Detecting changes in phosphorylation events during the cell cycle with AlphaLISA *SureFire Ultra* technology.

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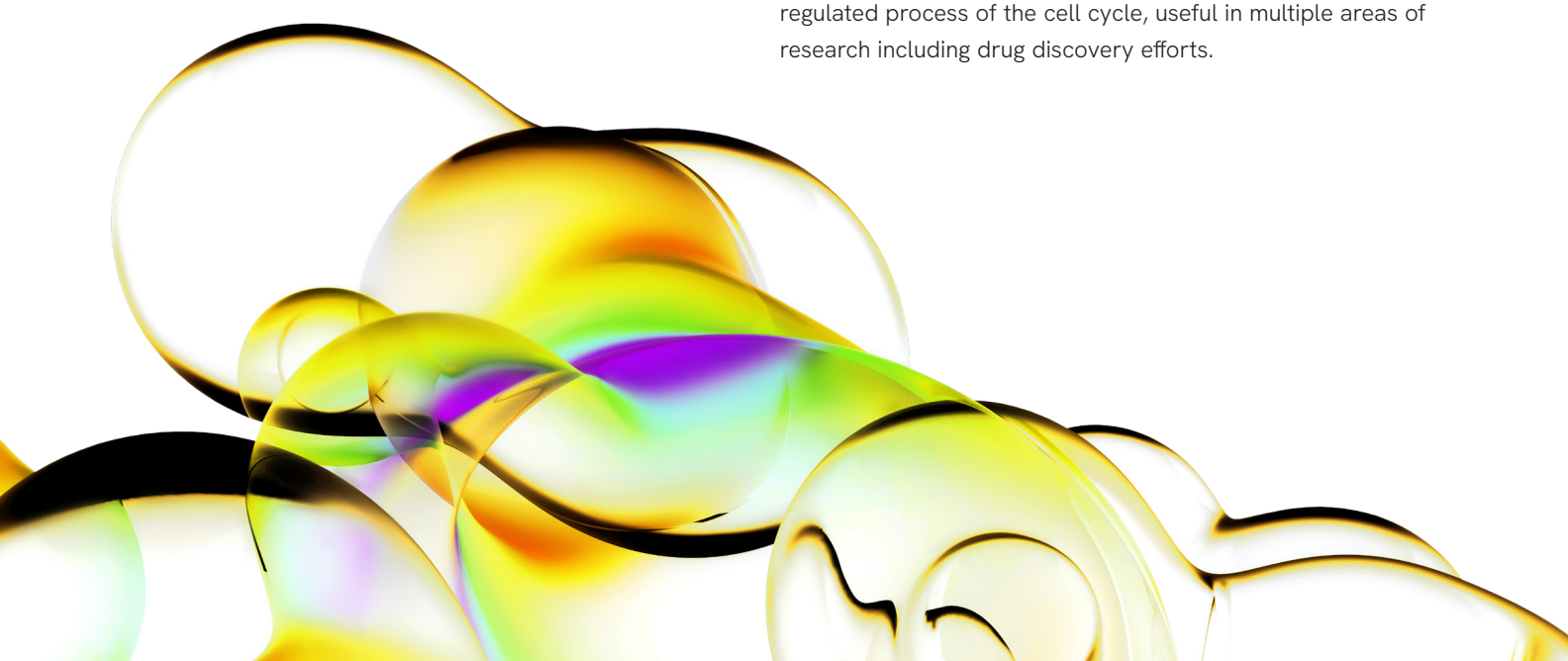
Introduction

Lamin A/C is a nuclear protein involved in multiple cellular processes including chromosome organization, transcriptional regulation, DNA repair, cell signalling and cell cycle regulation[1]. Lamin A/C phosphorylation plays a role in its incorporation into the nuclear lamina as well as disassembly during mitosis[2]. Phosphorylation at the so-called "mitotic sites" (Ser22 and Ser392) reaches the maximum level at the onset of mitosis and is triggered by the complex formed by CDK1 and Cyclin B1. The phosphorylation/dephosphorylation events surrounding the CDK1-Cyclin B1-Lamin A/C pathway are associated with alterations in the cell cycle that have an impact not only on the cell morphology and structure but also on the cell fate[3]. The dysregulation in the levels of these phosphorylated proteins and their total counterparts has been associated with increased cell growth, genomic instability and cell senescence in different types of cancers, becoming a key area for the discovery of efficient therapies to prevent tumor progression[1,2].

AlphaLISA™ *SureFire™ Ultra™* technology offers a rapid and reliable method for tracking and characterizing changes to the cell cycle and cellular homeostasis. Due to its superior sensitivity, low cell numbers can be used to capture cellular events that may go undetected by other less sensitive immunoassay techniques. This application note validates the utility and functionality of the AlphaLISA *SureFire Ultra* platform in monitoring and characterizing changes in phosphorylated and total cell cycle regulatory proteins from the same cell lysate sample. This study highlights *SureFire Ultra* technology as a powerful tool to dissect the complex cell biology and highly regulated process of the cell cycle, useful in multiple areas of research including drug discovery efforts.



Materials and methods

Cell culture and reagents:

A549 cells (ATCC, CCCL-185) and HeLa cells (ATCC, CCCL-2) were grown at 37°C with 5% CO2 in Minimum Essential Medium (MEM, Gibco 11095) supplemented with 10% FCS, 1% penicillin/streptomycin (Gibco, 15140122). In this study, two reagents well-known for their role in cell cycle regulation were used, Nocodazole (Sigma, M1404) and Aurora Kinase Inhibitor (Sigma, #189406).

Cell-based experiments:

The two-plate method was used for AlphaLISA *SureFire Ultra* assays in this study.

Nocodazole experiments: A549 cells (passage 85) were seeded at 20,000 cells/well in a 96-well culture plate (ThermoFisher, NUN167008) and incubated overnight at 37 °C with 5% CO2. A dose-response curve of nocodazole was prepared in cell culture media and cells were treated for 18 hours. Cells were then lysed for assaying of multiple targets (see Table 1).

Aurora kinase inhibitor experiments: HeLa cells (passage 30) were seeded at 20,000 cells/well in a 96-well plate and incubated overnight at 37°C with 5% CO2. Cells were pre-treated with 333 nM nocodazole in cell culture media for 18 hours. Media was removed and cells were treated with Aurora Kinase Inhibitor at different concentrations for 2 hours. Cells were then lysed with Lysis Buffer for assaying of multiple targets (see Table 1).

For both sets of experiments, cells were lysed in a variable final volume of AlphaLISA *SureFire Ultra* Lysis buffer (see Table 1) depending on the target of interest following the recommended procedure in the AlphaLISA *SureFire Ultra* manual for adherent cells. Briefly, media was removed from the cells and a volume of Lysis Buffer was added to each well (Table 1). Assay plates were placed on an orbital shaker at 350 rpm for 10 minutes. From these plates, 10 µL of lysate was used from each sample following the assay procedure outlined in the AlphaLISA *SureFire Ultra* manual.

Table 1: AlphaLISA *SureFire Ultra* targets assayed and lysis buffer volume

Target	Catalog no.	Lysis buffer volume (uL)	Cells/data point
Lamin A/C p-S22	ALSU-PLAM-A500	400	500
Lamin A/C Total	ALSU-TLAM-A500	1000	200
Cyclin B1 p-S126	ALSU-PCYCB1-A500	100	2000
Cyclin B1 Total	ALSU-TCYCB1-A500	100	2000
CDK1 p-T14	ALSU-PCDK1-A500	200	1000
CDK1 p-Y15	ALSU-PCDK1-B500	200	1000
CDK1 p-T161	ALSU-PCDK1-C500	200	1000
CDK1 Total	ALSU-TCDK1-A500	200	1000

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.

Principle of AlphaLISA *SureFire Ultra* assays

AlphaLISA technology is a fast, highly sensitive, homogeneous, no-wash assay platform that can be performed in a microplate format. AlphaLISA assays require two bead types: Donor beads and Acceptor beads. In the AlphaLISA *SureFire Ultra* assay, Donor beads are coated with streptavidin to capture one of the detection antibodies, which is biotinylated. Acceptor beads are coated with a proprietary Capt*Sure*™ agent that immobilizes the other

antibody, labeled with a Capt*Sure*™ tag. In the presence of target protein, the two antibodies bring the Donor and Acceptor beads close together. Upon excitation at 680 nm, a photosensitizer inside the Donor bead converts ambient oxygen to an excited singlet state. Singlet oxygen diffuses up to 200 nm to produce a chemiluminescent reaction in the Acceptor bead, leading to light emission at 615 nm. If an Acceptor bead is not in proximity of a Donor bead, little to no signal is produced over background. AlphaLISA signal was measured on the EnVision 2105 Multimode Plate Reader using default settings for AlphaLISA with excitation at 680 nm and emission at 615 nm. A schematic of an AlphaLISA *SureFire Ultra* assay for a phosphorylated target is shown in Figure 1.

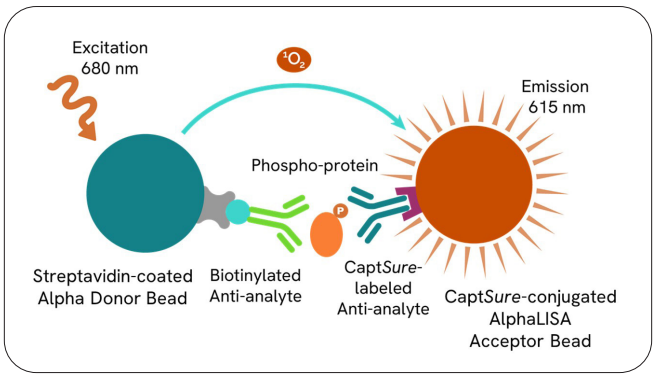


Figure 1: AlphaLISA *SureFire Ultra* Assay schematic demonstrating capture of a phosphorylated protein. The phosphorylated protein is detected in a sandwich assay using specific antibodies. One antibody is directed against a specific phospho-epitope on the analyte, while the other antibody is directed against another, non-phosphorylated, epitope on a distal part of the analyte. The resulting AlphaLISA signal in the immunoassay is directly proportional to the amount of phosphorylated analyte present in the sample.

Results and discussion

Activation of CDK1 and Cyclin B1 triggers upregulation of Lamin A/C phosphorylation

During the late G2 phase at the onset of mitosis, the gradual accumulation of Cyclin B1 promotes the formation of the Cyclin B1-CDK1 complex. At this point CDK1 is in an inactive state maintained by the phosphorylation on Thr14 and Tyr15[4].

In this study cells were treated with nocodazole, a well-known microtubule inhibitor that has been widely used as a synchronization tool of the cell division cycle[5].

Nocodazole treatment of A549 cells caused a significant activation of CDK1 by triggering phosphorylation of Thr161 and dephosphorylation of the inhibitory residues Thr14 and Tyr15 (Figure 2). In addition, and in agreement with the literature[5], higher doses of nocodazole led to an induction of Total and phosphorylated (Ser126) Cyclin B1 levels (Figure 2). Together these events resulted in an increase of Lamin A/C phosphorylation at Ser22 with no significant changes in the total levels (Figure 2).

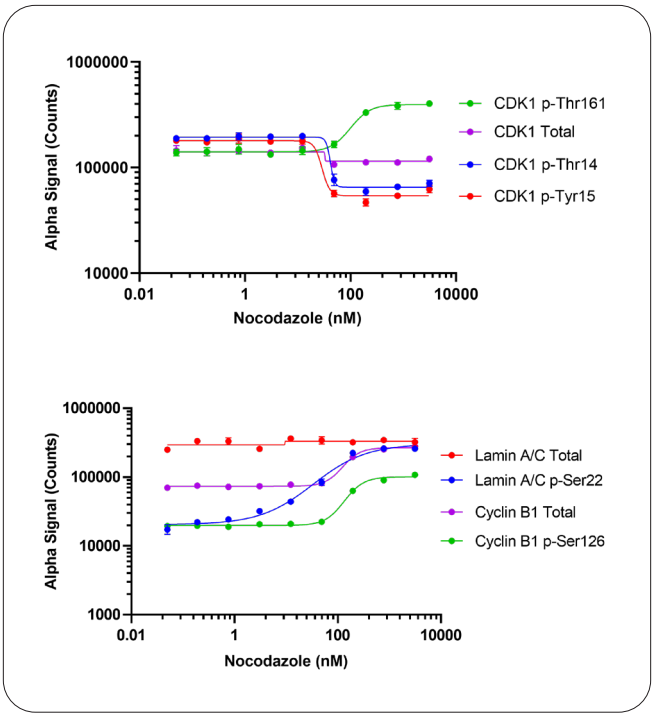


Figure 2: Nocodazole effects on CDK1, Cyclin B1 and Lamin A/C in A549 cells

Inhibition of CDK1 and Cyclin B1 induces downregulation of Lamin A/C phosphorylation

Further, the Aurora Kinase Inhibitor, a compound with opposite effects to nocodazole was evaluated in HeLa cells. Aurora kinases are essential for the onset and progression of mitosis. Specifically, Aurora A activates and targets the CDK1-Cyclin B1 complex[6]. The use of Aurora Kinase Inhibitor, which is highly potent against Aurora A kinase, should therefore lead to an inhibition of mitosis which can be measured by the opposite effects on CDK1, Cyclin B1 and Lamin A/C phosphorylation.

Results in HeLa cells treated with the inhibitor showed dephosphorylation of CDK1 at the positive regulator site Thr161 and phosphorylation of inhibitory residues Thr14 and Tyr15 (Figure 3), together with a modest increase in total CDK1 levels. In line with these results, a significant drop in both total and phosphorylated Cyclin B1 (Ser126) as well as phosphorylated Lamin A/C (Ser22) was observed (Figure 3). Once again, Total Lamin A/C levels remained unchanged.

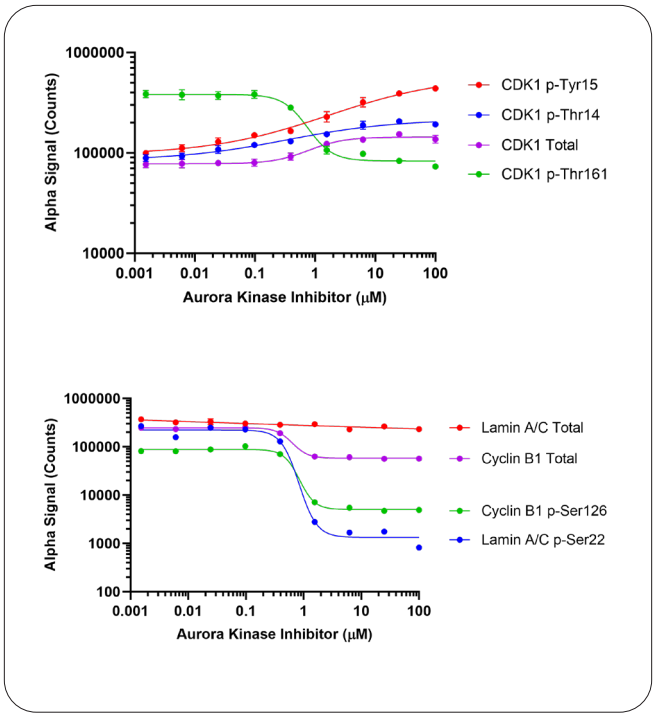


Figure 3: Aurora Kinase Inhibitor effects on CDK1, Cyclin B1 and Lamin A/C in HeLa cells

Summary

AlphaLISA™ is a bead-based technology that offers a rapid and reliable method for studying biomolecular interactions in a microplate format. This study demonstrates that AlphaLISA *SureFire Ultra* is a powerful platform to measure changes in the expression and phosphorylation status of key cell regulatory proteins.

Here we observed measurable changes in A549 and HeLa cells treated with two compounds with opposite effects: Nocodazole and Aurora Kinase Inhibitor. The low cell numbers used for each assay data point (Table 1) demonstrates the sensitivity of *SureFire Ultra* kits along with the capability to lyse cells and test multiple targets from the same lysate. This can reduce any technical errors seen with running parallel cell treatments across multiple assay plates. The AlphaLISA *SureFire Ultra* platform has the ability to dissect complex cell biology and highly regulated processes of the cell cycle and is useful for many areas of research in the drug discovery and screening process.

References

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