NanoBRET Approach for Characterizing the Impact of RAS Pathway Inhibitors on Protein Interactions in Live Cells

Kristin M. Riching, Jennifer Serriere, Amy Landreman , Nancy Murphy, and Marjeta Urh

Corporation, 2800 Woods Hollow Rd, Madison, WI, 53711

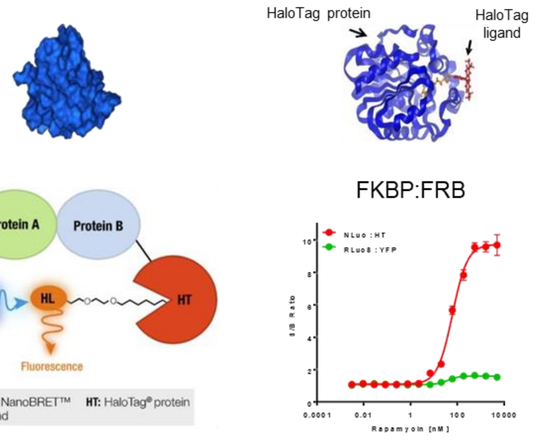
1. Introduction

The Ras family of proteins is frequently altered in many human cancers and is a significant driver of disease. Drugging this pathway has historically been very challenging due to lack of suitable binding pockets and significant pathway cross- talk, often contributing to poor patient response. The recent identification of novel Ras inhibitors has spurred renewed interest in this pathway for development of novel agents for treatment. Of vital importance to this goal is the availability of suitable assays with which to reliably assess disruption of Ras pathway protein:protein interactions in living cells. We show here the application of NanoBRET technology to study Ras pathway interactions and successful modulation with various commercially available inhibitors. NanoBRET is a proximity-based assay that can detect protein interactions by measuring energy transfer from a fusion protein with a bioluminescent donor, NanoLuc, to a second fusion protein with a fluorescent acceptor, HaloTag. The optimized blue-shifted NanoLuc donor paired with the red-shifted HaloTag acceptor minimizes spectral overlap within the assay, resulting in improved signal:background when calculating the NanoBRET ratio. Furthermore, the NanoBRET ratio is independent of cell number, and similar to other ratiometric assays, shows low variability and high reproducibility. Using NanoBRET, we demonstrate the ability to monitor changes in dynamic protein interactions within the Ras pathway, including induction or inhibition with various inhibitors and treatments, and the capability to monitor kinetics in live cells. We further highlight the therapeutic potential in expansion of Ras inhibitors to degraders using the KRas(G12C)-specific LC-2 PROTAC. Taken together, NanoBRET is a powerful live-cell assay for use in small-molecule screening and can be used to advance programs aimed at identifying novel Ras targeting agents.

2. NanoBRET for Measuring Protein:Protein Interactions (PPIs) in Live Cells

NanoLuc Luciferase

- 19kD, monomeric fusion partner
- ~150X brighter than other luciferases



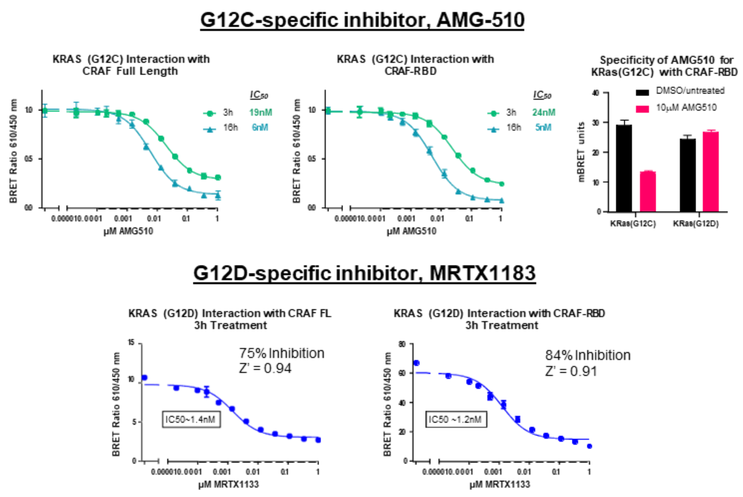
Key characteristics

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

3. Studying Activation of Ras Pathway PPIs

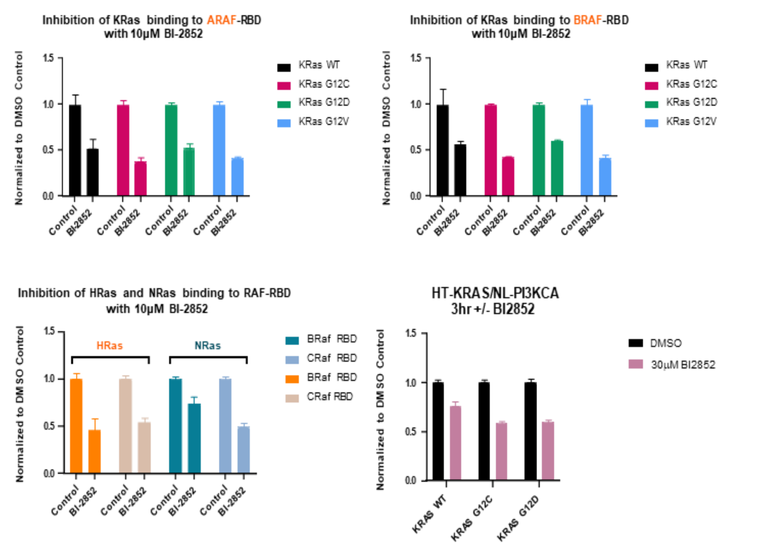
- KRas:CRAF interaction rapidly increases upon EGF stimulation
- Extended kinetics show decline toward basal levels within 60 minutes, consistent with transient RTK signaling.
- RAF homo- and heterodimers increase in response to GDC0879 or overexpressed KRas (G12C).

4. Disruption of KRas:CRAF Interaction with G12C and G12D Specific Inhibitors



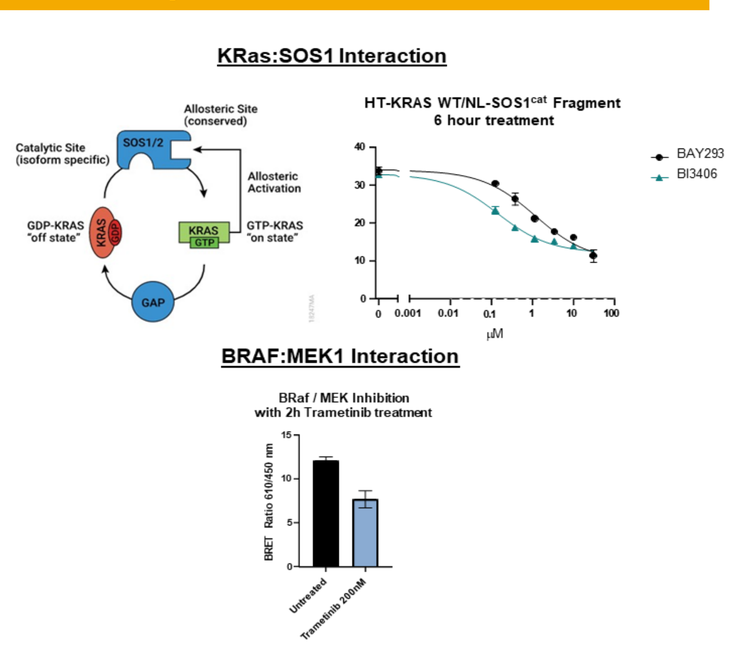
- KRas(G12C):CRAF full length and RBD (Ras Binding Domain) interactions are potently disrupted with AMG-510 with near complete inhibition after 16 hours (top panel). Kras(G12D):CRAF full length and RBD interactions are potently disrupted with MRTX1183 (bottom panel).
- AMG-510 shows expected specificity for G12C over G12D
- Similar IC50 values and maximal inhibition observed with full length CRAF and RBD
- Assays show excellent reproducibility, CVs and Z' factors for screening

5. Pan-Inhibition of Ras Isoforms and Downstream Effector Binding with BI-2852



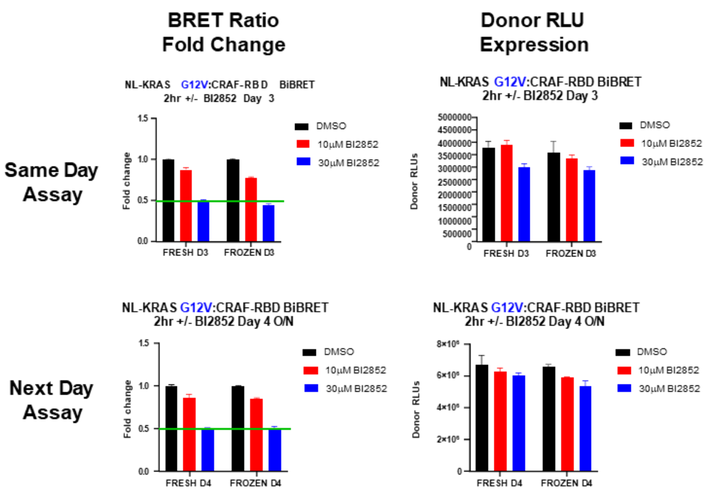
- BI-2852 results in inhibition of KRas, NRas and HRas isoforms for binding to Raf-RBDs
- Similar inhibition of KRas WT and G12 mutants observed for both ARAF, BRAF, and CRAF (CRAF data not shown)
- Additional disruption of effector binding in the PI3K signaling axis observed

6. Targeting Other Interactions in the Ras Pathway: KRas:SOS1 and BRAF:MEK



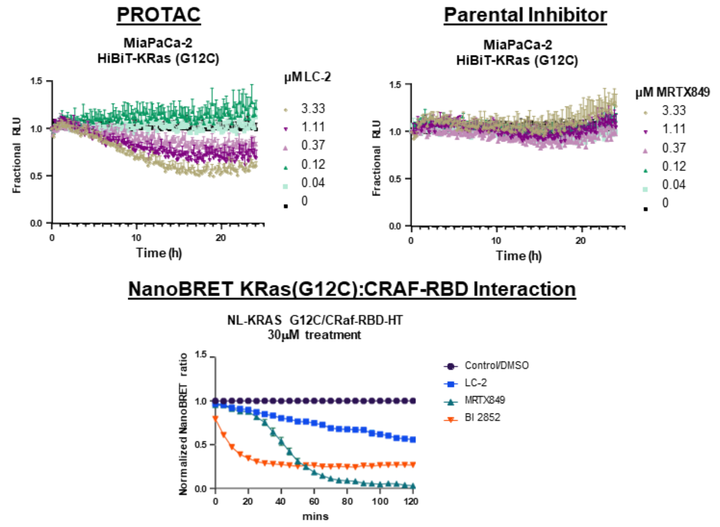
- SOS1 inhibitors BI3406 and BAY293 show dose-dependent disruption of KRas binding to the catalytic domain of SOS1 following a 6-hour treatment.
- BRAF:MEK1 interaction shows inhibition with 200nM Trametinib following a 2-hour treatment.

7. "Thaw & Use" Cells Provide Excellent Assay Reproducibility and Ease of Use



- Frozen transfected cells with KRas (G12V) and CRAF-RBD compared to fresh, unfrozen cells show identical assay performance, similar donor RLU expression levels, and fractional inhibition with BI-2852.
- Assay can be run the same day after thaw, or next day after allowing cells to adhere overnight
- Excellent batch to batch reproducibility and assay robustness

8. Degradation of Endogenous KRas(G12C) and Disruption of PPI with LC-2 PROTAC



- KRas(G12C) endogenously tagged with HiBiT (11aa peptide) in MiaPaCa-2 cells with overexpressed LgBiT shows kinetic and dose-dependent degradation with LC-2 PROTAC degrader compared to MRTX849 covalent parental inhibitor
- Investigation of kinetics of Kras(G12C):CRAF-RBD inhibition reveals slower inhibition of MRTX849 and even slower for LC-2 compared to BI2852, suggesting potentially reduced intracellular permeability.

9. Conclusions

We have developed a suite of NanoBRET assays within the Ras signaling network, which enable rapid identification and screening of new candidate therapeutic inhibitors of Ras pathway signaling.

NanoBRET Technology:

- Recapitulates the native biology of protein interactions
- IC_{50} values of commercial Ras pathway inhibitors similar to those reported in literature
- Enables monitoring of dynamic pathway interactions and signaling mechanisms in live cells
- Amenable to kinetic monitoring
- Ratiometric and high assay robustness – advantageous for screening

Corresponding author: kristin.riching@promega.com