Parkinson's Disease: Development of a Quantitative and Qualitative Real-Time Homogeneous TR-FRET Assay to Assess Parkin Activity

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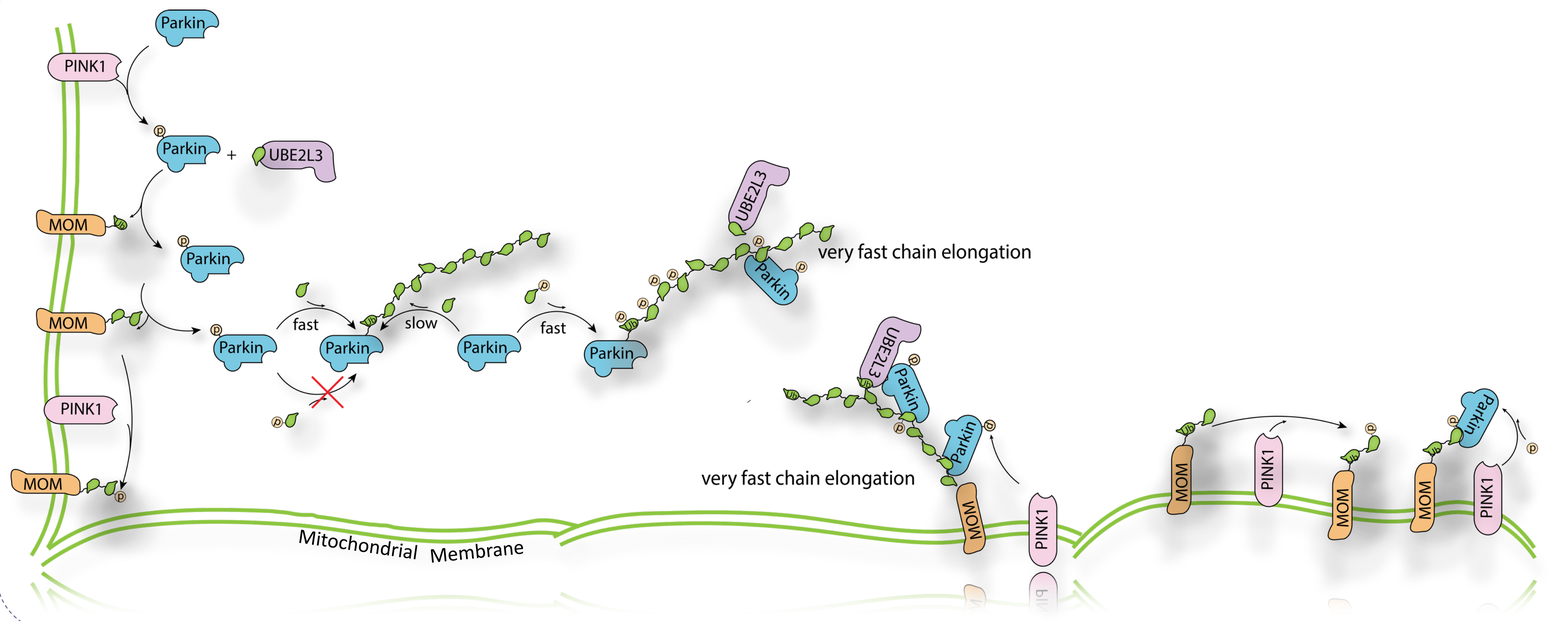
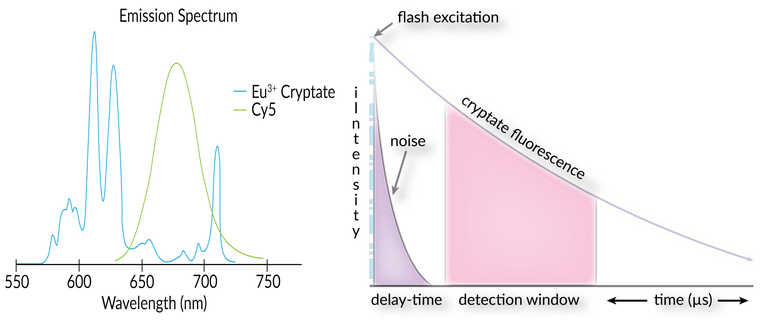
Introduction

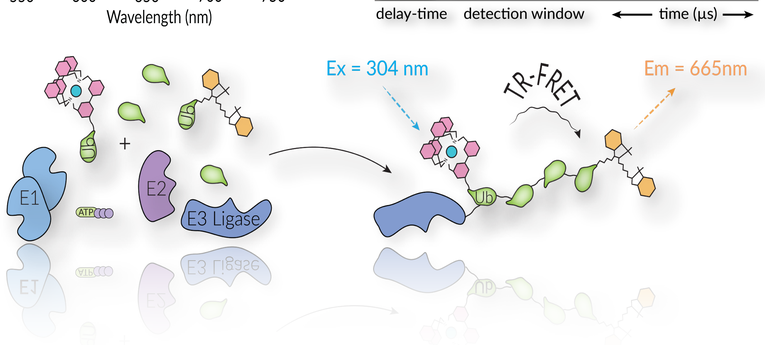
Parkinson's Disease (PD) is the second most common neurodegenerative disorder behind Alzheimer's in the industrial world with a prevalence of 1-2% among individuals 65 years and older (de Rijk *et al.*, 2000). The disease is partially characterized by cytoplasmic inclusions consisting of insoluble protein aggregates (Lewy bodies) and mitochondrial dysfunction as underlying factors in PD pathogenesis (Dawson., *et al.* 2003, Valente, E.M., *et al.* 2004). Defects or mutations in the Phosphatase and tensin homolog (PTEN)-induced ligase 1 (PINK1) and PARK2/Parkin mutations have also been shown to cause autosomal recessive juvenile parkinsonism (AR-JP), where Parkin and PINK1 have been shown to be critical for directing damaged mitochondria for mitophagy (Valente, E.M., *et al.* 2004, Ordureau, A. *et al.,* 2014). Their recruitment to the mitochondrial outer membrane (MOM) facilitates ubiquitination of MOM proteins, with the specifics of this biology being further unraveled to gain a better understanding of the biology and the underlying molecular mechanisms of PD pathology. Defining individual steps in the pathway and developing therapeutics have been challenging due to a lack of research tools able to assess either Parkin ubiquitin ligase and or PINK1 kinase activity, and how they work in concert. Here, we show the performance and capabilities of a newly developed homogeneous TR-FRET Parkin/PINK1 ubiquitination assay in support of the proposed feed-forward mechanism (Ordureau, A. *et al.,* 2014) and model of the Parkin/PINK1 pathway. Utilizing a real-time kinetic readout system, we show a dose-dependent activation of WT Parkin by PINK1. We also demonstrate similar PINK1 dependent activation of the Parkin mutant W403A. In contrast, autoubiquitination of pS65-Parkin can be inhibited using high concentrations of pS65-ubiquitin, which is in alignment with the proposed feed-forward mechanism. Additionally, Parkin activation can also be significantly accelerated by using phosphorylated ubiquitin chains instead of mono-pS65-Ub. In summary, our platform enables researchers to quickly and reliably interrogate the biology and enzyme kinetics of this critical drug-discovery pathway. The TR-FRET based assays have also 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.

The Feed Forward Mechanism



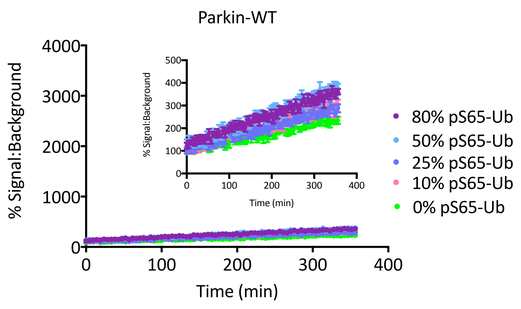
TR-FRET Ubiquitination Basics



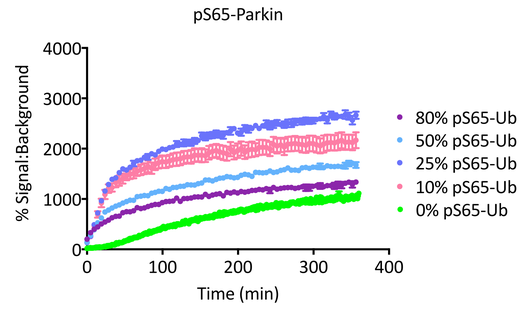


Ubiquitin E3-Ligase Conjugation Detection via TR-FRET: TR-FRET uses the extended fluorescence emission decay lifetimes typical of rare-earth lanthanides to impart a short time-delay between FRET donor excitation and emission. This delay provides a means to separate "true" signal from short-lived background fluorescence, and reduce interference from compound fluorescence and other assay artifacts. This is possible due to the extended emission half-life characteristic of rare-earth lanthanide cryptates compared to traditional donor fluorophores. As a result our homogeneous TR-FRET conjugation assays enable detection of ubiquitin chain formation in real-time. When reactions are initiated ubiquitin chain formation is detected via measurement of Cy5 and cryptate emission.

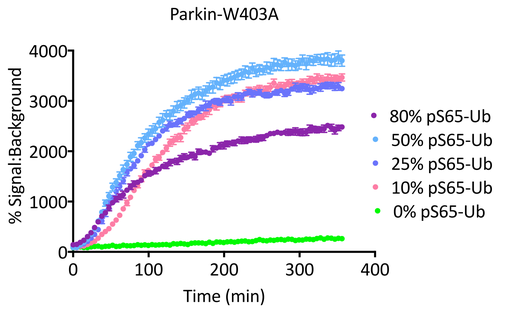
Parkin & PINK1 Activity Measured Using Real-Time TR-FRET



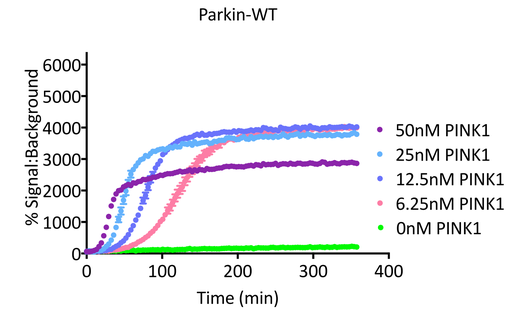
WT Parkin Activation with pS65 Ubiquitin: Increasing concentrations of pS65 ubiquitin activate WT Parkin and enable autoubiquitination. While detectable, activation of Parkin by up to 80% pS65 ubiquitin is minimal and slow. Reactions were initiated with addition of Mg-ATP, excited at 304 nm, and emission detected at 620 nm, and 665 nm.



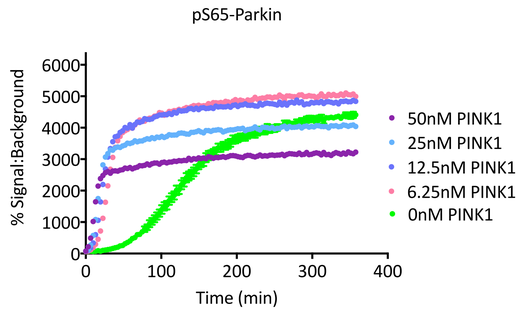
pS65-Parkin Activation with pS65 Ubiquitin: Increasing concentrations of pS65 ubiquitin activate purified phosphorylated Parkin and increase autoubiquitination almost 10x higher compared to WT Parkin . Interestingly, concentrations of pS65-Ub >25% of the total ubiquitin inhibit Parkin ubiquitination, as previously observed (Ordureau, A. *et al.,* 2014)



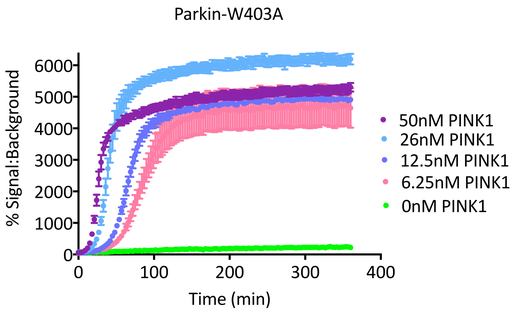
Parkin-W403A Activation with pS65 Ubiquitin: Increasing concentrations of pS65 ubiquitin activate Parkin mutant W403A even further compared to phosphorylated Parkin. Again, higher concentrations of pS65-Ubiquitin inhibit Parkin ubiquitination.



WT Parkin Activation by PINK1: Increasing concentrations of PINK1 kinase significantly activates WT Parkin and enables rapid autoubiquitination. Reactions were initiated with addition of Mg-ATP, excited at 304 nm, and emission detected at 620 nm, and 665 nm.

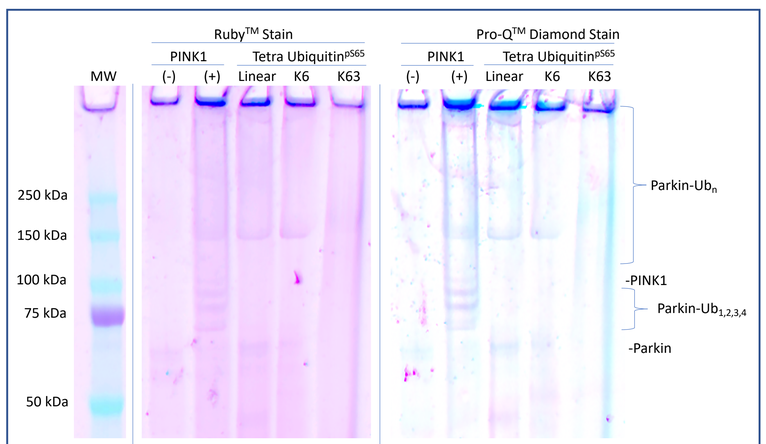


pS65-Parkin Activation by PINK1: Increasing concentrations of PINK1 kinase significantly activates purified pS65-Parkin and enables rapid autoubiquitination compared to pS65-Parkin alone. Although, pS65-Parkin has significantly increased activity, addition of PINK1 further accelerates Parkin activity.



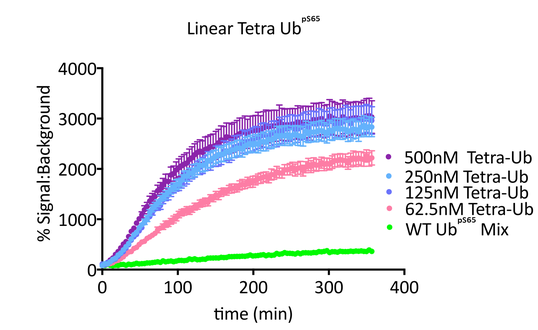
Parkin-W403A Activation by PINK1: Increasing concentrations of PINK1 kinase significantly increases the activity of mutant Parkin W403A. Similar to phosphorylated Parkin, PINK1 addition activated Parkin significantly more then only increasing the amount of pS65 ubiquitin.

PINK1 Promotes Longer Ub-Chains

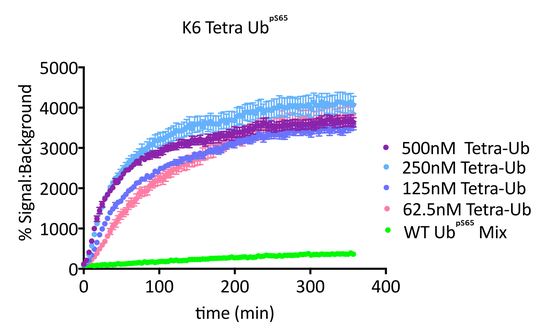


PINK1 Addition Promotes Longer Phosphorylated Ubiquitin Chains on Parkin: (Left) 100nM E1, 1uM UBE2L3, 1uM Parkin, 50uM Ubiquitin, 10mM ATP. Samples were incubated for 60 minutes at 37C, and developed with SYPRO™ Ruby Protein Gel Stain. Comparison of the first 2 lanes shows significant levels of higher MW Parkin ubiquitination in the presence of PINK1 vs no-PINK1 (first lane). (Right) The same gel stained with Pro-Q™ Diamond Phosphoprotein Gel Stain, which fluoresces when bound to phosphorylated proteins. It is clear that with PINK1 addition the Parkin Ub chains are not only running at a much higher MW, but are also phospho-stain reactive. Also important to note is that substituting previously phosphorylated and purified phospho tetra-Ub^{pS65} chains for PINK1 yield similar levels of high MW Parkin ubiquitination.

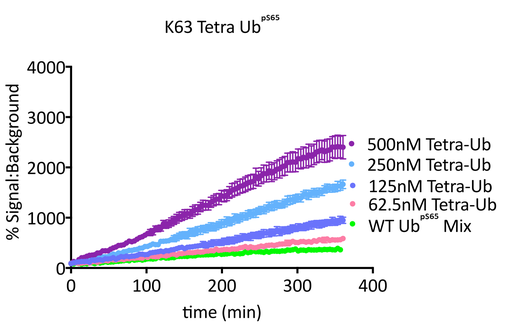
Parkin Activation using Purified Phosphorylated Tetra Ubiquitin Chains



TR-FRET WT Parkin Activation using Phosphorylated Linear Tetra-Ub^{pS65}: Addition of phosphorylated linear tetra-ubiquitin (pS65) chains significantly activates unphosphorylated Parkin compared to only adding phosphorylated mono ubiquitin (pS65) in the absence of PINK1.



TR-FRET WT Parkin Activation using Phosphorylated K6 linked Tetra-Ub^{pS65}: Addition of phosphorylated K6 linked tetra-ubiquitin (pS65) chains significantly activates unphosphorylated Parkin compared to only adding phosphorylated mono ubiquitin (pS65) in the absence of PINK1.



TR-FRET WT Parkin Activation using Phosphorylated K63 linked Tetra-Ub^{pS65}: Addition of phosphorylated K63 linked tetra-ubiquitin (pS65) chains significantly activates unphosphorylated Parkin compared to only adding phosphorylated mono ubiquitin (pS65) in the absence of PINK1.

Summary

We show and describe the capabilities of a novel TR-FRET based platform for probing the activities of the E3 Parkin ligase in a real-time kinetic system. In agreement with previous studies (Ordureau, A. *et al.,* 2014) we show relative activities between non-modified Parkin (WT), phosphorylated Parkin (pS65), active-mutant Parkin (W403A), and their activation by PINK1. The results indicate, PINK1 phosphorylates higher MW ubiquitin chains, likely localizing Parkin and E2 to these areas, resulting in longer and more rapid chain elongation. This is further supported by experiments where purified phosphorylated tetra ubiquitin (pS65) chains are spiked into conjugation reactions (without PINK1), yielding to similar levels of parkin activation as if PINK1 were present. 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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