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Real-Time Bio-Layer Interferometry Ubiquitination Assays as Alternatives to Western Blotting

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Abstract

Ubiquitination is a crucial cellular pathway enabling normal cellular functions. Abnormalities in the ubiquitination process can lead to cellular dysfunction and cause a range of diseases. Efforts to screen and develop small molecule inhibitors targeting portions of the ubiquitination cascade require rapid and robust methods for detecting ubiquitination. Enormous efforts have been made in the field to detect ubiquitination using various techniques including fluorescence, spectrophotometry, chemiluminescence, NMR, and radioactive tracers. The most common method to detect ubiquitination is western blotting. However, western blotting is time-consuming and difficult to use when seeking fine-grained time course experiments. Here we present the use of bio-layer interferometry to rapidly assay ubiquitination in real-time. An E3 ligase auto-ubiquitination system and a substrate ubiquitination assay have been applied as tests for the newly developed assay. The developed BLI ubiquitination assay provides 1-second time resolution and detects the formation of polyubiquitin chains directly on a biosensor-bound target. Results are returned instantaneously, and reagent concentrations are identical to those used by traditional western blot-based ubiquitination assays. The developed BLI ubiquitination assay is a viable alternative to traditional western blot assays to detect ubiquitination in a rapid real-time manner.

Keywords

Auto-ubiquitination; Western blotting; Bio-Layer Interferometry

1. Introduction

Over the last decade, the development of technologies for analyzing biomolecular interactions in real-time has significantly advanced [1]. One such promising technology is bio-layer interferometry (BLI). This powerful optical analytical technique utilizes a

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biosensor to measure the interference pattern of white light reflected from a bio-layer and an internal reference layer at the tip of a biosensor (Figure 1) [2].

Once a biosensor is dipped in a solution with a molecule of interest, molecules are immobilized to the bio-layer coated at the tip of the biosensor through various interactions with capture moieties on the biosensor surface. Consequently, by dipping the biosensor tip in a second solution with the second molecule of interest, the analyte, the interaction between the two molecules of interest can be measured [1,2]. The binding of molecules causes a variation in the thickness of the bio-layer at the tip of the biosensor. As the bio-layer thickness at the tip of the biosensor increases, the bio-layer components move away from the internal reference layer resulting in a shift in the interference pattern of the white light. The shift in the interference pattern is proportional to the variation of binding thickness at the biosensor tip [3] and is reported on a sensorgram as an output of binding thickness in nm versus time in seconds [2].

Many different types of biosensors are commercially available with capture moieties including anti-GST antibody, anti-Penta-HIS antibody, amine-reactive functional groups, streptavidin, and anti-Mouse IgG Fc. These sensors, and many others, permit the study of biomolecular interactions such as protein-protein interactions [1,4], protein-liposome interactions [5], DNA-protein interactions [1,6–8], RNA-protein interactions [1], and various other small molecule interactions [9]. Analysis of the BLI sensorgram trace readily yields kinetics of association and dissociation, and affinities of binding interactions [2,4,10]. Only the molecules associated to or dissociating from the biosensor contribute to the BLI response and the signal is unaffected by unbound molecules present in the solution. Therefore, BLI is able to perform experiments within cell lysates or crude samples to quantify the concentration of the target molecule or to screen a small molecule of interest [9,11–13]. BLI is emerging as a flexible and versatile tool for assisting numerous applications such as the development of vaccines, designing epitopes, determining affinities for antibodies, and the discovery of drugs including screening for inhibitors as it offers a simple dip-and-read format in a real-time platform [10,14]. Even though BLI is extensively used to study biomolecular interactions, it has not been utilized to track a processive biomolecular pathway. Here, for the first time, a robust and rapid BLI assay for detecting real-time auto-ubiquitination of an E3 ubiquitin ligase and substrate ubiquitination by an E3 ubiquitin ligase is presented. The E3 ubiquitin ligase CHIP (C-terminus of Hsc70-interacting protein) and the substrate Hsc70 are provided as a test platform for the new assay.

Ubiquitination is a post-translational modification that plays a major role in eukaryotic protein quality control systems [15–17]. Within protein quality control processes, ubiquitination results in the covalent attachment of a ubiquitin molecule to a misfolded protein targeting it for proteasomal degradation [18,19]. Defects or failures in ubiquitination lead to fatal neurodegenerative diseases such as Alzheimer’s and Parkinson [20–22]. Ubiquitination is a multi-enzyme cascade consisting of three main steps. Initially, ubiquitin is activated in an ATP-dependent manner by an E1 ubiquitin activating enzyme forming a thioester bond between ubiquitin and the E1. Next, ubiquitin is transferred to an E2 ubiquitin conjugating enzyme in a thiol exchange reaction. Finally, ubiquitin is transferred to the target substrate protein via an E3 ubiquitin ligase, in some cases targeting it for

proteasomal degradation [23–25]. Certain E3 ubiquitin ligases such as CHIP and Mdm2 (Mouse double minute 2) are also capable of ubiquitinating themselves in a process termed auto-ubiquitination (self-ubiquitination) which can serve to regulate the level of E3 ubiquitin ligase when it exceeds the threshold level in the presence or absence of a substrate [26–28]. In certain instances, auto-ubiquitination of E3 ligases (RING1B) is identified as a pre-requisite for the activation of its ligase activity and sometimes serves as a mechanism to recruit target substrates for E3 ubiquitin ligases such as NEDD4 and TRAF6 [27].

The traditional and the most common method to detect ubiquitination is the use of a western blot based on an immuno-interaction between an antibody and an antigen. At the completion of a ubiquitination assay, samples are subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by the transfer to a membrane to be probed with primary antibodies specific for the proteins of interest and labeled secondary antibodies to enable detection [29,30]. The western blotting process is time-consuming as at least 1 and one-half days are needed to obtain the results and the method offers limited resolution in time. Here we describe a cutting-edge BLI ubiquitination assay as a rapid and viable alternative to traditional western blots to detect ubiquitination in real time. Using similar reagent concentrations to those used by traditional western blot-based assays, our BLI ubiquitination assay is able to generate instantaneous results with fine-grained one-second time resolution. The assay produces robust signals for detecting ubiquitination and provides substantial time savings. The user-friendly BLI ubiquitination assay requires very small volumes of reagents and eliminates the need for labeled reagents. The developed BLI assay is demonstrated for the CHIP auto-ubiquitination and CHIP-mediated ubiquitination of Hsp70. The assay opens the doors to exploring other E3 ubiquitin ligase-mediated ubiquitination systems in the future.

2. Materials and Methods

2.1 Antibodies, proteins, and expression constructs

Rabbit anti-CHIP polyclonal antibodies raised against amino acids 73–303 of CHIP (were obtained from Santa Cruz Biotechnology Inc and Rabbit anti-GST polyclonal antibodies raised against GST tag were obtained from Thermo Fisher. Goat anti-Rabbit secondary antibody labeled with 680RD IR-Dye was purchased from LI-COR. Recombinant Human Ubiquitin E1 Enzyme (UBE1), wild-type ubiquitin, Mg-ATP solution, K48R ubiquitin, and K_0 ubiquitin were purchased from R&D Systems. The expression constructs of His_6-tagged CHIP, His_6-tagged E2 (UbcH5b), and GST-Hsc70 were prepared as previously reported [31,32]. The His_6 and GST tags were used for the purification of the proteins as previously reported [32]. A TEV (tobacco etch virus) protease cleavage site in the E2 protein was used for proteolytic removal of the His_6 tag after purification. The His_6 tag on CHIP and GST tag on Hsc70 were not proteolytically removed.

2.2 Mutagenesis

S94K-E2 (S94K-UbcH5b) mutant construct was constructed using the His_6-tagged UbcH5b plasmid as the template in a mutagenesis reaction using a QUIKCHANGE II XL (Agilent Technologies) site-directed mutagenesis kit. Mutant plasmids were purified using a miniprep

plasmid kit (27109, Qiagen) and the sequence was confirmed by Sanger sequencing (Eurofins Genomics).

2.3 Protein expression

The constructs for His_6-tagged CHIP, His_6-tagged E2, His_6-tagged S94K-E2, and GST-tagged Hsc70, were each transformed into BL21 (DE3) chemically competent *Escherichia coli* cells (C2527H, New England BioLabs) for expression. After screening for expression induced by isopropyl β-D-1-thiogalactopyranoside (IPTG), the best-expressing colony for each was selected and cultured overnight in LB Broth (BP9732, Fisher BioReagents). Terrific broth media (500 ml) was inoculated with overnight culture and incubated with shaking at 37 °C until the OD_{600} reached 1.0, then transferred to 20 °C. After 30 mins, 0.1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to the cultures to induce the overexpression of the proteins. Cultures were grown at 20 °C for 12–15 hours and the cells were collected via centrifugation at 3,381 ×*g* for 20 mins at 4 °C, followed by washing with the resuspension buffer (180 mM Tris, pH 8.0, 450 mM NaCl, 5 mM 2-mercaptoethanol, and 10% glycerol). To protect against protease cleavage, 100 μg/mL 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF; Gold Biotechnology) was added to the resuspended cells. The resuspended cells were stored at −80 °C after flash-freezing in liquid nitrogen.

2.4 Protein purification

The frozen cell lysate was thawed, sonicated, then centrifuged at 4 °C, 18,186 ×*g*, for 45 minutes. The supernatant was collected and filtered using a 0.45 μm filter. His_6-tagged CHIP, His_6-tagged E2, and His_6-tagged S94K-E2 were purified by Ni^{2+} affinity chromatography using 5 mL HisTrap HP columns equilibrated and loaded with buffer A (20 mM HEPES, 150 mM NaCl, 5 mM β-ME, pH 7.0) and eluted with buffer B (20 mM HEPES, 150 mM NaCl, 5 mM β-ME, 500 mM imidazole, pH 7.0). GST-tagged Hsc70 was purified by glutathione affinity chromatography using a 5 mL GSTrap HP column (GE Healthcare) equilibrated and loaded with buffer A (25 mM HEPES (pH 7.6), 50 mM NaCl) and eluted with buffer B (25 mM HEPES (pH 7.6), 10 mM reduced glutathione). The His_6-tags of E2 proteins (wild type and mutant) were proteolytically cleaved by digestion with TEV protease. The tag-cleaved proteins were repurified by Ni^{2+} affinity chromatography and all the proteins were further purified by size exclusion chromatography using a HiLoad 16/600 Superdex 75 pg column (Cytiva). Finally, purified proteins were concentrated, and the protein concentration was determined using a Bradford protein assay (Bio-Rad). The concentrated proteins were frozen dropwise in liquid nitrogen and stored at −80 °C.

2.5 Western blot-based assays

2.5.1 Detecting ubiquitination of CHIP—Ubiquitination reaction mixture containing 0.5 μM E1, 20 μM E2, 100 μM Ubiquitin, and 5 mM ATP-Mg were incubated at 37 °C for 30 min in a buffer containing 50 mM HEPES, 50 mM NaCl, pH 7.0, and 0.5 mM dithiothreitol (DTT). This pre-charged E1~E2~Ubiquitin reaction mixture was then added to 4 μM CHIP in 50 mM HEPES and 50 mM NaCl, pH 7.0. Ubiquitination reactions were incubated at 37 °C for different time intervals; 0 min, 5 min, 10 min, 20 min, 30 min,

and 60 min, and the reactions were stopped by the addition of 2x SDS sample loading buffer containing 50 mM EDTA. The quenched reactions were then subjected to SDS-PAGE followed by western blotting on a nitrocellulose membrane (Bio-Rad). Blots were blocked using a blocking buffer designed for fluorescence-detected Western blotting (item MB-070, Rockland Immunochemicals) and then probed with Rabbit anti-CHIP primary antibody (1:1000) and 680RD IR-Dye labeled Goat-anti-Rabbit secondary antibody (1:8000) diluted accordingly using blocking buffer. The Western blot was imaged using a LI-COR Odyssey Fc Imager using near-infrared detection at 700 nm. The multi-mono ubiquitination reaction mixtures containing K48R ubiquitin or K_0 ubiquitin instead of wild-type ubiquitin were performed identically with the exception of the ubiquitin construct used.

2.5.1 Detecting ubiquitination of Hsc70—A pre-charged E1/E2/Ubiquitin reaction mixture, described above, was added to 4 μM CHIP and 4 μM Hsc70 in 50 mM HEPES and 50 mM NaCl (pH 7.0). Ubiquitination reactions were incubated at 37 °C for different time intervals; 0 min, 5 min, 10 min, 20 min, 30 min, and 60 min, and the reactions were stopped by the addition of 2x SDS sample loading buffer and 50 mM EDTA. The quenched reactions were then subjected to SDS-PAGE followed by western blotting as described above, with exception that the primary Rabbit anti-GST (1:5000) antibody was used.

2.6 BLI assay general parameters

The BLI ubiquitination assay utilized identical reagents and concentrations as used for the traditional western blotting described above. The BLItz instrument (ForteBio) was used for the BLI assays, and ubiquitination assays were performed with customized step type and duration run settings. Biosensor tips were immersed in 50 mM HEPES and 50 mM NaCl, pH 7.0 for a minimum of 10 min prior to each run. For each BLI assay, a table is provided below with the step type, time, position, and step description. All the assays were performed at 37 °C by housing the BLItz in a Peltier incubator. All runs were performed in triplicate (biological replicates). As the initial step of every assay, a buffer run was performed, and this was used as the negative control for all the steps in the assay.

2.7 BLI ubiquitination assay parameters to detect CHIP auto-ubiquitination

Anti-Penta-HIS biosensors were used for the assay. The ubiquitination assay started with an initial baseline step (step 1) to wash the sensor with buffer (50 mM HEPES, 50 mM NaCl, pH 7.0) used in western blotting. In step 2, His_6-tagged CHIP was loaded onto the sensor followed by another baseline step (step 3). In the association step (step 4), the sensor tip was dipped in a reaction mixture containing 0.5 μM E1, 20 μM E2, 100 μM ubiquitin, and 20 mM ATP-Mg which had been incubated at 37 °C for 30 min immediately prior to the BLItz assay, to monitor ubiquitination of CHIP. Finally, the dissociation step (step 5) was carried out in buffer (50 mM HEPES, 50 mM NaCl, pH 7.0) to remove any unbound molecules from the biosensor. (Table 1 and Figure 2A). Similar assays were performed substituting S94K-E2, K48R-ubiquitin, and K_0-ubiquitin.

2.8 BLI assay to detect Hsc70 ubiquitination

Anti-GST biosensors were used for the assay. The ubiquitination assay started with an initial baseline step (step 1) in buffer (50 mM HEPES, 50 mM NaCl, pH 7.0) identical as described

above. Next, GST-tagged Hsc70 was loaded onto the sensor (step 2) followed by another baseline step (step 3) in buffer (50 mM HEPES, 50 mM NaCl, pH 7.0). In the association step (step 4), the sensor tip was dipped in a reaction mixture containing 0.5 μM E1, 20 μM E2, 100 μM ubiquitin, and 20 mM ATP-Mg which had been incubated at 37 °C for 30 min and mixed with CHIP immediately prior to the BLItz assay, to monitor ubiquitination of Hsc70. Finally, the dissociation step (step 5) was carried out in buffer (50 mM HEPES, 50 mM NaCl, pH 7.0) to remove any unbound molecules from the biosensor. (Table 2)

2.9 Data analysis for BLI ubiquitination assays

After performing the BLI ubiquitination assays, raw data recorded by the BLItz were exported as Excel files. Initially, data was step corrected to the 180 second data point (step corrected data) to analyze the buildup of ubiquitin on CHIP (step 4 – 300 seconds). The mean of the buffer run was then subtracted from each reaction to correct the baseline. Step and baseline corrected data was exported to Prism (GraphPad) and the mean of three replicates for each reaction was plotted along with the standard deviation.

3. Results and Discussion

3.1. BLI ubiquitination assay and traditional western blot-based assay on CHIP auto-ubiquitination system

Ubiquitination can take place by the addition of a single ubiquitin molecule (mono-ubiquitination), several individual ubiquitin molecules at multiple sites on the target (multi-mono-ubiquitination) or by the formation of one or more polyubiquitin chains on the target (polyubiquitination) [33]. The seven lysine residues (K6, K11, K27, K29, K33, K48, K63) and the N-terminal amine present in the ubiquitin molecule are each possible target sites for building polyubiquitin chains with a range of possible linkage types [34]. Several studies have developed real-time assays to monitor ubiquitination. Fluorescence polarization (FP) was used to design a real-time assay termed UbiReal to monitor the conjugation of ubiquitin utilizing a fluorescently labeled ubiquitin. Although a powerful assay for examining ubiquitination, key drawbacks of the UbiReal assay were the requirement of fluorescent labeling of a specific protein and the reliance on significant differences in the molecular weights of the proteins involved in the assay in order to generate significant FP signals [35]. Confocal fluorescence nano-scanning was used as well to develop an assay named ubiquitin proteasome system-confocal fluorescence nano-scanning (UPS-CONA) utilizing a fluorescently labeled ubiquitin, to track the ubiquitination cascade, in a real-time manner. However, it was time-consuming and susceptible to negative impacts of fluorescence bleaching or quenching [36]. Bioluminescence resonance energy transfer (BRET) was used to monitor ubiquitination in real-time which facilitated the detection of ubiquitination in living cells, however, the assay was labor-intensive and requires the use of relatively large fluorescent protein tags [37]. Even though BLI technology has been applied in a wide range of applications, it has not been used as the basis for an assay to monitor ubiquitination.

3.1.1 Autoubiquitination of CHIP—Once a CHIP loaded biosensor is dipped into the pre-charged E1/E2/ubiquitin reaction mixture with wild type ubiquitin, autoubiquitination of CHIP takes place. The buildup of ubiquitin chains on CHIP results in a corresponding

increase in the thickness of the biolayer at the tip of the sensor causing an increase in the BLI signal. The BLI data in Figure 3 was step corrected for the 300 second reaction taking place in step 4 (Figure 3, Table 1) so as to aid visualization of CHIP auto-ubiquitination. The step-corrected data for a reaction with His_6-tagged CHIP bound to the sensor and all other ubiquitination reaction components present suggests robust ubiquitination with a sharp rise in BLI response (Figure 3A). In contrast to control reactions, autoubiquitination of CHIP on the biosensor surface results in the covalent attachment of ubiquitin. The Z-factor calculated for the assay is 0.642, indicating an excellent assay.

With regard to the larger jump and slow plateau in the autoubiquitination of CHIP, we postulate that this may be an example of differential reaction rates observed for E3 ubiquitin ligases in which the addition of the first ubiquitin is relatively quick and the addition of each subsequent ubiquitin to a growing chain is progressively slower [38]. The continued growth of the ubiquitin chains results in a steadily increasing BLI response, contrasted with the saturated response observed in control reactions which allow for protein-protein interactions and do not allow for ubiquitination. Specifically, control reactions with no E1, with no ubiquitin, and no ATP-Mg (Figure 3A), exhibit a small BLI response saturating at 0.1nm likely due to the binding of E2 with the His_6-tagged CHIP on the sensor. Conversely, control reactions with no E2, with S94K-E2, or with no His_6-tagged CHIP bound to the sensor exhibit no clear increase in BLI response, indicative of no observable ubiquitination and no observable protein-protein interactions at the sensor tip. For reactions with no E2 or with no His_6-tagged CHIP bound to the sensor, the minimal BLI response is consistent with the expectation that no biomolecules within the sample would be capable of specific interactions at the bio-layer. The S94K-E2 control reaction used a mutant UbcH5b with the Ser_{94} to Lys (S94K) mutation in the conserved ‘SPA’ motif which is essential for interacting with CHIP [39,40]. Prior reports found that E2 enzymes with a KPA motif are not functional with CHIP [39,41], despite conflicting findings from these prior references with regard to the binding of S94K-E2 to CHIP. In separate BLI assays, we were not able to detect the binding of S94K-E2 to CHIP (Table 3), consistent with the S94K-E2 ubiquitination reaction data detected by BLI and western blot (Figure 3). For each reaction observed by BLI (Figure 3A), the sensorgram traces are consistent with levels of ubiquitination observed by western blot (Figure 3B).

3.1.2 Multi-monoubiquitination of CHIP—Ubiquitination reactions utilizing ubiquitin constructs with mutation of the 7 Lys residues responsible for the formation of ubiquitin chains are valuable tools for discerning between multi-monoubiquitination and polyubiquitination, or for determining the linkage type used within a polyubiquitin chain [42]. The mutation of Lys_{48} to Arg (K48R-ubiquitin) eliminates the ability of ubiquitin to form ubiquitin chains through Lys_{48}. This results in either monoubiquitination, the attachment of a single ubiquitin molecule, multi-monoubiquitination, the attachment of a single ubiquitin molecule at multiple sites, or polyubiquitination if the ubiquitin chain linkage type is not K_{48} [43]. For K_0-ubiquitin, the mutation of all the Lys residues to Arg (K_0-ubiquitin) renders the mutant unable to form ubiquitin chains, with the exception of those using the amino terminus, typically leading to either monoubiquitination or multi-monoubiquitination [44]. Ubiquitination reactions with K48R-ubiquitin and K_0-ubiquitin

monitored using the BLI ubiquitination assay each display a nearly identical increase in the BLI response over time (Figure 4A). Each of these sensorgram traces were smaller in magnitude than the trace for wild-type ubiquitin (Figure 4A). Taken alongside the western blot ubiquitination assays for K48R-ubiquitin and K_0-ubiquitin (Figure 4B) in comparison to the BLI sensorgram (Figure 3A and 4A) and western blot for wild type ubiquitin (Figure 3B), the data suggest that both K48R-ubiquitin and K_0-ubiquitin allow for multi-monoubiquitination. The identical performance for K48R-ubiquitin and K_0-ubiquitin, suggests that the polyubiquitin chains built within the CHIP autoubiquitination assays are K_{48}-linked chains. This is consistent with a prior study that utilized mass spectrometry to determine that CHIP, in concert with the E2 UbcH5, catalyzes the formation of K_{48}-linked chains [45]. The results (Figure 4A) demonstrate that the BLI ubiquitination assay is a viable assay for examining chain linkage type in a rapid 10-minute assay format that uses protein reagent concentrations identical to those for traditional western blots. The results further demonstrate the viability of the BLI ubiquitination assay obtained by assaying a 4 μl drop, a total sample volume significantly smaller than required for traditional western blots.

3.2. Application of the BLI ubiquitination assay to detect ubiquitination of Hsc70 in a chaperoned ubiquitination system

We extended the application of the developed assay to monitor the ubiquitination of a substrate by an E3 ubiquitin ligase. This test examined chaperoned ubiquitination of Hsc70 by CHIP. Once the biosensor is loaded with Hsc70, it is dipped in the pre-charged E1/E2/ubiquitin reaction mixture and immediately mixed with CHIP. Ubiquitination of Hsc70 then occurs directly on the biosensor. This buildup of ubiquitin chains on Hsc70 corresponds to an increase in the binding thickness at the tip of the sensor causing a significant increase in the BLI signal (Figure 5A). The measurement of ubiquitin chain assembly directly at the surface of the biosensor, as opposed to within the entire solution, provides the further advantage that confounding signals are not generated by other ubiquitination processes such as CHIP autoubiquitination which occurs simultaneously alongside ubiquitination of Hsp70 for chaperoned ubiquitination [45]. The Z-factor calculated for the assay is 0.584, indicating an excellent assay.

The control reactions with no E1, no E2, no ubiquitin, and no ATP-Mg each displayed a BLI response saturating around 0.2 nm. In contrast to the controls for the autoubiquitination experiments (Figure 3A), the BLI response saturates at a higher response value due to the larger size of CHIP compared to the E2. In fact, control reactions with both CHIP and E2 present saturate at higher response values than the no E2 control or the no CHIP control due to the binding of the CHIP/E2 complex. Thus, the no E2 control allows for the binding of CHIP, while the no CHIP control does not result in the binding of CHIP, E2, or the CHIP/E2 complex to Hsc70. Furthermore, the BLI sensorgram for chaperoned ubiquitination of Hsc70 by CHIP is smaller in magnitude than the CHIP autoubiquitination assays (Figure 3A, 4A) due to the lower degree of ubiquitination of Hsc70 (Figure 5B) compared to autoubiquitination of CHIP (Figure 3B).

4. Conclusion

Western blotting is a well-established standard method that has been used to assess ubiquitination in numerous studies. Prior efforts have utilized differently labeled secondary antibodies featuring a fluorescent tag, radiolabeled tag, or coupled to an enzyme to produce a detectable signal via colorimetry or chemiluminescence [46–49]. However, it is difficult to obtain fine-grained time resolution using western blots. Additionally, the western blot process is time-consuming and requires several labor-intensive steps and lengthy incubations. The goal of this study was to develop and demonstrate an assay to monitor ubiquitination in a robust and rapid manner compared to the traditional western blot-based assay. The BLI ubiquitination assay markedly reduces time and workload requirements while generating results with substantially improved time resolution. The fine-grained time resolution, collecting data every second over the time course of the experiment, is one to two orders of magnitude better than can be readily achieved by a traditional western blot-based assay. The BLI ubiquitination assay monitors the ubiquitination reaction for 5 minutes, with a total assay time of 40 minutes, the bulk of which is used for pre-charging E1/E2/ubiquitin reaction mixture. The user is also able to track the reaction in real time and data processing is straightforward. The BLI ubiquitination assay uses identical protein reagent concentrations as used in traditional western blots, though with the advantage that smaller volumes can be used. For example, using the BLItz bio-layer interferometer with a 4 μl drop holder allows for the ubiquitination assay to be run using a total sample volume at least one order of magnitude smaller than that required for a corresponding western blot-based assay with multiple time points.

Furthermore, the developed assay is readily scalable and allows for higher throughput with the use of multi-sensor BLI instrument, such as the Octet (Sartorius) or Gator Pro (GatorBio) platforms that utilize a standard microplate format with automated processing capacity for 96 or 384 samples. Our data for the CHIP E3 ubiquitin ligase system demonstrates that the BLI ubiquitination assay can be used to monitor autoubiquitination or ubiquitination of a substrate by an E3 ubiquitin ligase. It is anticipated that the assay will have reasonable generalizability. A prior study found that the rate of ubiquitination initiation for cullin-RING ligases was 0.5 s^{-1}, 0.2 s^{-1}, for the second ubiquitin transfer, and 0.08 s^{-1} for the third ubiquitin transfer [38]. This shows that three ubiquitin molecules could be added within 132 seconds, or slightly shorter than half of the time window for the BLI assay. Given the substantive differences in sensorgrams for wild type versus K48R- or K_0-ubiquitin, it is anticipated that the assay is capable of monitoring polyubiquitination, despite the relatively short 300 second assay window compared to traditional assay windows of up to one hour when utilizing a Western blot for detection. Even for a hypothetical E3 ligase that is 100 times slower than the studied cullin-RING ligase [38] the sensorgrams would yield detectable monoubiquitination.

Several limitations are noted for the current assay format. First, the small drop size used, 4 μl, limits the duration of the ubiquitination that could be monitored due to potential evaporation. Second, trials loading the ubiquitination reaction samples used for Western blots in Figure 3 (data not shown) onto empty biosensors suggest that the BLI response saturates after the 10-minute ubiquitination sample time point. Thus, although the

ubiquitination reaction continues to add ubiquitin molecules to the growing polyubiquitin chains, the BLI becomes insensitive to the concomitant increase in biolayer thickness. Although these limitations for the current assay lead to a recommended 5-minute time course, a notable positive aspect to the shorter timeframe experiment is the connection to physiologically relevant timescales. It is known that a chain of four ubiquitin molecules is sufficient to target a K48-polyubiquitinated protein to proteasomal degradation [50], thus our assay monitors a physiologically relevant window of ubiquitination, as opposed to traditional Western blot-based approaches which regularly assemble chains many times larger than 4 ubiquitin molecules due to the extended time courses used, often up to 1 hour. Additionally, it was reported that in some cases, mono-ubiquitination is sufficient and necessary for targeting a protein for proteasomal degradation [51]. Thus, monitoring ubiquitination reactions for timescales longer than 5 minutes may not be physiologically relevant. However, in the case where there is a need to monitor ubiquitination for 30 minutes or more, such as for slower ligases, fine-tuning the BLI ubiquitination assay could overcome the 5-minute time limitation by using a tube instead of low volume drop for the association step, thereby limiting the evaporation of the solution. Thus, the BLI ubiquitination assay is a compelling and rapid alternative to traditional western blotting and enables detecting ubiquitination in real-time.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Richard C. Page reports financial support was provided by the National Institutes of Health.

Data availability

Data deposited in the Miami University Scholarly Commons at http://hdl.handle.net/2374.MIA/6904

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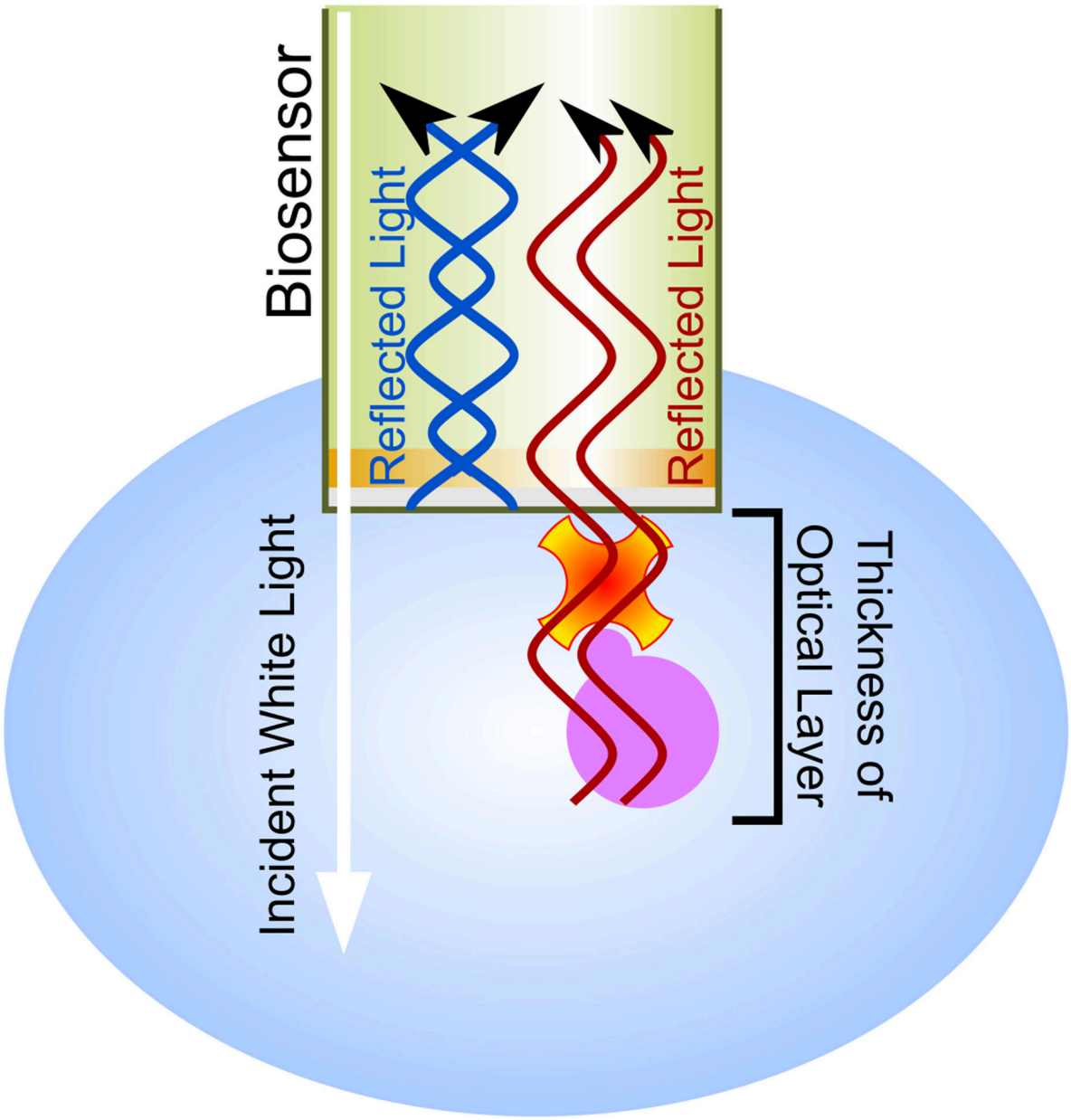

Figure 1.

Representation of a biosensor (*green*) with a capture moiety (*orange*) and bound analyte (*magenta*) in a drop of sample (*light blue*). The bio-layer interferometer analyzes the interference pattern of various wavelengths of light reflected from the bio-layer (*orange and magenta*) and an internal reference layer (*grey*) at the tip.

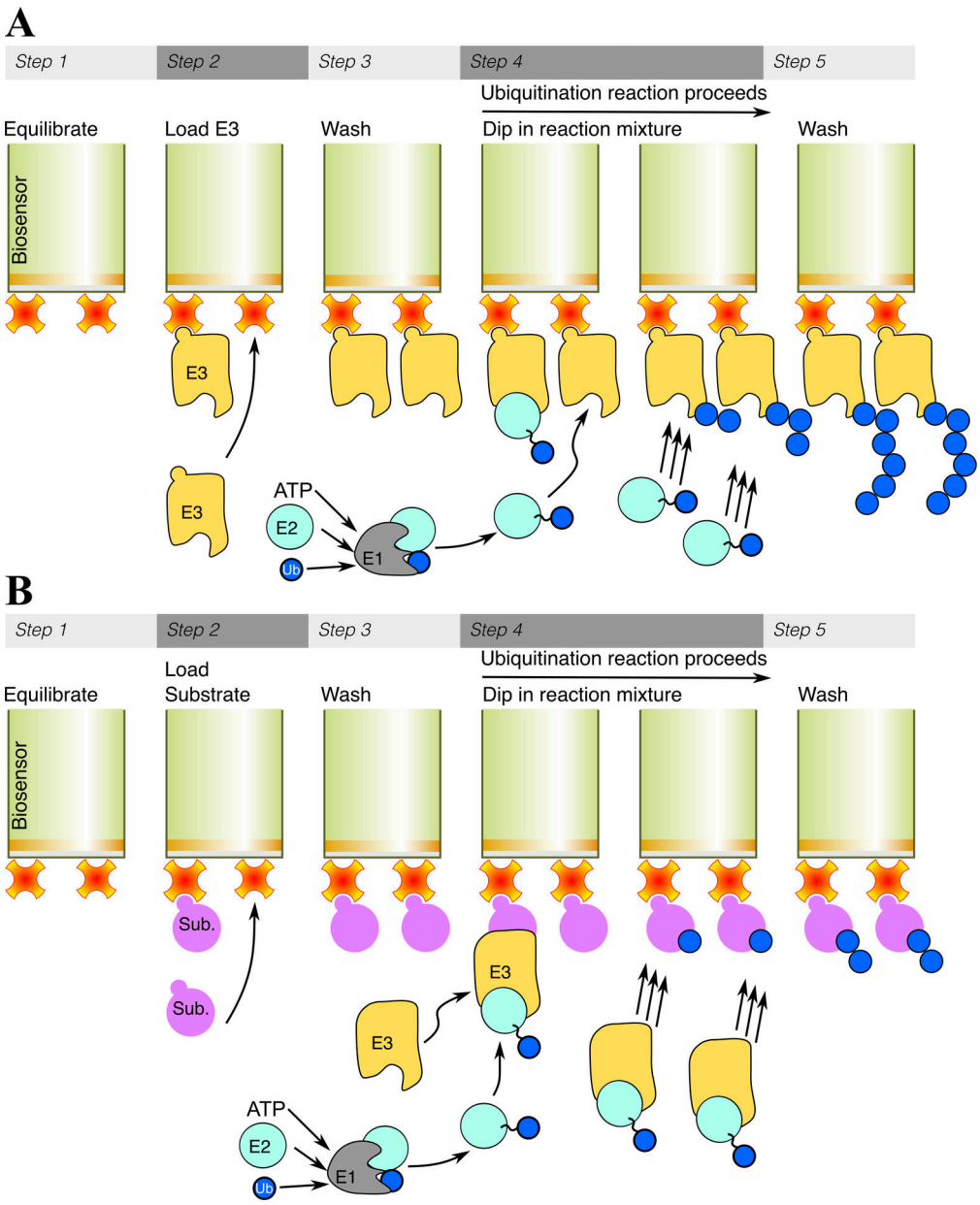

Figure 2:

Overview of the BLI ubiquitination assay used in this study. (A) Schematic diagram representing sequential steps of auto-ubiquitination of CHIP that are monitored using the BLI ubiquitination assay. The biosensor (*green*) is shown with a capture moiety (*orange*) for binding the E3 ubiquitin ligase (*yellow*) and subsequent ubiquitination involving the E1 ubiquitin-activating enzyme (*grey*), E2 ubiquitin-conjugating enzyme (*cyan*), and ubiquitin (*blue*) resulting in auto-ubiquitination of the E3. (B) Schematic diagram representing sequential steps of substrate-ubiquitination by CHIP that are monitored using the BLI ubiquitination assay. The biosensor (*green*) is shown with a capture moiety (*orange*) for binding the CHIP substrate Hsp70 (*magenta*), followed by a ubiquitination cascade involving the E1 ubiquitin-activating enzyme (*grey*), E2 ubiquitin-conjugating enzyme

(*cyan*), ubiquitin (*blue*), and the E3 ubiquitin ligase (*yellow*) resulting in ubiquitination of the substrate.

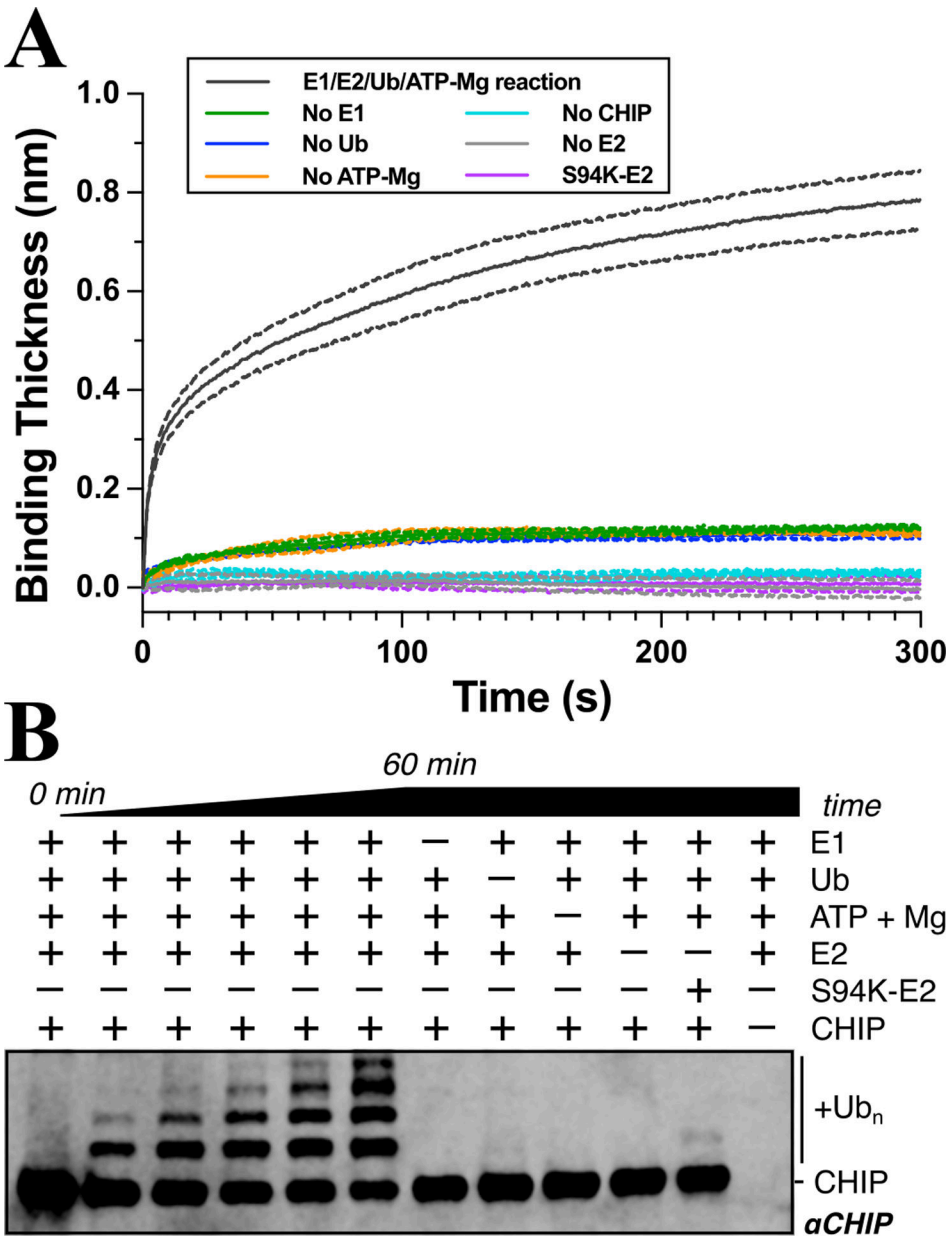

Figure 3:

Detection of autoubiquitination of CHIP using the BLI ubiquitination assay and the western blot-based assay. (A) Background corrected mean BLI signals (*solid line*) and the standard deviation of the BLI signals for triplicate polyubiquitination reactions (*dashed line*) of CHIP with all components (*black*) along with the controls lacking E1 (*green*), ubiquitin (*blue*), ATP-Mg (*orange*), E2 (*grey*), CHIP (*cyan*), or the use of S94K-E2 (*purple*). (B) Western blot with anti-CHIP (αCHIP) to detect polyubiquitination of CHIP followed by the controls used in part A. Time points used were 0, 5, 10, 20, 30, and 60 minutes.

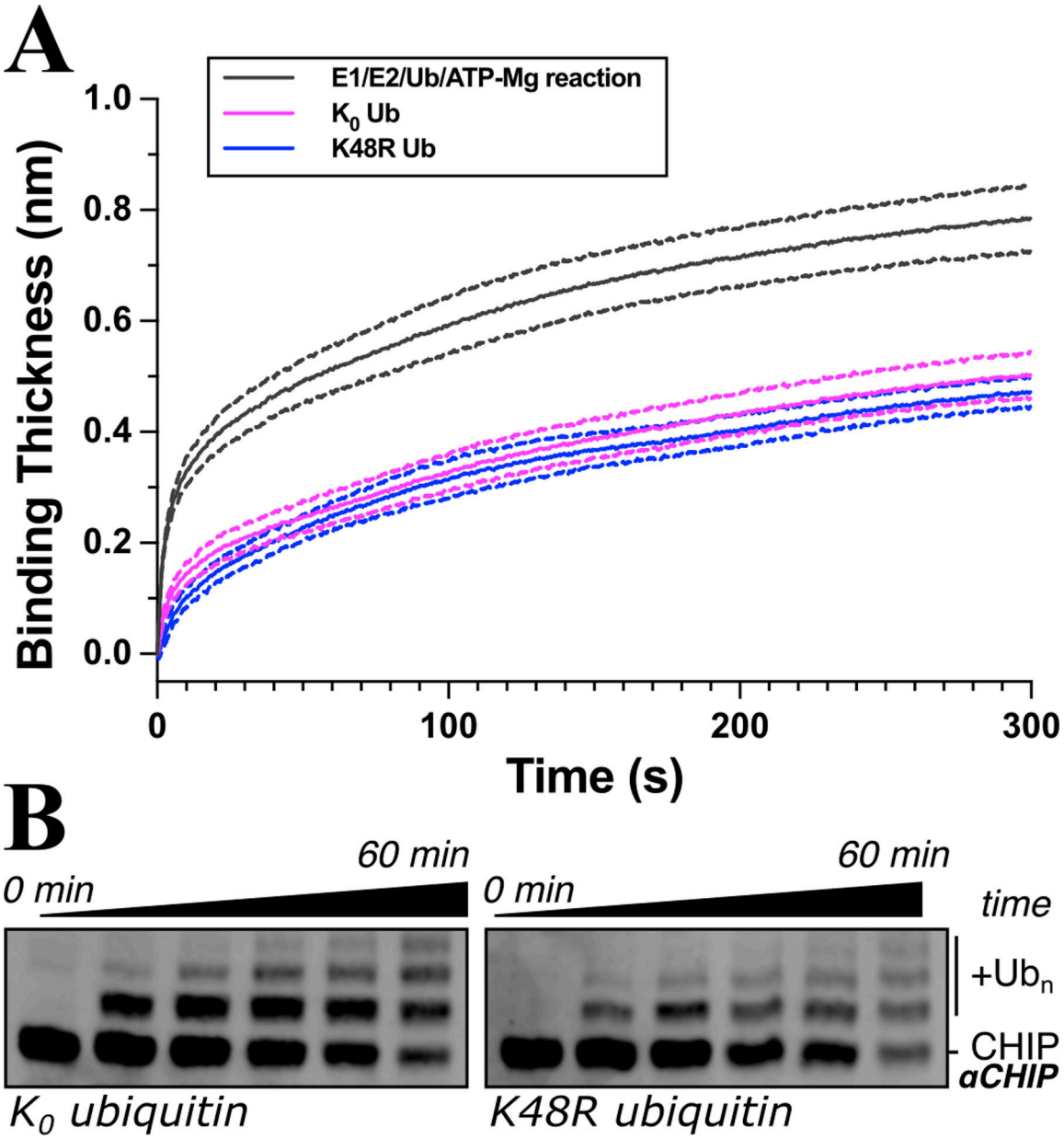

Figure 4:

Detection of multi-monoubiquitination of CHIP using the BLI ubiquitination assay and the western blot-based assay. (A) Background corrected mean BLI signals (*solid line*) and the standard deviation of the BLI signals for triplicate polyubiquitination reactions (*dashed line*) of CHIP with all components (*black*) along with the linkage type controls with K48R-ubiquitin (*blue*) or K_0-ubiquitin (*magenta*). (B) Western blot of CHIP autoubiquitination assays detected with anti-CHIP (αCHIP) indicates multi-monoubiquitination of CHIP using K_0-ubiquitin or K48R-ubiquitin. Time points used were 0, 5, 10, 20, 30, and 60 minutes.

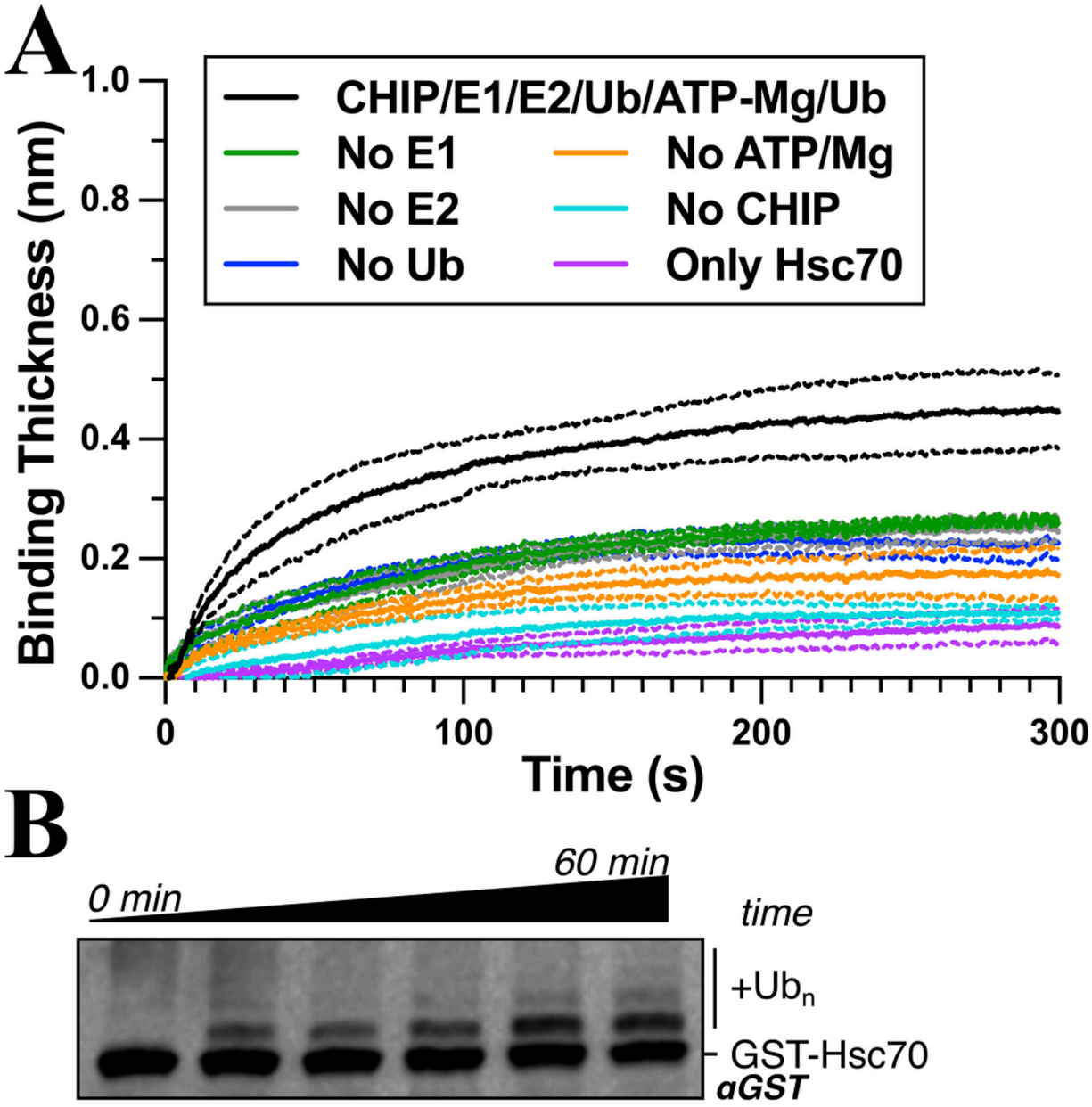

Figure 5:

Detection of Hsc70 polyubiquitination using the BLI ubiquitination assay and the western blot-based assay. (A) Background corrected mean BLI signals (*solid line*) and the standard deviation of the BLI signals (*dashed line*) for triplicate CHIP-mediated ubiquitination reactions of Hsc70 with all components (*black*) along with the controls lacking E1 (*green*), ubiquitin (*blue*), ATP-Mg (*orange*), E2 (*grey*), CHIP (*cyan*), or for sensors with Hsc70 only and no other reaction components (*purple*). (B) Western blot detected with anti-GST (αGST) to detect polyubiquitination of Hsc70. Time points used were 0, 5, 10, 20, 30, and 60 minutes.

Table 1:

Summary of BLI run settings used to detect auto-ubiquitination of CHIP.

Step	Step Type	Time (sec)	Position	Step Description
1	Initial Baseline	30	Tube	Washing
2	Loading	120	Drop	Attachment of His-CHIP to the sensor
3	Baseline	30	Tube	Washing
4	Association	300	Drop	Dipping the sensor in the reaction mixture
5	Dissociation	120	Tube	Washing

Table 2:

Summary of the run settings used in the BLI assay to detect polyubiquitination of Hsc70.

Step	Step Type	Time (sec)	Position	Step Description
1	Initial Baseline	30	Tube	Washing
2	Loading	120	Drop	Attachment of GST-Hsc70 to the sensor
3	Baseline	30	Tube	Washing
4	Association	300	Drop	Dipping the sensor in the reaction mixture
5	Dissociation	120	Tube	Washing

Table 3:

Summary of binding kinetics data for E2-wildtype and the S94K mutant against CHIP.

E2	K_d (M)	k_{on} (1/Ms)	k_{off} (1/s)
Wild-type	6.28×10^{-6} ($\pm$ 3.15×10^{-6})[a]	1.94×10^{-3} ($\pm$ 6.92×10^{-4})	5.61×10^{-3} ($\pm$ 4.63×10^{-3})
S94K mutant	N.D.[b]	N.D.	N.D.

[a] Data reported as the mean ± standard deviations of triplicate experiments.

[b] No detected binding.