Detection and Quantification of Autophagy Using the AlphaLISA p62 Assay

Introduction
Lysosomal dysregulation is the hallmark of many diseases including neurodegenerative diseases, lysosomal storages diseases, and aging.¹ The lysosome is responsible for eliminating cellular waste in a multistep pathway called autophagy. This process is well regulated and can be affected by a number of stimulants including nutrient starvation, physiological stress, and chemical induction.² The first step in autophagy is the accumulation of cellular debris which is then encapsulated to form autophagosomes. The autophagosome then fuses with the lysosome to create an autolysosome which is proteolyzed or exocytosed. Autophagy can be inhibited by treatments that block individual steps in this pathway, or modulated by factors that upregulate this system to increase turnover or flux.
Tracking the proteins associated with the lysosome allows one to investigate autophagy. Sequestosome-1, also known as p62, is incorporated into autophagosomes then subsequently degraded in the final steps of autophagy. Assessing this protein can help decipher a block or increased flux in autophagy. Cellular imaging strategies have often been used to visualize autophagosomes and lysosomes where the fused autophagosome-lysosome compartment accumulates and creates a punctate pattern in the cell. Quantification of p62 levels within cellular lysates is often performed with labor intensive wash-based ELISA assays. In this study, chloroquine is used as an inhibitor of autophagy to show how the homogeneous no-wash AlphaLISA® p62 assay can detect changing levels of this lysosomal protein (Figure 1). While first investigated as an antimalaria drug, chloroquine lowers the pH in the lysosome to block the formation of the autolysosome. p62 then accumulates in the autophagosomes which are unable to be cleared. AlphaLISA p62 quantification is corroborated by the visualization of p62 puncta which show the blocked fusion of autophagosome and lysosomes. Additionally, nuclear staining is used to normalize the cell numbers in each treatment group to show changing AlphaLISA p62 levels are due to the autophagic block and not an alternation of cell number.

For the AlphaLISA p62 assay (PerkinElmer, #AL3097), the media was removed and the cells were washed twice with PBS and finally lysed in 50 µL AlphaLISA Lysis Buffer (PerkinElmer, #AL003). The assay was run in accordance to Figure 2 where 5 µL of cell lysate was added to an AlphaPlate™-384 (PerkinElmer, #6005350) with a 20 µL mixture of 25 µg/mL anti-p62 Acceptor beads and 2.5 nM biotinylated anti-p62 antibody. This was incubated at ambient temperature for 60 minutes with a TopSeal-A™ plate seal (PerkinElmer, #6050185). Then, 25 µL of 80 µg/mL streptavidin Donor beads were added to the plate and incubated once more for 30 minutes in the dark.

p62 puncta and nuclei were visualized by fixing cells with 4% paraformaldehyde, permeabilizing with 0.1% Triton™ X-100 (ThermoFisher, #28314), then incubating in blocking buffer (PerkinElmer, #ARD1A01EA) for one hour. Cells were incubated with a 1:800 dilution of p62 antibody solution (Cell Signaling Technology, #88588) overnight. After washing three times with PBS, cells were incubated with a 1:1000 dilution of secondary antibody (FITC-labeled Goat anti-mouse antibody, JacksonImmuno Research, #115-095-062) and 0.2 µg/mL Hoechst (ThermoFisher, #H3570) and finally washed three times with PBS before imaging.

Materials and Methods

Reagents
HeLa cells (ATCC®, CCL-2™) were cultured in EMEM (ATCC®, 30-2003™) supplemented with 10% FBS (ThermoFisher, #26140079). Cells were plated at 10,000 cells/well in ViewPlate™-96 F TC treated (PerkinElmer, #6005182) and incubated at 37°C in 5% CO₂. The next day, cells were treated with and without 50 µM chloroquine (Tocris, #4109) in fresh media for 18 hours.

Figure 1. AlphaLISA p62 assay principle. A biotinylated anti-p62 antibody is coupled to a Streptavidin Donor bead, while an anti-p62 antibody is coupled to an Acceptor bead. Binding p62 brings the Acceptor and Donor bead in close proximity, so that when the Donor bead is excited at 680 nm, the Acceptor bead produces an emission at 615 nm. The AlphaLISA p62 detection assay generates results in under two hours, with no-wash steps.

Figure 2. A workflow for the no-wash AlphaLISA p62 assay.
Instrumentation
The AlphaLISA p62 assay and immunofluorescence imaging were read on the EnSight™ multimode plate reader (Figure 3). The EnSight multimode plate reader provides standard detection as well as cellular imaging capabilities to monitor fluorescence intensity. Standard imaging parameters for Hoechst and FITC were used, as well as standard Alpha settings for the AlphaLISA p62 assay.

Data Analysis
Curves were plotted in GraphPad Prism® version 7.0 according to a nonlinear regression using the four parameter logistic equation (sigmoidal dose-response curve with variable slope) and 1/Y^2 data weighing. All data shown are the average of at least triplicate measurements.

Results
Increased p62 Levels are Seen with Chloroquine Treatment
Untreated and treated cell lysates were run in the AlphaLISA p62 assay alongside a p62 standard curve prepared in AlphaLISA Lysis Buffer (Figure 4). The concentration of p62 in each condition was interpolated from the analyte standard curve. Untreated cells in complete media have a calculated p62 basal level of 33.15 ng/mL as autophagic flux is not impaired. Chloroquine treatment acidifies the autophagosome/lysosome to trap p62 in this vesicle and a corresponding increase in p62 to 135.75 ng/mL was detected. This verifies that the AlphaLISA can be used to accurately quantify p62 expression levels before and after treatment.

The EnSight Allows for Cell Normalization and Visually Confirms Changing p62 Levels
Cells treated under the same conditions as quantified by the AlphaLISA p62 assay were fixed and stained for p62 to visualize the increase of autophagosome/lysosome vesicles. The p62 and nuclei staining were visualized with the EnSight and confirmed chloroquine treatment does increase p62 levels (Figure 5). The FITC-p62 (green) appears as cytoplasmic puncta in the chloroquine treated cells while untreated cells do not show a strong staining pattern. These immunofluorescence images validate the AlphaLISA p62 plate-based assay data generated in this study.
To ensure chloroquine was not impacting the viability of the cells, nuclear staining with Hoechst was used to quantify cell number. From visual inspection, there appears to be little cell loss due to treatment; however, imaging quantification shows a small impact on cell number (16%) between the treatment groups. While the difference in cell number may not seem significant, cell number was used to normalize the AlphaLISA p62 results (Figure 6). This results in 98.9 ng/mL p62 per cell number/10⁴ in the chloroquine treated condition versus the untreated group yielding 20.3 ng/mL p62 per cell number/10⁴. The assay window is improved from 4.9 as compared to 4.1 without normalization and gives better confidence in the data.

**Conclusion**

The study of autophagy has many complex steps that have traditionally been studied by lower throughput immunofluorescence with specialized cell lines. Here, it is shown that the AlphaLISA p62 assay can provide a high throughput alternative to monitor flux or the inhibition of autophagy. HeLa cells treated with a common inhibitor of autophagy showed an increase in p62 after 18 hours of treatment with the AlphaLISA p62 assay. Further, the p62 concentrations were normalized to cell number which was calculated from the Hoechst nuclei staining which improves the overall assay window. The EnSight multimode plate reader is capable of reading AlphaLISA assays and can be used to image and quantify cell number for normalization.

Traditionally, a block in autophagy has been studied by the examination of p62 puncta. This visualization can be timely, require specialized instrumentation, and is lower throughput. Utilizing a high throughput plate-based assay can limit tedious staining steps and long incubations that are typical in immunofluorescence workflows resulting in a quicker turnaround time for results. The AlphaLISA p62 assay has a large dynamic range and can provide quantitative p62 data faster than immunofluorescent staining. The workflow is adaptable to a variety of cell types and can be a multifaceted tool in autophagy research.

**References**