Development of a Real-Time Homogeneous TR-FRET Ubiquitin Conjugation and Deconjugation Assay Platform

By South Bay Bio, 5941 Optical Court, Suite 229, San Jose CA, 95138
Contact info@southbaybio.com, Tel. (415) 935-3226

Introduction

Ubiquitin, a highly conserved 76 amino acid protein, is an important post-translational modifier responsible for regulating a wide variety of cellular functions including but not limited to protein degradation and recycling, self-cyclical control, and DNA damage repair. Ubiquitination, the modification of proteins by the attachment of ubiquitin polypeptide chains, is catalyzed by a three-step process in which ubiquitin is activated by an ATP-dependent E1 activating enzyme, transferred to an E2 conjugating enzyme, and then finally conjugated to a target substrate by an E3 ubiquitin ligase. The process is reserved by ubiquitin-conjugating enzymes (UBEs). Thus far, away from the lab, the in vivo ubiquitination process is still a task of tasks for providing full activity of the conjugation pathway. Herein, we report the development of a novel assay platform capable of measuring ubiquitin conjugation and deconjugation in real-time, in a homogeneous, single-step assay, utilizing TR-FRET technology.

TR-FRET Tech

TR-FRET (Time Resolved Fluorescence Resonance Energy Transfer) uses the extended fluorescence emission decay lifetime of rare-earth lanthanides to determine the distance between two fluorophores. Hoechst33258 is used as the energy donor and EosFluor623 as the energy acceptor. The fluorescence intensity decay is detected at 620 nm, and 665 nm. The antigen of interest is either conjugated to biotin or AlexaFlour647 and detected using streptavidin-TR-FRET. EosFluor623, trf-Ub, and Mg-ATP were added to the reaction mixture for 3 hours. TR-FRET MDM2-Ub was initiated with addition of Mg-ATP at 20 M and trf-Ub mix at 50 nM, and then followed for 8 hours at 37°C.

Activity of All Types of E3 Ubiquitin Ligases can be Measured in a Real-Time Homogeneous TR-FRET Assay

Endpoints E3 Ubiquitin-Ligase Autoubiquitination: Conjugation reactions can also be measured in an endpoint configuration. Optimal incubation time has been selected from real-time experiments: 1000 nM Ub-Aldehyde titrated to 50 nM wt-Parkin shown for comparison.

Deconjugation

Decoupling enzymes (DUBs) catalyze the removal of ubiquitin from substrate proteins. This reversal of the conjugation cascade affects critical events in the cell proteome such as protein degradation through the proteasome. To date, more than 100 DUBs have been annotated, and many studies have shown their multiple functions in human disease. Current DUB screening requires largely consist of namely trypsin, trypsin inhibitors such as ubiquitin rhodamine 110 and AMC. However, a growing need for more physiologically relevant substrates is emerging as we discover more DUBs with preferential lysine linkage specificities or multimeric ubiquitin chain requirements.

Summary

In this report, we show the capabilities of a novel TR-FRET based platform for probing the activities of E3 ubiquitin ligases and deubiquitinating enzymes in real-time and end-point systems. This technology fills a long-standing gap in the ability of researchers to conduct drug discovery with simple assays tailored for the ubiquitin proteasome system. Unlike existing technologies, the TR-FRET platform does not rely on secondary detection methods such as antibodies. We demonstrate this platform’s robustness with caspase-3 using a TR-FRET assay, and show its adaptability to miniaturized formats, making it a platform for high throughput screening experiments. We further provide a proof-of-concept for the platform’s utility and simplicity in making K determinations, and demonstrate that the platform holds its weight against a common dual substrate, Ubiquitin Rhodamine 110.

References


