A Homogeneous Bioluminescent Immunoassay Approach to Interrogate Cellular Signaling Pathway Activation and Deactivation

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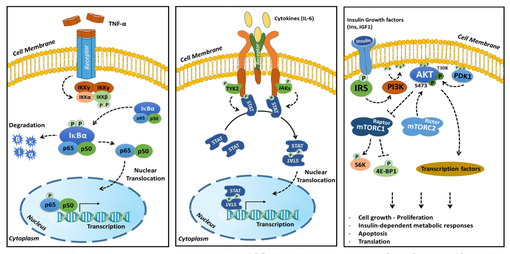
1. Introduction

Cellular responses such as gene expression, enzyme activities, protein synthesis and translocation are orchestrated through activation of diverse cell signaling pathways. In these pathways, phosphorylation of specific proteins by specific kinases constitute important **nodes** by which the signal is transduced from an upstream activation event to downstream cellular responses

Monitoring these signaling events using cell-based methods is essential to better understand normal cell behavior and disease states.

Immunoassays such as ELISA and Western blots are routinely used for protein detection and PTM analysis (e.g phosphorylation). Although sensitive, these methods are tedious, require multiple washing steps, and not easily adaptable to HTS.

Cancer and Inflammatory Response Signaling Pathways

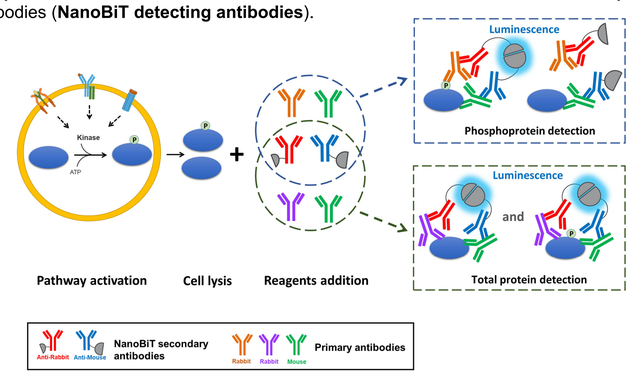


NF-κB **JAK/STAT** **mTOR/PI3K/AKT**

Here we describe the utility of a simple homogeneous bioluminescent cell-based platform to interrogate signaling pathways by detecting phosphorylation of specific proteins.

2. Principle of The Homogeneous Cell-Based Kinase Immunoassay

The bioluminescent cell-based kinase assays are based on NanoLuc® Binary Technology (NanoBiT) two-subunit system (SmBiT; 11 aa peptide and LgBiT; 18 kDa fragment). In this assay, the NanoBiT subunits are fused to an anti-mouse and an anti-rabbit secondary antibodies (**NanoBiT detecting antibodies**).



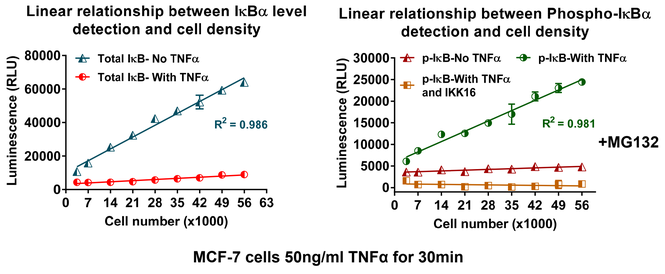
1. Activate pathway
2. Lyse Cells
3. Add antibody mix
4. Incubate 90 minutes
5. Add Nano-Glo reagent
6. Read Luminescence

Pairs of 1° Abs that recognize separate epitopes on a single protein bring NanoBiT-labeled 2° Abs into proximity to form an active NanoLuc luciferase that makes light in proportion to the amount of target protein. When the 1° Ab pair includes a phosphospecific antibody, the luminescence reflects the level of target protein phosphorylation.

3. Linearity and Sensitivity of Bioluminescent Cell-Based Kinase Immunoassays

Activate Pathway → Lyse Cells → Add Antibody Mix → Incubate 1.5hr → Add NanoGlo Reagent → Measure Luminescence

Detection of Total and Phospho IκBα in Different Cell Densities

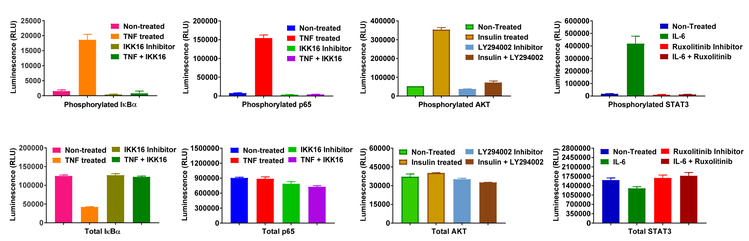


MCF-7 cells 50ng/ml TNFα for 30min

- Bioluminescent detection of IκBα protein and its phosphorylation upon NF-κB pathway activation is linear with increasing cell number.
- Assay is sensitive to detect total and phospho protein levels in low cell density.

4. NanoBiT Cell Based Immunoassays Detect Total and Phosphorylated Target Proteins

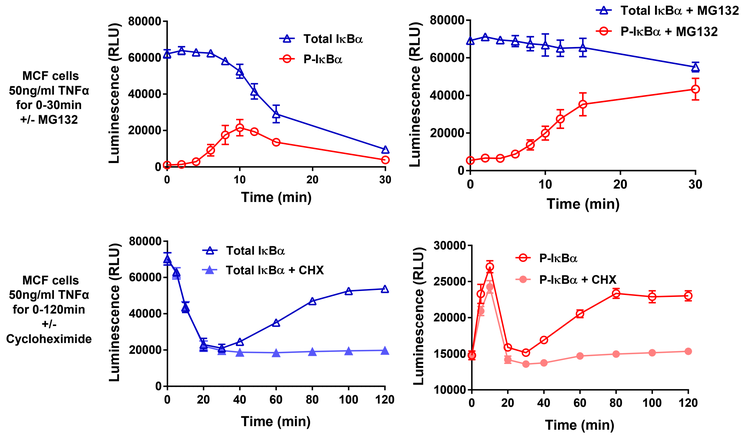
Detection of Total and Phosphorylated targets upon signaling pathway activation and deactivation



- NanoBiT cell based immunoassays reveal the predicted biology of NF-κB, PI3K/mTOR/AKT and JAK/STAT signaling pathways upon ligand mediated activation: Quick **Phosphorylation** of the pathway **nodes** IκBα (S32), P65 (S536), AKT (S473) and STAT3 (Y705).
- Detection of the predicted response of the signaling pathways to **node kinase** inhibitor treatment: IKK complex, PI3K, and JAK2 inhibitors abolish IκBα/P65 , AKT and STAT3 phosphorylations, respectively.
- Except for IκBα which is known to be degraded upon phosphorylation, the total level of the proteins remains unchanged upon ligand or inhibitor treatments.

5. Deciphering NF-κB Pathway Activation Through Total and Phospho IκBα Detection

Detection of Total and Phospho IκBα upon TNF Treatment



- NanoBiT IκBα immunoassay reveals the predicted response of NF-κB pathway to the proteasome inhibitor treatment: decrease in IκBα degradation and accumulation of phosphorylated IκBα.
- Cycloheximide inhibits de novo IκB protein expression in response to long NF-κB pathway activation and the NanoBiT immunoassay can detect easily this event.

6. NanoBiT immunoassays generate data similar to Western blot with faster and easier protocol

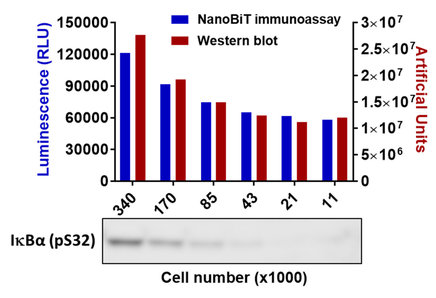
Western blot protocol (Heterogeneous)

Activate cellular pathway | Prepare Cell lysates | Add Loading buffer and Boil | Separate by SDS-PAGE | Transfer proteins to membrane | Block membrane | Wash membrane (3X) | Incubate with 1st antibody | Wash membrane (3X) | Incubate with 2nd antibody | Wash membrane (3X) | Incubate with reagent | Signal reading

NanoBiT immunoassay protocol (Homogeneous)

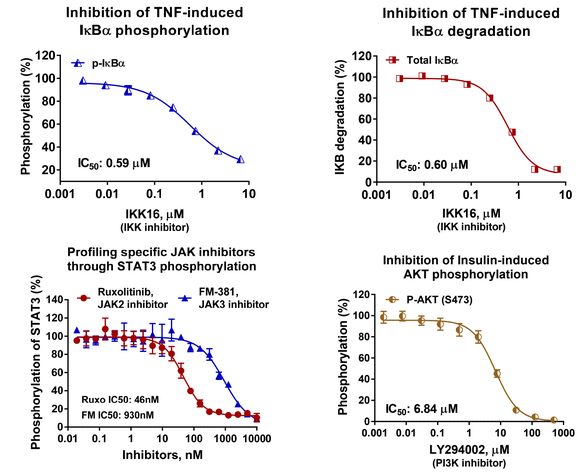
Activate Cellular Pathway | Add Lysis Buffer to Cells | Incubate and Shake for 20 min | Add Antibody Mix | Incubate for 90 min | Add Nano-Glo Reagent | Luminescent Signal Reading

Comparison of NanoBiT immunoassay and Western in detecting phospho IκBα



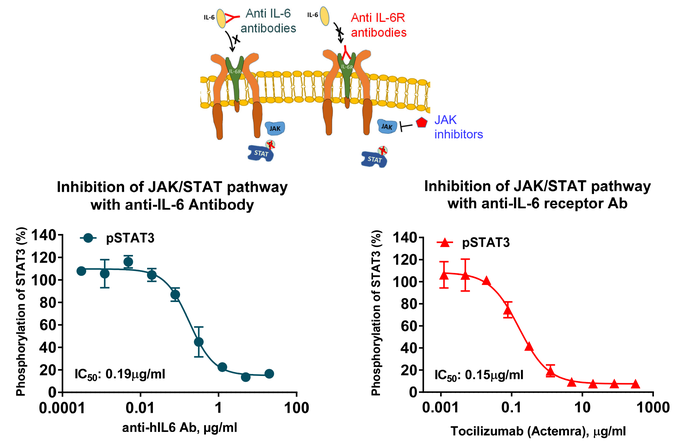
- Homogeneous NanoBiT Cell based immunoassays are easier and quicker than traditional Western to generate the same data.

7. Detection of Small Molecule Inhibition of Pathway Node Kinases With NanoBiT Immunoassays



- The bioluminescent NanoBiT pathway assays reveal the expected pharmacology of each pathway **node kinase** inhibitor.
- The cell-based immunoassays can be used to screen inhibitors of cancer, immune and inflammatory response pathways in fast and homogeneous fashion.

8. Pathway Modulation with Large Molecules Detected with NanoBiT Kinase Immunoassays



- Bioluminescent NanoBiT kinase immunoassays can be used to identify small or large molecule inhibitors of signaling pathways.
- Pathway node phosphorylation can be used as a reporter for biologics activity at the receptor level.

9. Conclusions

Benefits of the bioluminescent cell-based NanoBiT kinase immunoassays:

- **Bioluminescent,** less interference from chemical compounds
- **Homogeneous,** "Add and Read" format
- **No cell engineering required,** detection of endogenous substrates phosphorylation
- **No special instrument or plate requirement.** Only a luminometer is required
- **Less complex,** quicker with less steps than Western, ELISA, or fluorescent based technologies
- **Amenable to HTS formatting**
- **"Do It Yourself" format**, the NanoBiT detecting antibodies can be adapted to any pathway of interest

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