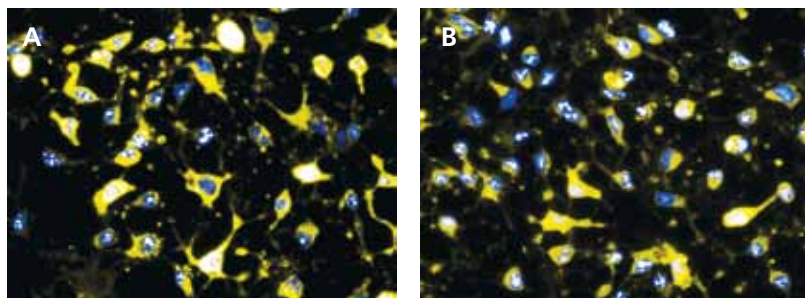
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Assay	**Condition**	**Staining Method**	**Primary Ab Concentration**	**Exposure Time**	**Average Intensity Primary Ab Stain**	**Signal Amplification**
Apoptosis Assay	Untreated	HCA ImagAmp 546	0.025 µg/mL	35 ms	2781 ±112	20-fold reduction in primary Ab combined with 10-fold reduction in exposure time
		Alexa Fluor®546	0.5 µg/mL	350 ms	2585 ±387	
	Treated	HCA ImagAmp 546	0.025 µg/mL	35 ms	2402 ±191	
		Alexa Fluor®546	0.5 µg/mL	350 ms	2185 ±197	

Figure 2. A comparison of imaging conditions for HCA ImagAmp 546 and Alexa Fluor®546 in the apoptosis assay. The antibody dilution and exposure times were adjusted to obtain similar staining intensities with both techniques. A and B) HeLa cells after apoptosis induction. Primary Ab anti-Cytochrome C (yellow). Overlay with Hoechst®33342 counterstain (blue). A) SecAb goat anti-mouse HRP (1 µg/mL) + HCA ImagAmp 546. B) SecAb goat anti-mouse Alexa Fluor®546 (5 µg/mL). C) Comparison of imaging conditions: The average intensity of the Cytochrome C staining is measured in a perinuclear ring. ± represents standard deviation.

DNA Damage Assay

In the DNA damage assay, after staining with HCA ImagAmp, virtually all nuclei of cells treated with NCS were strongly positive for phospho-Histone H2A.X, while untreated cells showed mostly much weaker or no staining (Figure 3, A and B). Staining with an Alexa Fluor®-labeled secAb under otherwise exactly the same conditions produced no significant signal (Figure 3, C and D). To calculate the percentage of DNA damage positive cells in the images stained with HCA ImagAmp, the average intensity of nuclear phospho-Histone H2A.X stain per cell was determined and a threshold applied to separate positive and negative cells (Table 2). On average, staining between untreated and NCS-treated cells increased by almost 9-fold. NCS treatment rendered 99.8% of the cells positive, in contrast to 3.1% without treatment. Several publications indicate the occurrence of DNA double strand breaks in a subpopulation of untreated cells (Kim *et al.,* 2011 and references therein). Therefore the small percentage of positive cells detected might indeed indicate such events in proliferating HeLa cells.

In the DNA damage assay, a 40-fold reduction of anti-phospho-Histone H2A.X Ab, combined with an 8-fold shorter exposure time, was sufficient to produce equivalent staining intensities with HCA ImagAmp (Figure 4). Therefore the improved sensitivity achieved by HCA ImagAmp significantly reduces exposure and plate reading times. The results suggest the technique is especially useful to produce robust fluorescence signals at a very low concentration of target-bound primary Ab.

In addition, the amplification mechanism of HCA ImagAmp might improve the detection of cellular targets. For example, it might allow detection of cellular changes at lower concentrations of an inducer than the standard indirect immunofluorescence approach. To compare the sensitivity of the two staining approaches, a DNA damage assay with a dilution series of NCS was performed. First, to be comparable, anti-phospho-Histone H2A.X Ab concentrations and exposure times were set at levels to produce equivalent average nuclear staining intensities at the highest concentration of NCS (1000 ng/mL). In the experiment shown in Figure 5, similar values were reached at 40-fold less primary Ab and

9-fold shorter exposure time for HCA ImagAmp. Using these settings, images were acquired at all NCS concentrations. The results show that, down to 30 ng/mL NCS, the average intensity of phospho-Histone H2A.X staining produced by HCA ImagAmp, remained very high. In contrast, the signal with Alexa Fluor®-labeled secAb was already significantly reduced at 100 ng/mL NCS (Figure 5 C). At NCS concentrations lower than 10 ng/mL and in untreated cells the intensity values obtained with both techniques were again similar. Therefore HCA ImagAmp, in comparison to Alexa Fluor®-labeled secAb, maintains a strong, specific signal at lower concentrations of NCS without an increase of unspecific signal.

At low levels of DNA damage, DNA double strand breaks are indicated by discrete nuclear spots of phospho-Histone H2A.X staining (Rothkamm and Loebrich, 2003). A low occurrence of such spots per nucleus will not have a significant impact on the average intensity measurements that were used in the experiment shown in Figure 5 to identify effective NCS concentrations. At 3 ng/mL of NCS, phospho-Histone H2A.X spots at varying intensities can be clearly seen (Figure 6, A and B), while the average intensity of the staining in these nuclei reached already control levels (Figure 5 C). To extend the analysis of NCS-induced DNA damage, nuclei of cells treated with low concentrations of NCS were evaluated with the SER-texture analysis module, a feature of the Harmony® image analysis software that allows the identification of defined structures, in this case spots, within an object. As the readout of this module, a value is assigned to each cell, depending on the frequency and intensity of spots in the nucleus. Higher values indicate a higher level of spots. In Figure 6 D, a comparison of the population distribution of spot-values is shown. The results indicate a shift of spot values toward higher values after treatment with 3 ng/mL NCS compared to untreated controls. In HCA ImagAmp-stained cells, this shift is much more pronounced than in cells stained with Alexa Fluor®-labeled secAb, allowing an improved separation between untreated and treated cells. This data indicates that HCA ImagAmp can improve the identification of relatively small structures in cells and thereby further increase the sensitivity of detection.
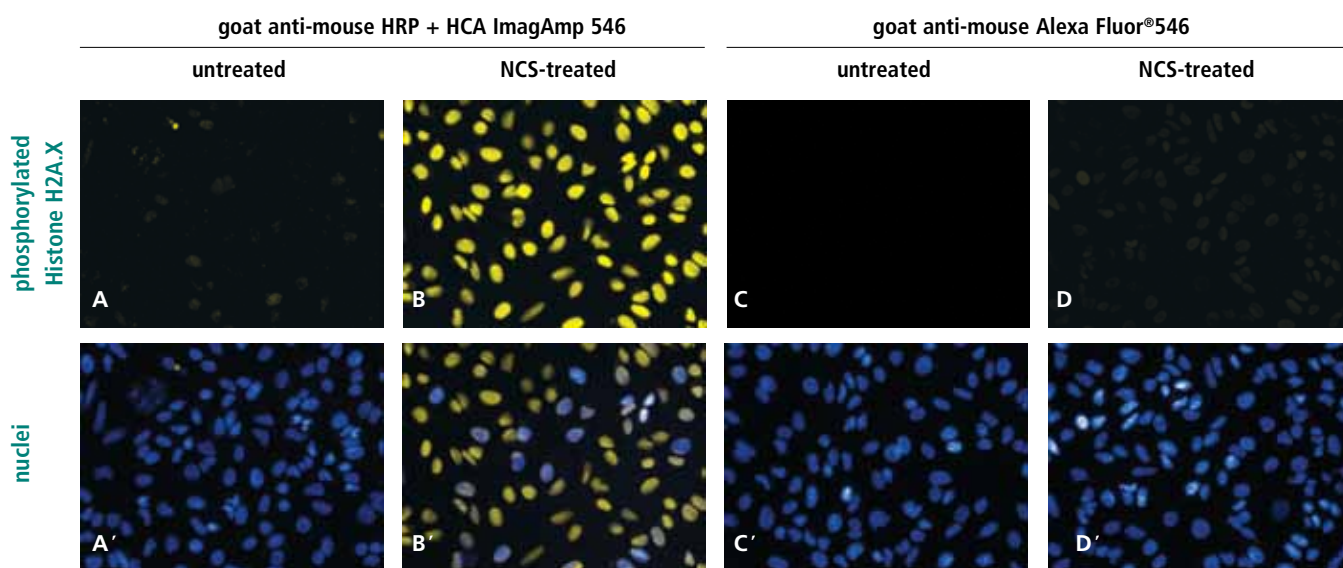

Figure 3. DNA damage induction imaged with HCA ImagAmp. HeLa cells were grown overnight in CellCarrier-384 plates, then incubated with 1 μg/mL NCS for 1 hour and fixed. All phospho-Histone H2A.X images (yellow) were taken at 25 msec exposure time. A-D) anti-phospho-Histone H2A.X Ab (0.5 μg/mL). A′-D′) overlay phospho-Histone H2A.X and nuclear counterstain (blue, Hoechst®33342). A and B) SecAb goat anti-mouse HRP (1 μg/mL) + HCA ImagAmp 546. C and D) SecAb goat anti-mouse Alexa Fluor®488 (5 μg/mL).

Table 2. **Phospho-Histone H2A.X positive cells were identified by an increase in average nuclear staining with HCA ImagAmp 546. ± represents standard deviation. Z′ was calculated comparing average nuclear phospho-Histone H2A.X intensities of treated and untreated wells (4 wells per condition).**

	Avg. intensity nuclear phospho-Histone H2A.X	% phospho-Histone H2A.X positive	Ratio avg. intensity NCS-treated/untreated	Z′ NCS-treated/ untreated
untreated	510 ± 104	3.1% ± 1.4	8.83	0.81
NCS-treated	4505 ± 145	99.8% ± 0.2		

HCA ImagAmp 546

Alexa Fluor®546
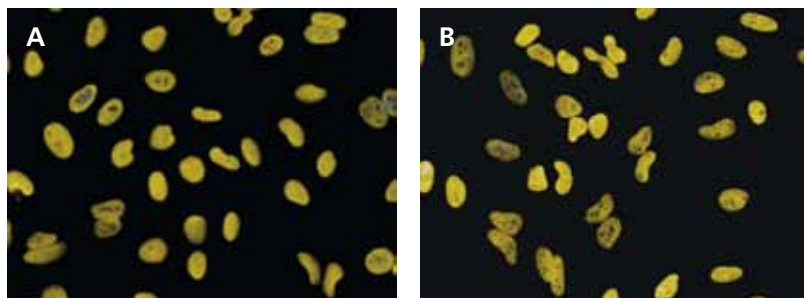
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Assay	**Condition**	**Staining Method**	**Primary Ab Concentration**	**Exposure Time**	**Average Intensity Primary Ab Stain**	**Signal Amplification**
DNA Damage Assay	Untreated	HCA ImagAmp 546	0.05 µg/mL	50 ms	288 ±34	20-fold reduction in primary Ab combined with 10-fold reduction in exposure time
		Alexa Fluor®546	2 µg/mL	400 ms	384 ±105	
	Treated	HCA ImagAmp 546	0.05 µg/mL	50 ms	6046 ±47	
		Alexa Fluor®546	2 µg/mL	400 ms	6655 ±262	

Figure 4. A comparison of imaging conditions for HCA ImagAmp 546 and Alexa Fluor®546 in the DNA damage assay. The antibody dilution and exposure times were adjusted to obtain similar staining intensities with both techniques. A and B) HeLa cells after induction of DNA damage. Primary Ab anti-phospho-Histone H2A.X (yellow). A) SecAb goat anti-mouse HRP (1 µg/mL) + HCA ImagAmp 546. B) SecAb goat anti-mouse Alexa Fluor®546 (5 µg/mL). C) Comparison of imaging conditions: The average intensity of the phospho-Histone H2A.X staining is measured in the nucleus. ± represents standard deviation.
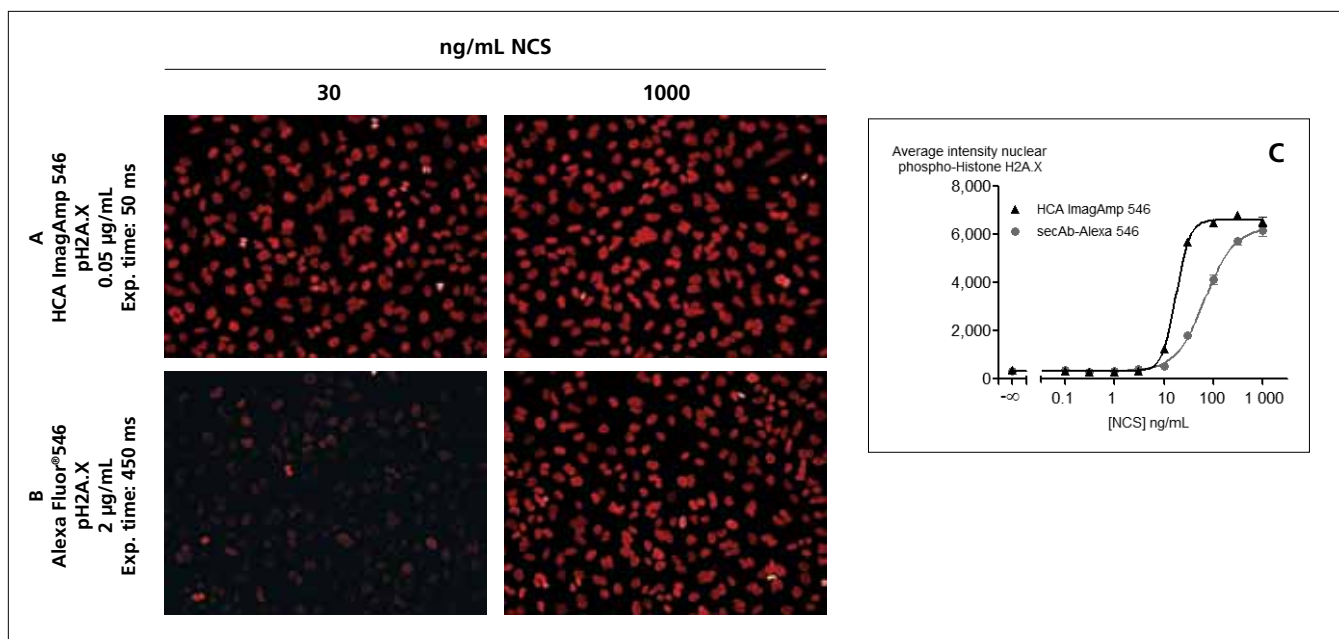

Figure 5. HCA ImagAmp increases sensitivity of detection. HeLa cells were cultured overnight in CellCarrier-384 plates, then incubated with different concentrations of NCS (ranging from 0.1 to 1000 ng/mL) for 1 hour and fixed. A) primary Ab phospho-Histone H2A.X, secAb goat anti-mouse HRP (1 µg/mL) + HCA ImagAmp 546. B) primary Ab phospho-Histone H2A.X, secAb goat anti-mouse Alexa Fluor®546 (5 µg/mL). Nuclear counterstain with Hoechst®33342 was omitted for clarity. C) NCS dilution series. Scale on x-axis is logarithmic.
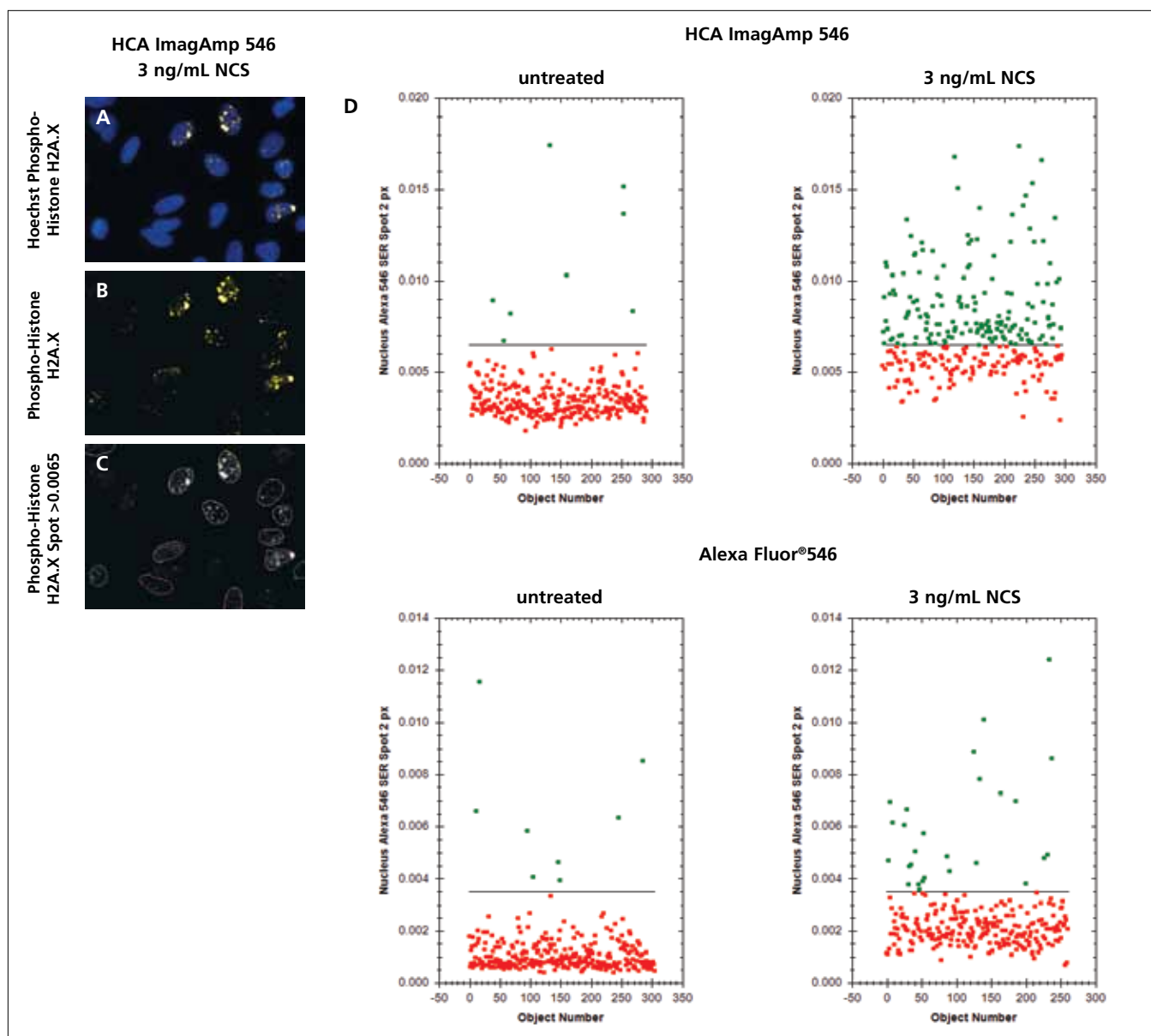
Figure 6. Texture analysis to evaluate DNA damage at low concentrations of NCS. Conditions are identical to Figure 5. A) HeLa cells treated with 3 ng/mL NCS. Overlay of nuclear counterstain (Hoechst®33342, blue) and phospho-Histone H2A.X stain (yellow). B) Phospho-Histone H2A.X stain alone. C) SER-spot analysis. Encircled nuclei show a spot index above the threshold of 0.0065. D) Examples for population distributions of cells according to their spot index. Each scatterplot represents one imaged field. Red dots depict cells below threshold; green dots are cells above threshold (threshold HCA ImagAmp 546: 0.0065, Alexa Fluor®546: 0.0035).

Conclusions

In this study, we analyzed the ability of HCA ImagAmp to increase sensitivity in immunofluorescence by applying it to two widely used HCA applications. The technique requires minimal adjustment to a standard immunofluorescence protocol, adding only a 10 min incubation period at the end, however, it can greatly enhance the ability to detect a given target. HCA ImagAmp delivers improved results with simultaneous reduction in primary antibody and exposure time. NCS-titration experiments show that HCA ImagAmp allows specific enhancement of signal intensity at low levels of DNA damage, thereby shifting the detection limit toward lower concentrations of NCS.

Higher sensitivity provided by HCA ImagAmp allows detection at lower concentrations of target and/or primary Ab. This advantage may be used for development of more efficient staining protocols and allow for quantitation of targets that previously escaped detection due to the availability of low affinity marker antibodies or relatively low target abundance.

Materials

Product	Company	Catalog No.
HeLa cells	ATCC	CCL-2
CellCarrier-384 plates	PerkinElmer	6007550
Staurosporine	Sigma	S6942
Neocarzinostatin	Sigma	N9162
Mouse anti-phospho-Histone H2A.X	Millipore	05-636
Mouse anti-Cytochrome C	BD Bioscience	556432
Goat anti-mouse IgG HRP	PerkinElmer	NEF822001EA
Goat anti-mouse IgG Alexa Fluor®488	Invitrogen	A-11029
Goat anti mouse IgG Alexa Fluor®546	Invitrogen	A-11030
HCA ImagAmp 488	PerkinElmer	NEL771B001KT
HCA ImagAmp 546	PerkinElmer	NEL774B001KT
Blocking Reagent	PerkinElmer	FP1012
Hoechst®33342	Invitrogen	H3570

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