A Fast and Sensitive Method of Monitoring Ubiquitin Conjugation Mediated by the Cullin4a/Rbx1/DDB1/CRBN E3 Ligase Complex using a Real-Time Homogeneous TR FRET Assay

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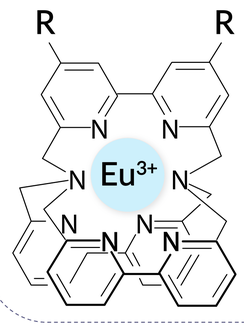
Introduction

Drug discovery in the ubiquitin proteasome system has the potential to create cancer-treating therapeutics with a high level of specificity and selectivity. While drugs such as Bortezomib and Carfilzomib can be somewhat selective for cancer cells, their cytotoxicity from inhibition of proteasomal degradation creates a need for more specific drugs. Using specific E3 ligases for inhibition is one such approach, aiming to find compounds with higher selectivity and less associated toxicity via degradation of cellular proteins regulated by E3s. Recently, the SCF E3 (Skip Cullin F-Box) ligase family of proteins, or the Cullin-RING ubiquitin Ligase (CRL's), has emerged specifically in targeted protein degradation widely known in an approach called **PRO**teolysis-**TA**rgeting **C**himeras (PROTAC's). This new field exploits the intracellular ubiquitin-proteasome system to selectively degrade target proteins. Lately, small-molecule PROTACs with high potency have been frequently reported. Yet it is difficult and cumbersome to validate and screen compounds in-vitro against the SCF due to a lack of proper tools probing the system. For the first time, we report the performance and capabilities of a real-time homogeneous TR-FRET Assay designed to provide a fast and sensitive method of monitoring ubiquitin conjugation mediated by the Cullin4a/Rbx1/DDB1/CRBN.

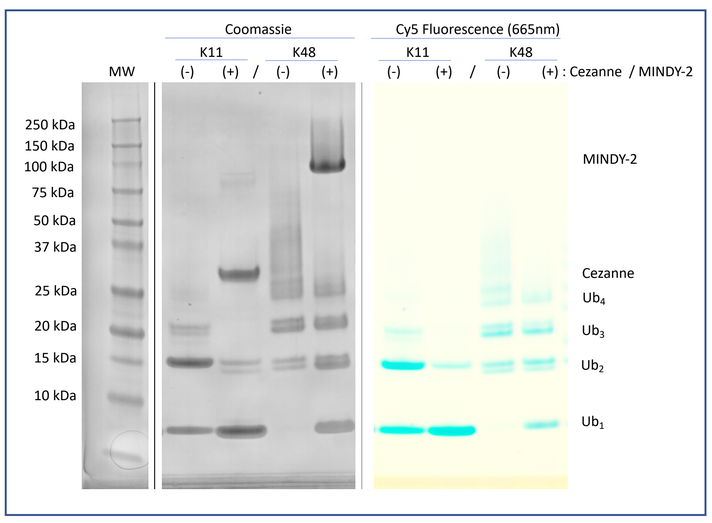
Similarly to improve discovery of selective DUB compounds we have developed several linkage specific TR-FRET ubiquitin free chains capable of acting as highly specific substrates to many linkage specific DUBs. Specifically we report the real-time kinetic-readout and cleavage of K48 chains digested with MINDY-2, and K11 linked chains digested with Cezanne. Unlike C-terminal derivatives these longer chain multimer TR-FRET DUB substrates can be processed by these very linkage-specific DUBS, with multimer substrate preferences. Our assay features robust high signal-to-noise ratios commonly exhibiting Z' ≥ 0.8. The platform shown has been optimized for low volume 384-well plates making them ideal and cost effective for HTS in a variety of academic and industry screening applications.

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).

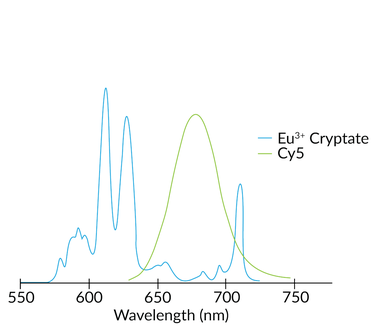


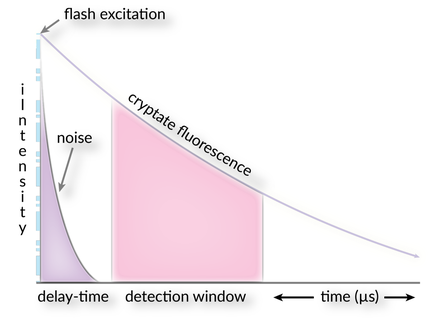
	Cryptates	Chelates
Stability	High	Low
Extreme pH Tolerant	Yes	No
Open Solvent Bonds	0 (optimal)	1 to 3
EDTA/EGTA Resistant	Yes	No
Divalent Cation Tolerant	Yes	No
Organic Solvent Tolerant	Yes	No
Stable at High Temp	Yes	No

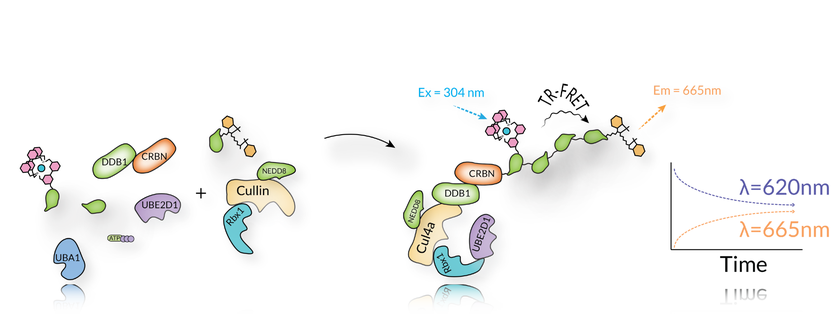


K11 and K48 TR-FRET Ubiquitin Chains cleaved with Cezanne and MINDY-2: (Left, Coomassie staining), 2µg K11-linked TR-FRET Ubiquitin chains were cleaved with 2µg Cezanne, and 2µg of K48-linked TR-FRET chains were cleaved with 2µg MINDY-2 for 30 minutes. Coomassie staining indicates majority of K11-linked Ubiquitin chains formed are di- and tri-ubiquitin multimers. Post hydrolysis, primarily monomer-Ubiquitin becomes visible. K48-linked TR-FRET chains consist mostly of di, tri, tetra, and higher MW multimers that hydrolyze down to lower MW ubiquitin species upon MINDY-2 addition (right panel). The same gel imaged using a fluorescence detection camera at 665nm reveals Cy5 incorporation into all chains.

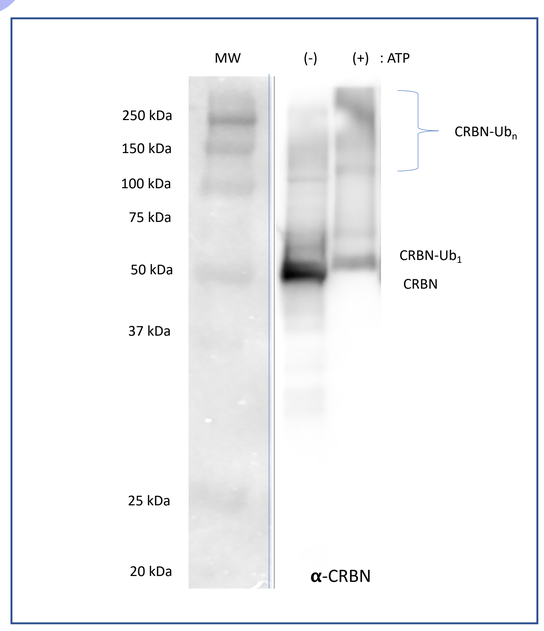
Cullin4a/Rbx1/DDB1/CRBN Ubiquitination



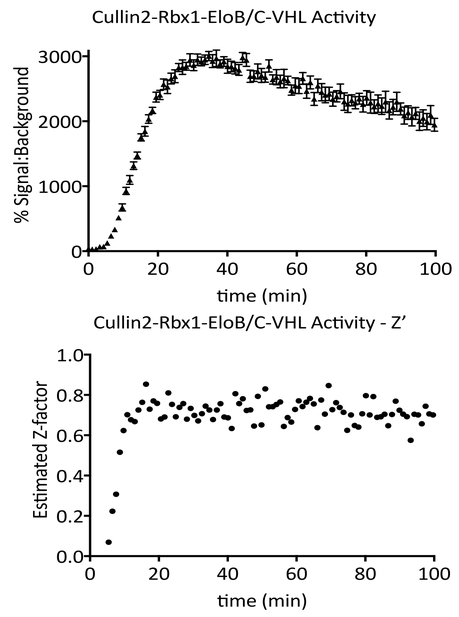




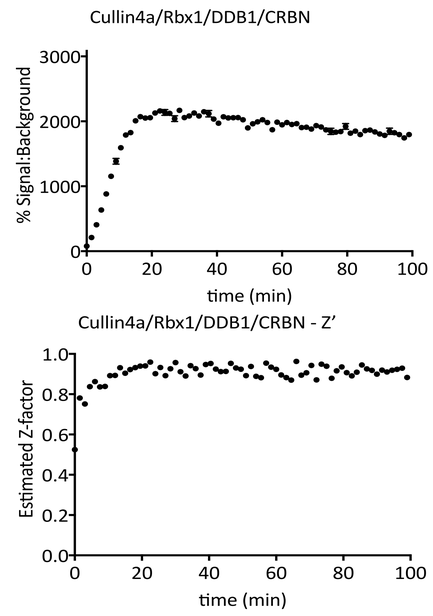
Cullin2-Rbx1-Elongin B/C-VHL & Cul4a-Rbx1-DDB1-CRBN Activity Measured Using Real-Time TR-FRET



TR-FRET Cullin4a/Rbx1/DDB1/CRBN Ubiquitination: 50nM of Cullin4a/Rbx1/DDB1/CRBN were mixed with UBA1, UBE2D1, TRF-Ub mix. Reactions were initiated with addition of Mg-ATP and quenched with SDS running buffer after 1 hour. Samples were run on SDS-PAGE and transferred onto PVDF membrane, and blotted against anti-CRBN. High MW CRBN ubiquitin multimers are observed with addition of ATP.



TR-FRET Cullin2-Rbx1-EloB/C-VHL Ubiquitination: 50nM of Cullin2-Rbx1-EloB/C-VHL were mixed with UBA1, UBE2D1, TRF-Ub mix. Reactions were initiated with addition of Mg-ATP, excited at 304 nm, and emission detected at 620 nm, and 665 nm. Reaction reaches maximum velocity in ~10 minutes.

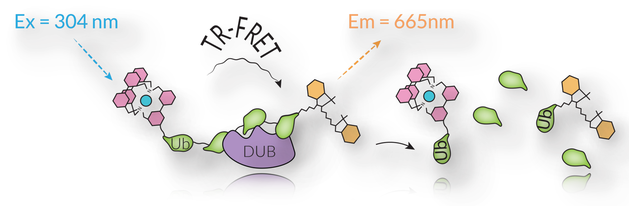


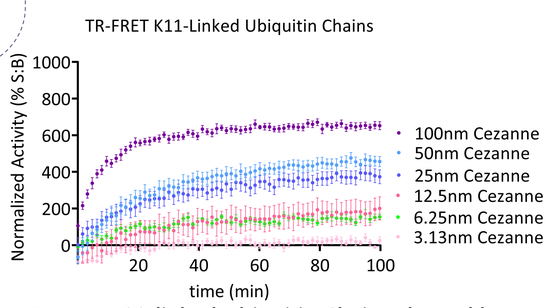
TR-FRET Cullin4a-Rbx1-DDB1-CRBN Ubiquitination: 50nM of Cullin4a-Rbx1-DDB1-CRBN were mixed with UBA1, UBE2D1, TRF-Ub mix. Reactions were initiated with addition of Mg-ATP, excited at 304 nm, and emission detected at 620 nm, and 665 nm. Reaction reaches maximum velocity in ~8 minutes.

Linkage Specific Unanchored TR-FRET Ubiquitin Chains

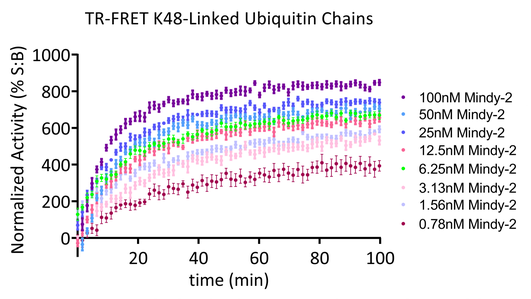
Deconjugation

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.

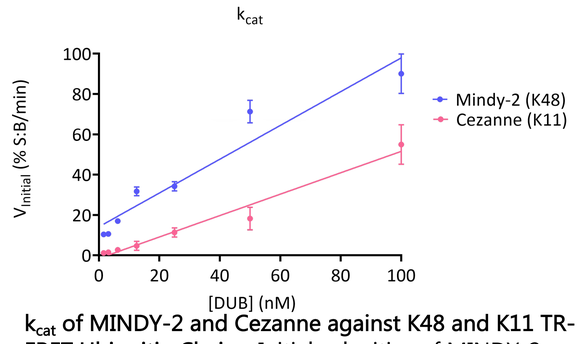




TR-FRET K11-linked Ubiquitin Chains cleaved by Cezanne : 1µM K11 TRF Ubiquitin chains were cleaved with a serial dilution of Cezanne from 100-3.13nM over 100 minutes. Cezanne preferentially cuts K11 chains. Rates of chain hydrolysis are concentration dependent on Cezanne, and initial velocities were used to calculate k_{cat}.



TR-FRET K48-linked Ubiquitin Chains cleaved by MINDY-2 : 1µM K48 TRF Ubiquitin chains were cleaved with a serial dilution of MINDY-2 from 100-3.13nM over 100 minutes. MINDY-2 is highly specific for multimeric K48 chains. Rates of chain hydrolysis are concentration dependent on MINDY-2, and initial velocities were used to calculate k_{cat}.



k_{cat} of MINDY-2 and Cezanne against K48 and K11 TR-FRET Ubiquitin Chains: Initial velocities of MINDY-2 with K48, and Cezanne with K11 TR-FRET ubiquitin chains exhibit a linear relationship against DUB concentration. MINDY-2 appears to process K48 chains ~50% faster then Cezanne processes K11 linked chains.

Summary

We report the performance and capabilities of a real-time homogeneous TR-FRET Assay designed to provide a fast and sensitive method of monitoring ubiquitin conjugation mediated by the Cullin4a/Rbx1/DDB1/CRBN as well as Cullin2/Rbx1/EloB/C/VHL. We demonstrate ATP dependent Cereblon ubiquitination in a real time assay readout typically occurring within 30 minutes, and quick achievement of Z' values of ~0.8 or higher. We also for the first time report the performance of a new series of purified TR-FRET multimer ubiquitin chains of specific lysine linkage useful for DUB kinetics analysis and or screening approaches. Both Cezanne and MINDY-2 exhibit linear concentration dependent increases in velocity with K11 and K48 chains respectively. Furthermore, MINDY-2 known for its very specific substrate specificity and preference for longer K48 chains, can quickly and conveniently be analyzed using our TRF-K48 substrate. Unlike existing technologies, our platform does not rely on secondary detection methods such as antibodies, and fills a longstanding gap in the ability of researchers to conduct drug discovery and mechanistic studies with simple assays tailored for the ubiquitin proteasome system.

References

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