Real-time Monitoring of PROTAC and Molecular Glue Targeted Degradation in Living Cells

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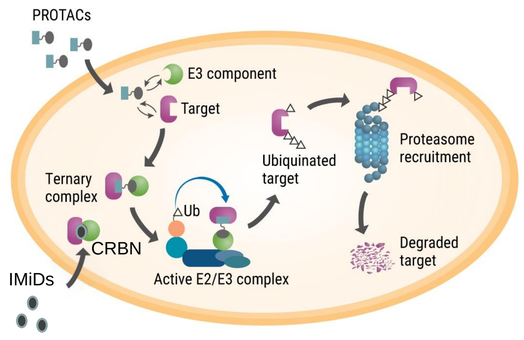
1. Introduction

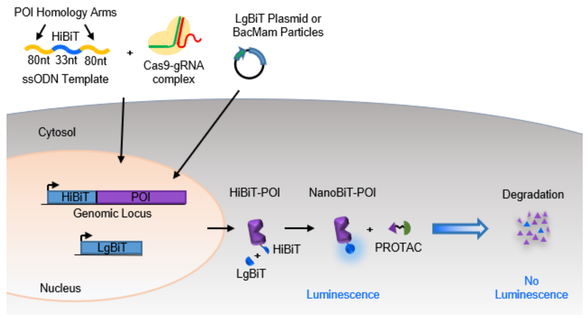
The emergence of targeted protein degradation as a broad, new therapeutic modality has greatly expanded the opportunities for treatments of many diseases. Currently, small molecule degrader compounds fall under two major categories; molecular glues and heterobifunctional PROTACs. The two types of compounds both facilitate and induce a ternary complex consisting of an E3 ligase-degrader-target protein, bringing into proximity the machinery proteins required to ubiquitinate and ultimately degrade the target protein. Significant challenges exist in characterization and rank-ordering of degradation compounds in live cells given the differences in dynamics of protein loss and recovery among compounds. Currently, the availability of technologies to interrogate real-time protein degradation is severely lacking. Here, we present a live-cell, luminescence-based technology platform with these capabilities. We use CRISPR/Cas9 endogenous tagging of target proteins with the small peptide, HiBiT, which has high affinity for and can complement with the LgBiT protein to produce NanoBiT luminescence. This allows for sensitive detection of endogenous protein levels in living cells and can also serve as a BRET energy donor to study protein:protein or protein:small molecule interactions. We demonstrate the power of this technology in continuous 24-hour monitoring of endogenous target protein levels, and the ability to quantify key degradation parameters for compound ranking including rate, Dmax, and Dmax50. We further show the ability to measure the mechanistic steps important for the degradation including kinetics of PROTAC- or molecular glue-induced ternary complex formation and target ubiquitination. These studies facilitate discernment of individual parameters required for successful degradation, ultimately enabling chemical design strategies for optimization and rank ordering of therapeutic degradation compounds.

2. Targeted Protein Degraders: PROTACs and Molecular Glues

Hijacking the UPS with PROTACs and Molecular Glues

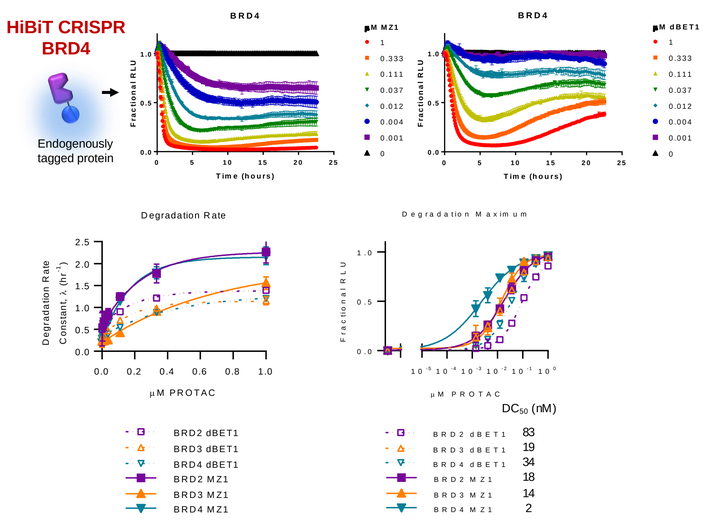


HiBiT-CRISPR Tagging Strategy and Experimental Approach



3. PROTAC-Induced Live Cell Degradation Kinetics

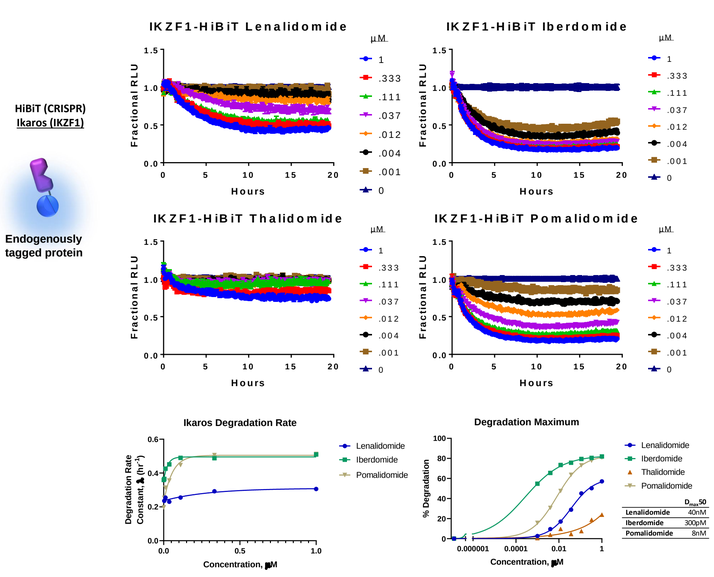
HiBiT Endogenous Tagging and Kinetic Degradation of BRD4



- BET family members were endogenously tagged using CRISRP/CAS9 with 11AA HiBiT fusion tag
- Complete cellular degradation profiles determined with continual luminescent reads on GloMax Discover
- Degradation rate, Dmax, and $Dmax_{50}$ determined to rank compounds and BET family members

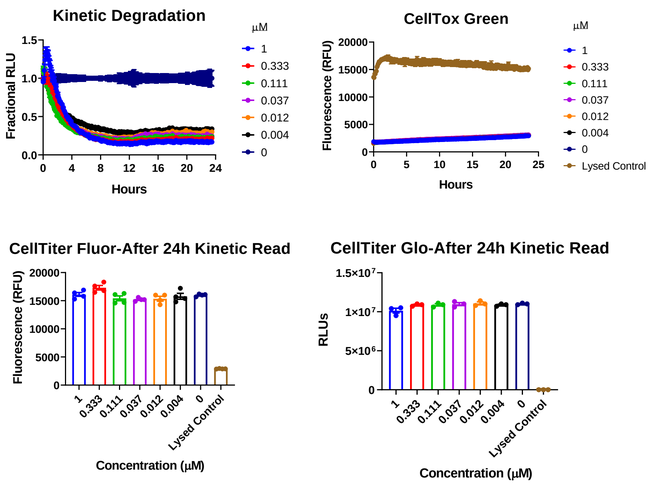
4. Molecular Glue-Induced Live Cell Degradation Kinetics

HiBiT Endogenous Tagging and Kinetic Degradation of Ikaros



- Observe degradation of molecular glue targets and can rank compounds by degradation rate, Dmax, and $Dmax_{50}$
- Iberdomide shows most potent degradation
- Can use for counter-screening PROTACs that have a glue handle

5. Multiplexing Ikaros Degradation by Iberdomide with Cell Viability

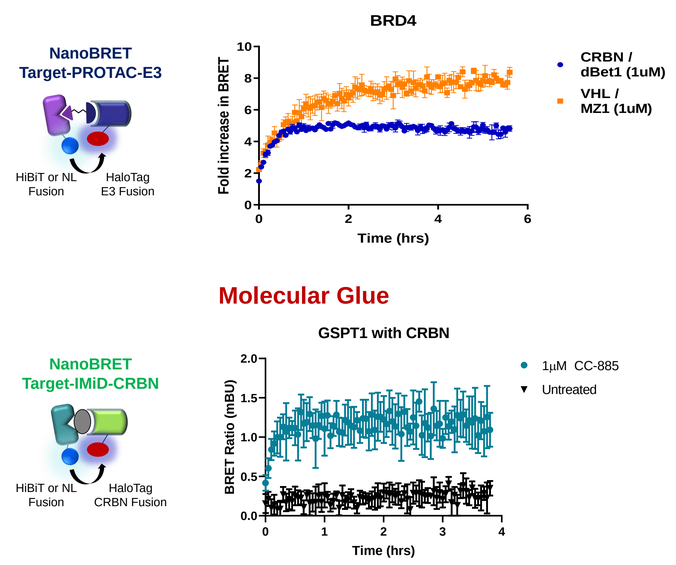


- Confirm loss of signal (degradation) is not due to impacts on viability
- Kinetics can help distinguish target degradation that may later lead to toxicity
- Different viability assays to measure different stages of toxicity

1. **Kinetic Multiplex:**
   - CellTox-Green: membrane integrity
2. **Endpoint Multiplex:**
   - CellTiter-Fluor: active cellular proteases
   - CellTiter-Glo: cellular ATP levels

6. Live Cell NanoBRET Ternary Complex Kinetics with PROTACs and Glues

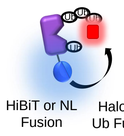
PROTAC



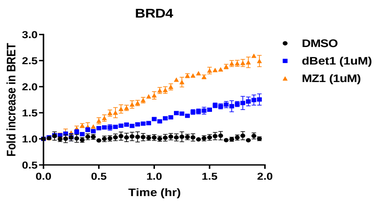
- Monitor ternary complex formation and degradation simultaneously in a single NanoBRET assay
- Use endogenous HiBiT-fused target protein, or ectopic expression
- Use of MG132 can increase signal window
- Kinetic analysis allows for understanding of cellular ternary complex stability

7. Live-Cell NanoBRET Ubiquitination Kinetics with PROTACs and Glues

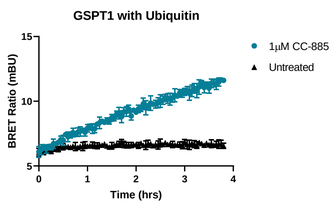
NanoBRET Target Ubiquitination



PROTAC

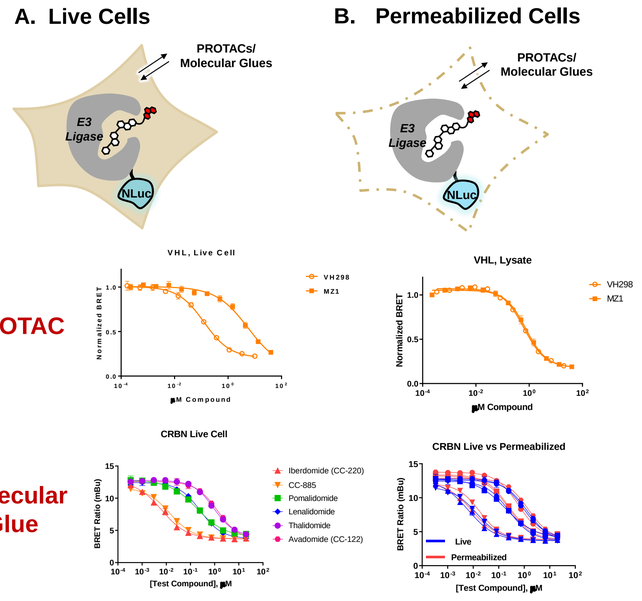


Molecular Glue



- Monitor target ubiquitination kinetics induced by PROTACs or molecular glues.
- Use endogenous HiBiT-fused target protein, or ectopic expression
- Differential target ubiquitination observed with compounds of different potency and E3 recruitment
- Ubiquitination levels correlate with degradation rate

8. NanoBRET E3 Ligase Binding Affinity and Permeability of PROTACs and Glues



- Run NanoBRET Target Engagement in live or permeabilized cells (A & B), enabling assessment of compound affinity and permeability.
- MZ1 binds to VHL with the same affinity (lysate) but has reduced permeability (live cell) compared to monovalent VH298.
- Panel of molecular glues shows range in binding affinities to CRBN, and all are readily cell-permeable (live vs permeabilized).

9. Conclusions

Differentiating cellular technologies to study key processes in PROTAC and Molecular Glue-mediated degradation for more rapid profiling of compounds

HiBiT and NanoLuc technology:

- Live cell kinetic degradation
- Amenable for use with CRISPR to study endogenous proteins
- Allows for quantitation of key degradation parameters

NanoBRET technology:

- Monitoring dynamic pathway interactions and signaling mechanisms in live cells
- Useful for assessment of PROTAC cellular permeability
- Follow induced interactions with E3 ligase components and target ubiquitination
- Provides mechanistic understanding of degradation kinetics

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NanoBRET™ in Live Cells as a Method to Assess E3 Ligase and Target Protein Occupancy for PROTACs

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1. Introduction

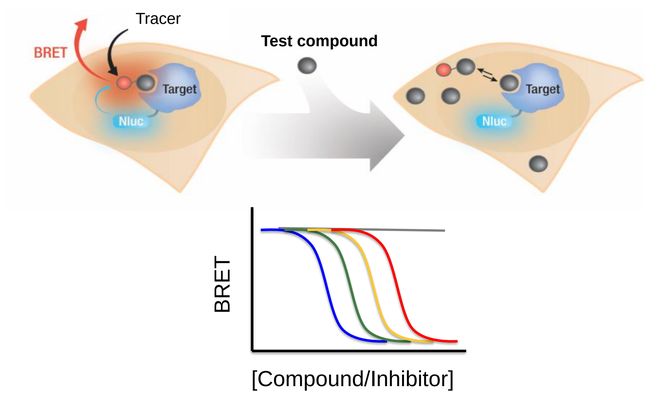
Proteolysis targeting chimeras (PROTACs) are bifunctional molecules that hijack ubiquitin E3 ligases and induce degradation of intracellular proteins through a tightly regulated proteasomal mechanism. Although several successful PROTACs have been developed against key intracellular target classes including bromodomains, kinases, and nuclear hormone receptors, these bivalent molecules often suffer from poor cell permeability due to high molecular weight.

To enable a high-throughput, quantitative readout for PROTAC cell permeability and E3 ligase occupancy in living cells, we have developed a panel of NanoBRET™ target engagement (TE) assays for key E3 ligase including CRBN, VHL, XIAP, cIAP, and MDM2. NanoBRET target engagement (TE) intracellular assays are the first biophysical method to enable the quantitative determination of compound occupancy, potency, and residence time for specific target proteins inside living cells using bioluminescent resonance energy transfer (BRET). This method has previously been applied to several other protein classes including kinases, bromodomains, and HDACs.

Here, we demonstrate that the NanoBRET platform allowed assessment of PROTAC cell permeability & E3 ligase occupancy. Using NanoBRET TE assays for proteins targeted by the E3 ligases, we obtained intracellular PROTAC binding kinetics. We further extend the analysis of PROTAC permeability as a dynamic process in real-time, using BRD-targeting MZ1 and dBET1 as a model system. Together these approaches allow a mechanistic interrogation of intracellular PROTAC permeability, E3 ligase occupancy, target occupancy, and target residence time.

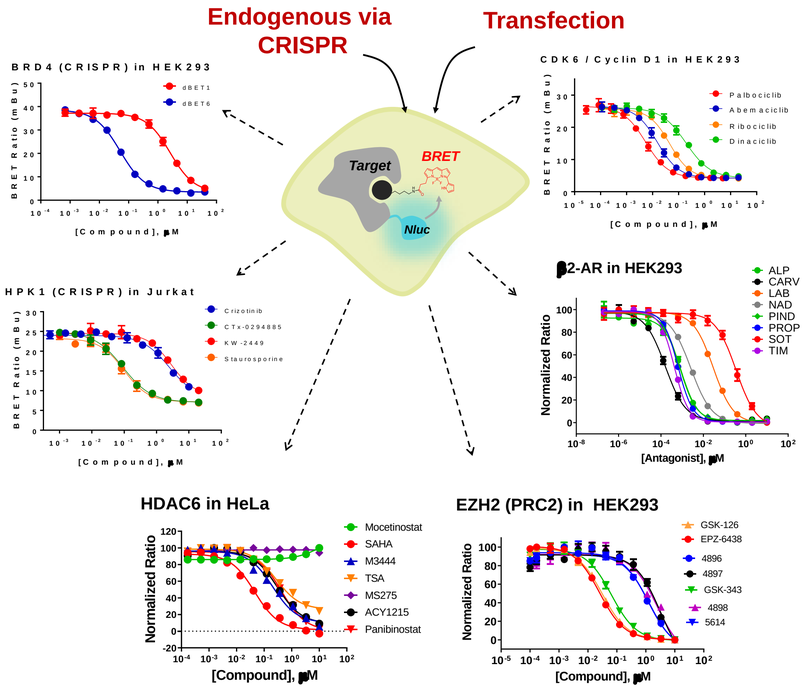
2. Target Engagement (TE) using BRET

Affinity / Potency Determinations



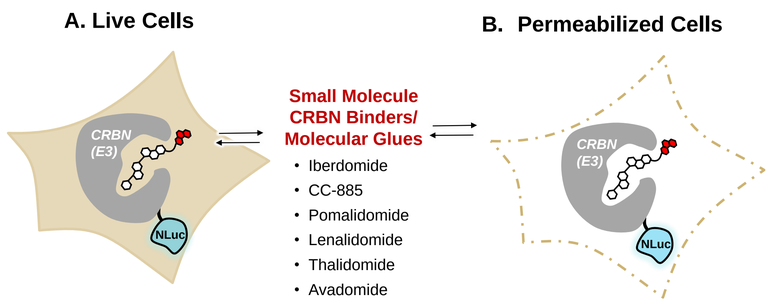
- BRET is achieved by the luminescent energy transfer from NanoLuc® luciferase to the fluorescent tracer that is bound to the target protein-luciferase fusion protein.
- The NanoBRET assay is specific for the target fused to NanoLuc, since BRET assays are governed by tight distance constraints between energy donor (NanoLuc) and energy acceptor (tracer).
- NanoBRET assays are conducted in live cells that allow equilibrium binding analysis and real time binding analysis.

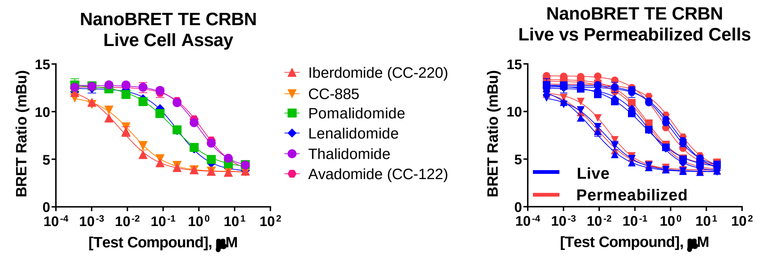
3. NanoBRET Target Engagement is Applicable to Multiple Target Classes



NanoLuc-target fusion gene delivery is most often done via transfection for transient or stable expression. Additionally, fusion genes can be introduced to cells by CRISPR editing or viral particles.

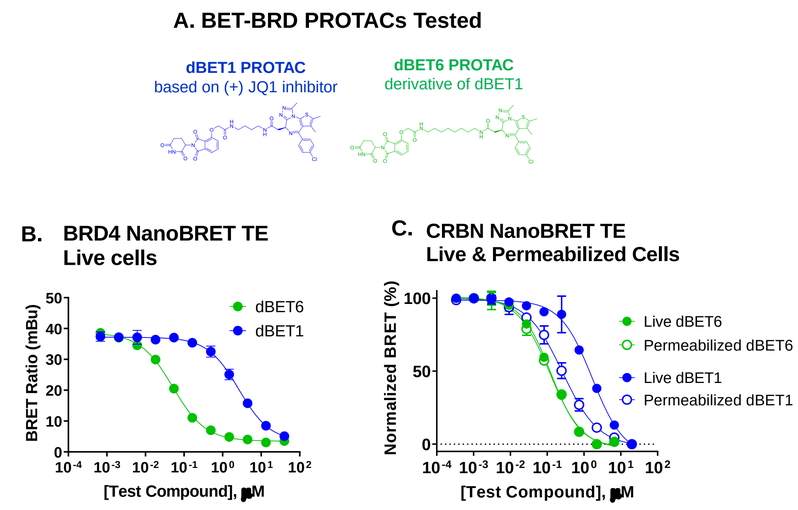
4. Assess Cellular Binding & Permeability of Small Molecules to CRBN





- NanoBRET TE CRBN assay can be run in live or permeabilized cells (A & B), enabling assessment of compound intracellular affinity and permeability.
- The live cell assay was used to quantify the apparent intracellular affinity of a series of CRBN binders that are also molecular glues (A).
- Using permeabilized cells (B), there was little change in IC_{50} observed compared to when the assay was run in live cells, indicating these compounds were readily cell permeable.

5. Quantitate PROTAC Occupancy at CRBN and Protein Targeted for Degradation

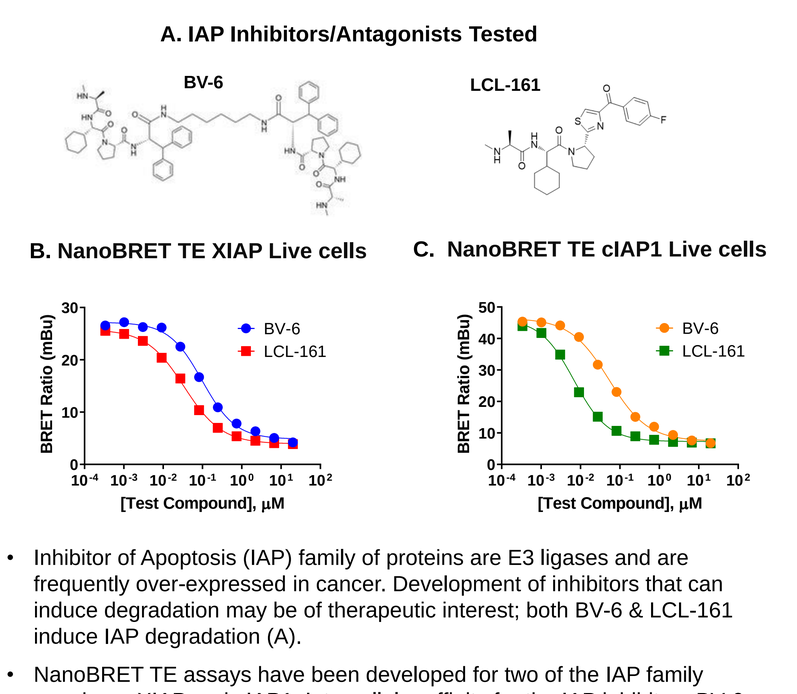


A. PROTACs tested vary in the linkers connecting CRBN & BRD ligands.

B. NanoBRET TE was used to quantitate PROTAC affinity for the BRD4 target in live HEK293 cells, showing dBET6 is 100-fold more potent. This can be a result of increased affinity for BRD4 target or cellular permeability.

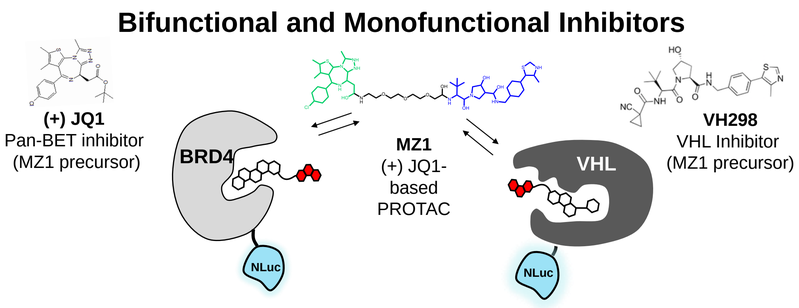
C. To understand PROTAC permeability, NanoBRET TE assays can be run in live or permeabilized cells. Comparing results from NanoBRET TE CRBN assay in live cells to permeabilized cells revealed that, 1) dBET6 is more permeable than dBET1; and 2) dBET6 is slightly more potent then dBET1.

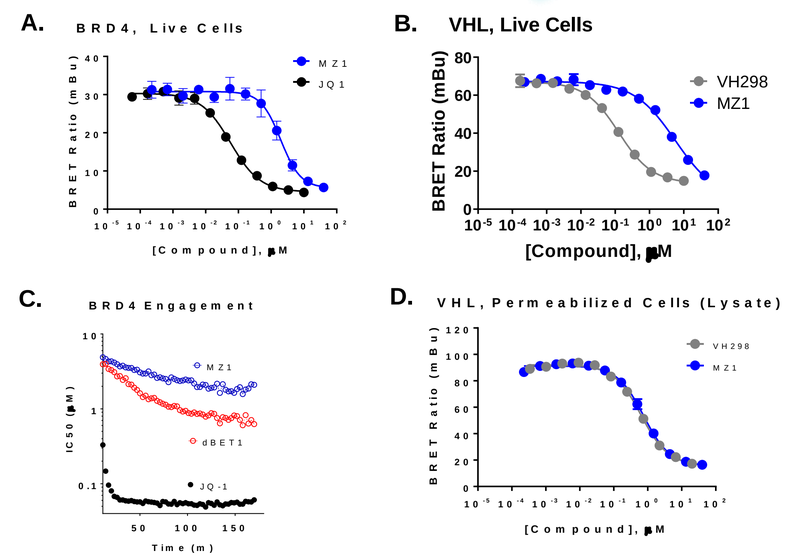
6. Intracellular Affinity of IAP Inhibitors for XIAP and cIAP in Live Cells



- Inhibitor of Apoptosis (IAP) family of proteins are E3 ligases and are frequently over-expressed in cancer. Development of inhibitors that can induce degradation may be of therapeutic interest; both BV-6 & LCL-161 induce IAP degradation (A).
- NanoBRET TE assays have been developed for two of the IAP family members, XIAP and cIAP1. Intracellular affinity for the IAP inhibitors BV-6 and LCL-161 were determined for both XIAP and cIAP1 in HEK293 cells (B & C).

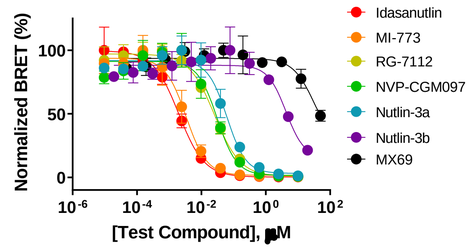
7. PROTAC & Precursor Permeability Measured with BRD4 & VHL NanoBRET TE Assays

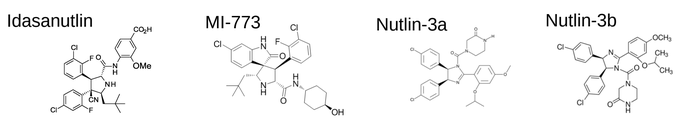




- Using target BRD4 NanoBRET TE assay in live cells, The PROTAC MZ1 is less potent compared to precursor JQ1 (A).
- NanoBRET TE VHL assays were used to assess permeability of PROTAC MZ1 and precursor VH298. In live cells, the MZ1 was ~40-fold less potent than VH298 (B). Since potency values are identical in permeabilized cells (D), the shift in live cell potency is due to decreased permeability of PROTAC MZ1.
- PROTAC & precursor potencies for BRD4 were measured in real time. Plotting compound IC_{50} vs time reveals slow equilibration of PROTACs MZ1 & dBET1 compared to JQ1 precursor (C).

8. Quantitative Measurement of MDM2 TE in Live Cells with Nutlin Derivatives





MDM2 Nanoluc fusions were expressed in HEK293 cells. Intracellular affinity for a series of compounds for MDM2 was quantified using NanoBRET TE MDM2 Assay.

9. Conclusions

NanoBRET TE assays broadly enable the quantitative determination of compound affinity/potency and occupancy for specific targets inside cells

- NanoBRET TE has been successfully used to interrogate live cell compound engagement for hundreds of intracellular targets, spanning a variety of protein classes in the human proteome.
- Cell permeable NanoBRET Tracers have been developed that allow TE assays for >200 full length kinases and five E3 ligases.

PROTACs, molecular glues, and small molecule inhibitors for E3 ubiquitin ligases can be assessed by NanoBRET TE assays

- Assays have been developed for the E3 ligases or adapter proteins including CRBN, VHL, XIAP, cIAP1, and MDM2
- NanoBRET TE assays can be run in live and lytic mode that aids in understanding compound permeability.
- NanoBRET TE live cell assays can be run in real-time to allow examination of kinetics of intracellular binding.

The NanoBRET TE method should facilitate the development of PROTAC and E3 ligase inhibitors with optimal cell permeability and target occupancy

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Broad Kinome Selectivity and Residence Time Analysis in Live Cells with NanoBRET

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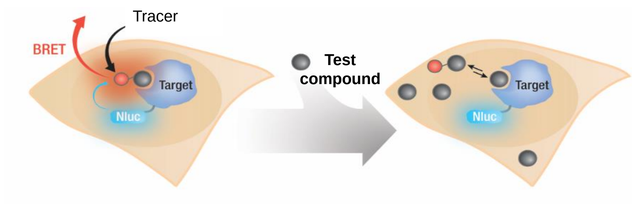
1. Introduction

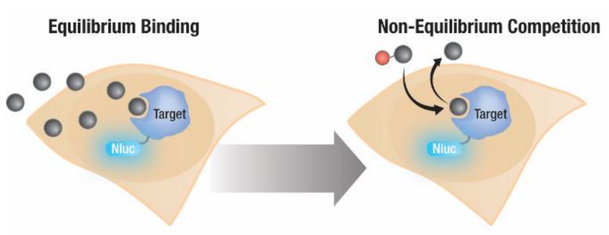
We present the first technique to broadly and quantitatively determine kinase inhibitor potency as well as profile kinase target engagement under physiological conditions, without disruption of cellular membrane integrity. NanoBRET™ enables a biophysical assessment of compound engagement and residence time for chosen intracellular targets. A quantitative capability is achieved in living cells, via energy transfer from cell-permeable tracers reversibly engaged to selected NanoLuc®-tagged target proteins. As the specificity of the BRET signal is dictated by the placement of NanoLuc on the chosen target, a diverse set of broad-coverage tracers support an HTS-compatible method to profile the isozyme-specific affinity and binding kinetics over entire enzyme classes. This technique has enabled a quantitative analysis of compound binding against >200 individual full-length protein kinases, including a key panel of integral membrane receptors. In-cell potency determinations for various types of kinase inhibitors were achieved, including type I, II, and allosteric compounds.. Time-dependent target-compound occupancy (or residence time) can also be obtained with this method. An assessment of kinetic and equilibrium selectivity of various clinically-relevant kinase inhibitors revealed different residence times for compounds with similar equilibrium affinity. The assay was extended to live cell broad kinome selectivity profiling using over 170 kinases. These cellular profiling results revealed an improved selectivity of the compound compared to results obtained by biochemical profiling.

2. Target Engagement (TE) using BRET

Affinity / Potency Determinations

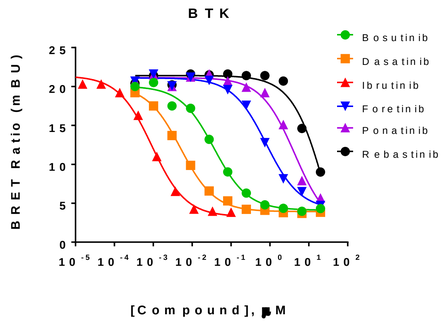


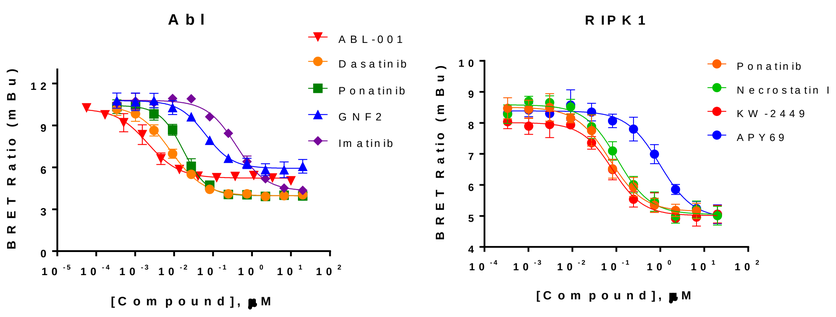
Residence Time Determinations



- Target fractional occupancy can be quantified in live cells with NanoBRET. Using the tracer at or below its apparent affinity in a competitive displacement mode, results in a compound IC50 that's a constant value and quantitative.
- Intracellular residence time can be evaluated in a simple format, where test compound is added prior to the tracer.

3. Interrogating Type I, Type II, and Allosteric Kinase Inhibitors



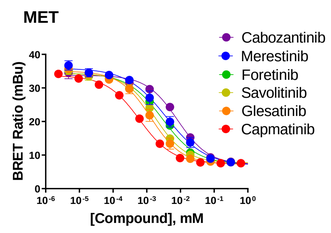


NanoBRET TE experiments were performed with HEK293 cells transiently transfected with Kinase/NanoLuc® fusion proteins. A fixed concentration of tracer approximating the apparent Kd was used.

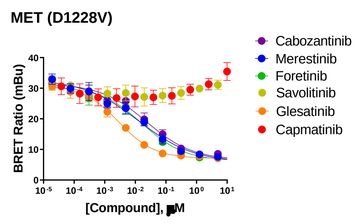
- **Top:** Characterization of type I & II kinase inhibitors at BTK
- **Bottom:** Characterization of type I, type II, & allosteric inhibitors at Abl and RIPK1 kinases

4. Diverse Applications to Explore Inhibitor Pharmacology

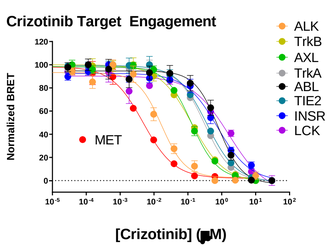
Evaluating diverse chemical matter



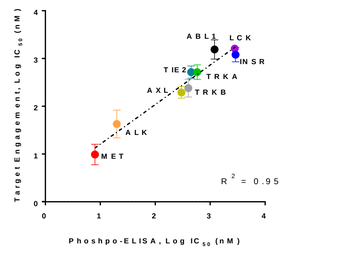
Impacts of clinical mutations



Selectivity among similar targets

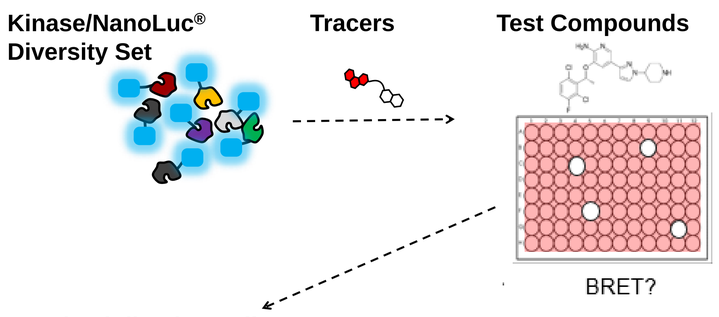


Correlation to phenotype

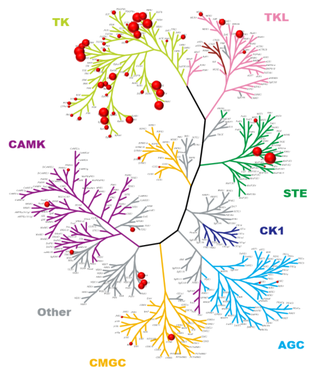


- NanoBRET can be used to evaluate both wildtype and mutant kinases, as shows for MET kinase
- In live cells, NanoBRET data can correlate well with cellular functional assays such as phospho-ELISA

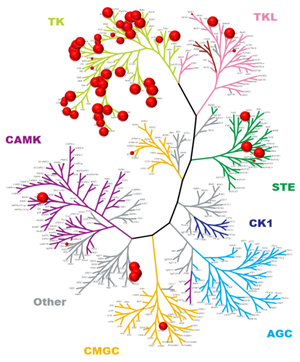
5. Broad Kinome Profiling In Live Cells Reveals Improved Selectivity



1 μM Crizotinib, Live Cell NanoBRET TE



1 μM Crizotinib, QuickScount™ Enzyme Activity

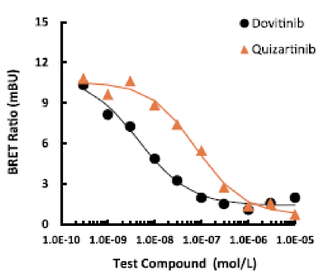


Broad kinome profiling workflow with NanoBRET TE:

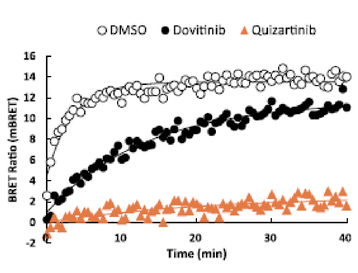
- A kinase library of NanoLuc fusion constructs (178) is reverse-transfected into cells of interest, with each well expressing a unique kinase/NanoLuc fusion. On next day,, target occupancy is determined via NanoBRET. Once occupancy is determined, compound affinity can be determined subsequently.
- A comparison of occupancy vs inhibition of 1μM crizotinib in NanoBRET versus cell-free (Carna Biosciences). In live cells, crizotinib is more selective for MET and ALK.

6. Intracellular Residence Time and Affinity May Not Always Correlate

FLT3: Equilibrium Binding

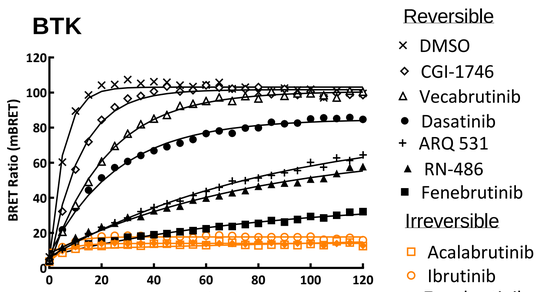


FLT3: Residence Time



- Equilibrium (steady-state) binding may not always correlate with binding kinetics.
- Despite weaker affinity, quizartinib exhibits more durable inhibition than dovitinib following a washout of live cells.

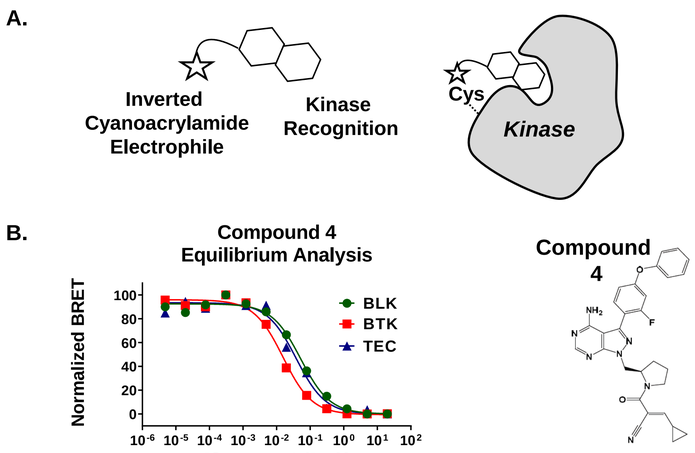
7. Residence Time Can Be Measured with Reversible and Irreversible Inhibitors

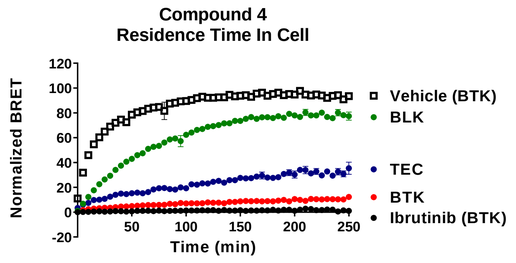


Compound	Binding mode	IC_{50} (nM)	Test concentration (nM)
Dasatinib	reversible	20.0	100
Ibrutinib	irreversible	0.84	3
Fenebrutinib	reversible	6.42	30
Acalabrutinib	irreversible	33.5	100
Zanubrutinib	irreversible	1.93	10
CGI-1746	reversible	66.6	1000
RN-486	reversible	6.46	30
Vecabrutinib	reversible	28.1	300
ARQ-531	reversible	33.4	300

HEK293 cells expressing BTK were preincubated with test compounds or vehicle (DMSO) for 2hrs at 37°C followed by a brief washout. NanoBRET Tracer K-4 was then added and BRET was repeatedly measured with the GloMax® Discover Multimode Reader (Promega) equipped with injector.

8. Kinetic Selectivity with a Reversible Covalent Inhibitor





Kinase	τ (hrs)
BTK	>10
TEC	9.1
BLK	1.1

A. The reversible covalent inhibitor compound 4 (Nature Chem. Bio. 11:525 (2015)), provided by Michael Bradshaw of Principia Biopharma, targets a non-catalytic cysteine in BTK.

B. It shows similar cellular potencies for BLK, BTK, & TEC using NanoBRET TE kinase assays.

C. Compound 4 shows kinetic selectivity for BTK using NanoBRET TE kinase residence time analysis. Ibrutinib was used as a control, as it's a covalent BTK inhibitor targeting the same cysteine in BTK.

9. Conclusions

Cell permeable Tracers have been developed that allow NanoBRET TE assays for >200 full length kinases. Carna Biosciences offers services to support kinase target engagement with NanoBRET.

NanoBRET TE Kinase assays broadly enable the quantitative determination of compound affinity inside cells, including various Type I, II, and allosteric kinase inhibitors.

Monitoring both potency and residence time in cells using NanoBRET TE assays can reveal equilibrium and kinetic selectivity of kinase inhibitors, offering unique opportunities.

For kinases, intracellular selectivity and affinity profiles can differ dramatically from those determined biochemically, underscoring the need for quantitative methods to measure compound engagement and occupancy in live cells.

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Examination of Intracellular Target Engagement for Clinically Relevant Inhibitors Across the CDK Family Using NanoBRET™ Assays

James Vasta, Cesear Corona, Jennifer Wilkinson, Morgan Ingold, Chad Zimprich, Marie Schwinn, Thomas Machleidt, Jim Hartnett, Mei Cong, Frank Fan, Michael Curtin, and Matthew Robers

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1. Introduction

Cyclin-dependent kinases (CDKs) play key roles in diverse cellular functions including cell cycle control, cell proliferation, and transcriptional regulation. There are >20 CDKs in the human kinome; CDK activity is tightly regulated by interactions with intracellular cyclins. Recent clinical successes of CDK inhibitors, such as Palbociclib and Abemaciclib for breast cancer, have helped drive interests in development of new CDK inhibitors as well as heterobifunctional degraders (e.g., PROTACs).

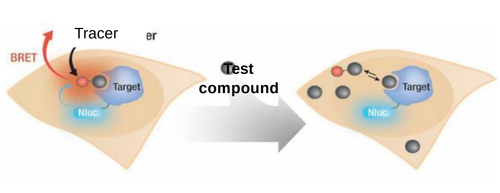
To aid in the inhibitor development for CDKs, we used NanoBRET™ target engagement (TE) technology to develop a panel of >30 specific CDK/cyclin pairings in live-cell assays. The NanoBRET TE technology enables quantitative assessment of target occupancy and residence time for kinase inhibitors and PROTACS in living cells. Using the panel of CDK-cyclin cellular assays enabled us to determine cellular compound potency and selectivity for various inhibitors. We also demonstrated cyclin dependent effect on inhibitor cellular potency, and we could quantitate intracellular binding of a promiscuous kinase PROTAC to several CDKs.

Time-dependent target-compound occupancy (or residence time) can also be obtained Using NanoBRET TE assays. An assessment of kinetic selectivity of compounds with similar equilibrium affinity for CDK9-cyclin T1 was examined for CDK9-cyclin T1. Despite the similar cellular equilibrium affinity, the compounds displayed different intracellular residence times.

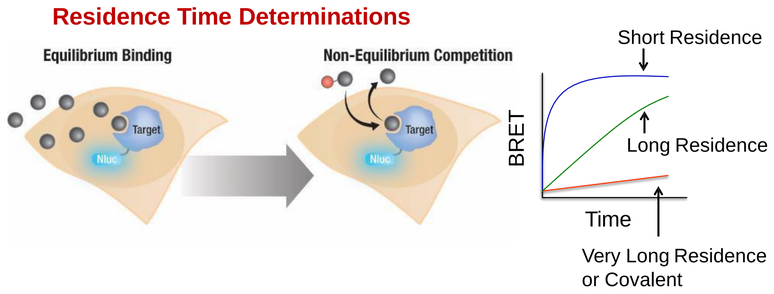
Our results show that NanoBRET TE CDK assays can interrogate test compound potency, selectivity, target occupancy and residence time under physiologically-relevant conditions.

2. Target Engagement (TE) using NanoBRET

Affinity / Potency Determinations



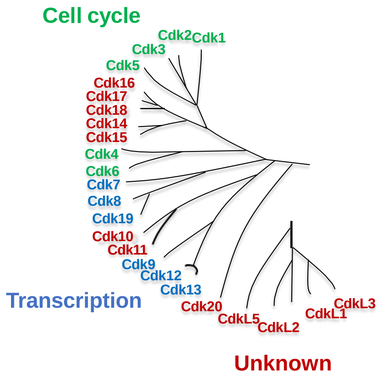
Residence Time Determinations



- The NanoBRET assay is specific for the target fused to NanoLuc since BRET assays are governed by tight distance constraints between energy donor (NanoLuc) and energy acceptor (tracer)
- NanoBRET assays are conducted in live cells that allow equilibrium binding analysis and real time binding analysis

3. Cyclin-Dependent Kinase Family

A. CDK Family



B. NanoBRET TE Cellular CDK Assays

CDK1 + Cyc B1	CDK5 + CDK5R1	CDK14 + Cyc Y
CDK1 + Cyc B2	CDK5 + CDK5R2	CDK15 + Cyc Y
CDK1 + Cyc E1	CDK6 + Cyc D1	CDK16 + Cyc Y
CDK1 + Cyc K	CDK6 + Cyc D3	CDK17 + Cyc Y
CDK2	CDK7	CDK18 + Cyc Y
CDK2 + Cyc A1	CDK7 + Cyc H	CDK19 + Cyc C
CDK2 + Cyc A2	CDK8 + Cyc C	CDKL1
CDK2 + Cyc E1	CDK9 + Cyc K	CDKL2
CDK3 + Cyc E1	CDK9 + Cyc T1	CDKL3
CDK4 + Cyc D1	CDK10 + Cyc L2	CDKL5
CDK4 + Cyc D3	CDK11A + Cyc K	
CDK5	CDK11A + Cyc L2	

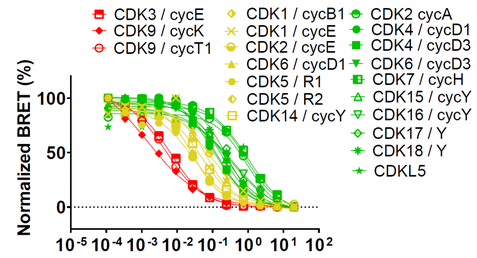
A. Over 20 CDKs and CDK-like proteins comprise the human kinome and play key roles in cell cycle control and transcriptional regulation.

B. By introducing exogenous cyclin with NanoLuc-CDK fusions, NanoBRET TE cellular assays have been developed for >30 specific CDK-cyclin pairings or CDKLs.

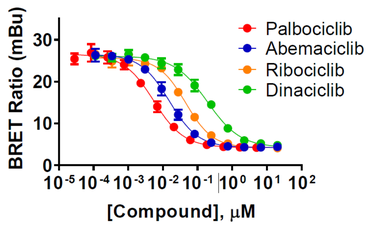
CDK activity is tightly regulated by interactions with numerous intra-cellular cyclins, resulting in many unique CDK-cyclin kinase pairs.

4. Cellular Compound Potency for CDK Family, using Numerous Cyclin Pairings

A. Representative CDK NanoBRET TE Assays



B. CDK6 / Cyclin D1 NanoBRET TE Assays

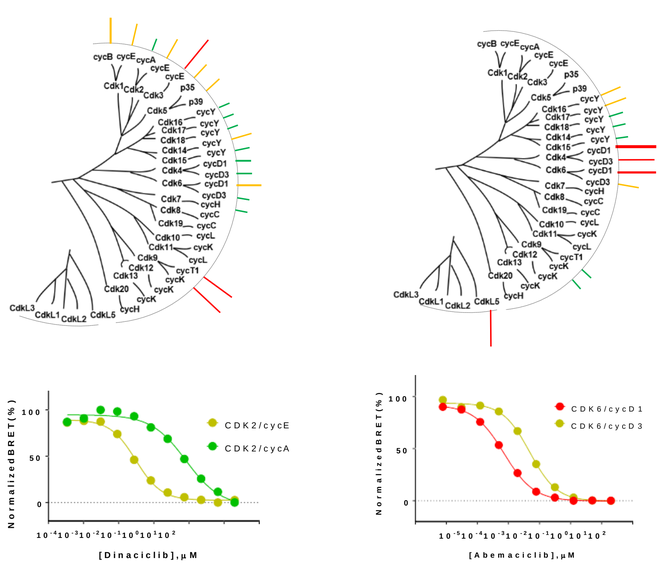


A. Dinaciclib is a CDK inhibitor in clinical trials with FDA orphan drug status. Potency determined for 20 CDK / cyclin pairs or CDKL using NanoBRET TE assays, in HEK293 cells.

B. Cellular potency of Dinaciclib and clinically relevant CDK inhibitors Abemaciclib (Verzenio®), Palbociclib (Ibrance®), & Ribociclib (Kasqali®) for CDK6 / cyclin D1.

5. Intracellular CDK Selectivity and Cyclin Bias for Clinical Drugs

A. Dinaciclib (SCH727965) **B. Abemaciclib (LY2835219)**

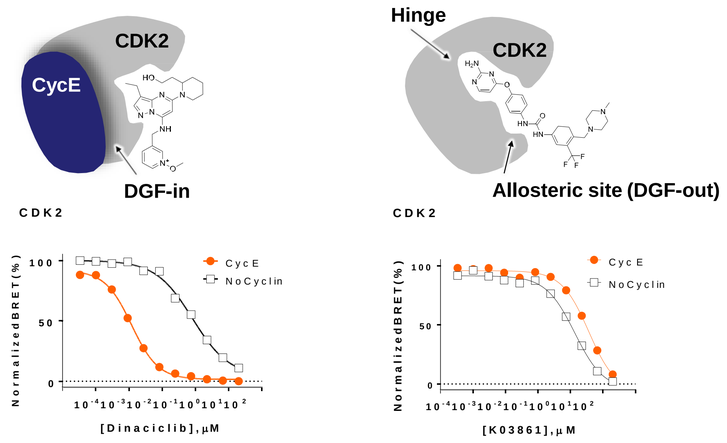


A. Cellular selectivity profile of Dinaciclib identifies primary targets of CDKs 1 - 6 & 9, & binding to several cyclin Y CDKs (CDKs 14-18). Dinaciclib intracellular potency can depend on cyclin pairing.

B. Cellular selectivity profile of the selective CDK inhibitor Abemaciclib reveals the primary targets CDKs 4 & 6. Also, it binds CDKs 14-18. Abemaciclib intracellular potency can depend on cyclin pairing.

6. Intracellular Potency of CDK Type I & II Inhibitors

A. Dinaciclib-Type I Inhibitor **B. K03861 – Type II Inhibitor**

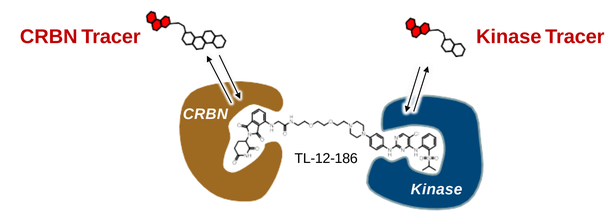


NanoBRET TE cellular assays enable the study of specific CDK-cyclin pairings by co-expression of CDK and cyclin pairs

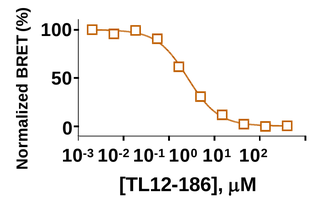
A. Higher intracellular potency for Dinaciclib with CDK2 / Cyclin E compared to CDK2 (lacking exogenous cyclin expression was observed. HEK293 cell were used (A).

B. No cyclin bias observed with the type II inhibitor K03861 that binds CDK2 in DFG-out conformation associated with catalytically inactive kinase. HEK293 cell were used (B).

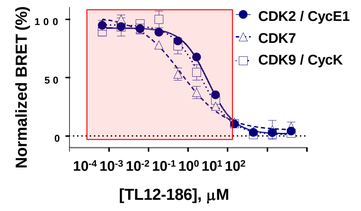
7. Kinase PROTAC TE at CRBN and CDKs can be Quantified via NanoBRET



CRBN NanoBRET TE



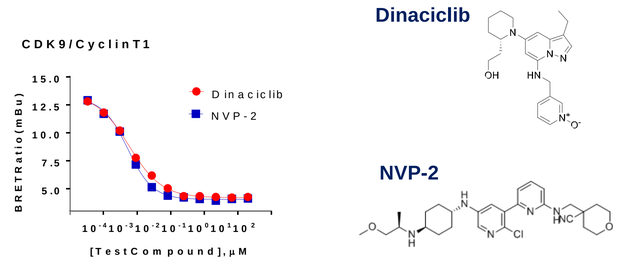
CDKs NanoBRET TE



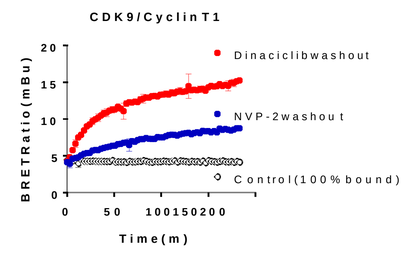
- TL-12-186 is a promiscuous kinase inhibitor conjugated to a cereblon (CRBN) E3 ubiquitin ligase ligand (pomalidomide)
- Intracellular binding of TL-12-186 to CRBN was quantified via CRBN NanoBRET TE assay. (left, IC_{50} = 0.3µM)
- Intracellular binding of TL-12-186 to CDK2, CDK7, & CDK9 was quantified via NanoBRET TE. (right, IC_{50} 0.07 – 0.3µM)

8. Using Cellular Affinity & Residence Time Measurements to Characterize Compounds

A. Equilibrium Binding



B. Residence Time



A. NVP-2 is CDK9 selective, while Dinaciclib inhibits several CDKs. NanoBRET TE showed both compounds had similar cellular affinity for CDK9 / cyclin T1 (A).

B. Despite the similar intracellular affinity, NVP-2 has a longer intracellular residence time compared to dinaciclib, demonstrating that affinity and residence time don't always correlate (B).

9. Conclusions

NanoBRET TE assays broadly enable the quantitative determination of compound affinity for specific targets inside cells

- Cell permeable NanoBRET Tracers have been developed that allow TE assays for >200 full length kinases
- For the CDK family, a suite of >30 NanoBRET TE assays enable interrogation of inhibitor potency against different CDK / cyclin pairs

Cellular potency of various kinase inhibitors types is measured using NanoBRET TE assays

- For CDKs, type I & II inhibitor cellular potencies have been determined
- For other kinases, NanoBRET TE has been used to quantitate type I, II and allosteric compounds cellular potency

Residence time for specific kinases in live cells is measured with NanoBRET TE: Using both equilibrium & residence time methods, selectivity may be revealed offering unique inhibitor development opportunities.

PROTACs cellular permeability and potency for E3 ubiquitin ligases CRBN and VHL can be quantified using NanoBRET TE assays: run in live and lytic mode to assess cellular permeability of PROTACs.

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A Versatile Bioluminescent Immunoassay Approach to Probe Cellular Signaling Pathway Regulation

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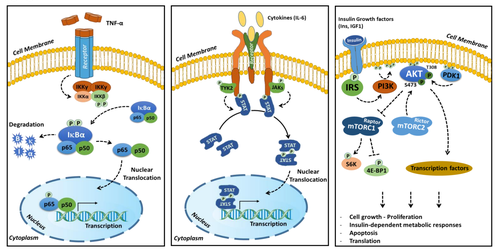
1. Introduction

Monitoring cellular signaling events can help better understand cell behavior in health and disease. Traditional immunoassays such as ELISA or Western blot, used to study proteins involved in signaling, can be tedious, require multiple steps, and are not easily adaptable to high throughput screening (HTS). Here we describe Lumit cellular immunoassay, a novel cell-based approach where immunodetection is combined with bioluminescent enzyme subunit complementation. It is solution based, does not include washing, liquid transfer, nor immobilization steps. Therefore, cells are lysed in the same well where antibody binding and luminescence generation steps occur. Lumit immunoassays take less than two hours to complete in a homogeneous "Add and Read" format and were successfully used to monitor the activation and deactivation of multiple signaling pathways through specific nodes of phosphorylation in unmodified cells.

Our results demonstrate that this technology can be broadly adapted to streamline the analysis of signaling pathways of interest or the identification of pathway specific chemical or biologic inhibitors.

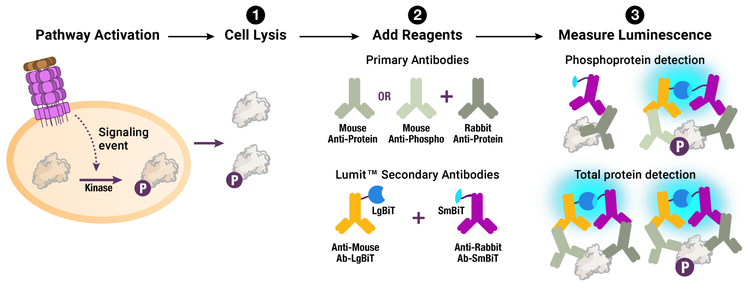
Cancer and Inflammatory Response Signaling Pathways



NF-κB **JAK/STAT** **mTOR/PI3K/AKT**

2. Principle of Homogeneous Lumit Immunoassay Cellular System

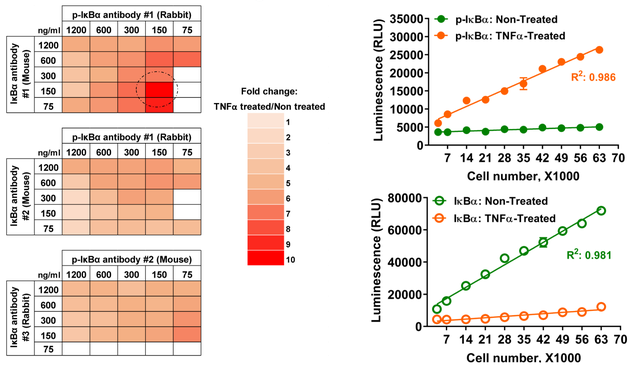
The bioluminescent Lumit immunoassays are based on NanoLuc Binary Technology (NanoBiT) two-subunit system (SmBiT; 11 aa peptide and LgBiT; 18 kDa fragment). In this assay, the NanoBiT subunits are fused to an anti-mouse and an anti-rabbit secondary antibodies (**Lumit™ secondary antibodies**).



Principle of Lumit™ Immunoassay cellular system. Phosphorylated (top panel) or total (bottom panel) target proteins in lysed cells after stimulation are recognized by each primary antibody pair. The Lumit secondary antibodies then recognize their cognate primary antibodies, resulting in close proximity of the NanoBiT subunits to form a functional enzyme that generates bright luminescence.

3. Optimizing Lumit Immunoassay Cellular System for Total and Phosphorylated Targets

Developing Lumit immunoassay to detect IκBα phosphorylation or degradation



- Selection and optimization of primary antibodies for Lumit immunoassay is fast and easy.
- 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. Validation of Lumit Cellular Immunoassay Detection of Phosphorylated and Total IκBα

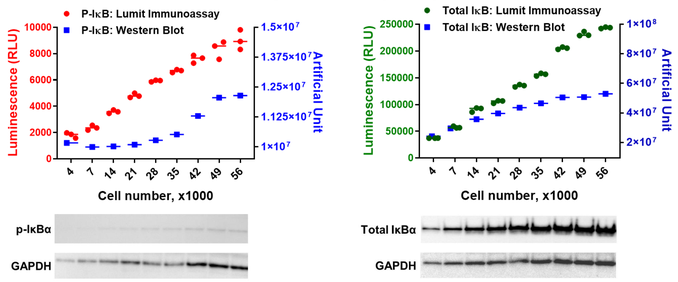
Western blot protocol (Heterogeneous)

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

Lumit immunoassay protocol (Homogeneous)

Activate pathway → Add lysis buffer to cells ① → 20 min → Add Antibody solution ② → 90 min → Add Detection reagent ③ → Measure Luminescence

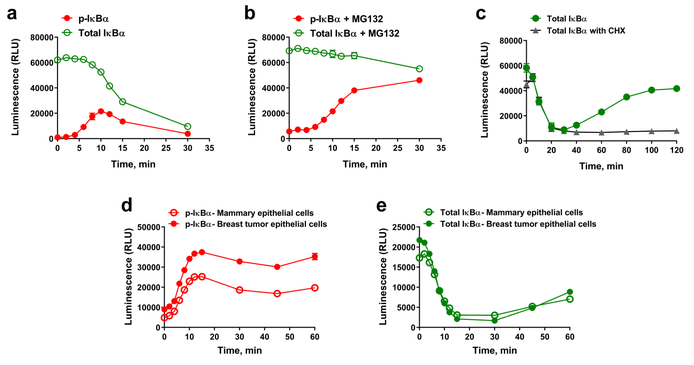
Comparison of Lumit immunoassay and Western blot in detecting total and phospho IκBα



- Homogeneous Lumit Cellular immunoassays are easier and quicker than traditional Western to generate the same data.
- Phosphorylation or degradation of IκBα can be measured using the Lumit immunoassay in small number of cells and in a more quantitative way.

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

Detection of total and phospho IκBα upon TNF Treatment



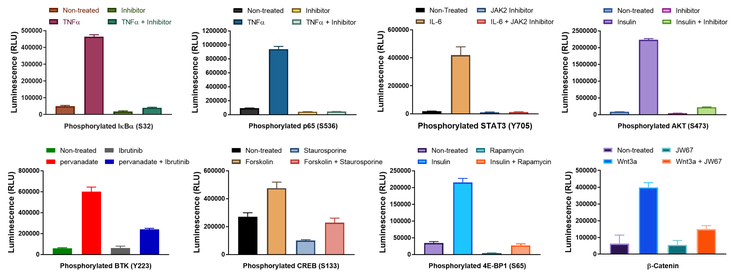
a. Lumit IκBα immunoassay reveals the predicted biology of NF-κB signaling pathway upon TNF treatment: IκBα phosphorylation (pS32) followed by its fast degradation. No cell engineering required: therefore same results in primary cells (d and e).

b. Lumit IκBα immunoassay reveals the predicted response of NF-κB pathway to the proteasome inhibitor MG132 treatment: decrease in IκBα degradation and accumulation of phosphorylated IκBα.

c. Cycloheximide inhibits de novo IκBα protein expression in response to long NF-κB pathway activation and the Lumit immunoassay can detect easily this event.

6. Detection of Diverse Signaling Target Proteins

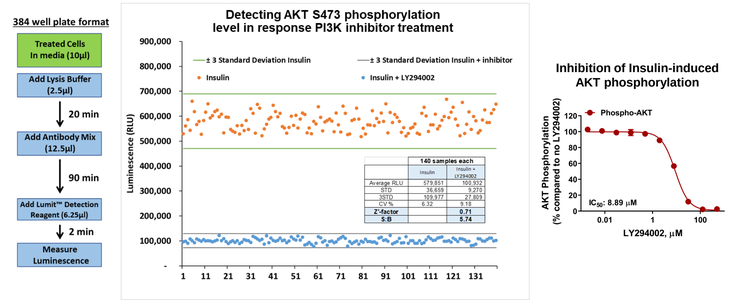
Detection of total and phosphorylated targets upon signaling pathway activation and deactivation



- Lumit cellular immunoassays reveal the predicted biology of multiple signaling pathways upon ligand mediated activation: Quick **phosphorylation** of the pathway **nodes** such as IκBα (S32), and STAT3 (Y705) or **degradation** of a **node** target such as β-Catenin with Wnt treatment.
- Detection of the predicted response of the signaling pathways to **node kinase** or **pathway** inhibitor treatment: such as inhibitors of IKK complex, PI3K, and JAK2 abolishing IκBα/P65, AKT and STAT3 phosphorylation, respectively.

7. Detection of Pathway Node Kinase Inhibition with Small Molecules

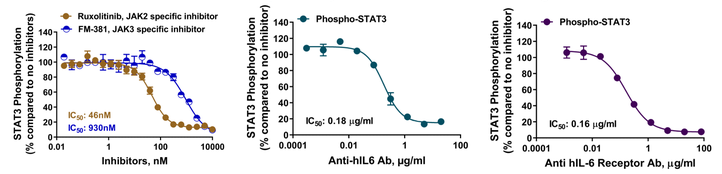
High Throughput Screening (HTS) utility of Lumit™ cellular immunoassay



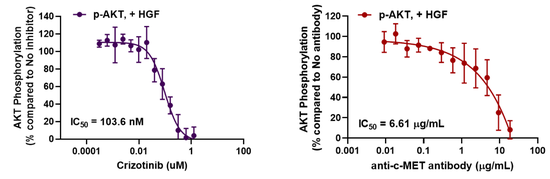
- The bioluminescent Lumit pathway assays reveal the expected pharmacology of pathway **node kinase** inhibitor.
- The Lumit immunoassays can be used to screen inhibitors of cancer, immune and inflammatory response pathways in fast and homogeneous fashion.
- High Z' value show a great applicability of Lumit Immunoassay Cellular systems to HTS.

8. Pathway Modulation with small and Large Molecules Detected with Lumit Immunoassays

Inhibition of JAK/STAT pathway with chemical and biologic inhibitors



Using p-AKT detection to analyze Inhibition of c-MET pathway with c-MET small and large molecule inhibitors



- Bioluminescent Lumit immunoassays can be used to identify small and large molecule inhibitors of signaling pathways.
- Pathway node phosphorylation can be used as a reporter for biologics activity at the receptor level.
- Lumit p-AKT node immunoassay can be used to screen anti c-MET large molecule drugs in non-engineered cells in fast (10 min activation) and easy way.

9. Conclusions

Benefits of the bioluminescent Lumit cellular 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 Lumit detecting antibodies can be adapted to any pathway of interest

Reference: Hwang, B., et al. (2020) A homogeneous bioluminescent immunoassay to probe cellular signaling pathway regulation. *Commun Biol* 3, 8 https://rdcu.be/b0jf0

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Lumit™ Immunoassays: Bioluminescent, Sensitive, and Homogeneous Analyte Detection Using Labeled Antibodies

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Abstract # 824424

1. Introduction

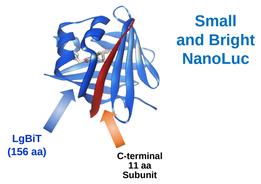
NanoLuc® Binary Technology (NanoBiT®), a two-part complementation system based on NanoLuc luciferase, is a proven technology for analyzing proteins at a cellular level. NanoBiT is comprised of an 11-amino acid subunit (low-affinity SmBiT or high-affinity HiBiT) that binds to its cognate large subunit partner (LgBiT) to form a bright luciferase that produces light when furimazine is added. We are building NanoBiT proximity immunoassays where complementary antibodies (or other affinity reagents) are labeled with NanoBiT subunits such that binding to analyte brings SmBiT and LgBiT into proximity, thereby producing signal proportional to analyte levels. This homogeneous detection chemistry has several advantages, including simple, add-and-read protocols, no requirement for sample transfer, no washes, and a broad linear dynamic range mitigating the need for sample dilutions. Moreover, time to assay completion is <30 to ≤ 90 minutes, depending on the specific assay. In development are assays for detection of cytokines (e.g., IL-1β), metabolic targets (e.g., Insulin), FcRn binding, cellular pathway analyses (total and phospho-protein levels), as well as labeling kits to build your own Lumit immunoassays.

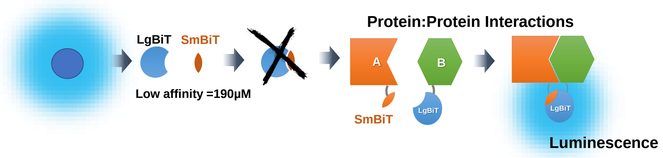
2. NanoLuc Binary Technology (NanoBiT)

The small NanoLuc luciferase (19kDa) was divided into two subunits and individually optimized for **assisted** complementation

Two subunits:

- Large BiT (LgBiT; 17.6kDa) and
- Small BiT (SmBiT; 11 amino acid peptide)

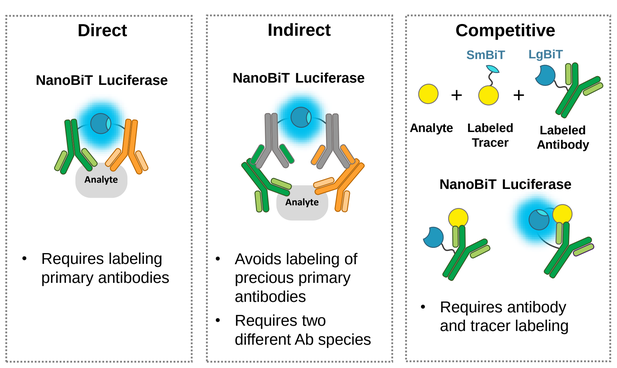




Dixon, A.S., *et al.* (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* **11**, 400-8.

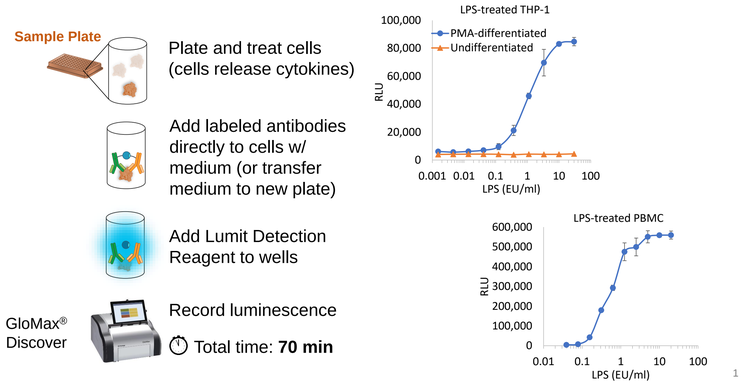
3. Lumit™ Immunoassay Configurations

Bioluminescent immunodetection platform based on NanoBiT technology



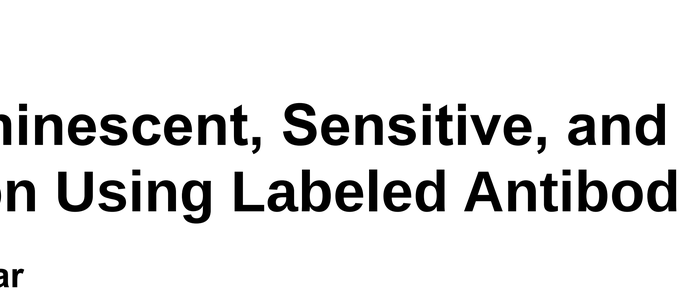
4. Lumit Cytokine Immunoassays

Protocol for Detecting Released IL-1β from Cells



Additional cytokine assays in development include, but are not limited to, **IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α, and VEGF**

5. Lumit Immunoassay Cellular System



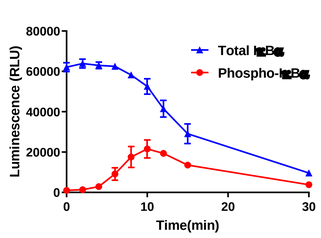
The assay is fast and homogeneous, with an easy "Add and Read" format

Hwang, B., Engel, L., Goueli, S.A. *et al.* A homogeneous bioluminescent immunoassay to probe cellular signaling pathway regulation. *Commun Biol* 3, 8 (2020) doi:10.1038/s42003-019-0723-9

6. NF-κB Signaling Pathway

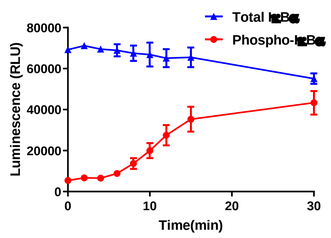
Activation of NF-κB Pathway with TNFα treatment in MCF-7 cells

Detection of Total and Phospho IκBα upon TNF treatment



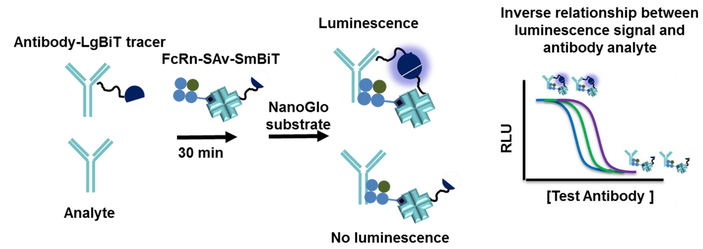
Lumit IκB detection reveals the predicted biology of NF-κB signaling pathway upon TNF treatment: **IκBα phosphorylation** (pS32) immediately followed by its **fast degradation.**

Detection of Total and Phospho IκBα upon TNF treatment in the presence of MG132



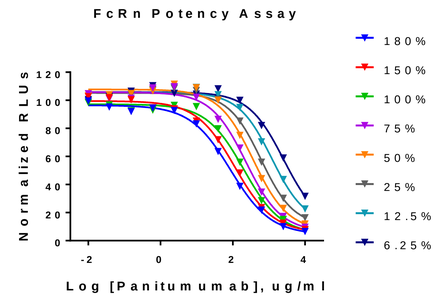
Detection of the predicted response of NF-κB pathway to the proteasome inhibitor treatment: **decrease** in IκBα degradation and **accumulation** of phosphorylated IκBα.

7. Lumit FcRn Immunoassay



- Lumit FcRn Immunoassay is solution based; minimizes artifacts introduced by immobilization
- Assay is homogeneous (add-and-read) and requires no washing
- Luminescence based detection provides wide dynamic range and a large assay window
- Assays are quick (30min) and require low sample volume (10-20µl)
- Use of 96/384 well white plates enables flexible throughput and automation capabilities

8. Measurement of Relative Antibody Potency



	180%	150%	100%	75%	50%	25%	12.5%	6.25%
IC50	86.66	113.9	200.4	231.1	369.9	604.7	1203	2553

Dose response curves for Panitumumab-FcRn binding corresponding to 180%, 150%, 100%, 75%, 50%, 25%, 12.5% and 6.25% of the nominal concentration, plotted versus nominal (100%) concentration values

9. Conclusions

Bioluminescent immunodetection platform based on NanoBiT technology

- No-wash assay can be performed directly on cells
- Fast, 30- to 90-minutes total time
- Scalable for high throughput use
- Large dynamic range reduces sample dilutions required
- Uses standard plate-reading luminometer
- Platform will include ready-to-use kits and reagents; user can create novel detection assays
- Custom antibody labeling and immunoassay development available; Contact: CAS@promega.com

Corresponding author: dan.lazar@promega.com

Novel Bioluminescent Tools to Study GPCR Pharmacology in Living Cells

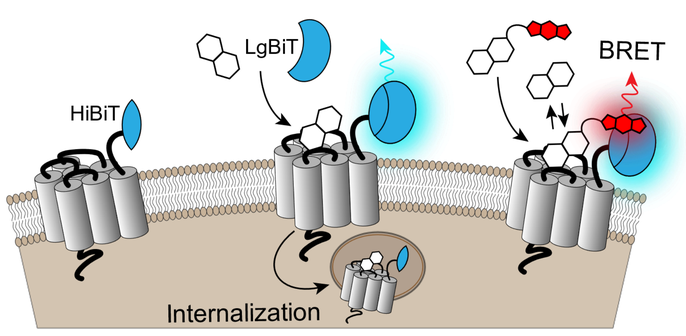
Steve Edenson[1], Brock Binkowski[1], Christopher Eggers[1], Braeden Butler[1], Rachel Friedman Ohana[1], Michelle Boursier[1], Sergiy Levin[2], Thomas Machleidt[1], Kevin Hsiao[1], Said Goueli[1], Keith Wood[1] & Frank Fan[1]

[1]Promega Corporation, 2800 Woods Hollow Rd, Madison; [2]Promega Biosciences LLC, 277 Granada Dr, San Luis Obispo, CA

1. Introduction

G-protein coupled receptors (GPCRs) continue to be prominent targets for new therapeutics. To support these efforts, we have developed a suite of bioluminescent assays to study GPCR pharmacology in real time in living cells. Quantification of ligand binding and receptor internalization relies on N-terminal fusion to High BiT (HiBiT), an 11 a.a. peptide. HiBiT binds with high affinity to Large BiT (LgBiT) to form NanoBiT Luciferase. For both applications, a non-lytic detection reagent is added containing cell-impermeable LgBiT and furimazine substrate. Ligand binding dynamics at the cell surface can be measured in real time through bioluminescent resonance energy transfer (BRET) between NanoBiT Luciferase and a fluorescent tracer. Receptor internalization and recycling are monitored in real time by measuring changes in the cell surface density of HiBiT-tagged GPCRs. Notably, the bright signal of NanoBiT Luciferase allows both assays to be run at endogenous levels of expression using CRISPR/Cas9 to introduce the HiBiT tag. Similarly, fusion proteins to LgBiT and Small BiT (SmBiT), an 11 a.a. peptide with low affinity binding to LgBiT, can be used to measure β-arrestin-1/2 recruitment to activated GPCRs in real time. Furthermore, changes in intracellular cAMP concentration can be sensitively measured using endpoint (cAMP-Glo) or real time formats (GloSensor cAMP) using thermal stable or circularly permuted forms of luciferase, respectively. These homogenous, cell-based assays, which are readily adaptable to laboratory automation, should be immensely useful in the study of GPCR pharmacology.

2. Using HiBiT to Monitor GPCR Ligand Binding



HiBiT tagging

- **Small:** 11 amino acids
- **Highly sensitive**
  Produces bright luminescence upon high affinity complementation with LgBiT, and allows analysis at endogenous expression levels
- **Selective cell surface detection:** due to cell-impermeability of LgBiT
- **Simplified CRISPR/Cas9 knock-in**

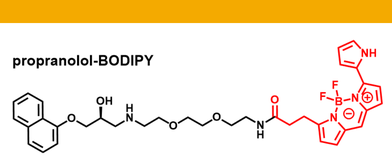
Monitoring ligand-induced internalization

- **Quantitative:** measures changes in cell-surface density of HiBiT-GPCRs

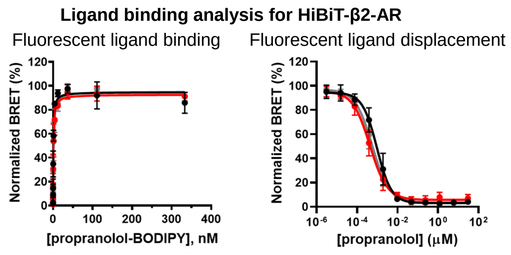
Measuring ligand binding by BRET

- **Highly specific:** due to the inherit distance constrains of BRET
- **Quantitative:** binding affinity & kinetics
- **Non-radioactive & homogeneous live cell assay**

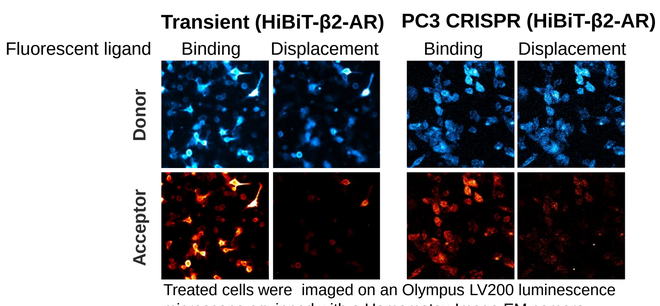
3. Analysis at Endogenous Expression Levels



Ligand binding analysis for HiBiT-β2-AR



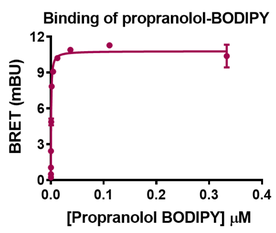
HiBiT β2-AR	K_D (nM)	IC50 (nM)
Transient (HEK293)	0.75 ± 0.10	0.95 ± 0.09
Transient (PC3)	1.09 ± 0.16	0.56 ± 0.05
PC3-CRISPR knock-in	0.95 ± 0.13	0.43 ± 0.05



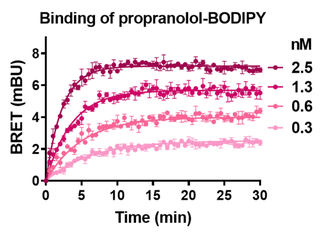
Treated cells were imaged on an Olympus LV200 luminescence microscope equipped with a Hamamatsu Image EM camera.

4. Ligand Binding Kinetics at β2-AR

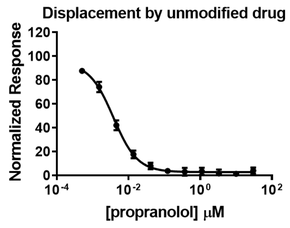
Binding at equilibrium



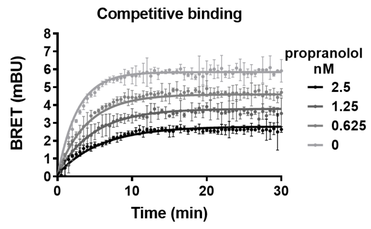
Binding kinetics



Displacement by unmodified drug



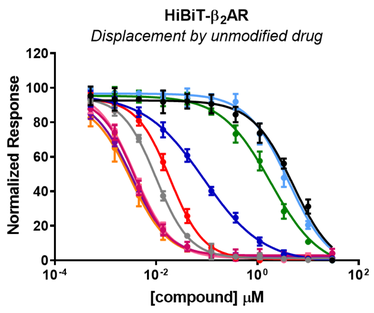
Competitive binding



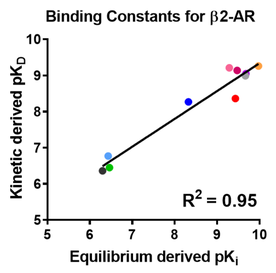
Analysis	Propranolol-BODIPY	propranolol
Equilibrium		
K_D (nM)	0.6	0.3
Kinetic		
K_D (nM)	0.9	0.7
K_{on} (M^{-1} min^{-1})	13 x 10^7	470 x 10^7
K_{off} (min^{-1})	0.12	3.4

Similar binding constants from equilibrium & kinetic analyses

5. Ligand Selectivity Profiling for β-ARs



	K_i (nM)
timolol	0.7
pindolol	0.7
propranolol	0.8
alprenolol	0.8
salmeterol	2.2
carvedilol	4.1
formoterol	19
xamoterol	370
isoproterenol	770
salbutamol	1100

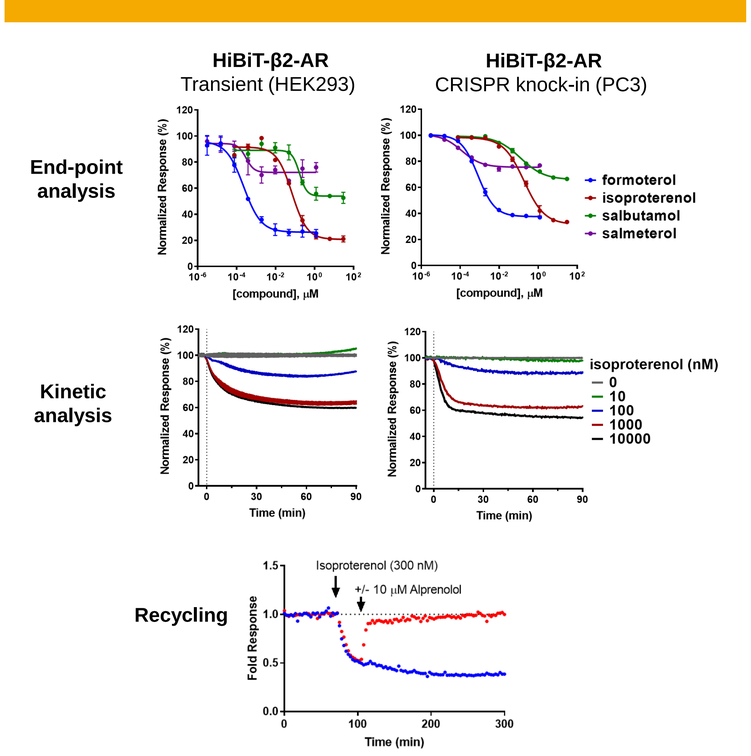


$K_i = (IC_{50}) / (1 + ([Tracer] / K_D)$

Cheng-Prusoff equation

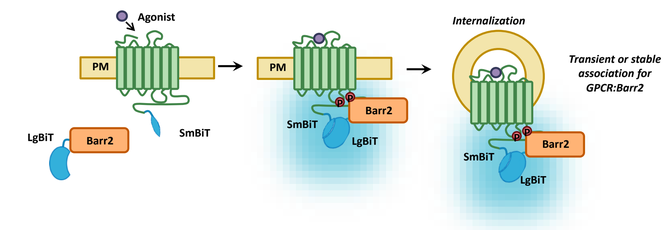
- **Rank order potencies are in general agreement with reported values.**
- **Equilibrium and kinetic derived binding constants for agonists and antagonists were generally consistent**

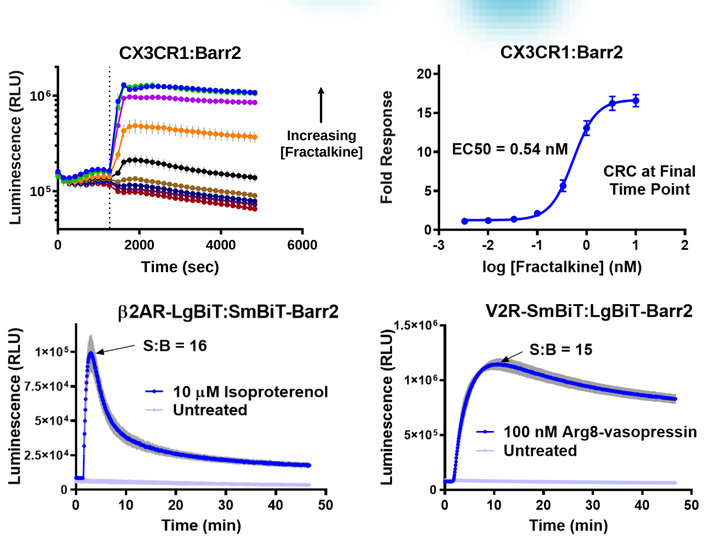
6. Ligand-Induced β2-AR Internalization



- **β2-AR internalization with various ligands is in general agreement with reported values**
- **Monitor receptor internalization and recycling in real time**

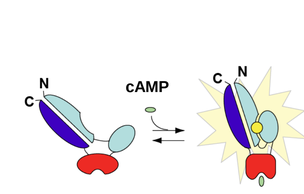
7. Monitor GPCR:β-arrestin-2 Interactions in Real Time



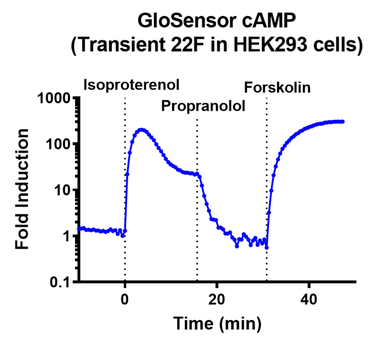


- **Monitor GPCR interactions with β-arrestin-1/2 in real-time**
- **Small tags: 11 a.a. peptide (SmBiT) or 17.6 kDa protein (LgBiT)**
- **Detect interactions using low levels of expression**

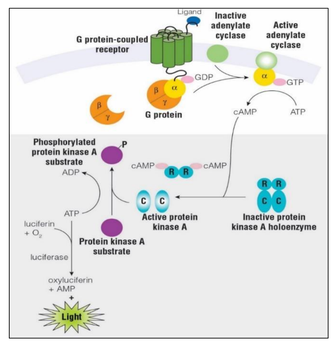
8. Real Time and Endpoint Assays for cAMP

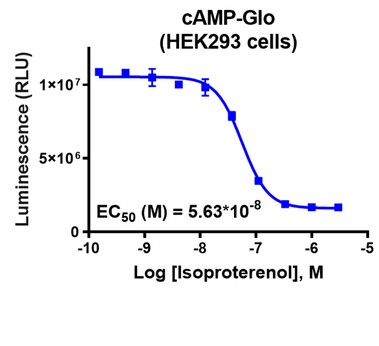


Firefly-luciferase-based biosensor



Real-time monitoring of changes in intracellular [cAMP]
Sensitive assay with wide dynamic range





Sensitive, endpoint assay for [cAMP] in lysates

9. Conclusions

Promega technology and assays for monitoring GPCR pharmacology:

NanoBiT technology

- Small tags minimize perturbation of fusion partner biology
- Bright luminescence offers extreme sensitivity, e.g. endogenous levels of expression

Measuring ligand binding by BRET

- Highly specific: due to inherit distance constraints of BRET
- Quantitative analyses of binding affinity & kinetics
- Non-radioactive

Monitoring ligand-induced internalization

- Quantification of GPCR internalization and recycling
- Kinetic and endpoint analyses

Monitoring GPCR interactions with β-arrestin-2

- Simple protocol with robust S:B

Real-time and endpoint assays for cAMP

Homogeneous cell-based assays

- Suitable for automation and high-throughput screening

Corresponding author: steven.edenson@promega.com

Detection of Insulin Action and Steatosis Using New Bioluminescent Metabolite Assays

Terry Riss, Maggie Bach, Mike Valley, Natasha Karassina, Donna Leippe, Jolanta Vidugiriene and Jim Cali

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

1. Introduction

The combination of high blood pressure, central obesity, elevated blood sugar, and high levels of triglycerides and cholesterol known as “metabolic syndrome” result in increased risk for diabetes and cardiovascular disease. The action of insulin and the accumulation of lipids (i.e. steatosis) are two related factors that are involved with these conditions. The presence of insulin stimulates glucose uptake, suppresses lipolysis, and inhibits gluconeogenesis whereas insulin resistance leads to increased gluconeogenesis, decreased glucose uptake, and steatosis. The long-term occurrence of steatosis (e.g. NAFLD or NASH) can lead to chronic inflammation, cirrhosis, and potentially carcinoma. We have developed a portfolio of in vitro cell-based assays to detect specific metabolites using: 1) a biochemical approach with dehydrogenase enzymes coupled to the production of NAD(P)H and the generation of light from firefly luciferase or 2) an immunoassay approach using subunit complementation to form active NanoBiT® luciferase. Examples of the function and performance of these assays systems will be presented.

2. Bioluminescent Metabolite Assays

Based on bioluminescent NAD(P)H detection and can be rapidly applied for multiple metabolite detection

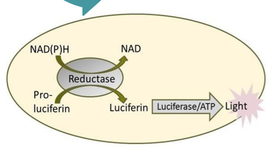
Metabolites
Glycogen
Glutamine
Aspartate
Triglycerides
Cholesterol Esters

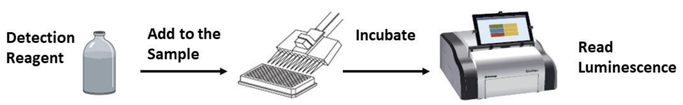
Enzymes

Metabolites for Dehydrogenases
Glucose
Glutamate
Lactate
Amino Acids (BCAA)
Glycerol
Cholesterol

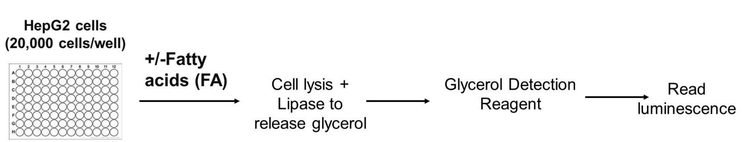
NAD(P)

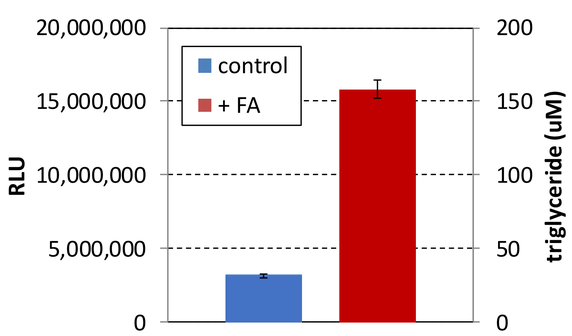
Specific Dehydrogenase





3. Model for NAFLD: Triglyceride Assay

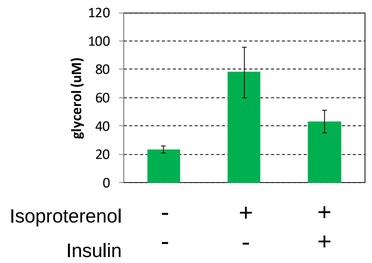




20,000 HepG2 cells per well were incubated overnight in the absence or presence of 0.3 mM BSA-bound linoleic and oleic acids. Lipid accumulation was measured using triglyceride detection assay.

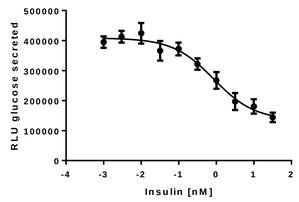
4. Models for Gluconeogenesis and Lipolysis: Glucose and Glycerol Assays

Insulin-mediated inhibition of lipolysis measured by glycerol secretion



Glycerol release into the medium in 3T3-L1 MBX adipocytes treated for 90 minutes with different combinations of isoproterenol (25nM) and insulin (150nM).

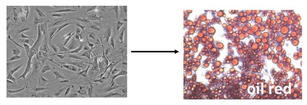
Insulin-mediated inhibition of gluconeogenesis measured by glucose secretion



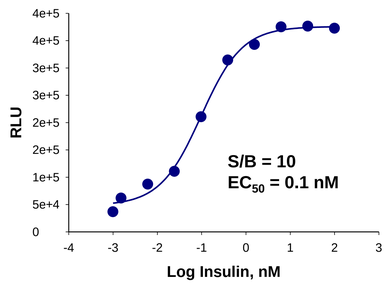
Spheroids formed from 2000 iCell® Hepatocytes 2.0 (CDI) were washed and incubated with 10mM lactate, 2µM forskolin, and a titration of insulin for 6hrs. An aliquot of medium was then assayed with the glucose detection assay.

5. Model for Studying Insulin Action: Glucose uptake Assay

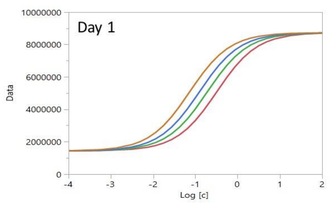
Differentiated 3T3 L1-MBX fibroblast
→ Adipocyte



Differentiated 3T3L1 adipocytes were treated with insulin, and the rate of glucose uptake was measured using bioluminescent detection. Insulin induces translocation of glucose transporters to the cell surface and increases glucose uptake in a dose-dependent manner.



Glucose Uptake-Glo as an Insulin Potency Assay using Differentiated 3T3 L1 MBX Adipocytes

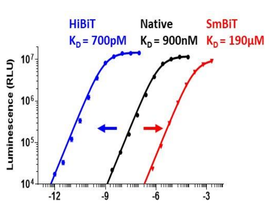


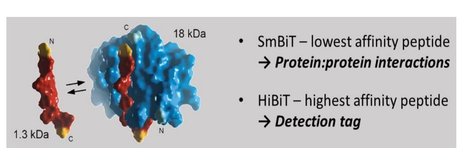
Expected relative potency		Measured relative potency
50%	day1	40.35%
	day2	38.80%
75%	day1	71.22%
	day2	64.59%
150%	day1	149.33%
	day2	170.94%

6. NanoBiT System

NanoBiT System is based on a small and very bright NanoLuc luciferase and consist of:

- 18kDa LgBiT with no luciferase activity
- 11 a.a. peptides with different affinity for LgBiTs

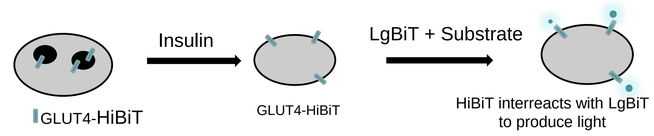




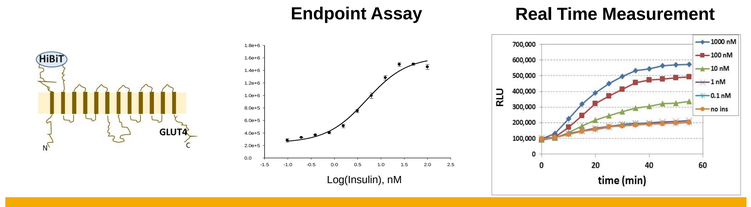
7. Model for Studying Insulin Action in Muscle Cells: NanoBiT GLUT4 Translocation

To monitor GLUT4 translocation to the plasma:

- HiBiT was inserted into extracellular loop of GLUT4
- Upon insulin stimulation, GLUT4-HiBiT is translocated to the plasma membrane and is measured by adding Detection Reagent containing LgBiT and NanoLuc substrate



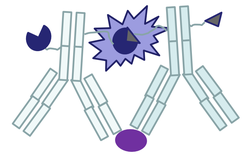
Measuring GLUT4-HiBiT translocation in C2C12 mouse myoblast stable clones



8. Model for Islet Function: Lumit™ Immunoassay

Anti-insulin antibody 1 with Large BiT

Anti-insulin antibody 2 with Small BiT

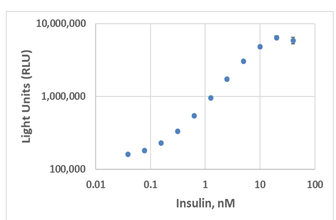


Complementation of BiTs in close proximity upon binding to insulin

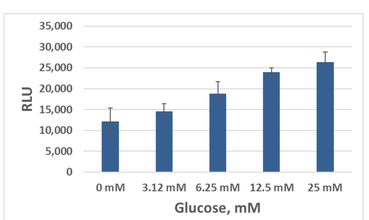
Active luciferase is formed and produces light

Insulin containing samples
↓
Add antibodies conjugated with BiTs
↓
Incubate 1hr
↓
Add NanoLuc Substrate and Read Luminescence

Insulin titration in 384-well plates



Glucose Stimulated Insulin Secretion in INS-1 rat insulinoma cells in 384-well plates



9. Conclusions

Bioluminescent metabolite assays and NanoBiT® technology are convenient tools for studying metabolic changes or evaluating biological activity of insulin and insulin analogues in cellular models:

- Applicable for multiple metabolites in different sample types (cells, media, tissues)
- Compatible with 96- and 384-well plates and standard plate readers
- Can be used for studying receptor/transporter translocation to plasma membrane
- Can be used to modify antibodies and provide an in-solution, no-wash Lumit™ immunoassay that is more rapid than conventional ELISA methods

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