Evaluating protein phosphorylation and cellular signaling cascades within the integrated stress response pathway with AlphaLISA *Surefire Ultra* assays.

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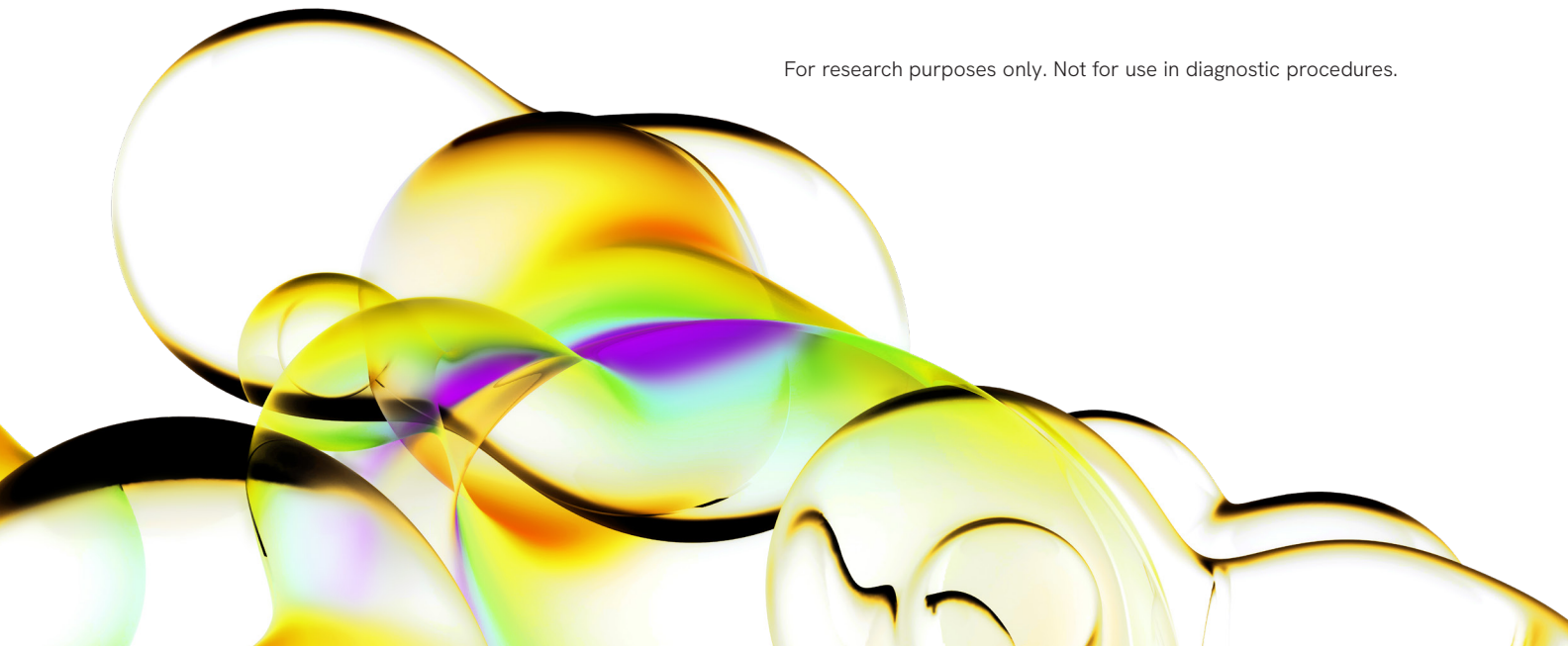
AlphaLISA *Surefire Ultra* Technology

Introduction

Protein phosphorylation is a critical regulatory mechanism for many cellular processes and is tightly controlled by kinase and phosphatase cascades. Therefore, monitoring phosphorylation events in a cellular model can be a useful approach to study the activity of compounds, as well as understand their mechanism of action or target modulation. Here we demonstrate the utility of AlphaLISA™ *SureFire™ Ultra™* (ALSU) assays to reliably measure a targeted phosphorylation event and total protein levels in cell-based experiments with a focus on the eIF2α/ATF-4 pathway.

Compatible with both cell and tissue lysates, ALSU kits are highly sensitive, no-wash (homogeneous) immunoassays well-suited for measuring levels of phosphorylated cellular proteins or total protein levels involved in various signaling pathways. ALSU assays rely on AlphaLISA (Amplified Luminescent Proximity Homogeneous Assay) technology, a bead-based proximity detection system, coupled with CaptSure™ immobilization technology. In an ALSU assay, Donor Beads are coated with streptavidin to capture one of the antibodies, which is biotinylated, and Acceptor Beads are coated with a proprietary CaptSure™ agent that immobilizes the other antibody, labeled with a CaptSure™ tag. When detecting phosphorylated proteins, one antibody is directed against a specific phospho-epitope on the analyte, while the other antibody is directed against another, non-phosphorylated, epitope on a distal part of the analyte (sandwich assay). This brings the Donor and Acceptor Beads into proximity, allowing for generation of Alpha signal (Figure 1), where the amount of light emitted is directly proportional to the amount of protein present in the sample.



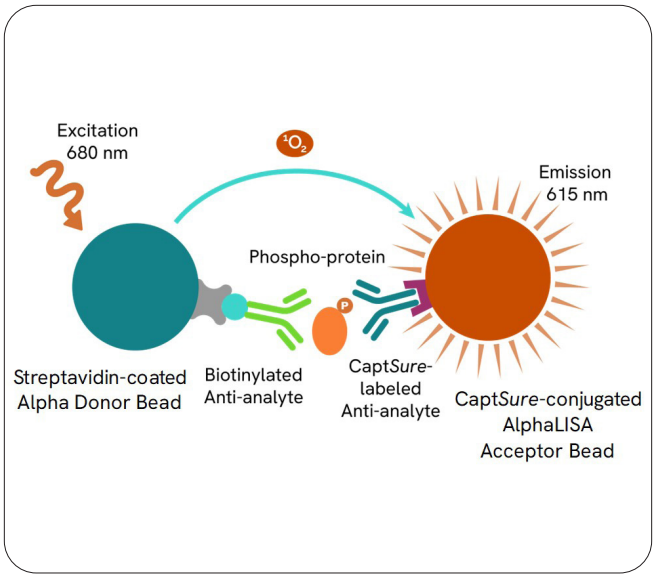


Figure 1: AlphaLISA SureFire Ultra Assay schematic demonstrating capture of a phosphorylated protein.

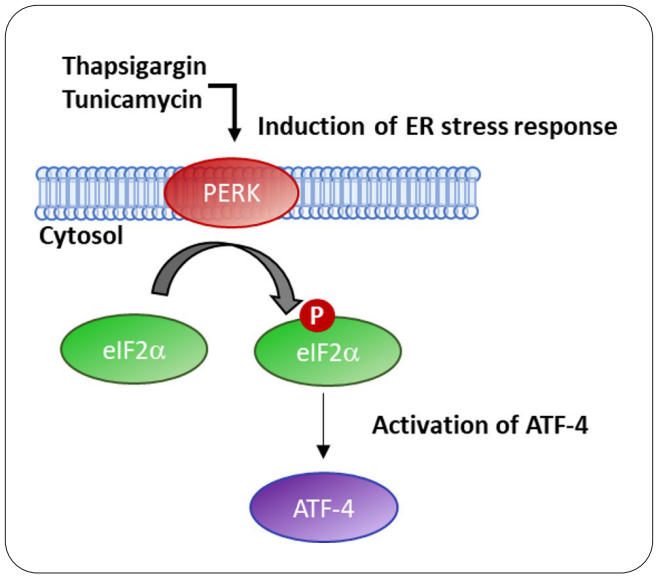


Figure 2: Depiction of the integrated stress response (ISR) pathway. In response to environmental stressors like Thapsigargin and Tunicamycin, PERK is activated leading to phosphorylation of eIF2α (Ser51) which induces selective translation of ATF-4 mRNA. ATF-4 regulates additional genes that promote cellular adaptation, survival or apoptosis.

The cellular response to environmental stressors, including nutrient deprivation, hypoxia, viruses, and endoplasmic reticulum (ER) stress (due to the accumulation of unfolded or misfolded proteins), is mediated through the integrated stress response (ISR) pathway. Through this highly conserved signaling pathway, the cell can respond adaptively to the stressor, or if necessary, trigger apoptosis.[4] The ISR pathway is initiated by four main kinases: PERK, GCN2, PKR and HRI, that converge on reversible phosphorylation of eIF2α at serine 51.[1-3] In turn, this phosphorylation event triggers a decrease in translation of most mRNAs, and thus, most protein synthesis. However, eIF2α phosphorylation also selectively increases translation of certain mRNAs, including ATF-4, which is a master regulator of genes that adaptively respond to stressors, with outcomes that include amino acid metabolism, metastasis, angiogenesis, resistance to oxidative stress, autophagy, or apoptosis.[3-5]

In this study, ALSU technology was used to study the activation of ISR pathway by measuring phosphorylation of eIF2α and subsequent activation of ATF-4 (Figure 2). Evaluating targets within the ISR pathway is valuable for a variety of applications, including understanding intrinsic and extrinsic stressors, cancer biology, and DNA damage.

Methods

Cell lines and agonists

SH-SY5Y cells (ATCC CRL-2266) and C2C12 (ATCC CRL-1772) were grown at 37 °C with 5% CO2 in Dulbecco's Modified Eagle's Medium (DMEM, Gibco 11965) supplemented with 10% FCS, 1% penicillin/streptomycin (Gibco, 15140122).

Two agonists were used to stimulate ER stress response in cells, Thapsigargin (Sigma, T9033) and Tunicamycin (Sigma, SML1287).

Treatment time course experiment

Time to maximal cellular phosphorylation varies based on cell type, pathway, target and stimulatory compound used. Thus, an initial time course experiment was run to determine the optimal agonist treatment time. SH-SY5Y cells (passage 15) and C2C12 cells (passage 10) were seeded in 96-well culture plates (ThermoFisher, NUN167008) at 40,000 cells/well and incubated overnight at 37 °C with 5% CO2. Cells were treated with 1 µM Thapsigargin or 10 µg/mL Tunicamycin across a series of nine timepoints (spanning from 0—6 hours). Cells were then lysed with 100 µL of Lysis Buffer and assayed by *SureFire Ultra* using respective *SureFire Ultra* kits (equivalent to approximately 4,000 cells/datapoint).

Agonist concentration dose-response curve

Based on optimal treatment times determined in the previous experiments, a concentration dose-response curve of each agonist was generated. SH-SY5Y cells and C2C12 cells were seeded in 96-well plates at 40,000 cells/well and incubated overnight at 37°C with 5% CO2. Cells were left untreated (basal levels) or treated with a dose range of Thapsigargin or Tunicamycin for 5 hours (SY-SY5Y cells) or 3 hours (C2C12 cells). All treatments were performed in complete media DMEM containing 10% FCS.

Following their respective treatment, media was removed and cells were lysed with 200 µL of Lysis buffer following the protocol described in the ALSU Guide, resulting in approximately 2,000 cells/data point.

Data Analysis

The assay plates (OptiPlate 384-well, Revvity, #6007290) were read on an EnVision 2105 Multimode Plate Reader with the default AlphaLISA settings. Results represent the mean ± SD for triplicate samples and are representative of three independent experiments. Data was analysed in GraphPad Prism (version 8, GraphPad Software Inc) using a non-linear four parameter logistic regression for the dose-response curves.

AlphaLISA® SureFire® Ultra™ Assays

Three Revvity AlphaLISA® *SureFire® Ultra™* kits were used, including phospho-eIF2α (Ser51) (phospho-eIF2α (Ser51); #ALSU-PEIF2); Total eIF2α (#ALSU-TEIF2); and Total ATF-4 (#ALSU-TATF4). The ALSU phospho-eIF2α (Ser51) assay is used for detection of phospho-eIF2α (phosphorylated on Ser51) in cellular lysates. The ALSU Total eIF2α assay is used for detection of Total eIF2α in cell lysates, which is useful to measure changes to Total eIF2a levels in response to agonist/antagonist treatment, but also a powerful normalization assay when used in conjunction with the ALSU phospho-eIF2α assay. Finally, the ALSU Total ATF-4 assay is used for detection of total ATF-4 in cellular lysates.

ALSU kits offer the option of 1- or 2-plate protocols, where cell culture and assay are conducted on the same, single plate or two separate plates, respectively. In this study, the 2-plate, 2-incubation protocol was followed for all assays as outlined in the provided kit datasheet. In brief, 10 µL of collected lysate was transferred to a white 384-well Optiplate (Revvity, # 6007299) for assay. Next, 5 µL of Acceptor Mix was added to the plate and then incubated for 1 hour at 22 ˚C. Next, 5 µL of Donor Mix was added and incubated again for 1 hour at 22 ˚C. Plates were then read using an EnVision 2105 multimode plate reader using Alpha settings.

Results & Discussion

Time-course activation of ISR pathway

In this study two main agonists effective at triggering ER stress response and able to induce ATF-4 expression were used: Thapsigargin and Tunicamycin.[7,8] The time required for peak phosphorylation of eIF2α (Ser51) and increases in Total ATF-4 varied based on cell type and agonist treatment (1 µM Thapsigargin or 10 µg/mL Tunicamycin), is shown in Figure 3. In SH-SY5Y cells, phosphorylation of eIF2α (Ser51) was increased almost 7-fold over basal levels by 0.25-hours post-treatment with Thapsigargin. Phosphorylation peaked at 1 hour (8-fold higher than basal) before decreasing slightly throughout the remaining time course; however, phosphorylation levels remained elevated at 5 hours compared to basal. eIF2a phosphorylation patterns were similar post Tunicamycin treatment. Phosphorylation again peaked at 1-hour post-stimulation (3-fold increase over basal), before decreasing slightly 5-hours post treatment. In contrast, the observed increase in Total ATF-4 was delayed compared to phospho-eIF2α (Ser51). This is expected, considering that ATF-4 is downstream in the ISR pathway and increased translation of ATF-4 is stimulated by phosphorylation of eIF2α. With both agonists, Total ATF-4 showed little to no change through the first hour of treatment, but then steadily increased, with levels 8-fold higher (Thapsigargin) or 6-fold higher (Tunicamycin) than basal at 5 hours post-treatment. For phospho-eIF2α (Ser51), Alpha signal was almost twice as high with Thapsigargin compared to Tunicamycin, but for ATF-4, the Alpha signal levels were generally similar across the two agonists. Considering the response times of both targets and both agonists, 5 hours was chosen as the dose time for subsequent experiments with SH-SY5Y cells, as Total ATF-4 signal was sufficiently elevated compared to baseline levels by 5 hours, and phospho-eIF2α levels remained elevated compared to basal, allowing for suitable measurement of both targets.

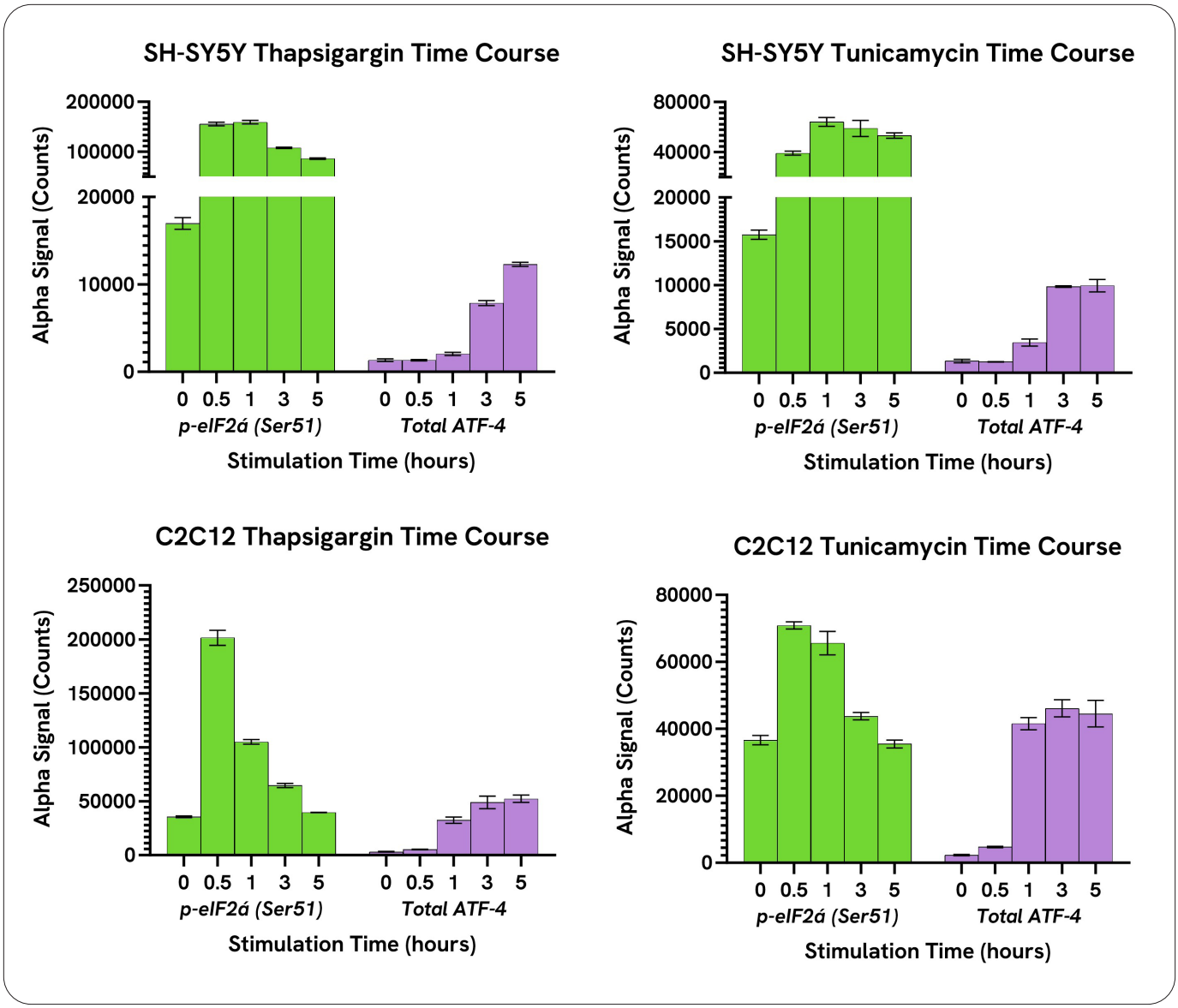


Figure 3: Results of time course experiments for SH-SY5Y cells (top row) and C2C12 cells (bottom row) for two different agonists: Thapsigargin (left panels) and Tunicamycin (right panels). Response time is shown for phospho-eIF2α (Ser51) (green bars) and Total ATF-4 (purple bars) across select stimulation timepoints, spanning from 0 hours (basal levels) to 5-hours post-stimulation.

In C2C12 cells, Thapsigargin stimulation resulted in peak phosphorylation of eIF2α (Ser51) by 0.5-hour post-stimulation, with approximately 5-fold increase over basal concentrations. Phosphorylation then declined over the remaining timepoints, returning to approximately baseline levels by 4-hours post treatment. Phosphorylation of eIF2α was much lower when stimulated with Tunicamycin compared to Thapsigargin, with Alpha signal peaking at only twice that of basal levels, before declining to near baseline levels by 4-hours post-treatment. As observed in SH-SY5Y cells, there was a slight delay before increases in Total ATF-4 were observed following stimulation with both agonists. At 0.5-hour post-treatment, Total ATF-4 levels were similar to basal levels, however by 1-hour post-stimulation, Total ATF-4 was increased 9-fold (Thapsigargin) or 17-fold (Tunicamycin) above baseline levels. Total ATF-4 was elevated through the remainder of the time course (up to 6 hour), with a peak at 5 hours for Thapsigargin treatment (16-fold above basal) and a peak at 4 hours (19-fold above basal) for Tunicamycin treatment. Overall stimulation levels for Total ATF-4 were similar across both agonists. For C2C12 cells, 3 hours was chosen as the stimulation time for subsequent experiments, as Total ATF-4 was sufficiently elevated by this time point, and although phospho-eIF2α had declined, levels were still elevated compared to basal, particularly for Thapsigargin treatment.

ER stressors stimulate the ISR pathway in a dose-dependent manner

To evaluate the effect of Thapsigargin and Tunicamycin in the induction of the ER stress response, a dose-response assay of both agonists in both cell lines was carried out with previously selected treatment times (5 hours for SH-SY5Y cells or 3 hours for C2C12 cells), Figure 4. In SH-SY5Y cells, at doses of 0.156 µM and greater, eIF2a Ser51 phosphorylation was significantly induced above background levels. Measurement of Total eIF2α remained consistent across all tested doses, indicating no increase in total expression of eIF2α protein, despite the increase in phosphorylation. The downstream marker Total ATF-4 increased substantially above basal levels at doses above 0.0156 µM Thapsigargin (coincident with phospho-eIF2α), with maximal signal obtained at concentrations of 0.0625 µM and above (10—12-fold greater than basal). As with Thapsigargin treatment, Tunicamycin treatment did not impact levels of Total eIF2α. Total ATF-4 was increased (4.8—7.8-fold higher) at doses of ≥0.156 µg/mL.

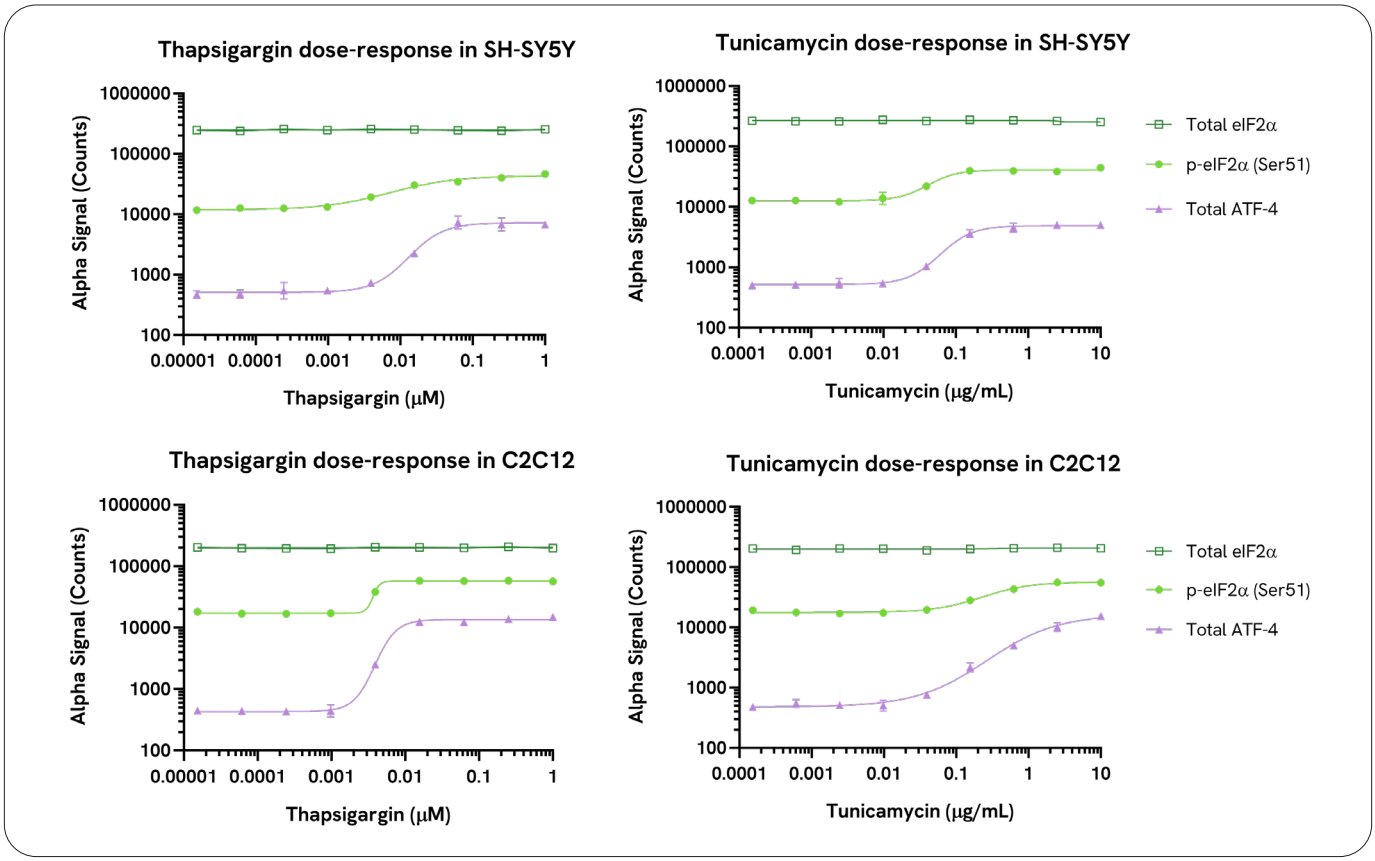


Figure 4: Dose-response curves of two agonists Thapsigargin (left panels) and Tunicamycin (right panels) in two cell lines: SH-SY5Y (top row) and C2C12 (bottom row). Changes in Alpha signal across various doses are shown for each of three targets: Total eIF2α (dark green, open squares), phospho-eIF2α (Ser51) (light green circles), and Total ATF-4 (purple triangles).

In C2C12 cells, Thapsigargin stimulated phosphorylation of eIF2α (Ser51) reaching maximal levels at doses of 0.0156 µM and greater. Total eIF2α levels remained unchanged across the entire range of tested dose (Figure 4). Total ATF-4 demonstrated a strong response to Thapsigargin, with increases of greater than 24-fold above baseline at concentrations of 0.0156 µM and greater. The dose-response to Tunicamycin for phospho-eIF2α (Ser51) and Total ATF-4 was more gradual compared to Thapsigargin. For both phospho-eIF2α (Ser51) and Total ATF-4, a moderate increase in signal was observed at a dose of 0.156 µg/mL Tunicamycin. At a dose of 0.625 µg/mL phospho eIF2α was increased to near maximal levels, with highest levels of stimulation at concentrations above 2.5 µg/mL. For Total ATF-4, signal increased gradually across the higher end of the dose curve, increasing to levels of 17-fold and 27-fold above basal levels for doses of 2.5 and 10 µg/mL, respectively. Total eIF2α did not change across the entire dose-response range.

Conclusion

Overall, these experiments demonstrate that AlphaLISA *Surefire Ultra* kits can be successfully used to measure critical targets within the integrated stress response. These experiments also offer an example for interpreting time course and dose-response curve data to select the appropriate stimulation time and doses for cell-based ALSU assays. Using agonistic compounds to stimulate the ISR pathway, we were able to measure phosphorylation of eIF2α and subsequent changes to downstream translation of other key proteins within the pathway, such as ATF-4. The sensitive nature of ALSU kits allowed for the detection of even subtle changes in phospho-eIF2α levels in two different cell lines. When paired with the phospho-eIF2α (Ser51) assay, the Total eIF2α kit was valuable for demonstrating that the agonists did not change overall expression levels of eIF2α even as phosphorylation increased. ALSU data showed the expression of ATF-4 was elevated following increases in eIF2α phosphorylation, as would be expected for this downstream target. This highlights the power of using multiple ALSU kits simultaneously to better elucidate the relationship of various targets within a pathway.

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