

Monitoring Protein Dynamics at Endogenous Levels with a Luminescent Peptide Tag

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1. Introduction

Intracellular signaling is largely mediated through protein dynamics, including changes in protein abundance, interactions, location, or post-translational modification. While the behavior of proteins is commonly studied using overexpressed reporter genes, CRISPR/Cas9 offers the possibility of tagging genes with reporters at the endogenous locus in order to maintain physiological expression levels, regulation, and stoichiometry with binding partners. The 11-amino-acid peptide, HiBiT, represents an ideal tag for studying the protein dynamics of endogenously expressed proteins. Its small size facilitates rapid knock-in of the tag using ribonucleoprotein complexes of Cas9 and gRNA along with synthesized single-stranded oligonucleotide donor DNA. High-affinity complementation of HiBiT with the 18 kDa LgBiT subunit generates the bright, luminescent NanoBiT™ enzyme, enabling sensitive quantification of HiBiT-tagged proteins over 7 orders of magnitude of linear dynamic range. Changes in protein abundance can be monitored in either a lytic endpoint assay using purified LgBiT protein or in a live-cell kinetic assay by expressing the LgBiT subunit in cells. Interactions of a HiBiT-tagged protein with protein fusions to HaloTag can be measured in live cells using bioluminescence resonance energy transfer (BRET). We apply these assays to the induced degradation by PROTAC compounds of the bromodomain-containing protein, BRD4, and show both a decrease of endogenously expressed HiBiT-BRD4 in cell lysates and live cells and increased protein interactions with the E3 ligase complex and ubiquitin. HiBiT can also be used to measure translocation of proteins to and from the cell surface in a live-cell assay that takes advantage of the membrane impermeability of LgBiT. We show that internalization of endogenously expressed HiBiT-EGFR and HiBiT-β2-AR can be measured in a matter of minutes as a loss in extracellular HiBiT signal. Furthermore, phosphorylation of HiBiT-EGFR upon activation can be monitored in a homogeneous assay by measuring BRET from the HiBiT/LgBiT complex to a fluorescently labeled secondary antibody to monitor binding of an anti-phospho antibody.

2. Overview of HiBiT and Endogenous Tagging

Attach HiBiT to protein of interest

Add LgBiT and Substrate

HiBiT and LgBiT interact to form bright luminescent enzyme

Rapid, Cloning-free CRISPR Knock-in Workflow

- Design and Order:**
 - Guide RNA (crRNA + tracrRNA)
 - HiBiT Donor DNA (ssDNA oligo)
 - Purified Cas9
- Assemble Ribonucleoprotein (RNP) Complex**
- Deliver RNP Complex & Donor DNA to cells (Electroporation)**
- Incubate 24-48hrs**
- Detect Edit by Luminescence**

- High-affinity interaction between HiBiT and LgBiT ($K_D = 700$ pM) drives spontaneous formation of a bright, luminescent enzyme with glow kinetics in the presence of furimazine substrate
- HiBiT can be fused to the N- or C-terminus of proteins or placed in accessible internal locations

3. Multiple Detection Formats to Measure Protein Dynamics

Directly measure HiBiT-tagged proteins

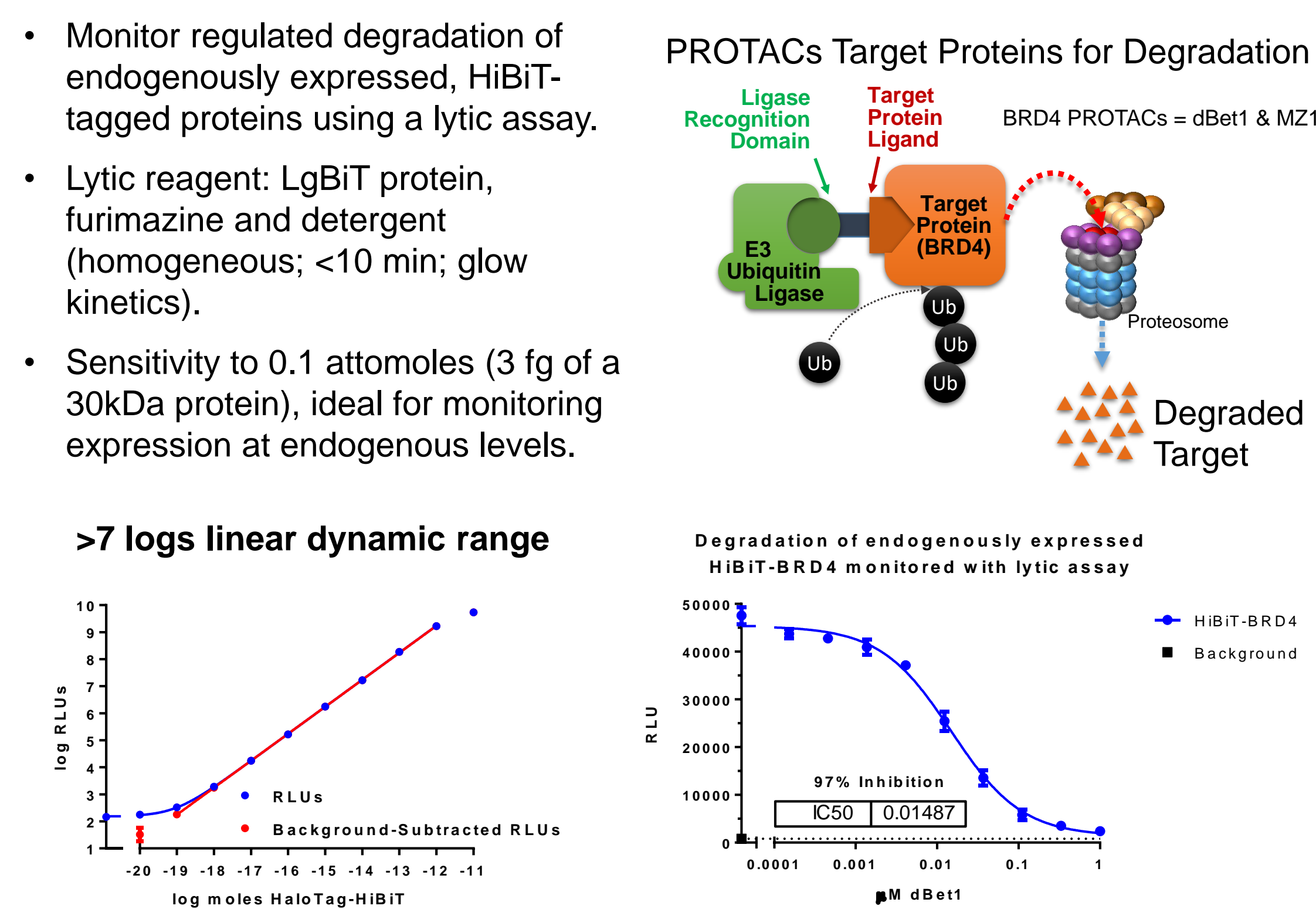
- HiBiT Lytic Assay:** sensitive detection of HiBiT-tagged proteins in cell lysates, IP complexes, or other samples.
- HiBiT Extracellular Assay:** Live-cell detection of secreted or cell-surface HiBiT-tagged proteins
- HiBiT Blotting:** simple and sensitive detection of HiBiT-tagged proteins on blots.
- Intracellular HiBiT Assay:** Live-cell, kinetic detection of intracellular HiBiT in LgBiT-expressing cells

Use complemented HiBiT as donor in BRET assays

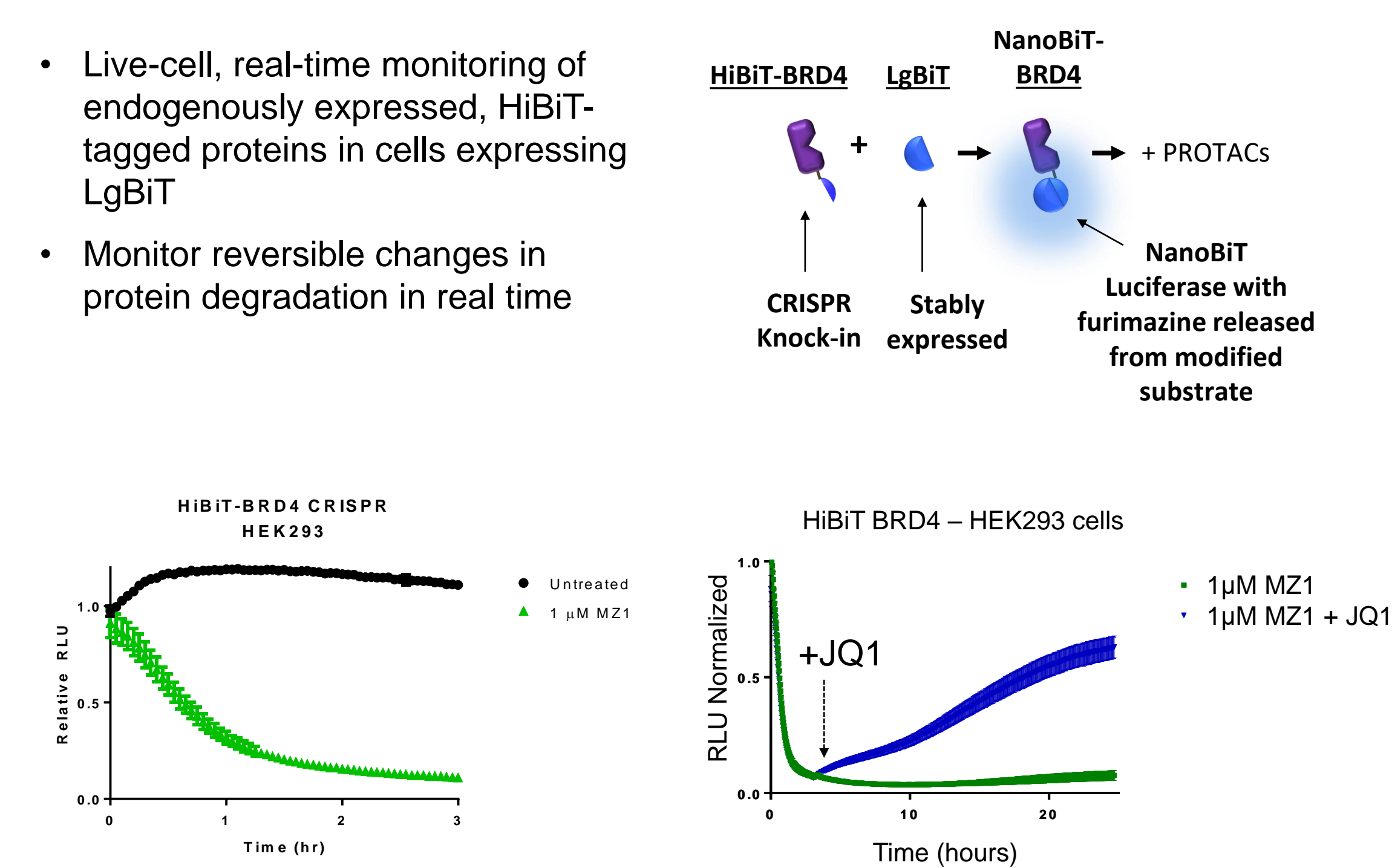
BRET acceptor can be:

- HaloTag fusion for protein interaction assays
- Fluorescent tracer compound for small molecule engagement
- Labeled 2^o antibody for post-translational modifications

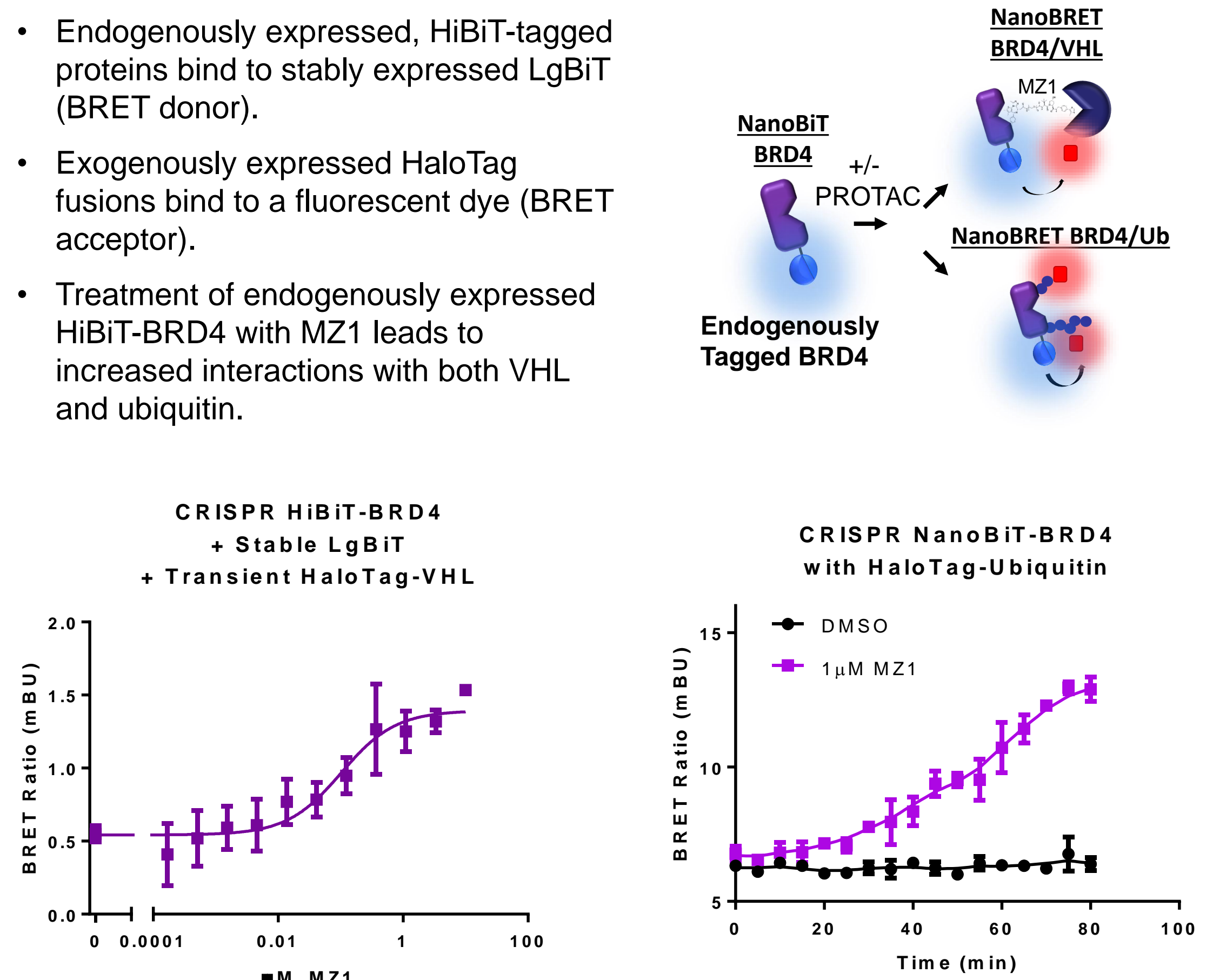
4. Quantify Protein Abundance Across Wide Dynamic Range with a Lytic Assay



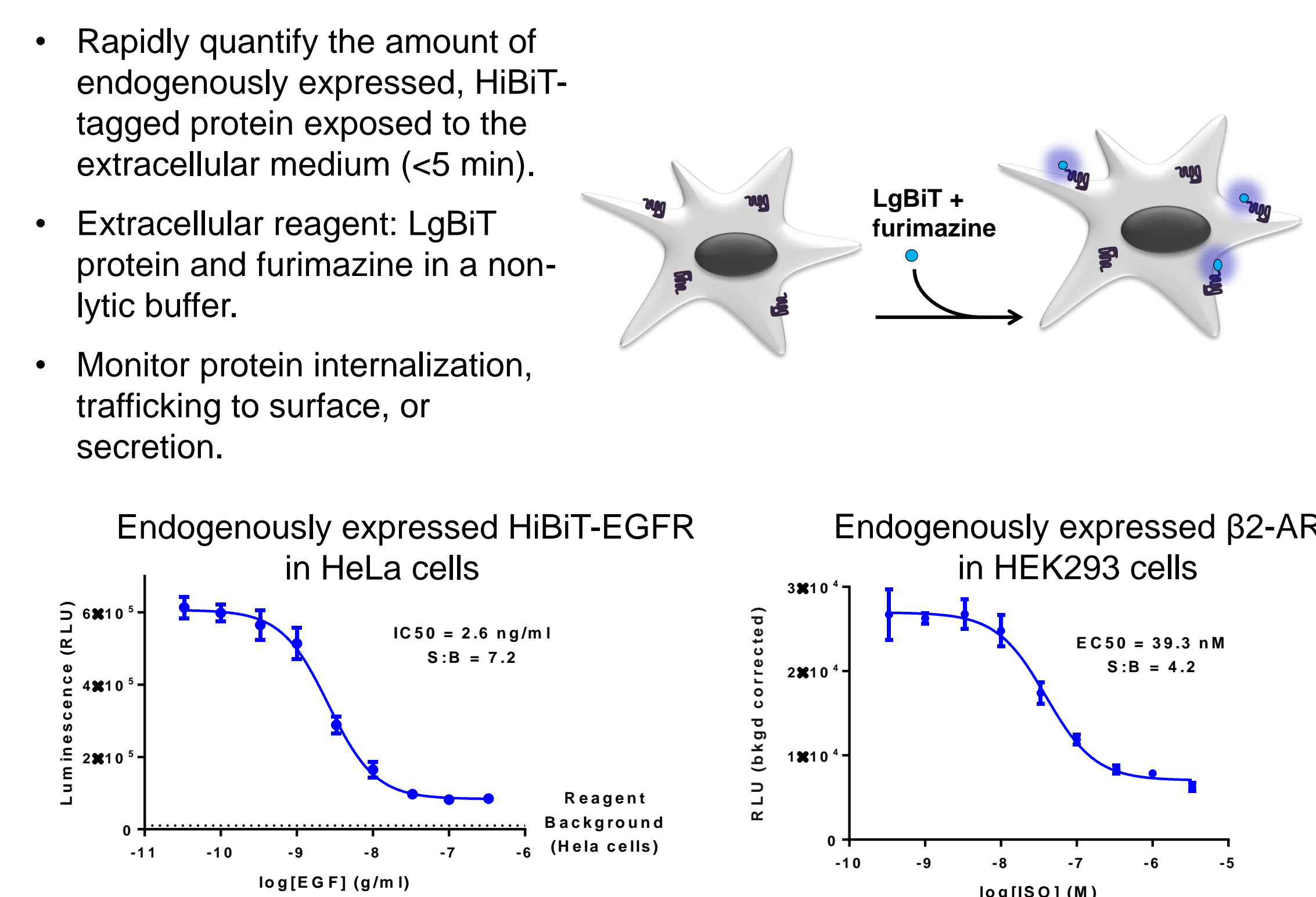
5. Quantify Protein Abundance in Live Cells



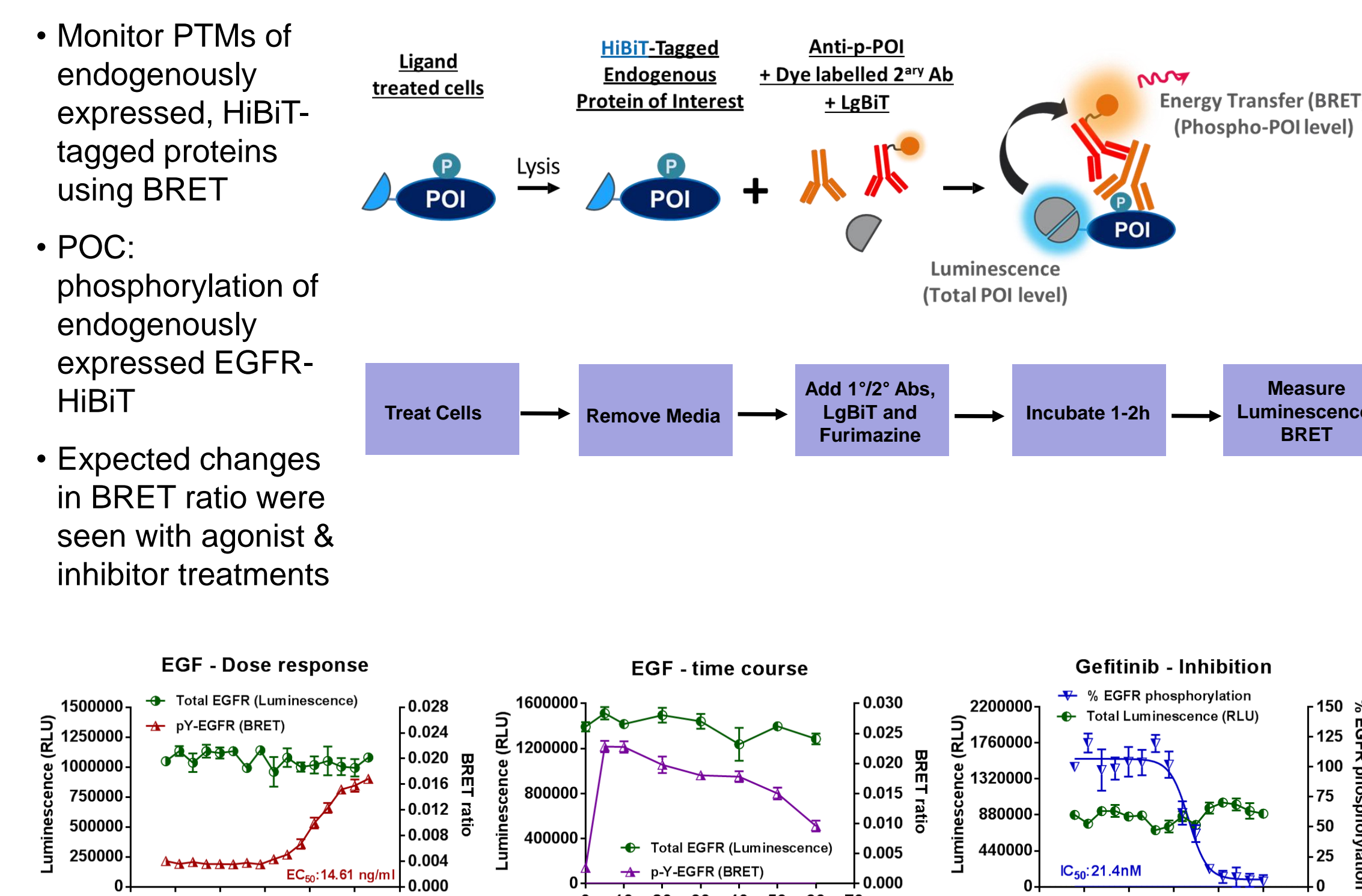
6. Measure Protein-Protein Interactions with NanoBRET



7. Measure Receptor Internalization



8. Measure Post-Translational Modifications



9. Conclusions

Monitor protein dynamics at endogenous levels of expression using HiBiT

- Tag genes at endogenous loci**
 - 11 amino acid size greatly facilitates CRISPR/Cas9 workflow for genomic knock-ins
 - Sub-attomole sensitivity enables measurement of proteins at endogenous levels
- Quantify protein abundance**
 - Homogeneous, add-mix-read assay protocols
 - >7-log linear dynamic range
 - Monitor changes in regulated protein stability or degradation
 - Lytic endpoint or live-cell kinetic assays
- Measure protein-protein interactions in live cells**
 - Expression of LgBiT converts HiBiT into a BRET donor
 - Fusions to HaloTag act as BRET acceptors
- Measure internalization or secretion of HiBiT-tagged proteins**
 - Quantify surface-expressed or secreted HiBiT-tagged proteins in < 4 min
- Measure post-translational modifications**
 - BRET in lysate from HiBiT/LgBiT complex to fluorescently labeled secondary antibody