Monitoring intracellular protein interactions using NanoLuc® Binary Technology (NanoBiT™)

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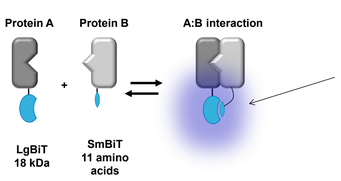
1. Introduction

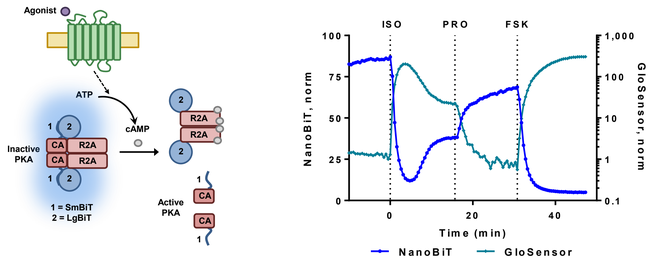
Protein:protein interactions (PPIs) are essential to the cellular signal transduction pathways that contribute to cancer. Although numerous approaches exist to monitor PPIs *in vitro*, methods for intracellular detection have been more limited. We developed NanoLuc® Binary Technology (NanoBiT), a two-subunit system based on NanoLuc® luciferase that can be applied to the intracellular detection of PPIs. Large BiT (LgBiT; 18 kDa) and Small BiT (SmBiT; 11 amino acid peptide) subunits are expressed as fusions to proteins of interest, where PPI facilitates subunit complementation to give a bright, luminescent enzyme. Unlike related approaches where an enzyme or protein is simply split, LgBiT was independently optimized for structural stability and SmBiT was selected from a peptide library specifically for the PPI application. The result is a subunit pair that weakly associates (K_D = 190 µM) yet still maintains 30% of the activity of full-length NanoLuc at saturation. In contrast to many split systems, the LgBiT:SmBiT interaction is reversible, allowing the detection of rapidly dissociating proteins. PPI dynamics can be followed in real-time in living cells using the Nano-Glo® Live Cell Reagent, a non-lytic detection reagent containing the cell-permeable furimazine substrate. Advantages over split systems include better sensitivity, reversibility, fusion to a peptide or a small, structurally stable protein domain, real-time measurements using a non-lytic assay format, and subunits with reduced affinity for self-association.

2. NanoBiT overview



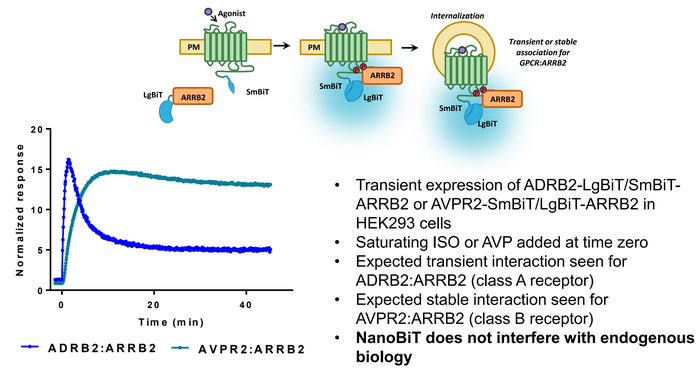
- LgBiT and SmBiT are fused to proteins A & B
- A:B interaction facilitates LgBiT:SmBiT interaction, generating a bright luminescent enzyme
- LgBiT:SmBiT with low affinity (K_D = 190 µM), limiting non-specific association and reducing assay background
- LgBiT:SmBiT interaction is reversible (kon = 500 $M^{-1}sec^{-1}$; koff = 0.2 sec^{-1})
- LgBiT evolved for increased structural stability making it a better fusion partner
- Non-lytic assay format allows real-time measurements of protein interaction dynamics for 1-2 hrs

3. Real time kinetics of PPI interaction dynamics



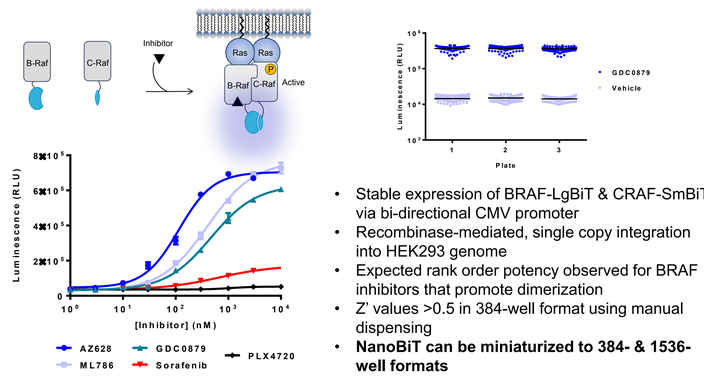
- Transient expression of SmBiT-PRKACA & LgBiT-PRKAR2A in HEK293
- Modulators of intracellular cAMP added sequentially at indicated time points
- Inverse correlation for NanoBiT vs. GloSensor cAMP 22F (cAMP biosensor)
- **NanoBiT can monitor reversible PPIs in real time**

4. GPCR interaction with β-arrestin-2



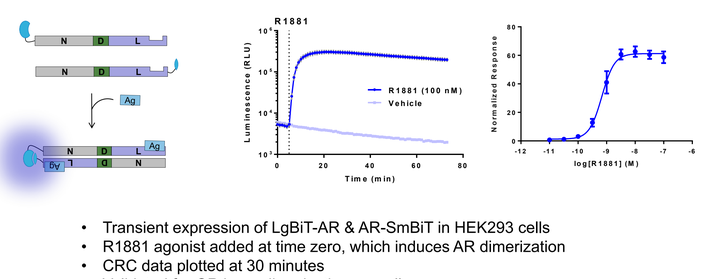
- Transient expression of ADRB2-LgBiT/SmBiT-ARRB2 or AVPR2-SmBiT/LgBiT-ARRB2 in HEK293 cells
- Saturating ISO or AVP added at time zero
- Expected transient interaction seen for ADRB2:ARRB2 (class A receptor)
- Expected stable interaction seen for AVPR2:ARRB2 (class B receptor)
- **NanoBiT does not interfere with endogenous biology**

5. RAF dimerization using stable expression



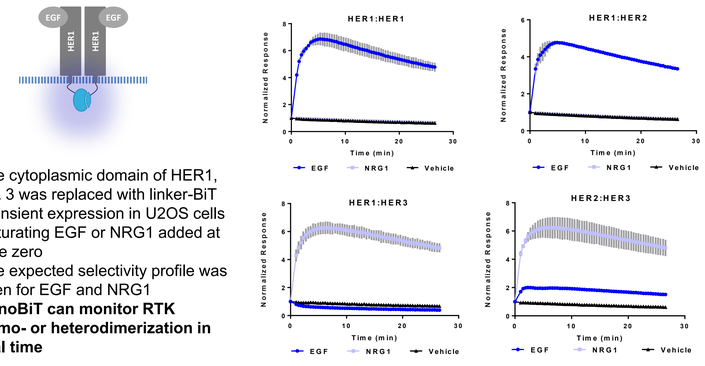
- Stable expression of BRAF-LgBiT & CRAF-SmBiT via bi-directional CMV promoter
- Recombinase-mediated, single copy integration into HEK293 genome
- Expected rank order potency observed for BRAF inhibitors that promote dimerization
- Z' values >0.5 in 384-well format using manual dispensing
- **NanoBiT can be miniaturized to 384- & 1536-well formats**

6. Nuclear hormone receptor dimerization



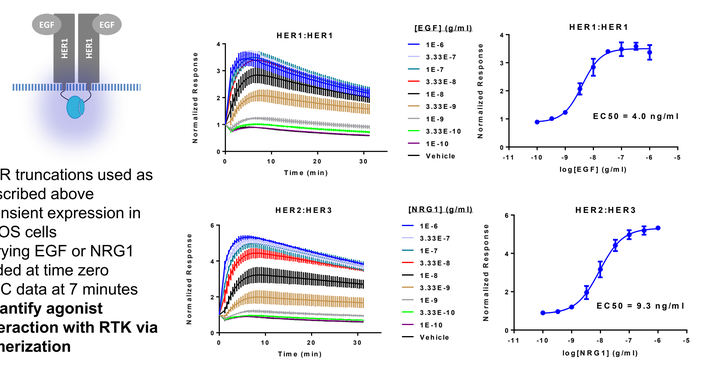
- Transient expression of LgBiT-AR & AR-SmBiT in HEK293 cells
- R1881 agonist added at time zero, which induces AR dimerization
- CRC data plotted at 30 minutes
- Validated for GR homodimerization as well
- **NanoBiT can monitor nuclear hormone receptor dimerization in real time**

7. Agonist-induced RTK dimerization



- The cytoplasmic domain of HER1, 2 & 3 was replaced with linker-BiT
- Transient expression in U2OS cells
- Saturating EGF or NRG1 added at time zero
- The expected selectivity profile was seen for EGF and NRG1
- **NanoBiT can monitor RTK homo- or heterodimerization in real time**

7. Quantifying agonist binding via dimerization



- HER truncations used as described above
- Transient expression in U2OS cells
- Varying EGF or NRG1 added at time zero
- CRC data at 7 minutes
- **Quantify agonist interaction with RTK via dimerization**

9. Summary

NanoBiT is extremely bright
- Fusion partners can be expressed at very low levels, minimizing potential artifacts
- 100-1,000 fold brighter than split firefly luciferase

NanoBiT components are small & stable
- LgBiT, 18 kDa; SmBiT, 11 amino acids
- LgBiT evolved for increased structural stability, providing a stable fusion partner

NanoBiT is reversible
- Monitor both protein association and dissociation events in real time

NanoBiT offers experimental flexibility
- Monitor protein interaction dynamics at a single time point or continuously for 1-2 hours
- Room temperature or 37 °C measurements
- Validated in 96-, 384- & 1536-well formats

Check http://www.promega.com/nanobit for new NanoBiT PPI expression vectors

For more information on NanoBiT, please see *ACS Chemical Biology*, 11(2), p. 400-408

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