Signal amplification with HCA ImagAmp reagent kits enhances signal intensity and sensitivity of immunofluorescence-based HCA

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1 Abstract

HCA ImagAmp™ technology is designed to greatly enhance target-specific fluorescence signals and to improve sensitivity in immunofluorescence-based assays. Signal amplification is achieved through enzyme-mediated deposition of multiple fluorophores in close proximity to a given antigen. The technology utilizes the ability of horse radish peroxidase (HRP) to convert fluorophore-labeled tyramide into a highly reactive molecule that will covalently bind to tyrosine residues close to the enzyme.

We utilized the HCA ImagAmp reagent kit in two typical high content analysis (HCA) assays for cytotoxicity: Cytochrome C release from mitochondria as a marker for apoptosis and phosphorylation of Histone H2A.X as an indicator of DNA damage. To evaluate the performance of HCA ImagAmp reagents, several staining and analysis parameters were compared to a conventional immunofluorescence 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. The HCA ImagAmp reagent kit also allowed a more robust detection of global H3K9 dimethylation levels following cell treatment with a selective chemical probe.

The results demonstrate that HCA ImagAmp reagent kits allow to significantly reduce the exposure time and the concentration of primary antibody required to produce a strong immunofluorescence signal, potentially providing significant savings in time and costs. Finally, signal amplification with HCA ImagAmp technology should be especially useful in applications such as the detection of early disease biomarkers, where the low number of available antigens renders conventional immunofluorescence staining often too weak to allow for a robust analysis.

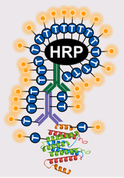
2 Materials

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	5415S
Goat anti-mouse (GAM) IgG HRP	PerkinElmer	NEF822001EA
Goat anti-mouse (GAM) IgG Alexa Fluor®488	Life Technologies	A-11029
Goat anti mouse (GAM) IgG Alexa Fluor®546	Life Technologies	A-11030
HCA ImagAmp 488	PerkinElmer	NEL771B001KT
HCA ImagAmp 546	PerkinElmer	NEL774B001KT
Blocking Reagent	PerkinElmer	FP1012
Hoechst®33342	Life Technologies	H3570

3 Methods

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 overnight incubation, 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 done 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 minute-incubation with HCA ImagAmp reagent. For comparative studies, cells were stained and imaged in parallel with an Alexa Fluor®-labeled secAb of the corresponding wavelength, under identical conditions. Imaging was performed with the Operetta® High Content Imaging System, using a 20X WD objective.

4 Signal Amplification Principle

Standard Detection	Tyramide Signal Amplification (TSA)
A common indirect immunofluorescence approach using fluorophore-labeled secondary antibodies (secAb).	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).
	

5 Apoptosis

Apoptosis was induced by treatment with staurosporine and the mitochondrial protein, Cytochrome C, was used as apoptosis marker. With HCA ImagAmp, 72.5% of the cells were found to be apoptotic, in contrast to only 3.9% in untreated controls. When imaged under exactly the same conditions, cells stained with Alexa Fluor®-labeled secAb did not yield any significant staining.

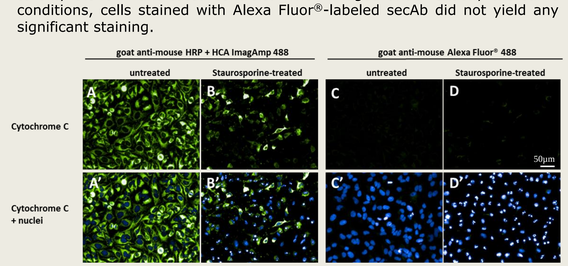


Figure 1: Apoptosis induction imaged with HCA ImagAmp 488. HeLa cells were grown overnight in CellCarrier-384 plates, then incubated with 1µM Staurosporine for 4 hours and fixed. Primary Ab: anti-Cytochrome C (0.5µg/ml). SecAb: GAM HRP (1µg/ml) + HCA ImagAmp 488 and GAM Alexa Fluor®488 (5µg/ml). In apoptotic cells, Cytochrome C localization changes from being predominantly mitochondrial to homogeneously distributed throughout the cell. All Cytochrome C images (green) were taken at 35ms exposure time.

To generate equal signal intensities with both staining approaches, primary antibody concentrations and exposure times were adjusted.

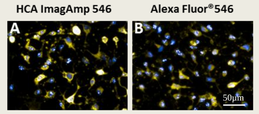


Figure 2: A comparison of imaging conditions for HCA ImagAmp 546 and Alexa Fluor®546. HeLa cells treated with 1µM Staurosporine for 4 hours, followed by Cytochrome C staining. Antibody dilutions and exposure times were adjusted to obtain similar staining intensities. SecAb: GAM HRP (1µg/ml) + HCA ImagAmp 546 (A) or GAM Alexa Fluor®546 (5µg/ml) (B).

Mean signal intensity of Cytochrome C staining in a perinuclear ring (±SD)

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

The increased sensitivity enabled by HCA ImagAmp allows for significant reduction in the concentration of primary Ab required and the exposure time.

6 DNA Damage

DNA damage is induced by Neocarzinostatin (NCS), which causes DNA double strand breaks and results in the phosphorylation of Histone H2A.X Staining for phospho-H2A.X with HCA ImagAmp shows that 99.8% of the cells were positive following NCS treatment compared to 3.1% of cells in control (untreated) samples. Staining with an Alexa Fluor®-labeled secAb under the same conditions resulted in negligible signal intensities.

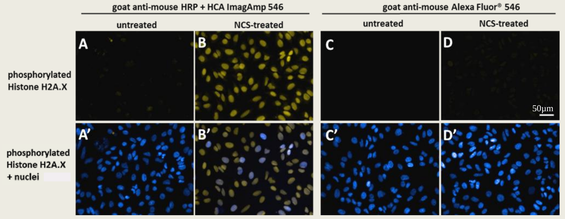


Figure 3: DNA damage induction imaged with HCA ImagAmp. HeLa cells were grown overnight in CellCarrier-384 plates, then incubated with 1µg/ml NCS for 1 hour and fixed. Primary Ab: anti-phospho-Histone H2A.X Ab (0.5µg/ml). SecAb: GAM HRP (1µg/ml) + HCA ImagAmp 546 and GAM Alexa Fluor®488 (5µg/ml). All phospho-Histone H2A.X images (yellow) were taken at 25ms exposure time.

Primary antibody concentrations and exposure times were adjusted.
Mean nuclear signal intensity of phospho-Histone H2A.X (±SD)

Condition	Staining Method	Primary Ab Concentration	Exposure Time	Average Intensity (1st Ab Stain)	Decrease 1st Ab/Exp. Time
Untreated	HCA ImagAmp 546	0.05µg/ml	50 ms	288 ± 34	x40 / x8
	Alexa Fluor®546	2µg/ml	400 ms	384 ± 105	
Treated	**HCA ImagAmp 546**	**0.05µg/ml**	**50 ms**	**6046 ± 47**	
	Alexa Fluor®546	**2µg/ml**	**400 ms**	**6655 ± 262**	

An NCS dilution series demonstrates that even at low levels of NCS (30ng/ml) the mean nuclear staining intensity of phospho-Histone H2A.X, as detected with HCA ImagAmp, remained high compared to that observed with the Alexa Fluor®-labeled secAb.

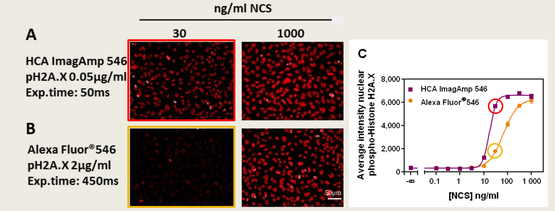


Figure 4: HCA ImagAmp increases sensitivity of detection. HeLa cells were cultured overnight in CellCarrier-384 plates, then incubated with different concentrations of NCS (ranging from 0.1 to 1000ng/ml) for 1 hour and fixed. (A) Primary Ab phospho-Histone H2A.X, secAb GAM HRP (1µg/ml) + HCA ImagAmp 546. (B) Primary Ab phospho-Histone H2A.X, secAb GAM Alexa Fluor®546 (5µg/ml). (C) NCS dilution series. 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 (1000ng/ml). Scale on x-axis is logarithmic.

To extend the analysis of NCS-induced DNA damage, the percentage of phospho-Histone H2A.X-spot-positive nuclei at low concentrations of NCS was evaluated. HCA ImagAmp enables the detection of a clear increase of the number of positive nuclei at 1-3ng/ml NCS.

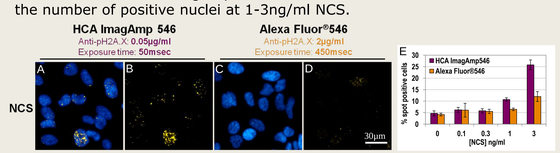


Figure 5: HCA ImagAmp spot analysis. HeLa cells were cultured overnight in CellCarrier-384 plates, then incubated with 3ng/ml NCS for 1 hour and fixed. (A-B) Primary Ab phospho-Histone H2A.X, secAb GAM HRP (0.05µg/ml) + HCA ImagAmp 546. (C-D) Primary Ab phospho-Histone H2A.X, secAb GAM Alexa Fluor®546 (2µg/ml). HCA ImagAmp increases S/B-related spot parameters, e.g. relative spot intensity (0.65±0.02) and spot contrast (0.53±0.02) compared to Alexa Fluor® staining (0.44±0.01 and 0.32±0.01, respectively). (E) Quantitation of spot positive cells under serial dilutions of NCS.

The HCA ImagAmp-enhanced signal improves the identification of the small, otherwise weakly stained phospho-Histone H2A.X spots, increasing sensitivity in the detection of nuclei affected by NCS treatment.

7 Histone H3 Epigenetic Modifications

Detection of modulations in H3K9me2 in HeLa cells treated with G9a and GLP methyltransferase inhibitor UNC0638.

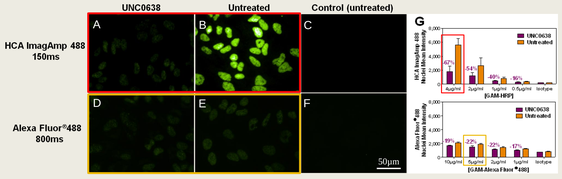


Figure 6: HeLa cells were grown overnight in CellCarrier-384 plates, then incubated with 1µM UNC0638 for 48 hours and fixed. Primary Ab: anti-H3K9me2 or mouse IgG1 Isotype control (5µg/ml), secAb: (A-C) GAM HRP (4µg/ml) + HCA ImagAmp 488 or (D-F) GAM Alexa Fluor®488 (5µg/ml). Images were taken at 150ms (A-C) and 800ms (D-F) exposure time. (G) Quantitative analysis of imaging results. Nuclear mean intensities using different concentrations of each secAb were measured. A 3-fold increase of intensity at a more than 5-fold shorter exposure time was observed using HCA ImagAmp 488.

Levels of H3K9me2 detected with Alexa Fluor®488 staining approximated background levels (isotype control) while that detected with HCA ImagAmp 488 were significantly above background. Furthermore, the S/B ratio and the signal ratio between untreated and UNC0638-treated cells was markedly higher, thereby demonstrating the technological advantages of the HCA ImagAmp reagent in detecting technically challenging targets such as H3K9me2.

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.

9 Summary

- These experiments demonstrate that HCA ImagAmp strongly increases sensitivity, in comparison to commonly used fluorophore-labeled secAb. This allows for a significant reduction in primary Ab concentration and exposure time.
- 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 lysine methyl marks, the HCA ImagAmp™ reagent kit clearly represents a markedly enhanced detection method over conventional immunofluorescence.