Novel AlphaLISA assays for the detection of autophagy

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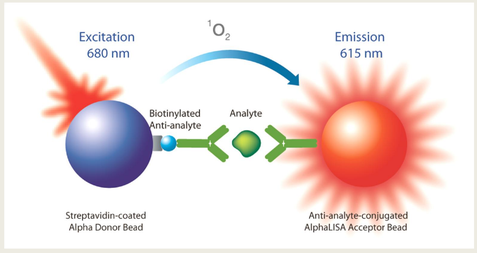
1 Introduction

Autophagy has gained considerable attention in the last few years due to its importance in disease processes such as tumorigenesis and neurodegeneration. Significant efforts are ongoing to identify modulators of the autophagy pathway. Sequestosome (p62) and Microtubule-Associated Protein 1 Light Chain 3 (LC3) are key players in the autophagy process. LC3 is involved in formation of the autophagosomal membrane and p62 sequesters polyubiquitinated proteins in autophagosomes. To date, LC3 has been a frequently used marker for autophagy. The modification of LC3 by attachment of phosphatidylethanolamine is detectable by Western blotting, and its cellular localization at the autophagosomal membrane can be visualized using immunofluorescence microscopy. However, there is a lack of high-throughput methods for monitoring autophagy. For this reason, AlphaLISA® no-wash assays have been developed for the detection of endogenous levels of p62 and LC3B in cell lysates. The cellular model system used was HeLa cells treated with chloroquine to inhibit the final autophagy step - autolysosomal degradation. This inhibition leads to an accumulation of the autophagosome marker proteins. Both Western blotting and immunofluorescence were performed to validate the model system. In a typical experiment, the cells were treated overnight with 50 µM chloroquine, following which the AlphaLISA assay was initiated by direct addition of lysis buffer to the wells of the microplate. The analyte was simultaneously captured by a biotinylated antibody and antibody-conjugated Alpha acceptor beads. The assay was then completed by addition of Streptavidin-coupled Alpha Donor beads and read on an EnVision® Multilabel Plate Reader. The levels of LC3B and p62 both increased significantly upon treatment with chloroquine. The autophagy markers could be detected using as little as 1 000 cells in a 384-well plate. These assays can accelerate the identification of therapeutics that modulate the autophagy process.

2 AlphaLISA Technology principle



The biotinylated anti-analyte antibody binds to the Streptavidin-coated Alpha Donor beads while another anti-analyte antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. The 680 nm laser excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads resulting in a sharp peak of light emission at 615 nm.

3 Materials and methods

AlphaLISA products (PerkinElmer):

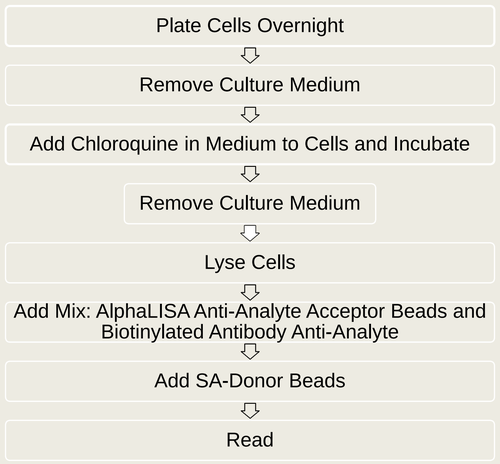
Standalone products:
- AlphaScreen Acceptor beads: Protein A Acceptor beads (#6760137M)
- Alpha Donor beads: Streptavidin Donor beads (#6760002S)

AlphaLISA kits:
- Human Sequestosome-1 (p62) Kit (#AL305)
- Human Microtubule-associated Protein 1 Light Chain 3 Isoform B (LC3B) Kit (#AL306)

Other material:
AlphaScreen SureFire GAPDH Assay kit (#TGRGDS500)

Detection of autophagy markers. Detection of either p62 or LC3B was performed on HeLa cells seeded in 384-well CulturPlate microplates at different cell densities per well. Cells were either treated over-night with 50 µM Chloroquine or left untreated. Normalization with GAPDH was performed using the SureFire GAPDH kit. Refer to the flow chart.

Protocol for AlphaLISA cell-based assays



Z'-factor determination. Z'-factor values were determined by comparing the AlphaLISA signal of HeLa cells (5 000 cells/well) untreated and treated with 50 µM Chloroquine. Cells were incubated overnight in 48 wells/condition in MEM/HBSS medium.

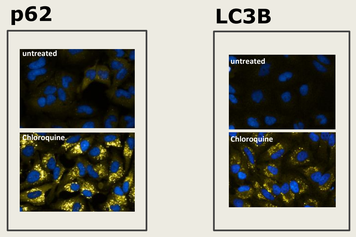
Dilutional linearity. The dilutional linearity was determined by serial dilutions of HeLa cell lysates in AlphaLISA Buffer. The samples were interpolated on a standard curve prepared using AlphaLISA Buffer. The recovery was calculated using the neat sample as the 100% value.

Western blot detection of autophagy markers. HeLa cells were seeded and treated as described above. The cells were washed in cold PBS and collected by scraping in an Epppedorf tube. After a short spin, PBS was removed and the cells lysed in M-RIPA lysis buffer containing protease inhibitor cocktail at concentration of $2x10^6$ cells/mL of lysis buffer for 10 min. Protein concentration was determined by a Bradford protein assay kit. Cell lysates were then separated by SDS-PAGE on a 10%-20% gradient gel. Following transfer to nitrocellulose, p62 and LC3B were detected using the same antibody present on the Acceptor beads. GADPH staining was used as loading control.

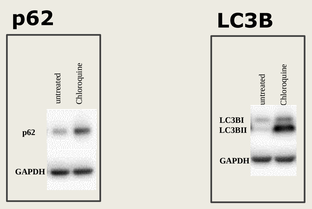
Immunofluorescence. HeLa cells were seeded at a density of 5 000 cells per well in CellCarrier-384 plates. After overnight incubation, the cells were treated for 18 to 24 hrs with 50 µM Chloroquine to induce autophagy. The cells were then fixed in 3.7% formaldehyde. For p62, permeabilization was done with a 0.1% Triton/PBS solution, for LC3B cells were treated with ice cold methanol. Nuclei were counterstained by adding 2 µg/ml Hoechst®33342 to the solution. Primary Ab and Alexa546–labeled secAb incubation steps were done in PerkinElmer blocking solution. Imaging was performed with the Operetta® High Content Imaging System, using a 20X WD objective.

4 Autophagy model validation

A *Autophagy marker detection by Immunofluorescence*



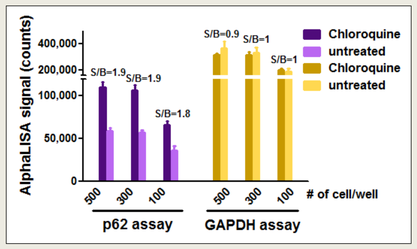
B *Autophagy marker detection by Western blotting*



Model validation. HeLa cells were treated and then immunofluorescence (A) and Western blotting (B) were performed as described in Methods. Chloroquine treatment clearly leads to a perinuclear accumulation of p62 or LC3B positive spots and an increase in the p62 or LC3B I and II bands.

5 Development of autophagy assays

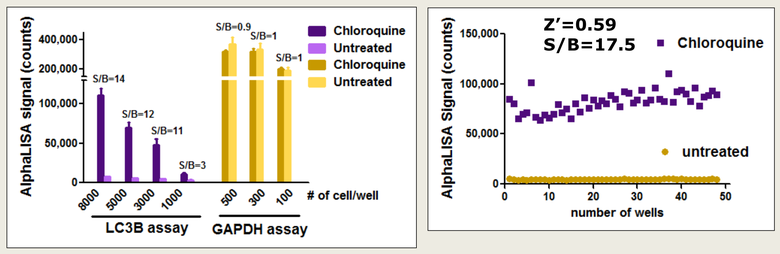
A *Development of p62 AlphaLISA assay*



Dilutional linearity

Dilution Factor	% Recovery		Interpolated [p62] (µg/mL)	
	Untreated	Chloroquine	Untreated	Chloroquine
1	100	100	2.47	3.62
2	106	100	2.62	3.61
4	109	102	2.68	3.69
8	112	107	2.76	3.89
16	108	110	2.67	3.99

B *Development of LC3B AlphaLISA assay*



Dilutional linearity

Dilution Factor	% Recovery		Interpolated [LC3B] (µg/mL)	
	Untreated	Chloroquine	Untreated	Chloroquine
1	100	100	0.02	0.16
2	106	93	0.03	0.15
4	98	80	0.02	0.13
8	-	88	-	0.14
16	-	95	-	0.15

Assay development. At first, biochemical AlphaLISA assays with recombinant p62 and LC3B were developed by screening for antibody pairs that gave an appropriate sensitivity for the analytes. The cell-based assays were then optimized by testing conditions such as lysis incubation time, assay detection buffer composition, and number of cells per well. The results in terms of S/B, Z', and dilutional linearity for untreated versus Chloroquine-treated cells are illustrated for the p62 (A) and LC3B (B) cellular assays. The number of cells per well was adjusted to provide counts below the saturation level of the assays. The p62 protein level was consistently shown to increase approximately 2-fold upon Chloroquine treatment, however, Z' values > 0.5 were not obtained for this assay (data not shown). The LC3B protein was accumu-lated to a level up to 18-fold higher than the untreated cells and a Z' value of 0.59 was obtained. The levels of the proteins in cell lysates could be accurately measured by interpolation of sample values on a standard curve, as illustrated by the %Recovery values between 80-110% for dilutional linearity experiments.

6 Summary

Highly efficient AlphaLISA assays were developed to measure p62 and LC3B in a cell-based assay. First, a HeLa cell autophagy model was validated using immunofluorescence and Western blotting to demonstrate the increased levels of these biomarkers after treatment with an autolysosomal degradation inhibitor, which halts the progression of autophagy and allows the accumulation of these proteins. Then, AlphaLISA assays were optimized for detection of p62 and LC3B in cell lysates. Consistent increases in the levels of p62 (2-fold) and LC3B (10 to 20-fold) were observed with Chloroquine treatment, whereas the GAPDH levels were not affected.

The Alpha technology provides a highly efficient immunoassay method to detect autophagy markers and will accelerate the identification of therapeutics that modulate the autophagy process.