Measuring Intracellular Target Engagement via BRET with Stable Cell Lines

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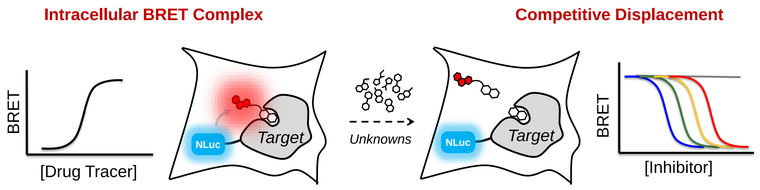
Abstract # 4025

1. Introduction

Acquiring cell-derived information on compound cell permeability, equilibrium binding affinity, and kinetic binding rates can be challenging. We present a bioluminescence energy transfer (BRET) target engagement method to directly measure these parameters within intact mammalian cells. In its simplest form, the assay relies on the cellular expression of the target protein fused to a very bright luciferase (NanoLuc); a cell-permeable fluorescent drug tracer that specifically binds to the target protein; and a substrate for NanoLuc. This BRET-based method has been applied to multiple key drug target classes including HDACs, bromodomains and kinases. Here we'll demonstrate applications of this technology for monitoring HDAC target engagement using transient transfection and cell lines stably expressing NanoLuc-HDAC fusion proteins. This BRET-based method significantly increases the type of data (permeability, equilibrium affinity, and residence time) that can be achieved in a cellular context. Furthermore, by generating stable cell lines expressing a Nanoluc-target fusion protein, the work flows for these assays are enhanced.

2. Measuring Intracellular Compound Permeability and Equilibrium Affinity with NanoBRET

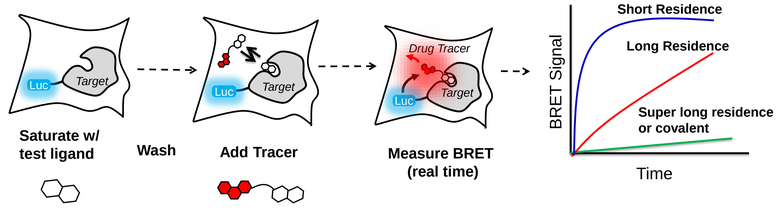
NanoBRET Target Engagement Configuration



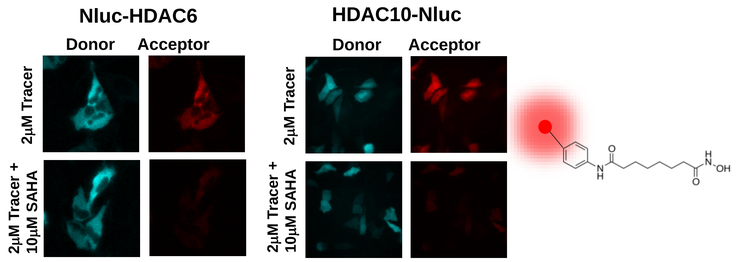
- **Ultra-specific:** Specificity governed by tight distance constraints of BRET (<5nM)
- **Physiologic Intracellular Measurement:** Achieved with intense bioluminescence emitted by NanoLuc donor plus optimized acceptor dye properties of tracer (spectral resolution)
- **Equilibrium Binding Analysis**: Allows assessment of compound permeability & affinity

3. Measuring Intracellular Compound Residence Time Using NanoBRET

Fast Binding Tracers & Long Signal Half-Life for NanoLuc: Allows real-time monitoring of binding kinetics and drug-target residence time.

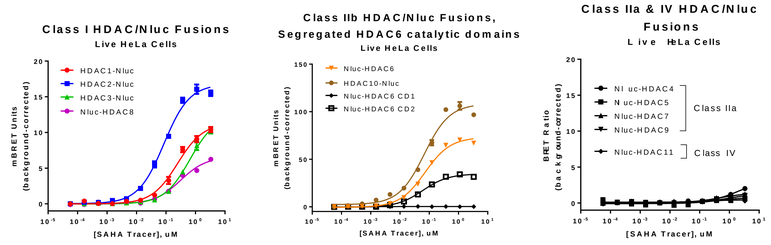


4. NanoBRET HDAC Tracer Permeates Live Cells and Reversibly Forms NanoBRET Complexes



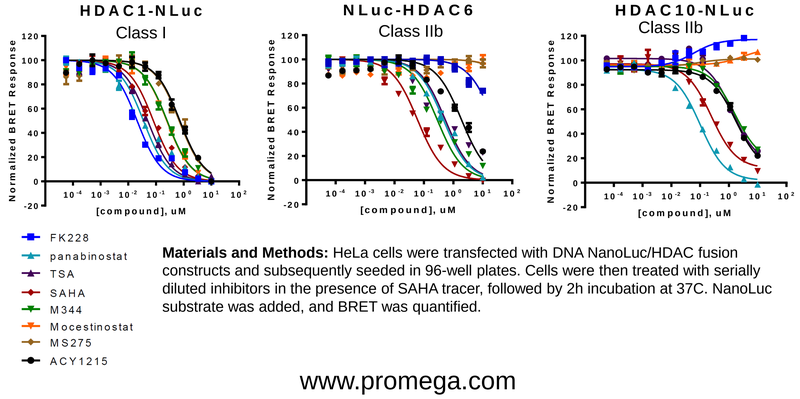
Materials and Methods: HeLa cells were transfected with HDAC-Nluc DNA constructs. Cells were seeded onto Ibidi Imaging dishes. Cells were treated with SAHA tracer in the presence or absence of 10µM SAHA, followed by a 2h incubation at 37C. NanoLuc substrate was added prior to imaging on an Olympus LV200 Microscope equipped with 460/80BP and 610LP filter sets. Image J used for analysis.

5. Class I and IIb HDACs Selective Engage the SAHA NanoBRET Tracer



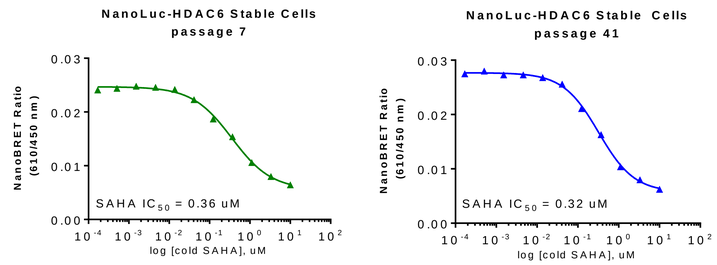
Materials and Methods: HeLa cells were transfected with DNA/FuGENE® HD complexes, using NanoLuc/HDAC fusion constructs. Twenty-four hours post-transfection, cells were seeded into white 96-well plate and then incubated for 2h at 37C with serially diluted HDAC tracer w/ or w/o 20µM SAHA. NanoLuc substrate was added, and BRET was quantified on a Clariostar luminometer equipped with 610LP (acceptor) and 450/50BP (donor) filters. BRET ratios of samples in the presence of 20µM SAHA were used to background-correct for non-specific BRET signals. mBRET units are raw BRET ratio values x 1000.

6. Profiling HDAC Inhibitor Affinities Against Class I and IIb HDACs in Cells



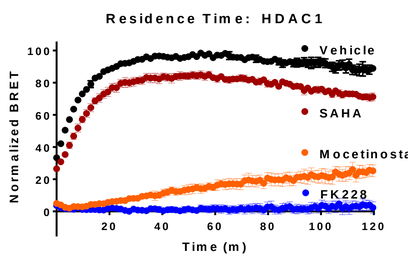
Materials and Methods: HeLa cells were transfected with DNA NanoLuc/HDAC fusion constructs and subsequently seeded in 96-well plates. Cells were then treated with serially diluted inhibitors in the presence of SAHA tracer, followed by 2h incubation at 37C. NanoLuc substrate was added, and BRET was quantified.

7. Stable NanoLuc-HDAC Expressing Cells Maintain NanoBRET Assay Reproducibility after >30 Passages



Materials and Methods: HeK293 parental cells engineered for single site integration were transfected with DNA for NanoLuc/HDAC 6 fusion with a PGK promoter. Antibiotic selected pools of cells were produced, passaged, and aliquots frozen back. Cells from two different passages were subsequently thawed and tested side-by-side in the NanoBRET HDAC target engagement assay.

8. NanoBRET Intracellular Residence Time Analysis Reveals Differences in Inhibitor Dissociation Rates



- SAHA has fast dissociation kinetics relative to Mocetinostat, as expected
- Pro-drug FK228 displayed very slow dissociation kinetics, providing a possible explanation for its prolonged functional effect

Materials and Methods: HeLa cells expressing HDAC1-Nluc were equilibrated with test ligand for 3 hours. Test ligands were separated from cells via centrifugation. BRET reagents were immediately added and BRET monitored in real-time on a Thermo Varioskan Luminometer, equipped similarly as described above.

9. Conclusions

NanoBRET enables direct measure of target engagement in live cells
- Compound permeability and affinity can be assessed
- Real-time monitoring of binding can be achieved

NanoBRET can be used to measure intracellular drug-target residence times
- This is the first method to measure intracellular drug-target residence time

Nanoluc-target fusion proteins can be expressed in cells via transient or stable methods
- Transient expression enables rapid testing and profiling of target engagement
- Stable expression allows work-flow simplifications that may be useful for high-throughput applications

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