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Monitoring functional mechanisms of protein degradation in living cells

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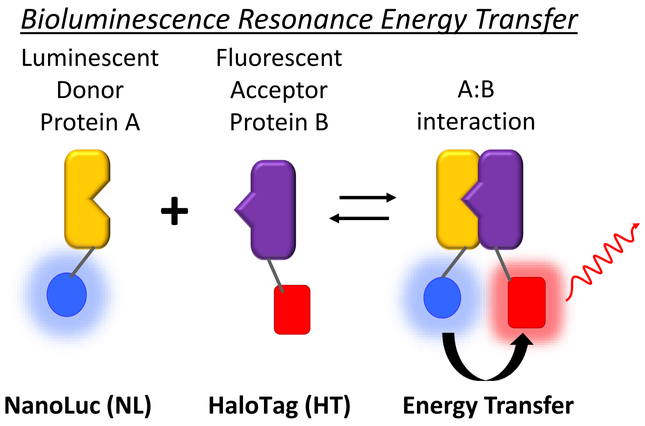
Abstract and introduction

Here we present mechanistic cellular studies on several proteins targeted for degradation via the ubiquitin proteasome pathway in mammalian cells. Using bioluminescence resonance energy transfer with NanoLuc® and HaloTag® fusions, termed NanoBRET, we can monitor changes in interactions of proteins targeted for degradation, including dynamic recruitment to E3 ligases and real-time trafficking to the 26S proteasome using inhibitors or PROTAC based compounds. We show also the ability to quantitate protein levels over a significant dynamic range by monitoring NanoLuc luminescent levels (RLUs) of several proteins, either expressed exogenously or as endogenous knock-in fusion proteins using CRISPR/Cas9. Live cell monitoring of protein:protein interactions and degradation is shown for BRD4, β-catenin, cMyc, and HIF1α. These combined approaches deconvolute the complicated processes involved in proteosomal recruitment and are a powerful strategy for understanding and following protein degradation.

Protein:Protein interactions in living cells

NanoBRET™ Protein:Protein Interactions

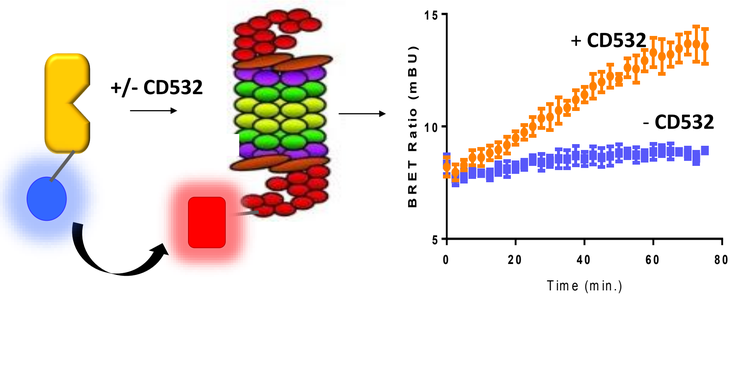
Bioluminescence Resonance Energy Transfer



- Improved signal: background, decreased overlap
- Greater assay window as compared to other BRET systems
- NanoLuc enables BRET at low expression levels
- Ratiometric, highly reproducible assay with excellent z' factor

Monitoring proteosomal trafficking

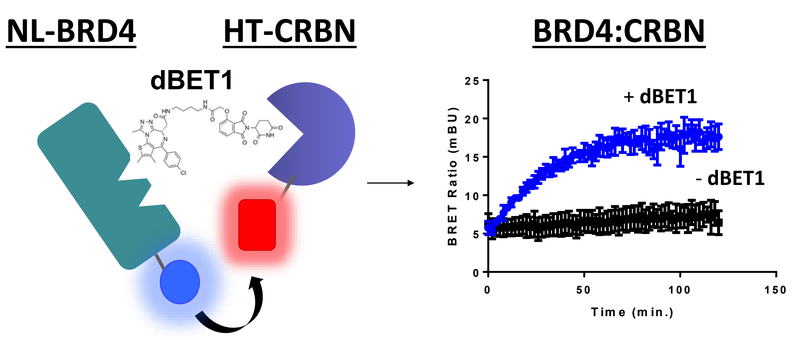
NanoBRET for real-time kinetic interactions with 26S Proteasome



- Measure changes in proteosomal recruitment of cMyc after addition of AURKA inhibitor, CD532
- CD532 known to increase ubiquitination of cMyc and promote degradation

Monitoring interactions with E3 Ligases

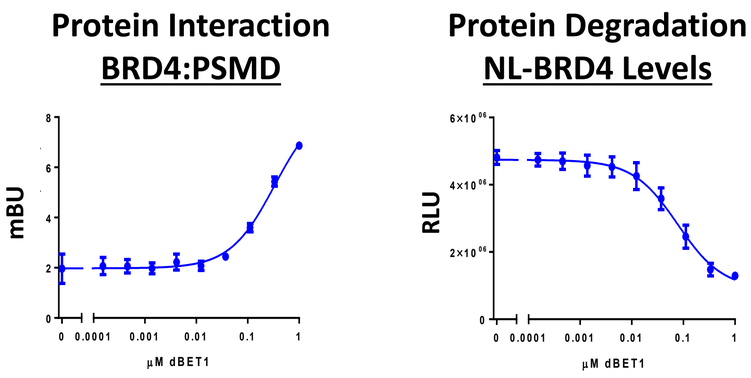
NanoBRET for real-time kinetic interactions with E3 Ligases



- Detect the induced and increased interaction of BRD4 with Cereblon E3 ligase after treatment with dBET1
- Able to use the technology to study small molecule or PROTAC induced interactions with E3 Ligases

Dose response degradation of BRD4

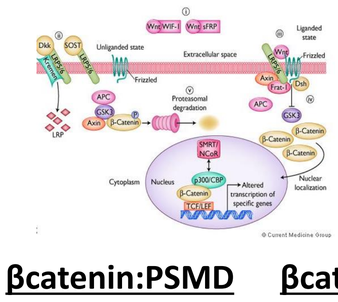
Simultaneous measurement of BRD4 proteasomal interaction with BRD4 protein levels +/- dBET1



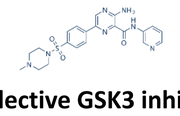
- Determine EC50 for BRD4:Proteasome and IC50 for loss of BRD4
- In the same experiment follow new interaction as well as protein level

Degradation studies with β-catenin

Following interactions of β-catenin in the Wnt signaling pathway

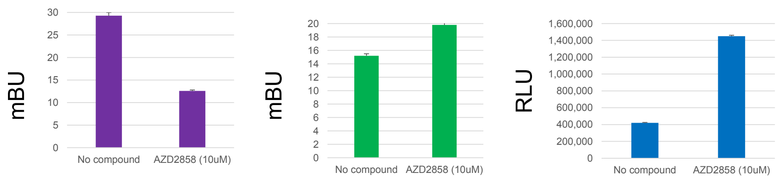


AZD2858



Selective GSK3 inhibitor and Wnt signaling activator

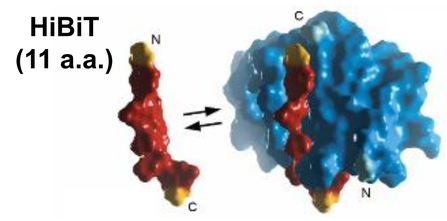
βcatenin:PSMD | βcatenin:Tcf3 | βcatenin Levels



- Measure decrease and correlative protein interaction increases after treatment with B-catenin stabilizer compound AZD2858

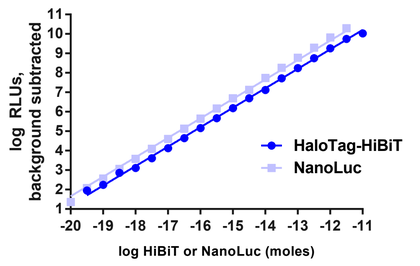
HiBiT Technology for endogenous studies

NanoLuc® Binary Complementation



Detection of HiBiT Fusion proteins in Lysates

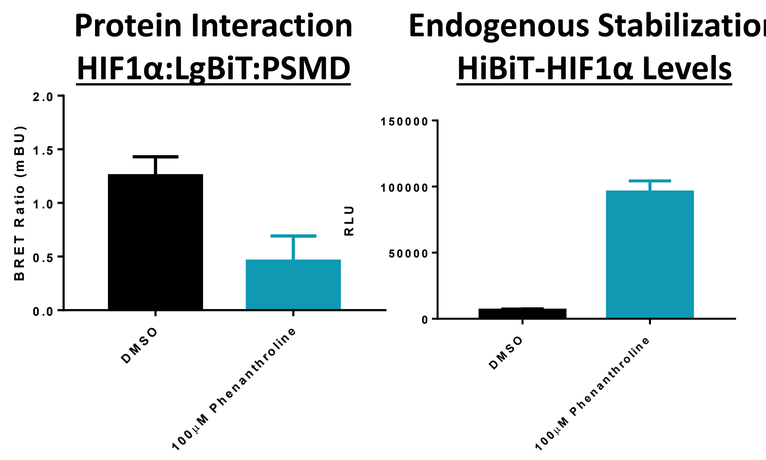
HiBiT technology consists of an 11amino acid high affinity peptide which complements with a larger fragment, LgBiT, to generate NanoLuc luminescence



- High affinity interaction between HiBiT and LgBiT (K_D = 700 pM) drives complementation in lysates or cells
- HiBiT can be an N- or C-terminus fusion tag expressed either exogenously or endogenously via CRISPR/Cas9
- Shows a 7-log range for detection of protein levels

Endogenously tagged HiBiT-HIF1α

Proteasome recruitment and cellular protein quantitation of CRISPR/Cas9 endogenously tagged clonal HiBiT-HIF1α



- NanoBRET sensitivity allows for detection of decrease of HIFα at proteasome after phenanthroline treatment
- Significant range of HIF1α stabilization measured
- HiBiT, 11aa, allows for rapid tagging of endogenous proteins via CRISPR/Cas9

Summary

- NanoBRET technology is a highly sensitive method for detecting live cell protein:protein interactions
- NanoBRET assays for protein degradation can monitor in real time:
  - Protein:E3 ligase recruitment
  - Protein:Proteasome trafficking
  - PROTAC induced interactions
- NanoLuc and/or HiBiT can be used for quantitative monitoring in cells of proteins targeted for degradation
- HiBiT technology is highly amenable for use in CRISPR/CAS9, allowing for the study of endogenously tagged proteins

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