

# Enhanced assay sensitivity with HCA ImagAmp™: An enabling signal amplification high content technology for technically challenging targets.

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## 1 Abstract

We utilized the HCA ImagAmp™ reagent kit in: (1) two typical high content analysis (HCA) assays for cytotoxicity, and in (2) a specific epigenetic assay monitoring the modulation of dimethylated lysine 9 residue on histone H3 (H3K9me2). Signal amplification with HCA ImagAmp is achieved through enzyme-mediated deposition of multiple fluorophores in close proximity to a given antigen. The technology utilizes the ability of horseradish peroxidase (HRP) to convert fluorophore-labeled tyramide into a highly reactive molecule that will covalently bind to tyrosine residues in close proximity to the enzyme.

For cytotoxicity assays, Cytochrome C release from mitochondria, as a marker for apoptosis, and phosphorylation of Histone H2A.X, as an indicator of DNA damage, were used to evaluate the performance of HCA ImagAmp™ reagents in HeLa cells. Several staining and analysis parameters were compared to a conventional immunofluorescence (IF) approach with a fluorophore-labeled secondary antibody. The results show that the HCA ImagAmp reagents can produce equivalent signal intensities with a 10-fold reduced exposure time and an up to 40-fold reduced concentration of primary antibody. Titration of the DNA damage inducing agent Neocarzinostatin (NCS) also demonstrates that HCA ImagAmp reagents increase assay sensitivity, allowing the detection and quantification of signs of DNA damage at lower concentrations of NCS than conventional immunofluorescence assays.

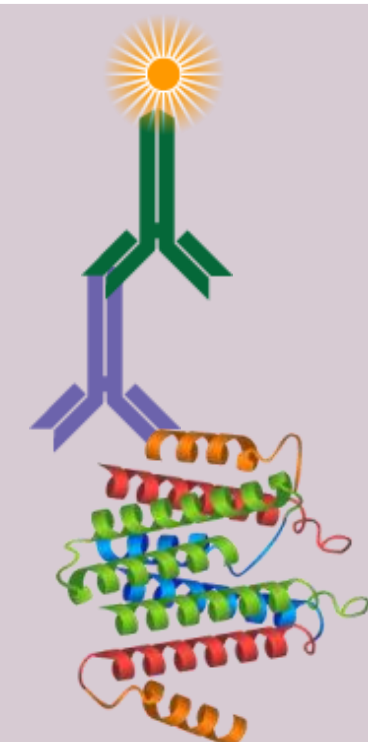
Amongst histone H3 epigenetic modifications, the methylated lysine marks are notoriously the most challenging to detect and monitor in biochemical as well as in cell-based assays, including imaging assays. Owing to the low cellular abundance of methylated lysine histone marks, compared to the prevalence of their acetylated counterparts, and to the modest decreases in global lysine methylation levels expected following treatment with methyltransferase inhibitors, monitoring the modulation of these marks in imaging and HC assays is technically quite difficult. Indeed, fluorescence signal intensities of H3K9me2 levels in untreated cells, detected by conventional approaches (primary antibody followed by fluorophore-labeled secondary antibody) were only minimally above background (isotype or no primary antibody) control levels. Conversely, using the HCA ImagAmp™ reagent kit, strong fluorescence intensity levels were obtained, and a higher extent of mark decrease following treatment with UNC0638 (a specific inhibitor of H3K9 methyltransferases G9a and GLP) was observed using HCA ImagAmp™. While an assay using conventional immunofluorescence performed poorly for the detection of H3K9me2, an assay using the HCA ImagAmp™ reagent was successfully developed and could detect the small decrease in a target that, at steady-state, is already in low abundance.

For technically challenging, low abundance, antigens including epigenetic histone methyl marks, HCA ImagAmp™ is an enabling, high performance imaging assay alternative to conventional IF approaches.

## 2 Assay Principle

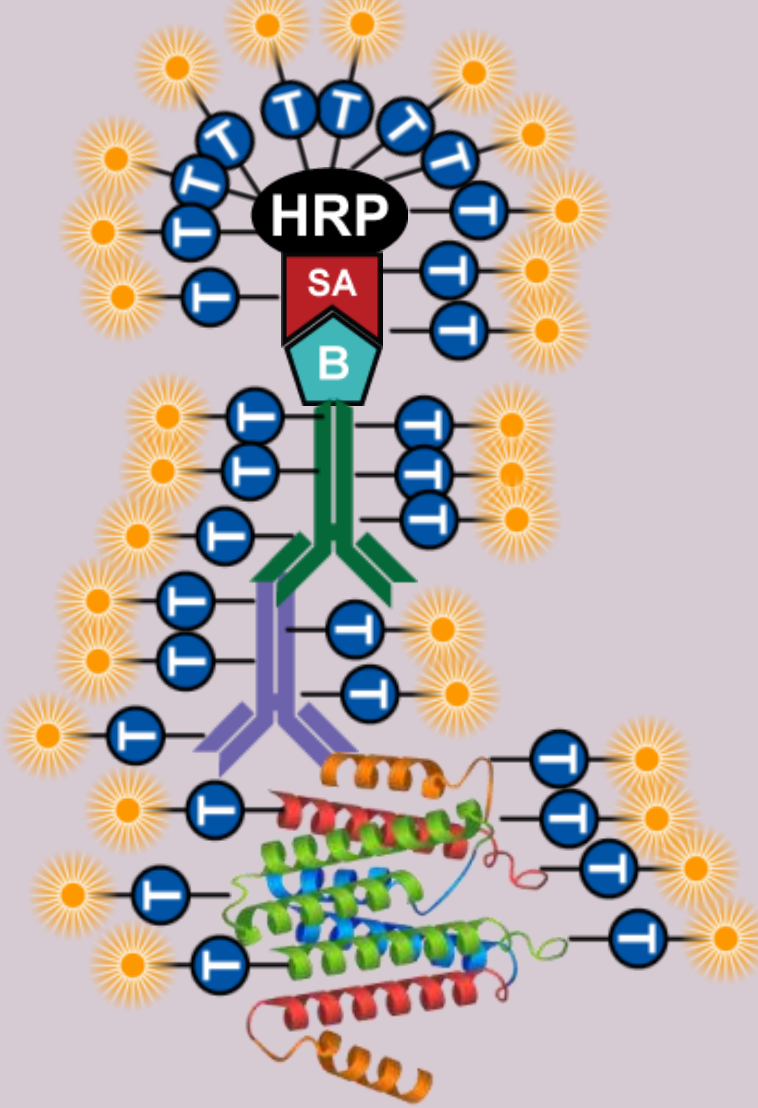
### Standard Detection

A common indirect immunofluorescence approach using fluorophore-labeled secondary antibodies (secAb).



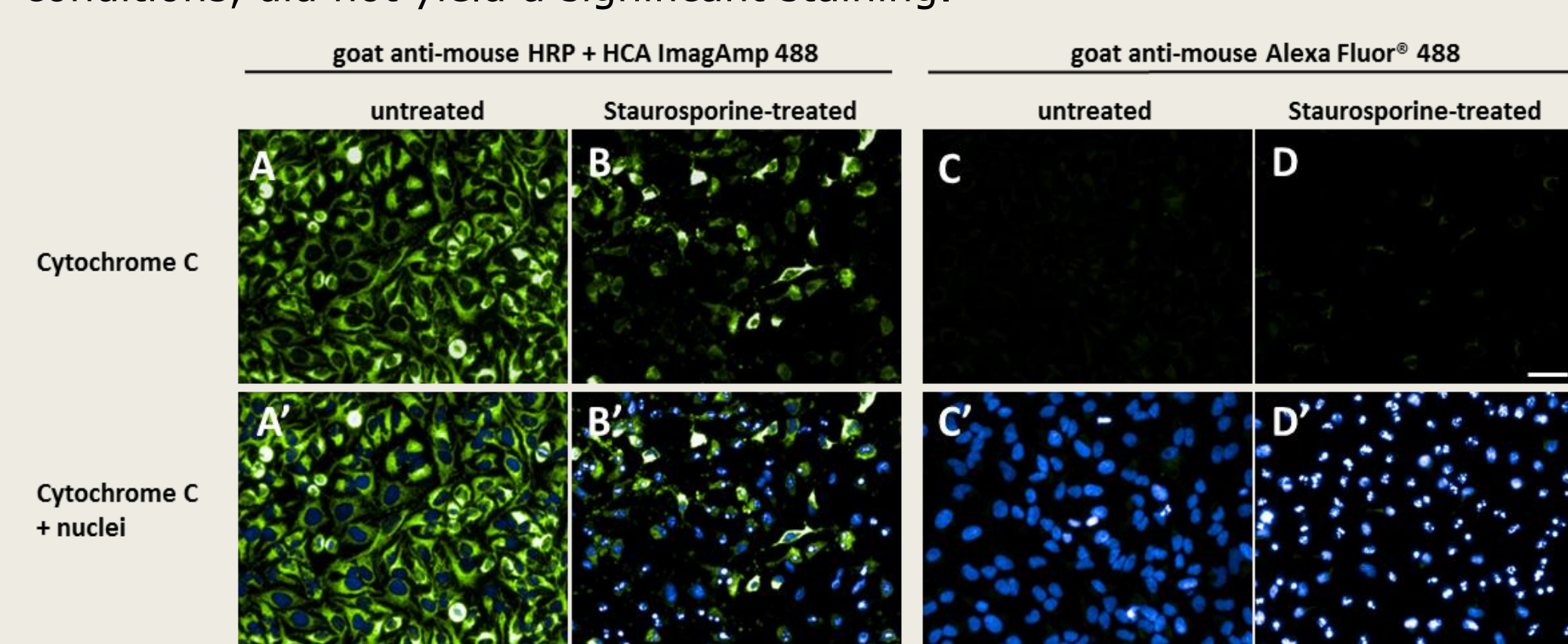
### Tyramide Signal Amplification (TSA) Detection

The technique utilizes the ability of horseradish peroxidase to convert fluorophore-labeled tyramide into a highly reactive molecule that will covalently bind to tyrosine residues in or very close to the enzyme (Hunyady *et al.*, 1996).



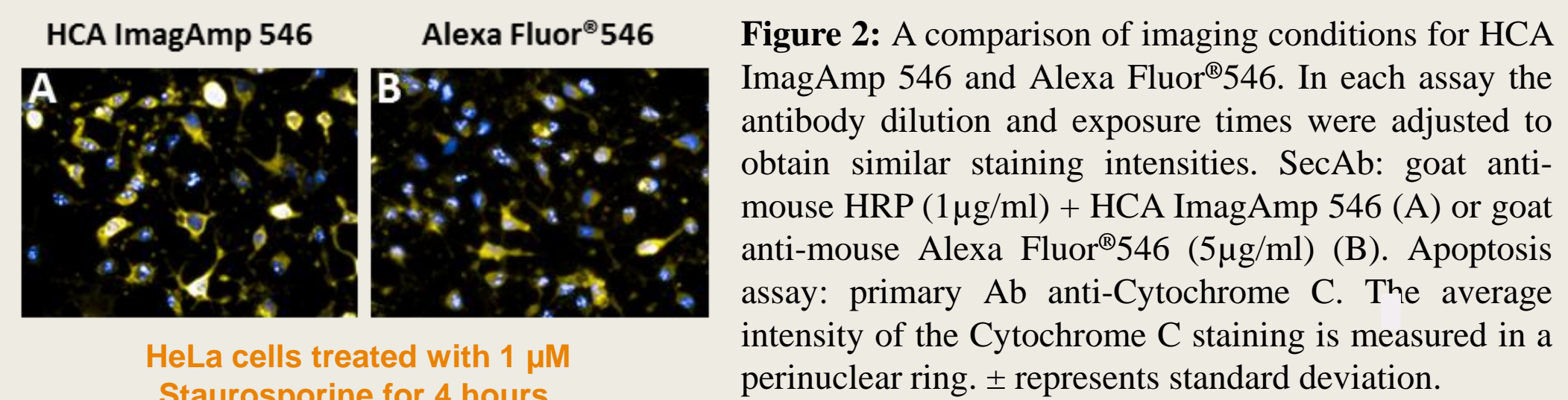
## 3 Apoptosis

Cytochrome C is a mitochondrial protein; during apoptosis it loses its specific localization and becomes more evenly distributed throughout the cell cytoplasm (Bossy-Wetzel *et al.*, 1998). Apoptosis was induced by treatment with staurosporine, and Cytochrome C staining was used as a marker. With HCA ImagAmp, 72.5% of the cells were found to be apoptotic, in contrast to only 3.9% in untreated controls. In contrast, Alexa Fluor®-labeled secAb stained cells, when imaged under exactly the same conditions, did not yield a significant staining.



**Figure 1:** Apoptosis induction imaged with HCA ImagAmp 488. HeLa cells were grown overnight in CellCarrier-384 plates, then incubated with 1µM Staurosporine for 4 hours and fixed. All Cytochrome C images (green) were taken at 35ms exposure time. (A-D) Anti-Cytochrome C Ab (0.5µg/ml). (A, B) secAb goat anti-mouse HRP (1µg/ml) + HCA ImagAmp 488. (C, D) secAb goat anti-mouse Alexa Fluor®488 (5µg/ml). (A'-D') Overlay of Cytochrome C staining with nuclear counterstain Hoechst®33342 (blue). Bar: 50µm.

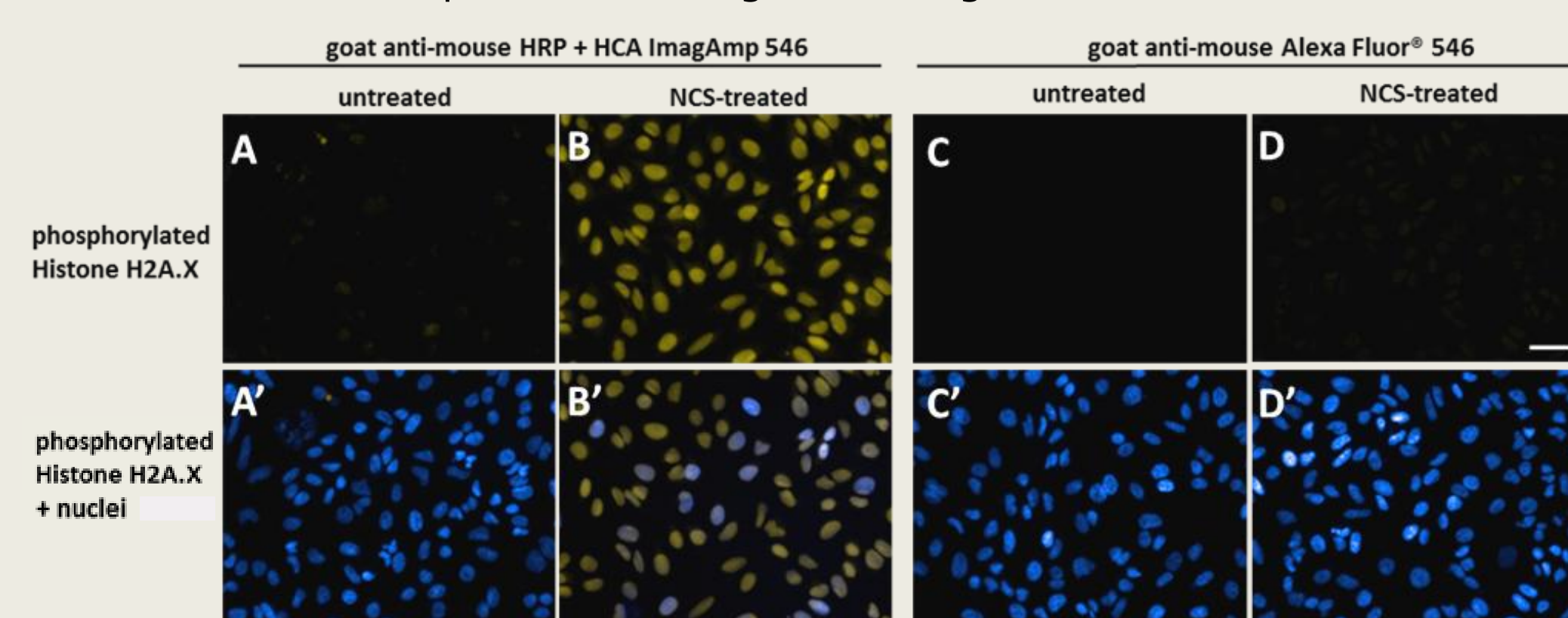
To quantify the extent of amplification by HCA ImagAmp compared to standard immunofluorescence, the concentration of the primary Ab and the exposure time were adjusted to produce equivalent intensity levels with HCA ImagAmp and an Alexa Fluor®-labeled secAb. The increased sensitivity mediated by HCA ImagAmp allows for significant reduction in the concentration of primary Ab required and the exposure time.



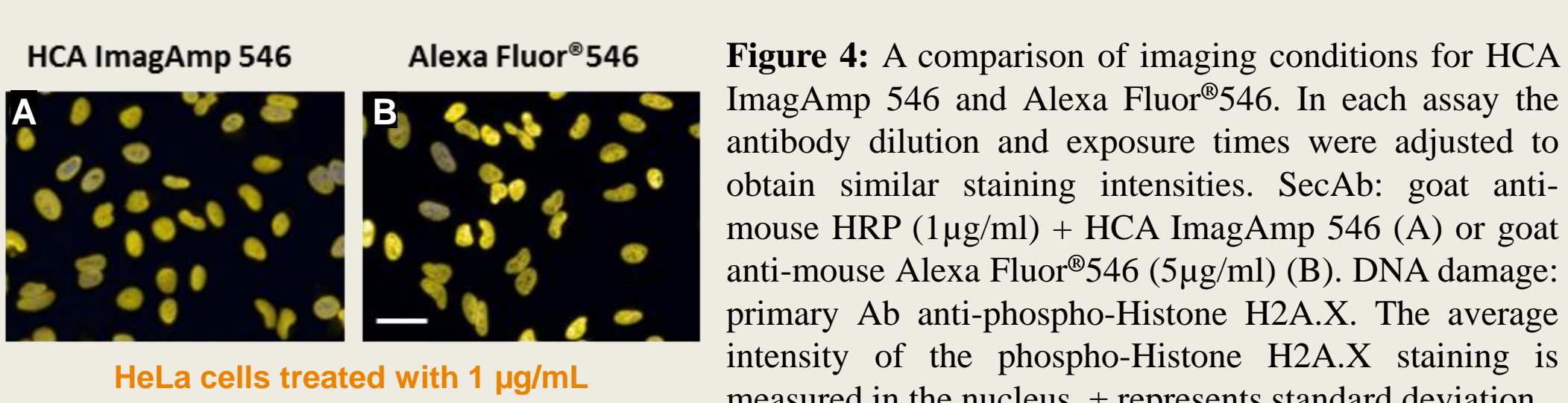
Condition	Staining Method	Primary Ab Concentration	Exposure Time	Average Intensity (1st Ab Stain)	Decrease 1st Ab/Exp. Time
Untreated	HCA ImagAmp 546	0.025 µg/ml	35 ms	2781 ± 112	<b>x20 / x10</b>
	Alexa Fluor® 546	0.5 µg/ml	350 ms	2585 ± 387	
Treated	HCA ImagAmp 546	0.025 µg/ml	35 ms	2402 ± 191	
	Alexa Fluor® 546	0.5 µg/ml	350 ms	2185 ± 197	

## 4 DNA Damage

DNA damage is induced by Neocarzinostatin (NCS), which causes DNA double strand breaks. As a result, phosphorylation of Histone H2A.X occurs, which can be detected with an antibody against the phosphorylated protein (Rogakou *et al.*, 1999). After staining with HCA ImagAmp, NCS treatment rendered 99.8% of the cells positive, in contrast to 3.1% without treatment. Again, staining with an Alexa Fluor®-labeled secAb under otherwise exactly the same conditions produced no significant signal.

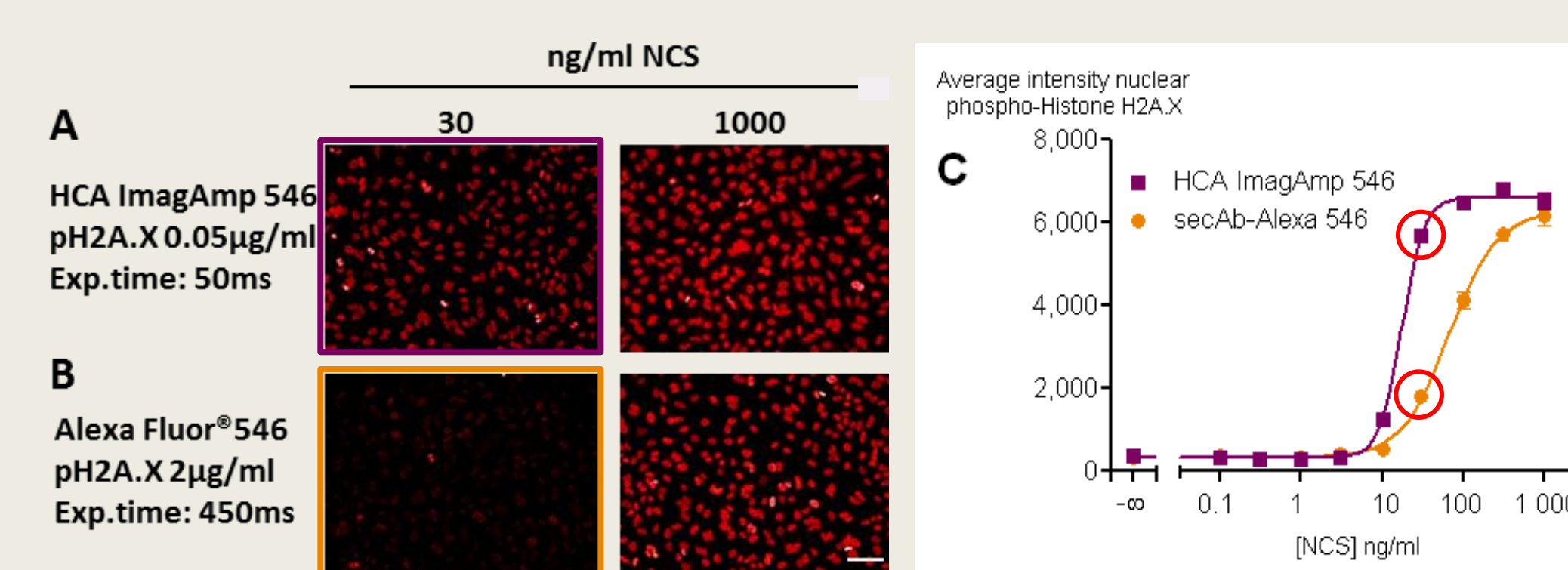


**Figure 3:** DNA damage induction imaged with HCA ImagAmp. HeLa cells were grown overnight in CellCarrier-384 plates, then incubated with 1µg/ml NCS for 1 hour and fixed. All phospho-Histone H2A.X images (yellow) were taken at 25ms exposure time. (A-D) anti-phospho-Histone H2A.X Ab (0.5µg/ml). (A'-D') overlay phospho-Histone H2A.X and nuclear counterstain (blue, Hoechst®33342). (A and B) SecAb goat anti-mouse HRP (1µg/ml) + HCA ImagAmp 546. (C and D) SecAb goat anti-mouse Alexa Fluor®488 (5µg/ml). Bar: 50µm.



Condition	Staining Method	Primary Ab Concentration	Exposure Time	Average Intensity (1st Ab Stain)	Decrease 1st Ab/Exp. Time
Untreated	HCA ImagAmp 546	0.05 µg/ml	50 ms	288 ± 34	<b>x40 / x8</b>
	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	

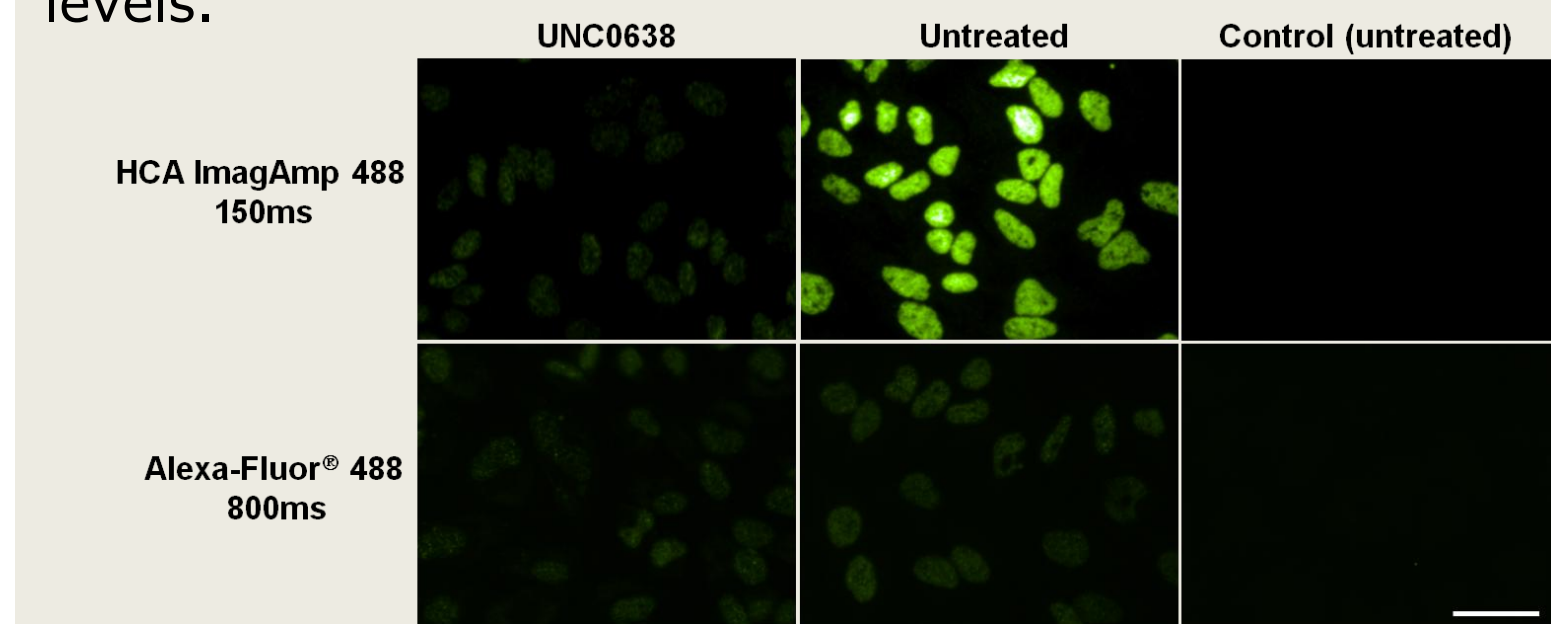
To evaluate the sensitivity of the two staining approaches, a DNA damage assay with a dilution series of NCS was performed. To be comparable, anti-phospho-Histone H2A.X Ab concentrations and exposure times were set at levels to produce equivalent average nuclear staining intensities at the highest concentration of NCS (1000 ng/ml). 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.



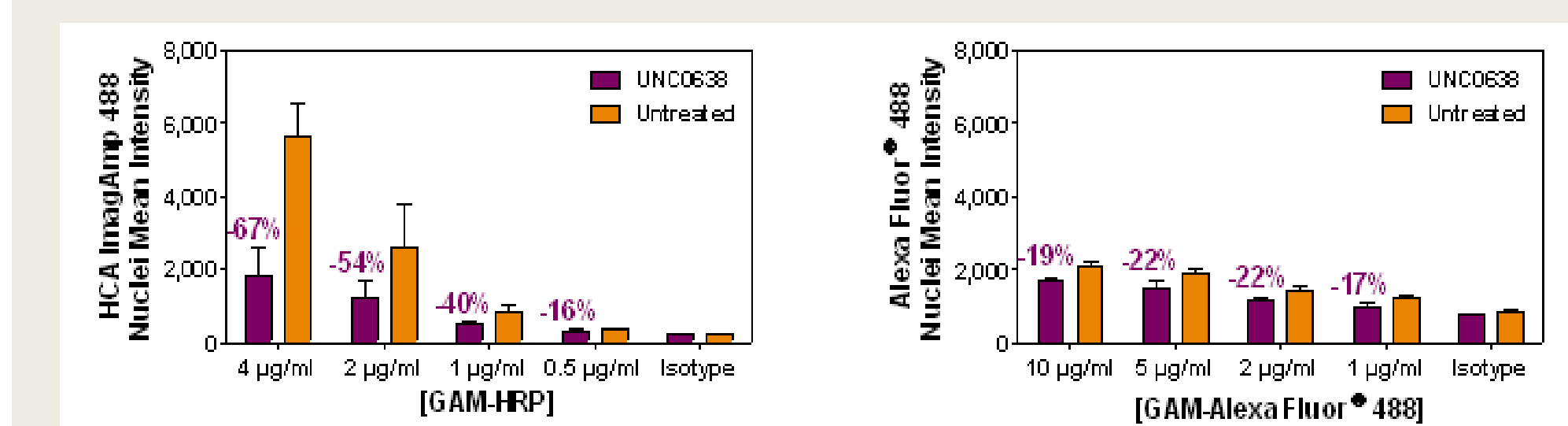
**Figure 5:** HCA ImagAmp increases sensitivity of detection. HeLa cells were cultured overnight in CellCarrier-384 plates, then incubated with different concentrations of NCS (ranging from 0.1 to 1000 ng/ml) for 1 hour and fixed. (A) primary Ab phospho-Histone H2A.X, secAb goat anti-mouse HRP (1µg/ml) + HCA ImagAmp 546. (B) primary Ab phospho-Histone H2A.X, secAb goat anti-mouse Alexa Fluor®546 (5µg/ml). Nuclear counterstain with Hoechst®33342 was omitted for clarity. Bar: 50µm. (C) NCS dilution series. Scale on x-axis is logarithmic.

## 5 Histone H3 Epigenetic Modifications

UNC0638 is a histone methyltransferase inhibitor. When the cells are treated with this compound, a lower level of H3K9me2 is observed (Vedadi *et al.*, 2011). In comparison to GAM-Alexa Fluor® 488 staining, HCA ImagAmp 488 strongly increased the H3K9me2 specific signal in untreated cells (3-fold increase of intensity at a more than 5-fold shorter exposure time). In addition the signal to background ratio and the signal ratio between untreated and UNC0638-treated cells increased significantly, thereby improving the ability to detect changes in H3K9me2 levels.



**Figure 6:** Evaluation of the efficacy of the HCA ImagAmp 488 compared to the Alexa Fluor®488. HeLa cells were grown overnight in CellCarrier-384 plates, then incubated with 1µM UNC0638 for 48 hours and fixed. Epigenetic modifications detection assay: primary Ab anti-H3K9me2 or mouse IgG1 Isotype control (5µg/ml), secAb: goat anti-mouse HRP (4µg/ml) + HCA ImagAmp 488 or goat anti-mouse Alexa Fluor®488 (5µg/ml). Images were taken at 150ms and 800ms exposure time with HCA ImagAmp 488 and Alexa Fluor®488 respectively. The average intensity of the H3K9me2 staining is measured in the nucleus. Bar: 50µm.



**Figure 7:** Efficacy of the HCA ImagAmp 488 compare to the Alexa Fluor®488 with different concentrations of each secondary antibody (GAM). Protocol same as figure 6.

## 6 Materials

Product	Company	Catalogue number
HeLa	ATCC	CCL-2
CellCarrier-384 plates	PerkinElmer	6007550
Staurosporine	Sigma	S6942
Neocarzinostatin (NCS)	Sigma	N9162
UNC0638 inhibitor	Sigma	U4885
Mouse anti-phospho-Histone H2A.X	Millipore	05-636
Mouse anti-Cytochrome C	BD Bioscience	556432
Mouse anti-H3K9me2	Active Motif	39683
Mouse (G3A1) IgG1 Isotype Control	CST	54155
Goat anti-mouse IgG HRP	PerkinElmer	NEF822001EA
Goat anti-mouse IgG Alexa Fluor®488	Life Technologies	A-11029
Goat anti mouse IgG Alexa Fluor®546	Life Technologies	A-11030
Goat anti-mouse IgG HRP	PerkinElmer	NEF822001EA
HCA ImagAmp 488	PerkinElmer	NEL771B001KT
HCA ImagAmp 546	PerkinElmer	NEL774B001KT
Blocking Reagent	PerkinElmer	FP1012
Hoechst®33342	Life Technologies	H3570

## 7 Methods

To perform immunofluorescence staining a standard protocol was used. Briefly, HeLa cells were seeded at a density of 6000 cells per well (cytotoxicity assays) and 2500 cells per well (epigenetics assays) in CellCarrier™-384 plates. After incubation overnight, the cells were incubated with the appropriate chemical agents to induce apoptosis (1µM staurosporine for 4 hours), DNA damage (1µg/ml NCS for 1 hour) or to inhibit histone methyltransferase (1µM UNC0638 for 48h). The cells were then fixed in 3.7% formaldehyde. Permeabilization was carried with a 0.1% Triton/PBS solution. Nuclei were counterstained by adding 2µg/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, in each experiment 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.

## 8 References

Hunyady *et al.* (1996): Immunohistochemical signal amplification by catalyzed reporter deposition and its application in double immunostaining. *J Histochem Cytochem*, 44: 1353-62.  
Bossy-Wetzel *et al.* (1998): Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J*, 17: 37-49.  
Rogakou *et al.* (1999): Megabase chromatin domains involved in DNA double-strand breaks in vivo. *J Cell Biol*, 146: 905-16.  
Vedadi *et al.* (2011): A chemical probe selectively inhibits G9a and GLP methyltransferase activity in cells. *Nat Chem Biol*, 7(8):566-74.

## 9 Summary

- The **increase in sensitivity** is highlighted by the fact that HCA ImagAmp, in comparison to commonly used fluorophore-labeled secAb, allows for significant reduction of **the concentration of primary Ab needed** and requires much shorter exposure times.
- In addition, the NCS-titration experiments show that the amplified signal **enables specific enhancement of signal intensity at low levels of DNA damage**, thereby shifting the detection limit towards lower concentrations of NCS.
- Finally, for **technically challenging targets** such as the detection of **low abundance epigenetic histone methyl marks**, the HCA ImagAmp™ reagent kit clearly represents a **markedly better detection method than conventional IF**.