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

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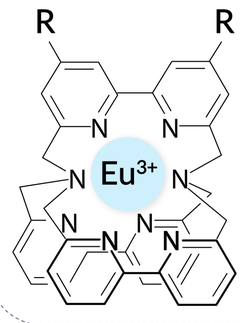
Introduction

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, cell cycle control, and DNA damage repair. Ubiquitination, the modification of proteins by the attachment of ubiquitin or polyubiquitin 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, then finally conjugated to a target substrate by an E3 ubiquitin ligase. The process is reversed by ubiquitin deconjugating enzymes (DUBs.) Thus far, assay development within the ubiquitin field has suffered a lack of tools for probing the 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 homogenous, single step assay, utilizing TR-FRET technology.

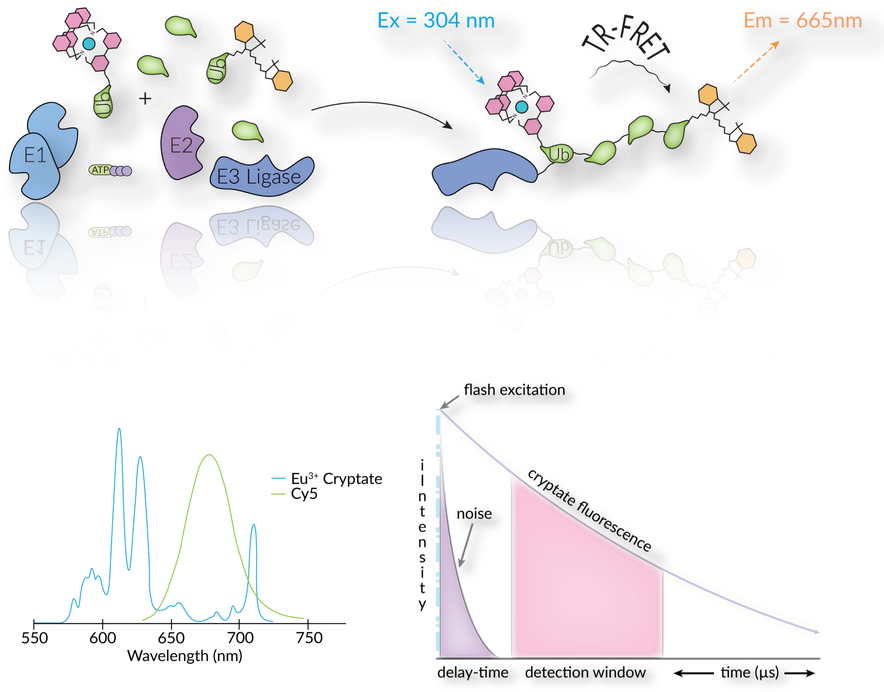
TR-FRET (Time Resolved Fluorescence Resonance Energy Transfer) uses the extended fluorescence emission decay lifetimes typical of rare-earth lanthanides to impart a short time-delay between FRET donor excitation and emission. This delay provides a means to separate "true" signal from short-lived background fluorescence and reduce interference from compound fluorescence and other assay artifacts. Using ubiquitin labeled with either Europium-Cryptate (donor) or Cyanine5 (acceptor), we demonstrate ubiquitin conjugation and deconjugation measured homogenously in real time, with assays commonly exhibiting Z' ≥ 0.8. We show examples of auto-ubiquitination kinetics of several human recombinant E3 ligases of significant interest: MDM2, Parkin, ITCH, NEDD4, and XIAP. Additionally, we are able to show the reversal of this process by deubiquitinating ubiquitinated MDM2 with the DUB USP2, and compare USP2's kinetics with this substrate versus Ubiquitin-Rhodamine 110.

TR-FRET Tech

Rare earth cryptates are macrocyclical structures encasing a rare earth lanthanide atom. Lanthanide ions don't exhibit suitable fluorescence properties on their own, and require incorporation with organic moieties functioning as "light-harvesting" antennas to collect and transfer energy by intramolecular non-radiative processes. Our cryptate is composed of a macrocycle in a cage-shaped assembly composed of three bipyridine arms, which complex a Eu^{3+} ion (Eu^{3+}TBP) as shown in the structure below. Unlike other common flavors of rare earth complexes like chelates, cryptates are extremely robust and stable structures that show no sensitivity to photobleaching. This is largely due to cryptates having no dissociation between the complexed ion and the macrocycle, contrary to chelates, which often exhibit uncoordinated bonds with solvent. These properties allow cryptates to perform in stringent conditions, e.g. in presence of strong chelators, of divalent cations (e.g. Mn^{2+}, Mg^{2+}), extremes of pH, organic solvents, and high temperatures (PCR or other thermoregulated processes).

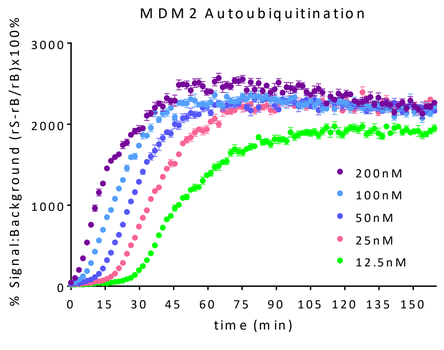


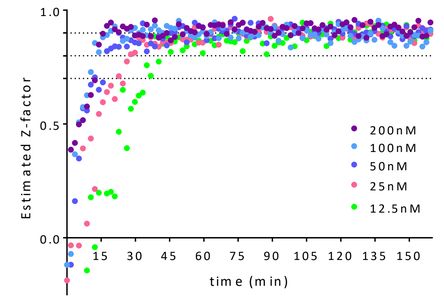
	Cryptates	Chelates
Stability	High	Low
Extreme pH Tolerant	Yes	No
Open Solvent Bonds	0 (optimal)	1 to 3
EDTA/EGTA Resistant	Yes	No
Divalent Cation Tolerant	Yes	No
Organic Solvent Tolerant	Yes	No
Stable at High Temp	Yes	No



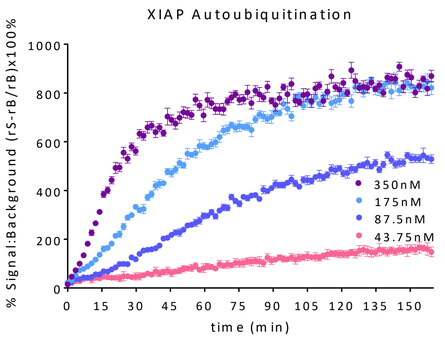
Activity of All Types of E3 Ubiquitin Ligases can be Measured in a Real-Time Homogeneous TR-FRET Assay

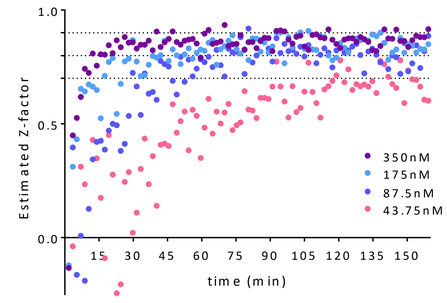
RING





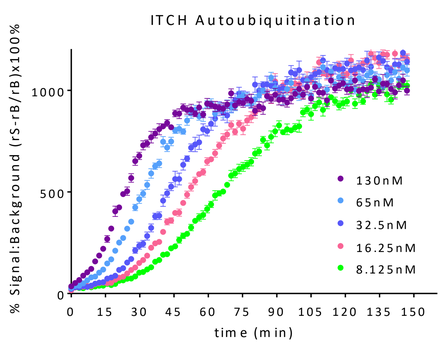
TR-FRET MDM2 Autoubiquitination: Serial dilutions of MDM2 from 200nM to 12.5nM mixed with UBA1, UBE2D3, trf-Ub mix. Reaction was initiated with addition of Mg-ATP, excited at 304 nm, and emission detected at 620 nm, and 665 nm.

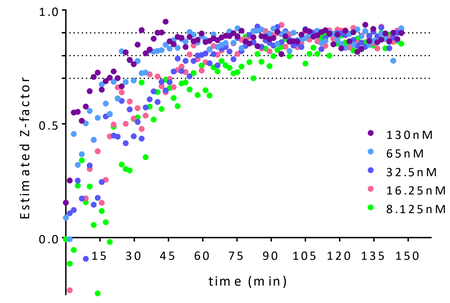




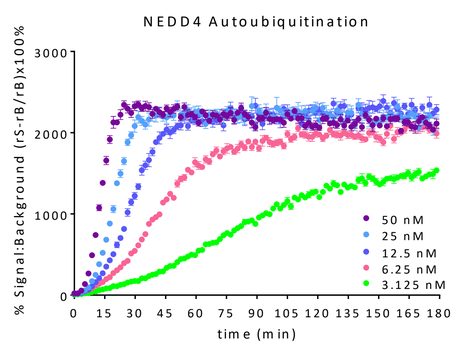
TR-FRET XIAP Autoubiquitination: Serial dilutions of XIAP from 350nM to 43.75nM mixed with UBA1, UBE2D2, trf-Ub mix. Reaction was initiated with addition of Mg-ATP, excited at 304 nm, and emission detected at 620 nm, and 665 nm.

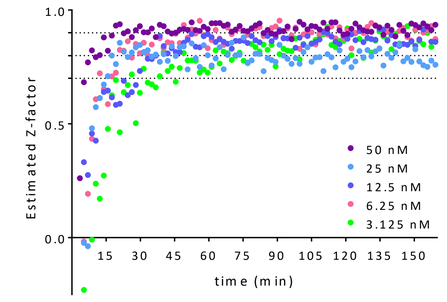
HECT





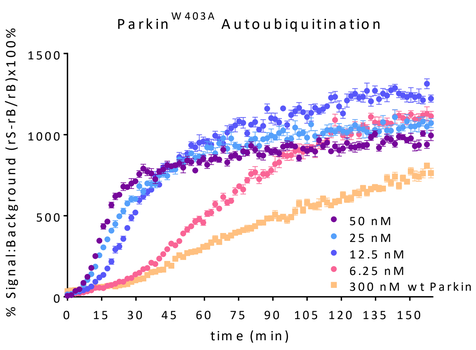
TR-FRET ITCH Autoubiquitination: Serial dilutions of ITCH from 130nM to 8.125nM mixed with UBA1, UBE2L3, trf-Ub mix. Reaction was initiated with addition of Mg-ATP, excited at 304 nm, and emission detected at 620 nm, and 665 nm.

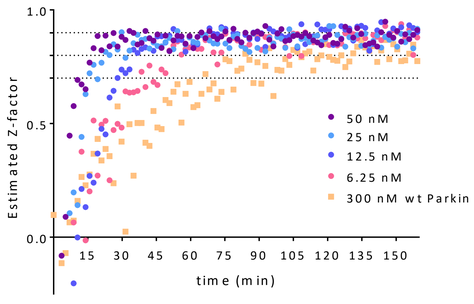




TR-FRET NEDD4 Autoubiquitination: Serial dilutions of NEDD4 from 50nM to 3.125nM mixed with UBA1, UBE2L3, trf-Ub mix. Reaction was initiated with addition of Mg-ATP, excited at 304 nm, and emission detected at 620 nm, and 665 nm.

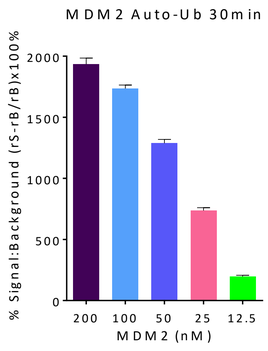
RBR

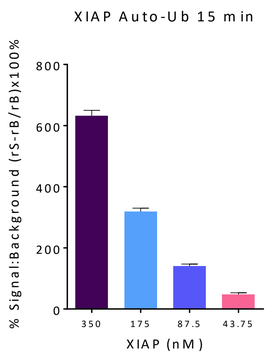


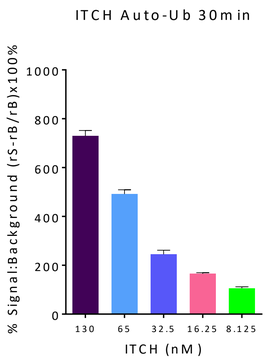


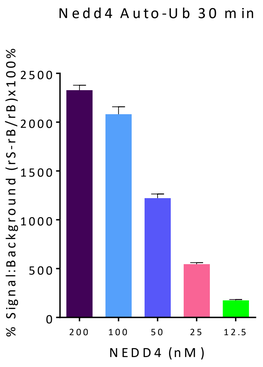
TR-FRET Parkin^{W403A} Autoubiquitination: Serial dilutions of Parkin^{W403A} from 50nM to 6.25nM mixed with UBA1, UBE2D3, trf-Ub mix. Reaction was initiated with addition of Mg-ATP, excited at 304 nm, and emission detected at 620 nm, and 665 nm. 300nM wt-Parkin shown for comparison.

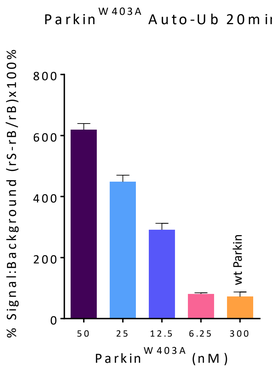
E3 Ligase Autoubiquitination Measured in an Endpoint TR-FRET Assay









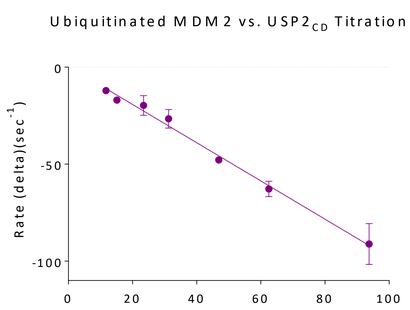


Endpoint E3 Ubiquitin Ligase Autoubiquitination: Conjugation reactions can also be measured in an endpoint configuration. Optimal incubation time has been selected from real-time experiments where the rate of conjugation is linear. Setup conditions were similar to a real-time reactions, except 10mM DTT and 2mM EDTA were added to quench the reactions before measurement.

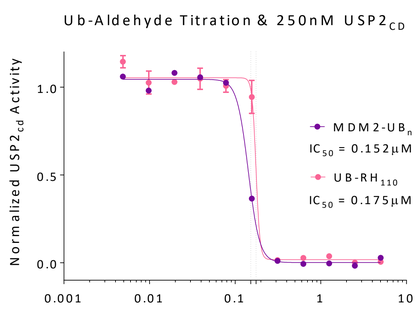
Deconjugation

Deconjugating 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 reagents largely consist of c-terminal derivatives 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

TR-FRET MDM2-Ub_n Digests with $USP2_{CD}$ & Measures K_i Values



TR-FRET MDM2-Ub_n Chains Titrated with $USP2_{CD}$: 50nM MDM2 was conjugated with trf-Ub chains using UBA1, UBE2D3, and Mg-ATP for 3 hours. TR-FRET MDM2-Ub_n chains were then digested using titrating amounts of $USP2_{CD}$, from 93nM to 11nM. Initial velocities (delta (sec^{-1})) are shown plotted as a function of $USP2_{CD}$ concentration. Increasing the DUB concentration increases the reaction rate linearly.



TR-FRET MDM2-Ub_n Chains & $USP2_{CD}$ Ub-Aldehyde Dose-Response: 50nM MDM2 pre-conjugated with trf-Ub chains was digested with 250nM $USP2_{CD}$ along with serial dilutions of Ub-Aldehyde. Initial rates of each inhibitor concentration were calculated and normalized relative to the no-inhibition control. Normalized activity was plotted against the log of inhibitor concentration, and fit with a 4th parameter agonist curve yielding an IC_{50} of 152nM. A similar dose-response using 500nM Ubiquitin-Rhodamine 110 substituted for trf-MDM2-Ub_n yields similar results.

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 endpoint systems. This technology fills a longstanding gap in the ability of researchers to conduct drug discovery with simple assays tailored for the ubiquitin proteasome system. Unlike existing technologies, our platform does not rely on secondary detection methods such as antibodies. We demonstrate this platform's robustness with assays commonly exhibiting Z' ≥0.8, and show its adaptability to miniaturized formats, making it ideal for high-throughput screening experiments. We further provide a proof-of-concept for the system's utility and simplicity in making K_i determinations, and demonstrate that the platform holds its weight against a common DUB substrate, Ubiquitin Rhodamine 110.

References

Amerik, A. Y., & Hochstrasser, M. (2004). Mechanism and function of deubiquitinating enzymes. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1695*(1), 189-207.

Koyano, F., Okatsu, K., Kosako, H., Tamura, Y., Go, E., Kimura, M., ... & Endo, T. (2014). Ubiquitin is phosphorylated by PINK1 to activate parkin. Nature, 510(7503), 162.

Magennis, S. W., Parsons, S., Pikramenou, Z., Corval, A., & Woollins, J. D. (1999). Imidodiphosphinate ligands as antenna units in luminescent lanthanide complexes. *Chemical communications*, (1), 61-62.

Zheng, N., & Shabek, N. (2017). Ubiquitin Ligases: Structure, Function, and Regulation. *Annual Review of Biochemistry*, (0).

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