1266-B

Assessing Autophagic Flux in 2D and 3D Cell Culture Models with a Novel Plate-Based Assay

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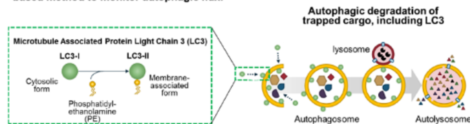
1. Introduction

We have developed a homogeneous plate-based assay to measure autophagic flux that works in 2D and 3D cell culture models. The LC3 protein was tagged on its N-terminus with a spacer sequence and a small subunit of a shrimp-derived luciferase (HiBiT). When stably expressed at low to moderate levels in mammalian cell lines, this novel LC3-based reporter is processed through the autophagic pathway. The cellular level of the autophagy reporter is determined by addition of a lytic detection reagent containing a large subunit of luciferase (LgBiT) and a luminogenic substrate. LgBiT rapidly associates with HiBiT in the cell lysate producing an active NanoBiT luciferase that generates a luminescent signal proportional to the amount of autophagy reporter. Cells stably expressing the autophagy reporter and treated with stimulators of autophagy will show a decreased luminescent signal. Treatment with inhibitors of autophagy results in a buildup in the level of LC3-based reporter and thus a higher luminescent signal. The autophagic flux assay can be multiplexed (on the same sample) with a cytotoxicity assay to serve as a control to detect cytotoxic effects of test compounds. The assay has been shown to have excellent performance in an automated 384 well high throughput screening format using U2OS and HEK293 autophagy reporter cells. The luminescent signal is stable for hours enabling batch processing of multiple 96- or 384-well plates in the same experiment. Both induction and inhibition of autophagic activity was easily observable following reference compound treatment of HEK293 cells grown as 3D spheroids.

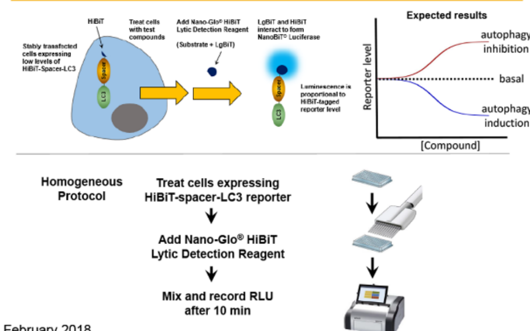
This novel assay method enables screening for modulators of autophagic flux in 2D or 3D culture model systems using a simple homogeneous assay procedure that is recorded with a plate reading luminometer.

2. LC3 protein dynamics provide a useful indicator of autophagic activity

- **LC3 autophagy marker protein exists mostly as free cytosolic form (LC3-I) under basal conditions.**
- **Induction of autophagic activity promotes LC3-PE conjugation (LC3-II) and membrane targeting.**
- **Substantial LC3-II protein is trapped with cargo upon closure of autophagosome.**
- **Subsequent degradation in the autolysosome results in a decrease in total LC3 protein.**
- **HiBiT-tagged LC3 reporter is a luminogenic LC3 surrogate that enables a simple, plate reader-based method to monitor autophagic flux.**



3. How the assay works



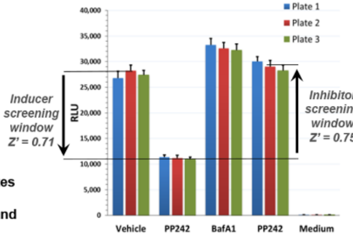
4. Automated assay in 384-well plates

30µl HEK293 cells (2000)
+ 10µl 4X cmpds or vehicle
40µl treatment volume

Incubate 6 hr @ 37°C

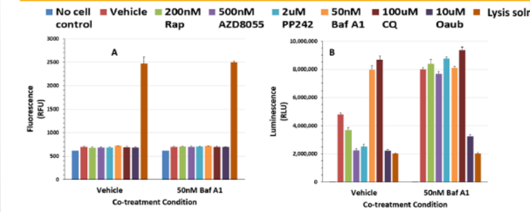
Add 40µl assay reagent and shake (wait 10min)

Read RLU



- High Z' in 384-well plates
- CVs = 3-5% for n = 60
- Consistent induction and inhibition responses

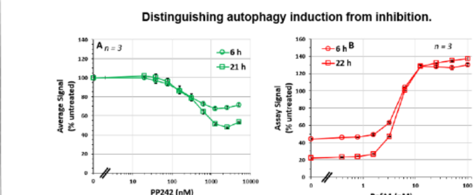
5. MOA confirmation for autophagy modulators



Autophagy induction blockade by BafA1 and cytotoxicity assessment. U2OS autophagy reporter cells (8000/well) in 96-well plates in medium with CellTox™ Green Dye. Attached cells were treated for 21 hr with test comps ± Baf A1. **(A)** Cytotoxicity was first assessed by CellTox™ Green fluorescence (only lysis soln killed cells). **(B)** Nano-Glo® HiBiT Lytic Rgnt added and RLU measured after 10 min. Treatments induced or inhibited autophagy as expected.

6. Dose-dependent modulation of autophagic flux

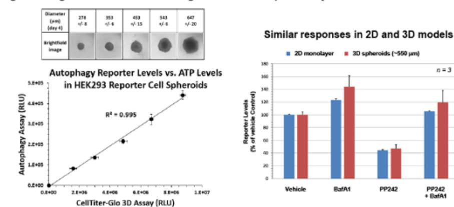
Distinguishing autophagy induction from inhibition.



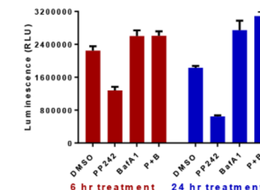
U2OS autophagy reporter cells (8000/well) were plated in white 96-well plates. After overnight attachment, cells were treated for the indicated times with (A) increasing concentrations of a reference autophagy inducer, PP242, or (B) increasing concentrations of a reference autophagy inhibitor, Bafilomycin A1, in the presence of a fixed concentration of PP242 (2µM). Nano-Glo® HiBiT Lytic Reagent was added and luminescence measured after 10 minutes. Assay signal is normalized to time-matched, vehicle-treated controls.

7. Autophagy assay applied to 3D cell culture

- **Autophagy assay signal from different sizes of HEK293 spheroids is proportional to ATP viability marker measured using CellTiter-Glo® 3D Assay in parallel wells**
- **longer mixing time with detection reagent enhanced spheroid lysis**



8. Autophagy reporter transient expression by BacMam transduction



Modulation of transiently expressed autophagy reporter activity. BacMam viral particles harboring the autophagy reporter cDNA were applied (6.25% vol/vol) to U2OS cells (5000/100µL/well) in a 96 well plate. 24 hrs later vehicle (DMSO), PP242 (2µM), BafA1 (50 nM), or PP242 + BafA1 were applied for 6 or 24 hrs before adding Nano-Glo® HiBiT Lytic Rgnt and measuring luminescence. Similar results were obtain with HEK293 cells.

9. Conclusions

NanoBiT™ luciferase technology enables detection of autophagic flux
- The LC3-based autophagy reporter can be stably expressed in desired cell lines
- The autophagy reporter HiBiT subunit is measured by adding LgBiT subunit to reconstitute a bright luciferase activity
- Homogeneous assay protocol: add reagent, mix, measure signal on plate reader
- Stimulation of autophagy results in signal reduction
- Inhibition of autophagy results in signal increase

Assay performance
- Signal is linear over wide range of reporter levels
- Half-life of luminescent signal is greater than 3 hours
- Quality assessment by Z' indicates suitability for HTS for autophagy modulators

This novel multi-well plate assay detects changes in autophagic flux and enables efficient screening for autophagy modulators in both 2D and 3D cell culture models.

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1323-D

Implementation of Acoustic Dispensing for Kinetic Monitoring of Glycolysis and Glutaminolysis.

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1. Abstract

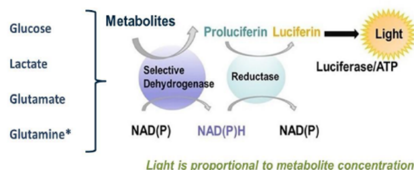
Glycolysis and glutaminolysis are two major energy metabolism pathways required for rapid cell proliferation. In the cancer microenvironment, cancer cells and activated T lymphocytes exhibit similar metabolic profiles as they compete for the same nutrients. Understanding and targeting these metabolic pathways, therefore, can provide valuable information on the function of those cells and lead to more effective cancer treatment strategies.

Contamination and carryover-free acoustic liquid handling technology has been widely implemented into high-throughput applications. Here we evaluated the novel application of small volume acoustic sampling for measuring cell metabolism using bioluminescent metabolite assays. Normal usage of these high sensitivity metabolism assays requires sample pre-dilution. In contrast, use of the Labcyte Echo® Liquid Handler eliminates this pre-dilution step as it is able to dynamically adjust to differing fluid properties and is able to transfer from any well to any well.

This type of rapid and small volume sampling has minimal impact on the cell growth conditions and in combination with bioluminescent metabolite assays that are well suited for miniaturization provides a unique approach for easy and fast repetitive cell metabolism monitoring.

2. Bioluminescence Metabolite Assays

Metabolites *Detection Reagent*



Light is proportional to metabolite concentration

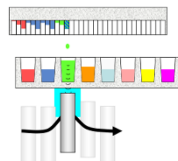
*Glutamine detection requires a two step protocol: glutamine is converted to glutamate before adding glutamate detection reagent

Leippe, D. et al. SLAS Discovery. 2017, 22, 366-377

3. Labcyte Echo

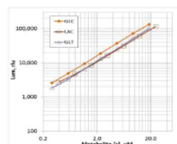
The Labcyte Echo® 500 series liquid handlers revolutionize liquid transfer by using acoustic energy to eject fluids. Transfer with Echo liquid handlers is completely touchless — no tips or nozzles, and no material contacts the sample as it moves from source to destination. The elimination of tips when using the Echo liquid handler provides additional cost savings and eliminates waste, carry-over effects, and cross-contamination. The Echo 555 liquid handler can transfer in 2.5 nL increments to allow miniaturization with accuracy and precision.

Echo 555 liquid handlers have a transducer that emits low energy sound waves to eject 2.5 nL droplets from a source plate to an inverted destination plate above. Droplets are retained in the destination plate by electrostatics and surface tension.



4. Experimental Design and Validation

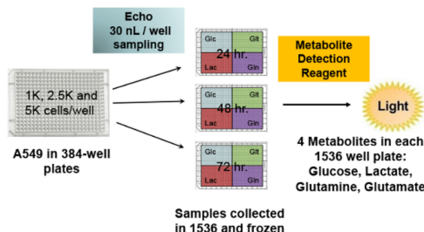
Experimental design and validation in 1536-well plates using metabolite standards (glucose, lactate and glutamine)



	Step	Volume	Details
1	Prepare Metabolite Standards	60 µl	Prepare in medium in 384-well plates
2	Prepare Sample collection plates	4.0 µl	Pre-dispense PBS into 1536-well plates
3	Collect samples	30 nl	Use Echo® to collect. Samples can be analyzed immediately or stored at -20C
4	Detect Metabolites	4.0 µl	Add appropriate detection reagent and read luminescence after 1hr incubation at room temperature

5. Measuring Metabolites in Culture Medium

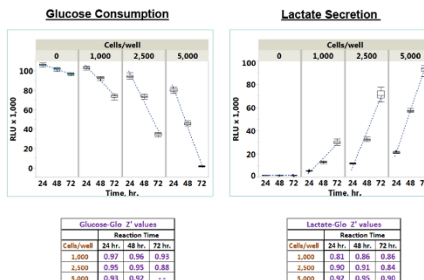
To detect changes in metabolite concentration during cell growth, media samples are collected at different time points, frozen and analyzed at the end of experiment



6. Glycolysis: Measuring Glucose Consumption and Lactate Secretion

A549 cancer cells were cultured in DMEM with 5mM glucose, 2mM glutamine and 4% dialyzed FBS:

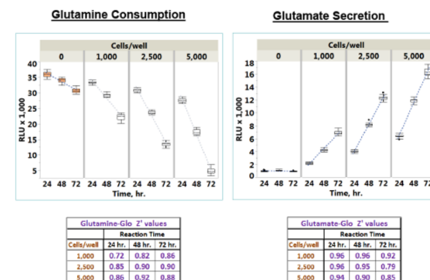
- Rapid glucose consumption with high levels of lactate production – consistent with glycolytic phenotype of cancer cells



Glucose-Glo Z' values			
	Reaction Time		
Cells/well	24 hr.	48 hr.	72 hr.
1,000	0.97	0.96	0.93
2,500	0.95	0.95	0.88
5,000	0.93	0.92	- -

Lactate-Glo Z' values			
	Reaction Time		
Cells/well	24 hr.	48 hr.	72 hr.
1,000	0.81	0.86	0.86
2,500	0.90	0.91	0.84
5,000	0.92	0.95	0.90

7. Glutaminolysis: Measuring Glutamine Consumption and Glutamate Secretion

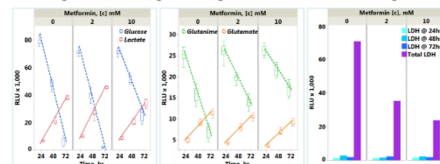


Glutamine-Glo Z' values			
	Reaction Time		
Cells/well	24 hr.	48 hr.	72 hr.
1,000	0.72	0.82	0.86
2,500	0.85	0.90	0.90
5,000	0.86	0.92	0.88

Glutamate-Glo Z' values			
	Reaction Time		
Cells/well	24 hr.	48 hr.	72 hr.
1,000	0.96	0.96	0.92
2,500	0.96	0.95	0.79
5,000	0.94	0.90	0.85

8. Metformin: Inhibition of Glycolysis and Glutaminolysis with Decrease in Cell Proliferation

Affect of metformin, an inhibitor of the mitochondrial respiratory-chain complex 1, on glucose, lactate, glutamine, glutamate metabolism and cell growth



Metformin treatment:

- No effect of cell toxicity - no change in LDH release into the medium
- Decrease in cell numbers - decrease in total LDH (LDH after cell lysis)
- Increase in aerobic glycolysis - No or slight decrease in glucose consumption with no significant change in lactate
- Decrease in glutamine consumption with no significant changes in glutamate secretion

9. Conclusions

Bioluminescent Cellular Energy Metabolism Assays

- Wider assay window, broader linearity and improved sensitivity as compared to colorimetric and fluorometric assays
- Amenability to automation with robust performance in 384-, 1536-well plates

Acoustic Dispensing

- Fast and accurate cell culture medium sampling at different time points of cell growth or treatment
- Nanoliter sampling volumes do not change the volume of total cell culture – multiple samples can be collected from the same well

Implementation of Acoustic Dispensing with Bioluminescent Metabolite Assays

- Minimal sample handling – samples can be collected at different time points and analyzed directly in 1536-well plates
- Changes in key metabolic pathways, glycolysis and glutaminolysis, can be analyzed rapidly in high-throughput format

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1236-B

Deciphering key cancer and inflammation signaling pathways with homogeneous bioluminescent cell based kinase activity assays

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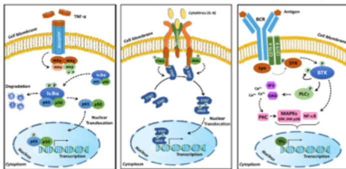
1. Introduction

Signaling pathway activation leads to a multitude of cellular responses including modulation of enzyme activity, altered gene expression, and protein translocation or degradation.

Specific phosphorylation events by specific kinases in cells constitute important **nodes** in signaling pathways. Monitoring these signaling events using cell-based methods is essential to better understand normal cell behavior and disease states.

Cancer and Inflammatory Response Signaling Pathways

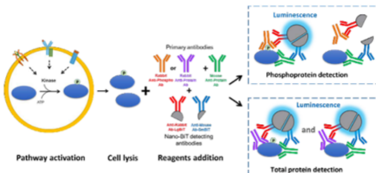


Here we describe the utility of a simple homogeneous cell-based platform to interrogate signaling pathways by detecting phosphorylation of specific proteins.

These bioluminescent cell-based pathway analysis assays can be used to analyze signaling pathways of interest, study the kinase cellular activity and regulation or identify specific kinase or pathway inhibitors

2. Principle of Homogeneous Cell-Based Kinase Assay

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



Assay Principle:

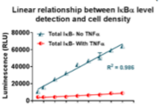
1. Activate pathway
2. Lyse Cells
3. Add antibody mix
4. Incubate 2 hours
5. Add Nano-Glo reagent
6. Read Luminescence

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

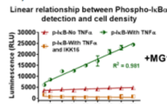
3. Linearity and Sensitivity of Bioluminescent Cell-Based Kinase Assay

Detection of Total and Phospho IκBα in Different Cell Densities
(MCF7 cells)

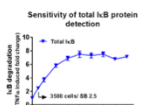
Linear relationship between IκBα level detection and cell density



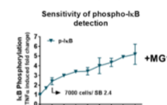
Linear relationship between Phospho-IκBα detection and cell density



Sensitivity of total IκB protein detection



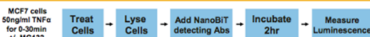
Sensitivity of phospho-IκB detection



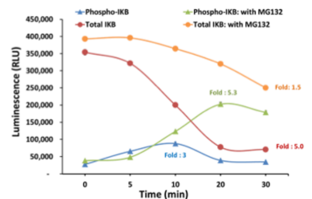
- Bioluminescent detection of IκBα protein and its phosphorylation upon NF-κB pathway activation is linear with cell number.
- Assay is sensitive to detect total and phospho protein levels in low cell density.

MG132: Proteasome inhibitor. IKK16 is IKK kinase inhibitor

4. NF-κB Pathway: TNF-Induced IκBα Phosphorylation and Degradation



Detection of Total and Phospho IκBα upon TNF Treatment



- NanoBiT IκB detecting reagents reveal the predicted biology of NF-κB signaling pathway upon TNF treatment: **IκBα phosphorylation** (pS32) immediately followed by its **fast degradation.**
- Detection of the predicted response of NF-κB pathway to proteasome inhibitor MG132 treatment: decrease in IκBα degradation and accumulation of phosphorylated IκBα.

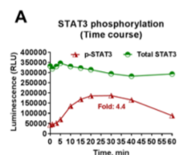
5. JAK/STAT Pathway: Monitoring IL6-Mediated Phosphorylation of STAT3

Detection of Total and Phospho STAT3 upon IL-6 Treatment

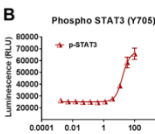
Protocol:

1. 50,000 A431 cells were treated with 50ng/ml IL-6 for various time points **(A).**
2. 50,000 A431 cells were treated with different concentrations of IL-6 for 25 min **(B and C).**
3. Total STAT3 and p-STAT3 (Y705) were detected using the protocol in panel 2 **(A, B, and C).**

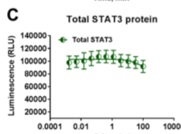
A



B



C



NanoBiT STAT3 detecting reagents reveal the known biology of JAK/STAT pathway after IL6 treatment: STAT3 **phosphorylation** (pY705) with **no effect** on STAT3 protein levels.

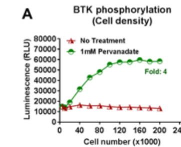
6. BTK Pathway: Activation of BTK Phosphorylation

Detection of Phospho BTK upon pervanadate Treatment

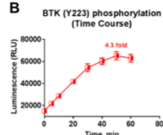
Protocol:

1. Ramos cells were plated at different densities and treated with 1mM pervanadate for 40 min **(A).**
2. 100,000 Ramos cells were treated with 1mM pervanadate for various time points **(B)** or treated with different Pervanadate concentrations for 40 min **(C).**
3. Phospho BTK (Y223) was detected using the phosphoprotein detection protocol in section 2 **(A, B, and C).**

A



B



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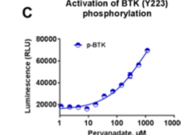
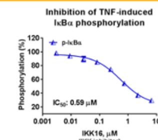
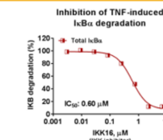
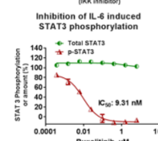


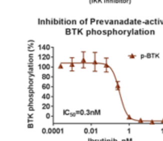
NanoBiT BTK detecting reagents show linear relationship between BTK phosphorylation (pY223) and cell number, incubation time and pervanadate (pY phosphatase inhibitor) concentration.

7. Modulation of Immune and Inflammatory Response Pathways with Small Molecules



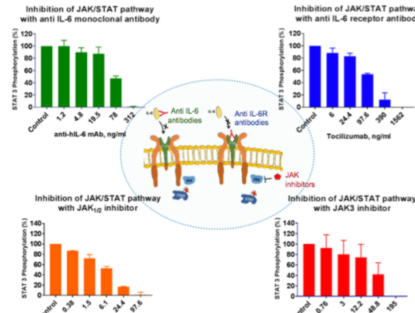






- The bioluminescent NanoBiT pathway assays reveal the expected pharmacology of the pathway node kinase inhibitors.
- These cell-based assays can be used to screen inhibitors of cancer, immune and inflammatory response pathways.

8. Pathway Modulation with Large and Small Molecules Detected with NanoBiT Assay

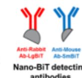


Bioluminescent NanoBiT kinase assays can be used to identify small or large molecule inhibitors of signaling pathways.

9. Conclusions

Benefits of the bioluminescent cell-based NanoBiT kinase assays:

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



Nano-BiT detecting antibodies

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1348-D

Monitoring functional mechanisms of protein degradation in living cells

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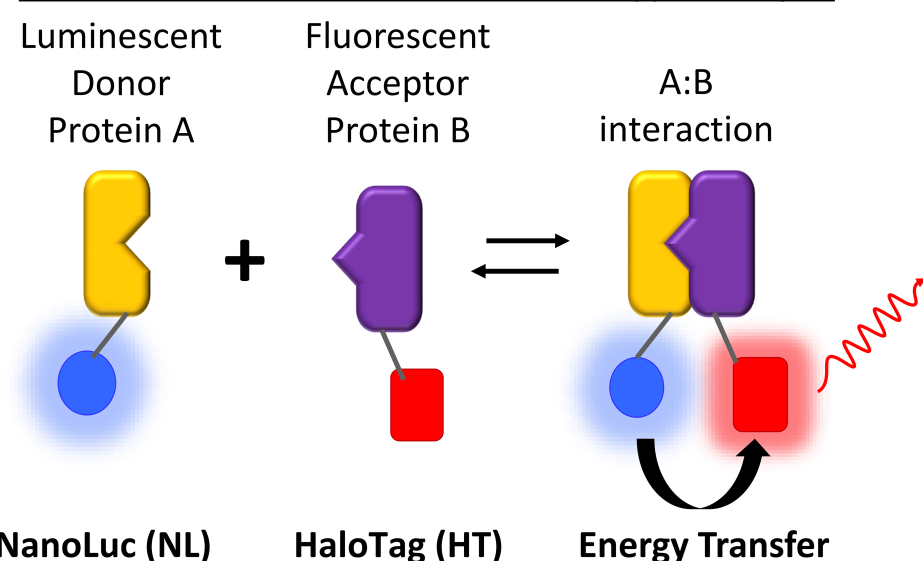
Abstract and introduction

Here we present mechanistic cellular studies on several proteins targeted for degradation via the ubiquitin proteasome pathway in mammalian cells. Using bioluminescence resonance energy transfer with NanoLuc® and HaloTag® fusions, termed NanoBRET, we can monitor changes in interactions of proteins targeted for degradation, including dynamic recruitment to E3 ligases and real-time trafficking to the 26S proteasome using inhibitors or PROTAC based compounds. We show also the ability to quantitate protein levels over a significant dynamic range by monitoring NanoLuc luminescent levels (RLUs) of several proteins, either expressed exogenously or as endogenous knock-in fusion proteins using CRISPR/Cas9. Live cell monitoring of protein:protein interactions and degradation is shown for BRD4, β-catenin, cMyc, and HIF1α. These combined approaches deconvolute the complicated processes involved in proteasomal recruitment and are a powerful strategy for understanding and following protein degradation.

Protein:Protein interactions in living cells

NanoBRET™ Protein:Protein Interactions

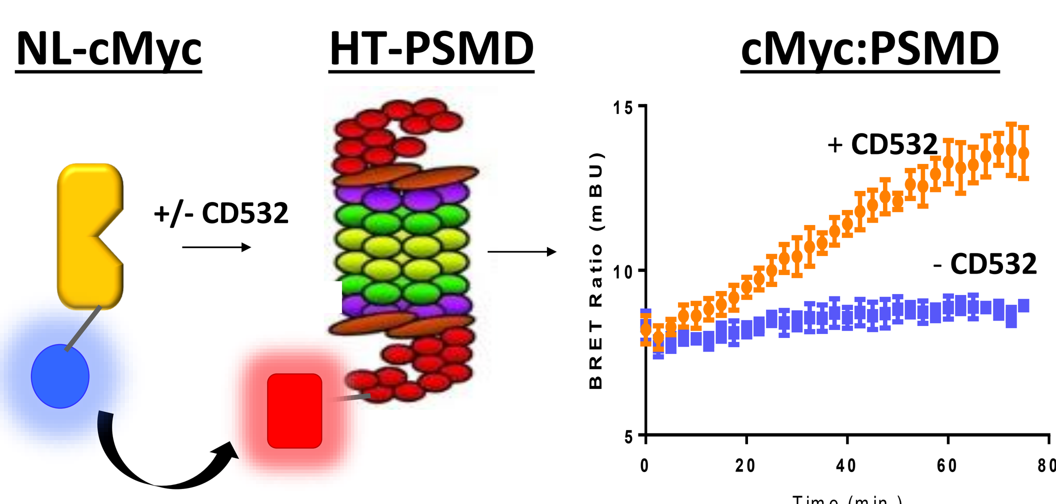
Bioluminescence Resonance Energy Transfer



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

Monitoring proteosomal trafficking

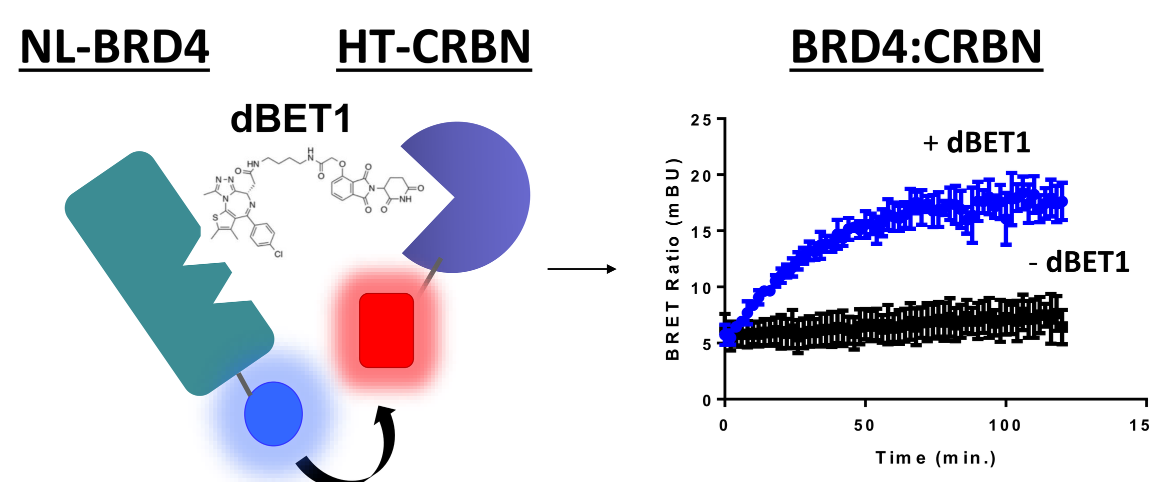
NanoBRET for real-time kinetic interactions with 26S Proteasome



- **Measure changes in proteosomal recruitment of cMyc after addition of AURKA inhibitor, CD532**
- **CD532 known to increase ubiquitination of cMyc and promote degradation**

Monitoring interactions with E3 Ligases

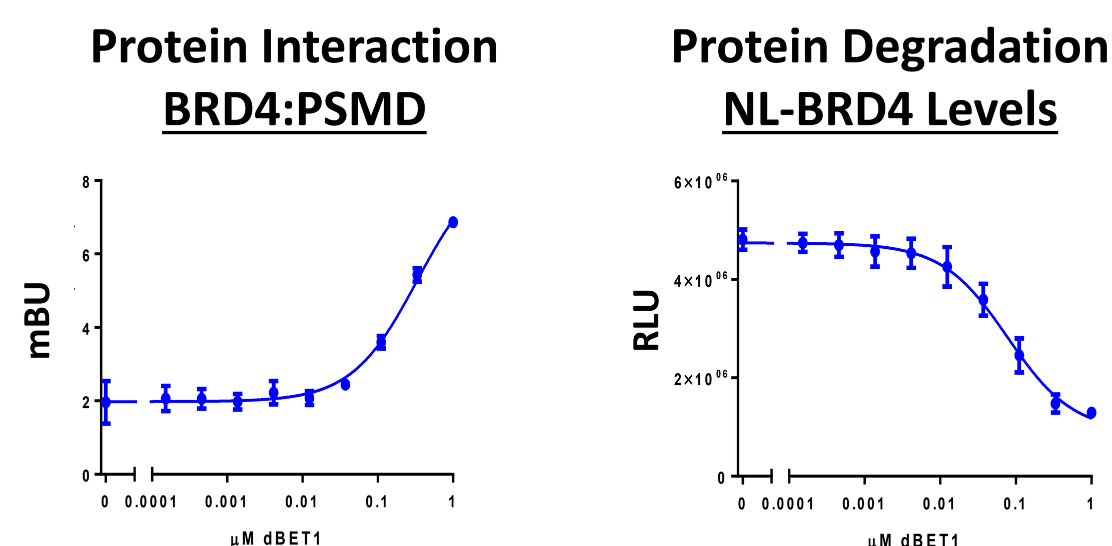
NanoBRET for real-time kinetic interactions with E3 Ligases



- **Detect the induced and increased interaction of BRD4 with Cereblon E3 ligase after treatment with dBET1**
- **Able to use the technology to study small molecule or PROTAC induced interactions with E3 Ligases**

Dose response degradation of BRD4

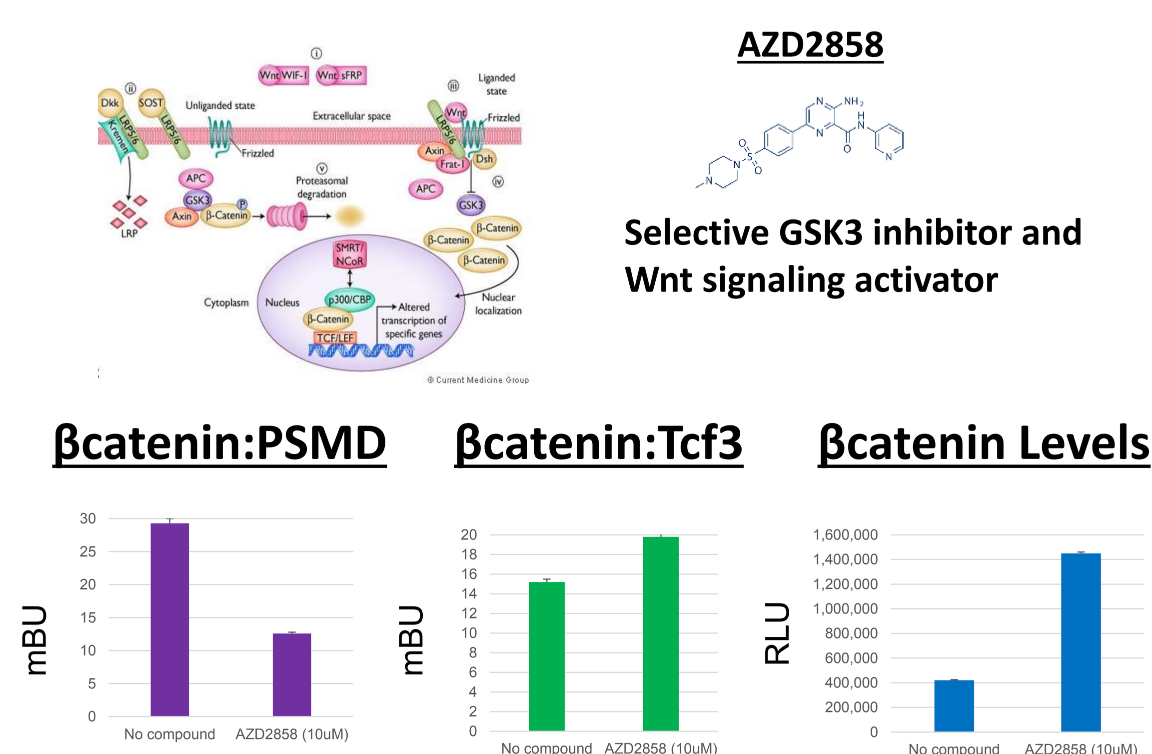
Simultaneous measurement of BRD4 proteasomal interaction with BRD4 protein levels +/- dBET1



- **Determine EC50 for BRD4:Proteasome and IC50 for loss of BRD4**
- **In the same experiment follow new interaction as well as protein level**

Degradation studies with β-catenin

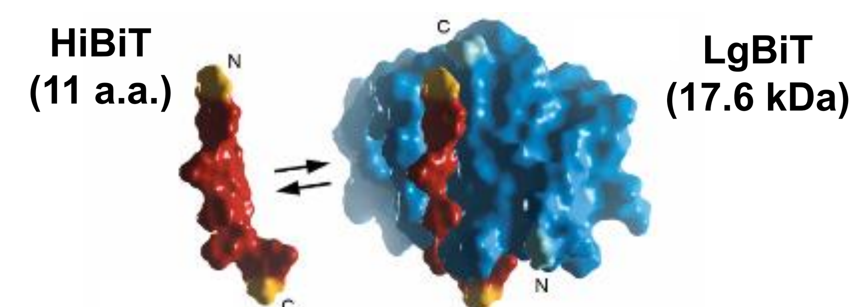
Following interactions of β-catenin in the Wnt signaling pathway



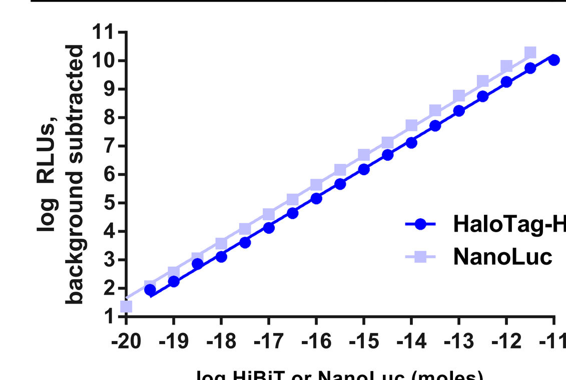
- **Measure decrease and correlative protein interaction increases after treatment with B-catenin stabilizer compound AZD2858**

HiBiT Technology for endogenous studies

NanoLuc® Binary Complementation



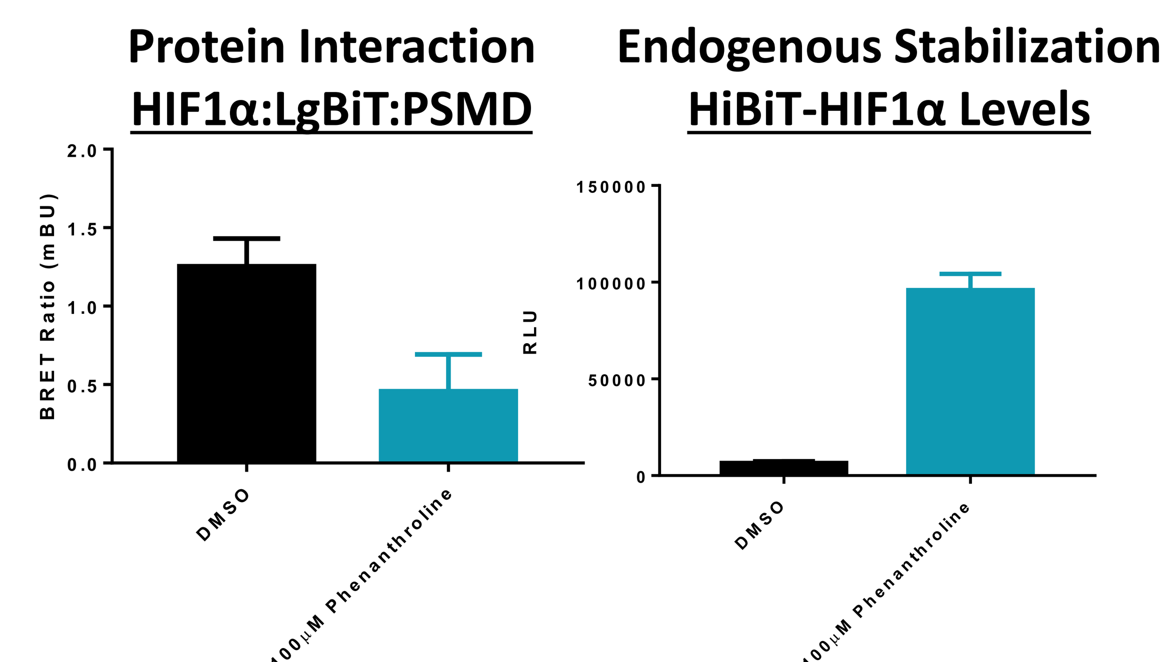
HiBiT technology consists of an 11amino acid high affinity peptide which complements with a larger fragment, LgBiT, to generate NanoLuc luminescence



- **High affinity interaction between HiBiT and LgBiT (K_D = 700 pM) drives complementation in lysates or cells**
- **HiBiT can be an N- or C-terminus fusion tag expressed either exogenously or endogenously via CRISPR/Cas9**
- **Shows a 7-log range for detection of protein levels**

Endogenously tagged HiBiT-HIF1α

Proteasome recruitment and cellular protein quantitation of CRISPR/Cas9 endogenously tagged clonal HiBiT-HIF1α



- **NanoBRET sensitivity allows for detection of decrease of HIFα at proteasome after phenanthroline treatment**
- **Significant range of HIF1α stabilization measured**
- **HiBiT, 11aa, allows for rapid tagging of endogenous proteins via CRISPR/Cas9**

Summary

- **NanoBRET technology is a highly sensitive method for detecting live cell protein:protein interactions**
- **NanoBRET assays for protein degradation can monitor in real time:**
  - **Protein:E3 ligase recruitment**
  - **Protein:Proteasome trafficking**
  - **PROTAC induced interactions**
- **NanoLuc and/or HiBiT can be used for quantitative monitoring in cells of proteins targeted for degradation**
- **HiBiT technology is highly amenable for use in CRISPR/CAS9, allowing for the study of endogenously tagged proteins**

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1349-E

Monitoring Protein Dynamics at Endogenous Levels with a Luminescent Peptide Tag

Christopher Eggers, Brock Binkowski, Kristin Riching, Sarah Mahan, Juliano Alves, Marie Schwinn, Braeden Butler, Danette Daniels, Hicham Zegzouti, Thomas Machleidt, Keith Wood, and Frank Fan

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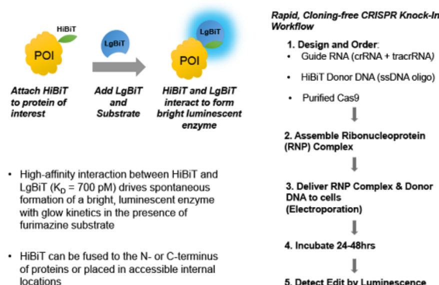
1. Introduction

Intracellular signaling is largely mediated through protein dynamics, including changes in protein abundance, interactions, location, or post-translational modification. While the behavior of proteins is commonly studied using overexpressed reporter genes, CRISPR/Cas9 offers the possibility of tagging genes with reporters at the endogenous locus in order to maintain physiological expression levels, regulation, and stoichiometry with binding partners. The 11-amino-acid peptide, HiBiT, represents an ideal tag for studying the protein dynamics of endogenously expressed proteins. Its small size facilitates rapid knock-in of the tag using ribonucleoprotein complexes of Cas9 and gRNA along with synthesized single-stranded oligonucleotide donor DNA. High-affinity complementation of HiBiT with the 18 kDa LgBiT subunit generates the bright, luminescent NanoBiT™ enzyme, enabling sensitive quantification of HiBiT-tagged proteins over 7 orders of magnitude of linear dynamic range. Changes in protein abundance can be monitored in either a lytic endpoint assay using purified LgBiT protein or in a live-cell kinetic assay by expressing the LgBiT subunit in cells. Interactions of a HiBiT-tagged protein with protein fusions to HaloTag can be measured in live cells using bioluminescence resonance energy transfer (BRET). We apply these assays to the induced degradation by PROTAC compounds of the bromodomain-containing protein, BRD4, and show both a decrease of endogenously expressed HiBiT-BRD4 in cell lysates and live cells and increased protein interactions with the E3 ligase complex and ubiquitin. HiBiT can also be used to measure translocation of proteins to and from the cell surface in a live-cell assay that takes advantage of the membrane impermeability of LgBiT. We show that internalization of endogenously expressed HiBiT-EGFR and HiBiT-β2-AR can be measured in a matter of minutes as a loss in extracellular HiBiT signal. Furthermore, phosphorylation of HiBiT-EGFR upon activation can be monitored in a homogeneous assay by measuring BRET from the HiBiT/LgBiT complex to a fluorescently labeled secondary antibody to monitor binding of an anti-phospho antibody.

2. Overview of HiBiT and Endogenous Tagging

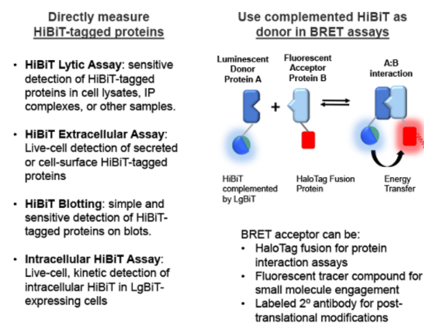


Rapid, Cloning-free CRISPR Knock-In Workflow

1. Design and Order:
   - Guide RNA (crRNA + tracrRNA)
   - HiBiT Donor DNA (ssDNA oligo)
   - Purified Cas9
2. Assemble Ribonucleoprotein (RNP) Complex
3. Deliver RNP Complex & Donor DNA to cells (Electroporation)
4. Incubate 24-48hrs
5. Detect Edit by Luminescence

- High-affinity interaction between HiBiT and LgBiT (K_D = 700 pM) drives spontaneous formation of a bright, luminescent enzyme with glow kinetics in the presence of furimazine substrate
- HiBiT can be fused to the N- or C-terminus of proteins or placed in accessible internal locations

3. Multiple Detection Formats to Measure Protein Dynamics



Directly measure HiBiT-tagged proteins

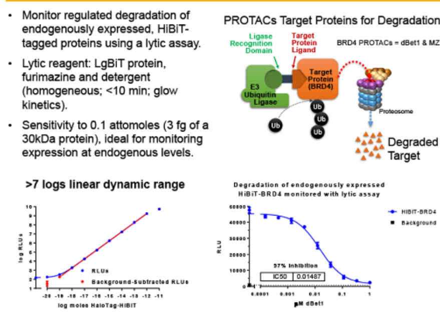
- **HiBiT Lytic Assay**: sensitive detection of HiBiT-tagged proteins in cell lysates, IP complexes, or other samples.
- **HiBiT Extracellular Assay**: Live-cell detection of secreted or cell-surface HiBiT-tagged proteins
- **HiBiT Blotting**: simple and sensitive detection of HiBiT-tagged proteins on blots.
- **Intracellular HiBiT Assay**: Live-cell, kinetic detection of intracellular HiBiT in LgBiT-expressing cells

Use complemented HiBiT as donor in BRET assays

BRET acceptor can be:
- HaloTag fusion for protein interaction assays
- Fluorescent tracer compound for small molecule engagement
- Labeled 2° antibody for post-translational modifications

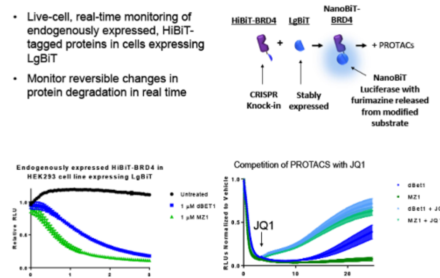
4. Quantify Protein Abundance Across Wide Dynamic Range with a Lytic Assay

- Monitor regulated degradation of endogenously expressed, HiBiT-tagged proteins using a lytic assay.
- Lytic reagent: LgBiT protein, furimazine and detergent (homogeneous; <10 min; glow kinetics).
- Sensitivity to 0.1 attomoles (3 fg of a 30kDa protein), ideal for monitoring expression at endogenous levels.



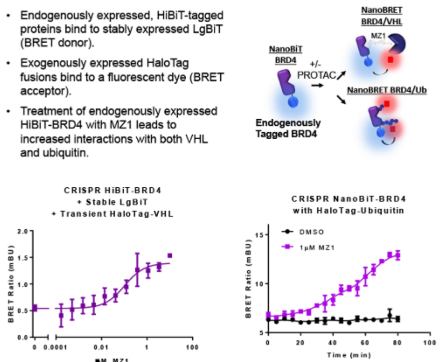
5. Quantify Protein Abundance in Live Cells

- Live-cell, real-time monitoring of endogenously expressed, HiBiT-tagged proteins in cells expressing LgBiT
- Monitor reversible changes in protein degradation in real time



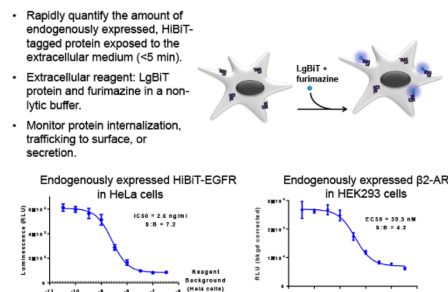
6. Measure Protein-Protein Interactions with NanoBRET

- Endogenously expressed, HiBiT-tagged proteins bind to stably expressed LgBiT (BRET donor).
- Exogenously expressed HaloTag fusions bind to a fluorescent dye (BRET acceptor).
- Treatment of endogenously expressed HiBiT-BRD4 with MZ1 leads to increased interactions with both VHL and ubiquitin.



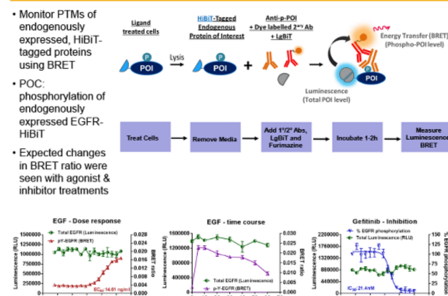
7. Measure Receptor Internalization

- Rapidly quantify the amount of endogenously expressed, HiBiT-tagged protein exposed to the extracellular medium (<5 min).
- Extracellular reagent: LgBiT protein and furimazine in a non-lytic buffer.
- Monitor protein internalization, trafficking to surface, or secretion.



8. Measure Post-Translational Modifications

- Monitor PTMs of endogenously expressed, HiBiT-tagged proteins using BRET
- POC: phosphorylation of endogenously expressed EGFR-HiBiT
- Expected changes in BRET ratio were seen with agonist & inhibitor treatments



9. Conclusions

Monitor protein dynamics at endogenous levels of expression using HiBiT

- **Tag genes at endogenous loci**
  - 11 amino acid size greatly facilitates CRISPR/Cas9 workflow for genomic knock-ins
  - Sub-attomole sensitivity enables measurement of proteins at endogenous levels
- **Quantify protein abundance**
  - Homogeneous, add-mix-read assay protocols
  - >7-log linear dynamic range
  - Monitor changes in regulated protein stability or degradation
  - Lytic endpoint or live-cell kinetic assays
- **Measure protein-protein interactions in live cells**
  - Expression of LgBiT converts HiBiT into a BRET donor
  - Fusions to HaloTag act as BRET acceptors
- **Measure internalization or secretion of HiBiT-tagged proteins**
  - Quantify surface-expressed or secreted HiBiT-tagged proteins in < 4 min
- **Measure post-translational modifications**
  - BRET in lysate from HiBiT/LgBiT complex to fluorescently labeled secondary antibody

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February 2018

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1362-C

Real-Time High Throughput Detection of Annexin V Binding and Caspase-3 Activity Using a Plate Reader

Terry Riss, Kevin Kupcho, John Shultz, Jim Hartnett, Robin Hurst, Wenhui Zhou, Michael R. Slater, Brock Binkowski, Ryutaro Akiyoshi and Andrew Niles   Promega Corporation, Madison, WI, 53711 and [2]Olympus Corp., Tokyo, Japan

Abstract

1. Introduction

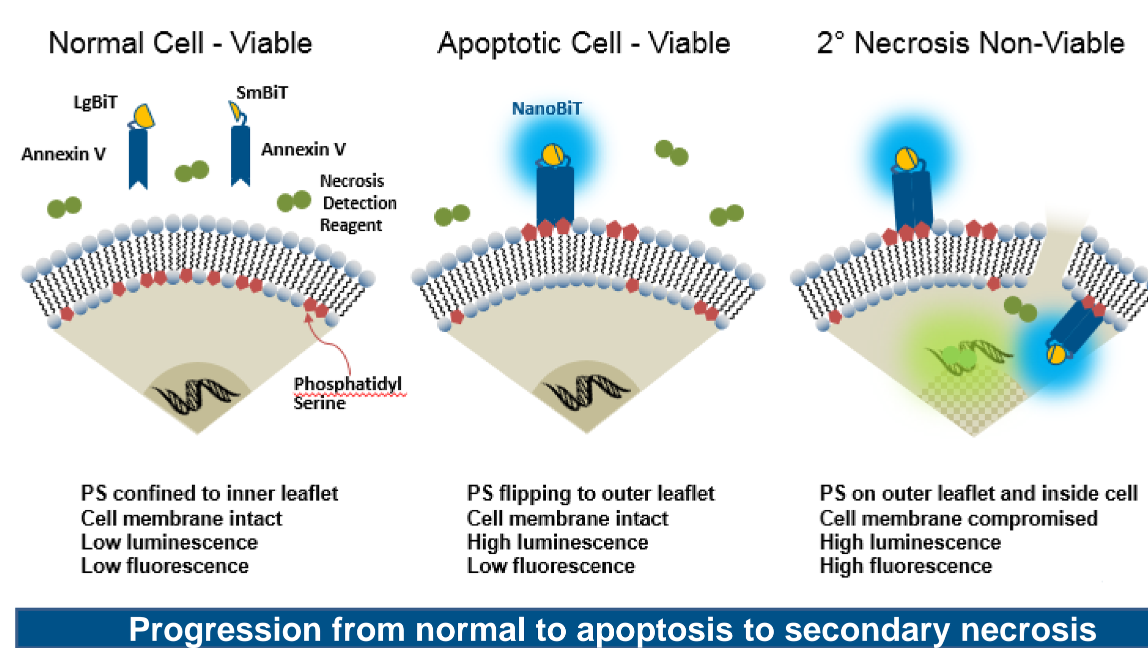
We developed two homogeneous real-time assays for detecting apoptosis that can be recorded using a plate-reading luminometer. The first assay is based on binding of annexin V to phosphatidyl serine (PS) which becomes exposed on the outer leaflet of the cell membrane during apoptosis. We engineered two fusion proteins composed of annexin V linked to a small or large subunit of NanoBiT® luciferase. When the fusion proteins bind in close proximity on the surface of apoptotic cells, the reconstituted NanoBiT generates light to report apoptosis in real-time. Multiplexing with a DNA binding dye reports 2° necrosis in real-time from the same sample.

The second approach relies on expression of a modified firefly luciferase (GloSensor™ Caspase-3/7 Biosensor) containing the DEVD amino acid sequence that is cleaved by caspase-3. GloSensor is inactive in viable cells. Upon induction of apoptosis, caspase-3 cleavage of the DEVD sequence enables GloSensor to fold into an active conformation and generate a luminescence in real-time as the population of cells undergoes apoptosis.

These real-time homogeneous apoptosis assay methods represent an improvement over endpoint assay methods by providing kinetic data from the same sample of live cells in real time using a standard plate reading luminometer.

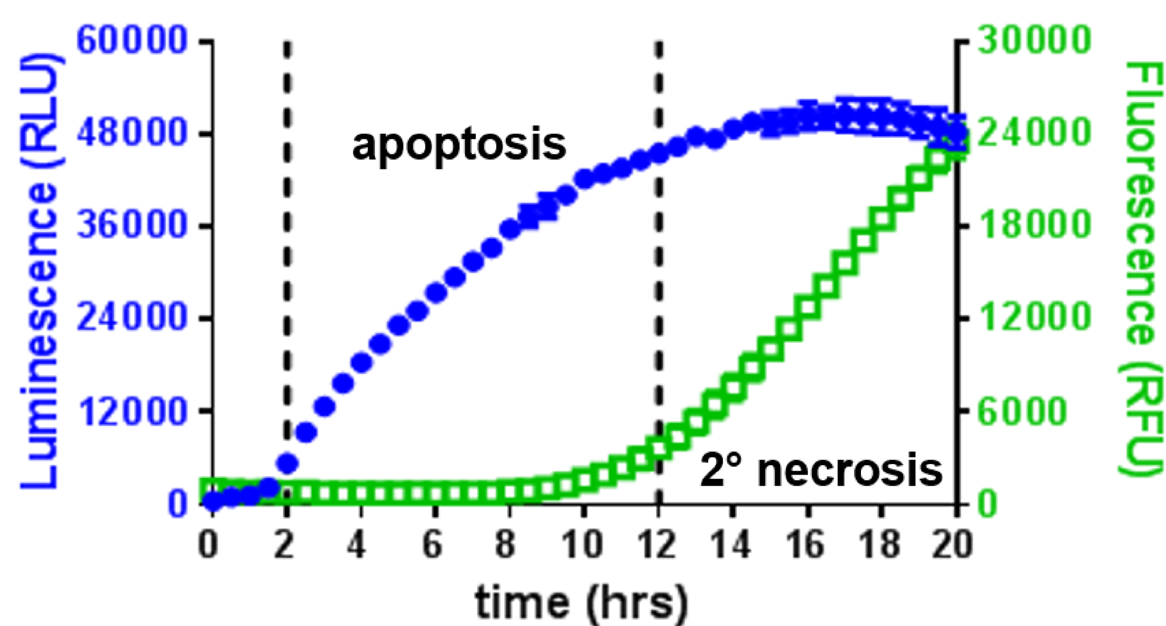
2. Real-Time Detection of Apoptosis using Annexin V Enzyme Complementation Assay

The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay is a homogeneous method to detect the kinetics of phosphatidylserine (PS) exposure as a marker of apoptosis and DNA staining to detect necrosis.



3. Real-Time Detection of Apoptosis and Necrosis

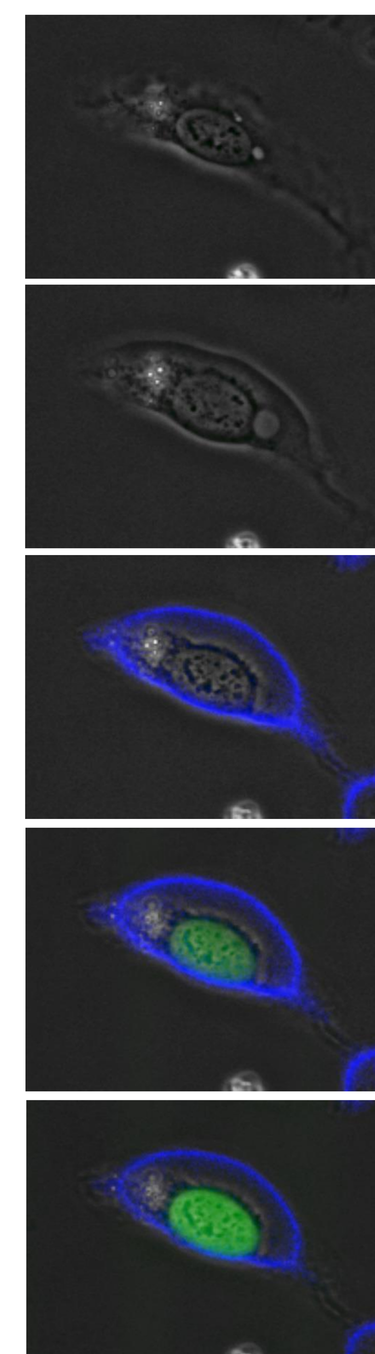
Multiplexing real-time apoptosis (annexin V binding to PS) and secondary necrosis (DNA staining dead cells) assays from the same sample of cells.



The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay reagent was added once at time zero to DLD-1 cells treated with rhTRAIL. Luminescence from annexin V binding indicating apoptosis (blue) and fluorescence from DNA staining indicating necrosis (green) were recorded repeatedly from the same samples.

4. Real-Time Imaging of Luminescent Annexin V Binding and Fluorescent DNA Dye Staining

U2OS cells were cultured in presence of Apoptosis and Necrosis Assay reagent, exposed to 1 µM staurosporine and photographed at indicated times using fluorescent and luminescent modes with an Olympus LV200 microscope. Data provided by Olympus.



1 hr after staurosporine exposure. No staining.

3:45 hr. Note slight cell shape change.

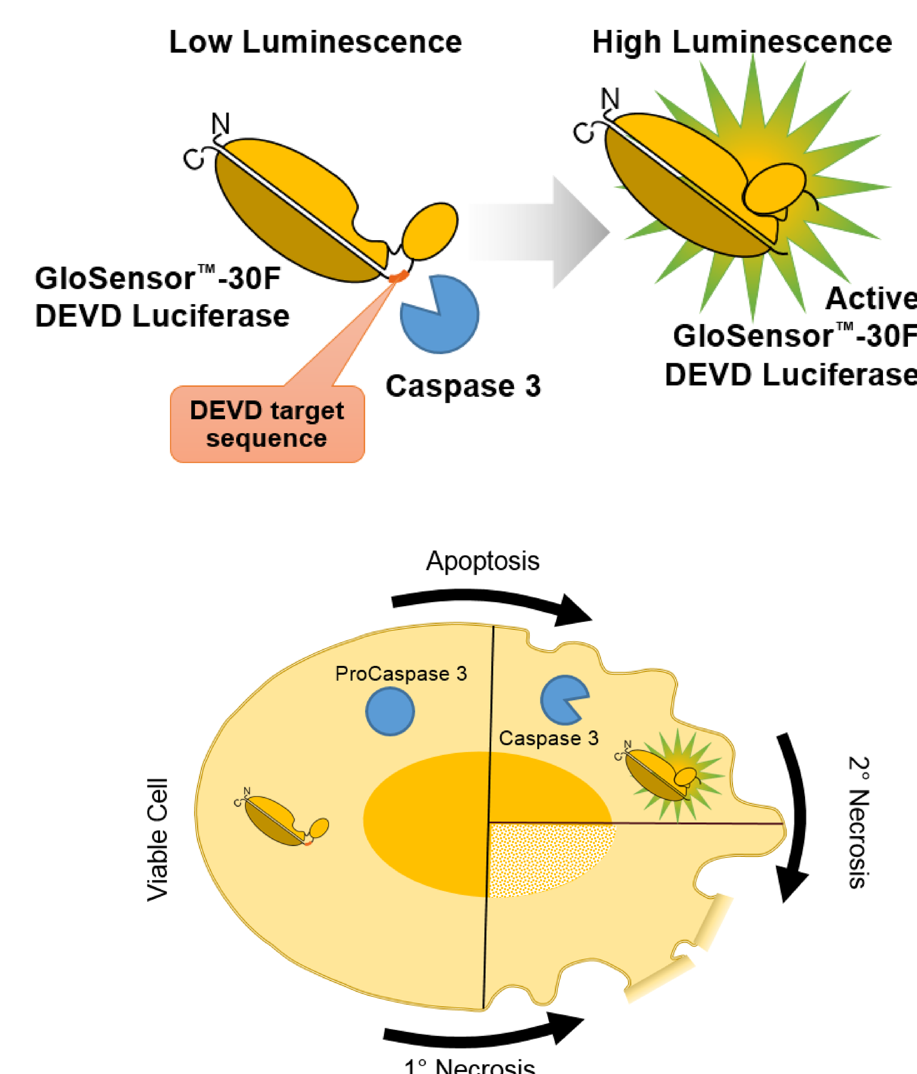
6 hr. Cell membrane is decorated with Annexin V fusion proteins enabling reconstitution of luciferase to generate light indicating apoptosis.

9 hr. Fluorogenic DNA dye has penetrated cell membrane to stain nucleus green indicating 2° necrosis.

12 hr. More intense staining of nucleus with DNA dye. Luminescent signal remains on outer membrane.

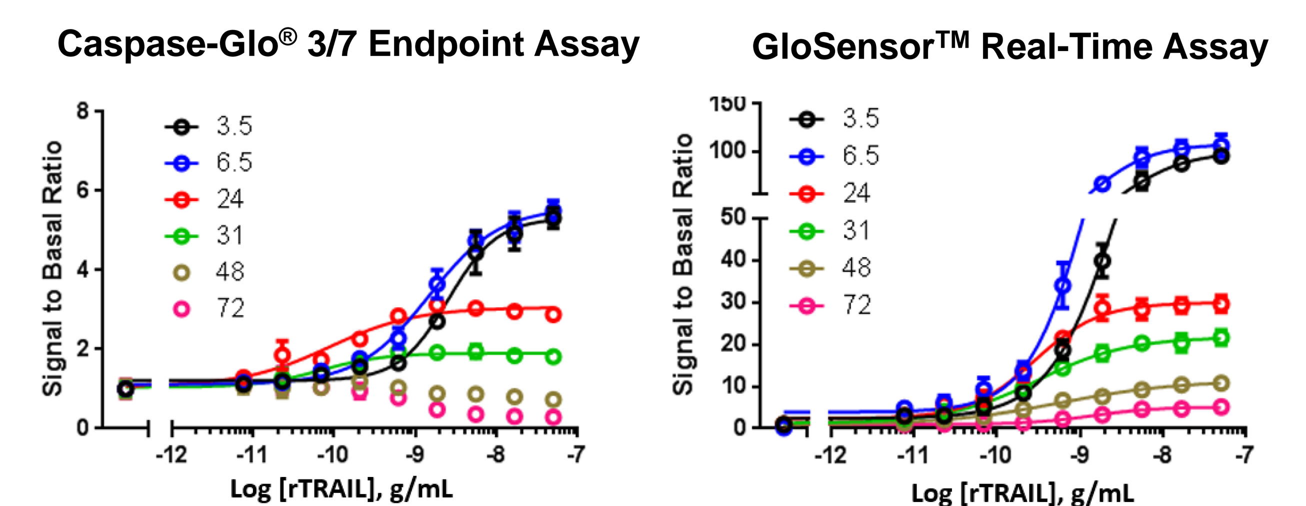
5. Real-Time Live Cell Detection of Caspase Activity

The GloSensor™ luciferase contains DEVD sequence that restricts the conformation of the molecule in an inactive confirmation. Caspase cleavage at DEVD activates luciferase to generate a real-time luminescent signal.



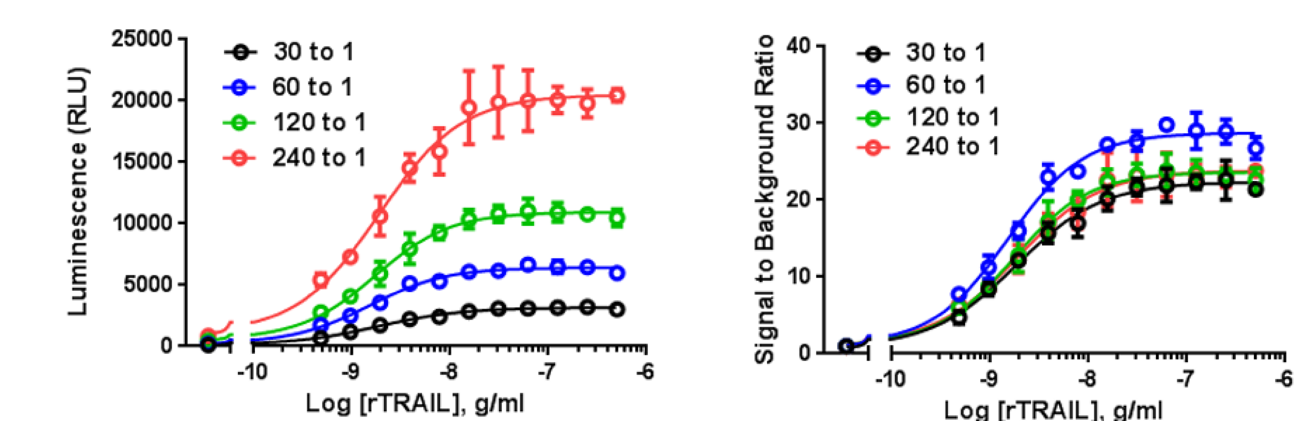
6. Comparison of Real-Time GloSensor™ 30F and Endpoint Caspase-Glo® 3/7 Assays

A plate containing U2OS cells was forward transduced for 18 hr with BacMam GloSensor™ 30F Caspase particles at a MOI of 60, then exposed to serial dilutions of rhTRAIL prepared in medium containing luciferin. Luminescence was recorded from the GloSensor™ plate at indicated times. Six parallel plates were prepared with U2OS cells treated with the same rhTRAIL concentrations. Caspase-Glo® 3/7 Reagent (endpoint lytic assay) was added to one of the six parallel plates at the indicated times (3.5-72hr) and luminescence recorded.



7. Performance of GloSensor™ Caspase Assay Delivered at Different MOI using BacMam

U20S were forward transduced by the addition of BacMam GloSensor™ 30F Caspase particles at ratios of 30, 60, 120 and 240 particles per cell (multiplicity of infection, MOI) for 16 h. Serial dilutions of recombinant human TRAIL were added to replicate wells to induce an apoptotic response during an 18 h exposure. Bright-Glo™ lytic reagent was added to reveal the magnitude of GloSensor™ 30F activation for each MOI.



8. Conclusions

- The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay detects the kinetics of apoptosis and secondary necrosis in real time using a plate reader.
- The assay requires one homogenous reagent addition step and no other washes or processing steps.
- The non-lytic assay reagents enable multiplexing with other assay chemistries such as endpoint methods to measure caspase-3/7 activity as an orthogonal marker of apoptosis.
- The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay enables luminescent time lapse imaging of cells undergoing apoptosis to illustrate heterogeneity among the population of cells and the “real-time” kinetics of apoptosis.
- The GloSensor™ Caspase-3/7 Biosensor containing the DEVD sequence is expressed in cells in an inactive form.
- During apoptosis, caspase-3/7 cleavage at the DEVD sequence enables folding of GloSensor™ luciferase to form an active enzyme to generate light and detection of apoptosis in real-time.
- Stable expression of GloSensor™ luciferase or BacMam delivery of plasmid can be used to generate cells for this real-time caspase assay.

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1363-D

Real-Time Image Analysis of Apoptotic to Necrotic Process in the Same Cells by Microscopy

Ryutaro Akiyoshi[1], Mitsunori Ota[2], Tsutomu Kudoh[2], Kevin Kupcho[2], Thomas Machleidt[2], Andrew Niles[2] and Hirobumi Suzuki[1]

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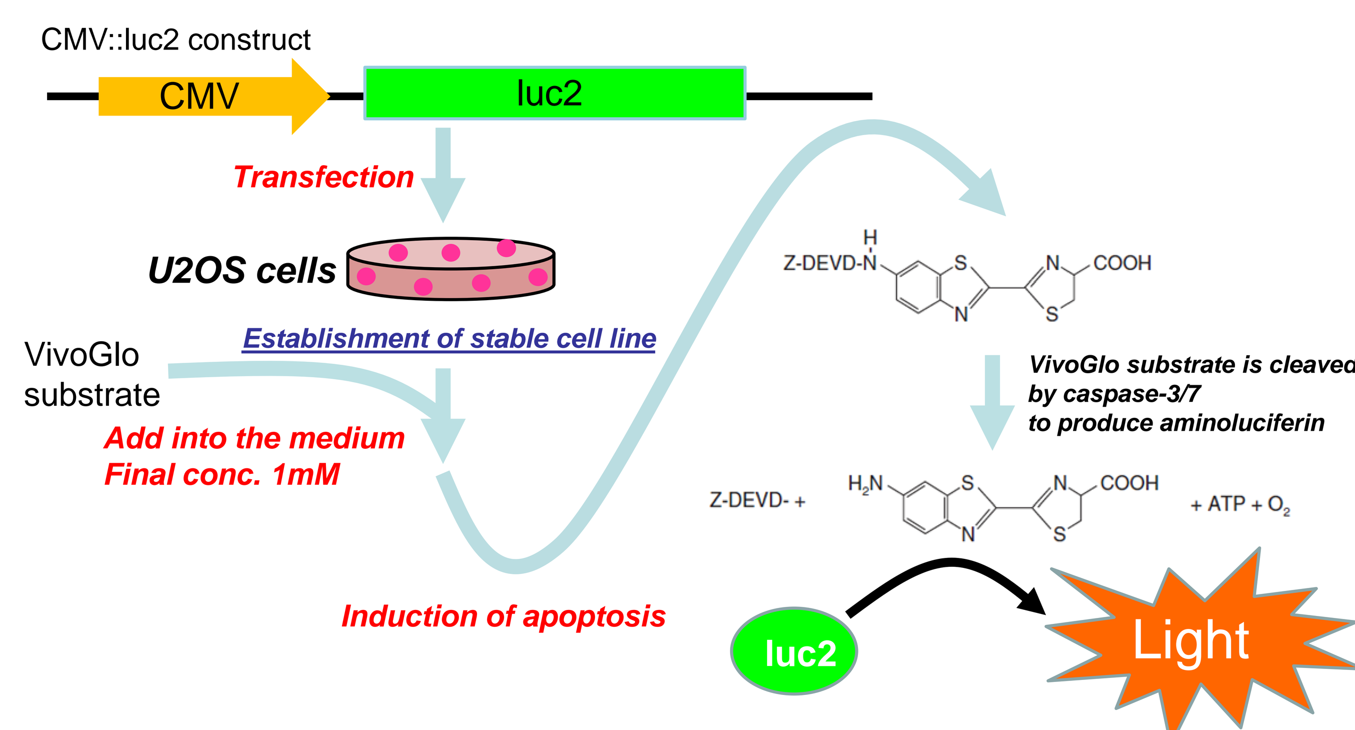
Abstract # 437650

1. Introduction

Apoptosis is an indispensable process for normal tissue development and homeostasis, which allows cells to undergo timely programmed cell death. When apoptosis is induced, in most cases, caspase-3/7 is activated and the cell membrane is compromised by rearrangement of phospholipid. As activation of caspase-3/7 always leads to apoptotic death of the cells, the caspase activity is considered to be a reliable apoptosis marker. In addition, externalization of phosphatidylserine (PS) in the cell membrane is also a typical apoptosis marker. Therefore, the two markers have been assessed by enzymatic and flow cytometric endpoint assays. However, major disadvantages of these assays are (1) it obtains only a single result for each set of endpoints, and therefore (2) it is impossible for real-time measurement of live cells. In order to overcome these disadvantages, we combined bioluminescence and fluorescence microscopy and tried to detect the apoptotic to necrotic process on the same live cell samples.

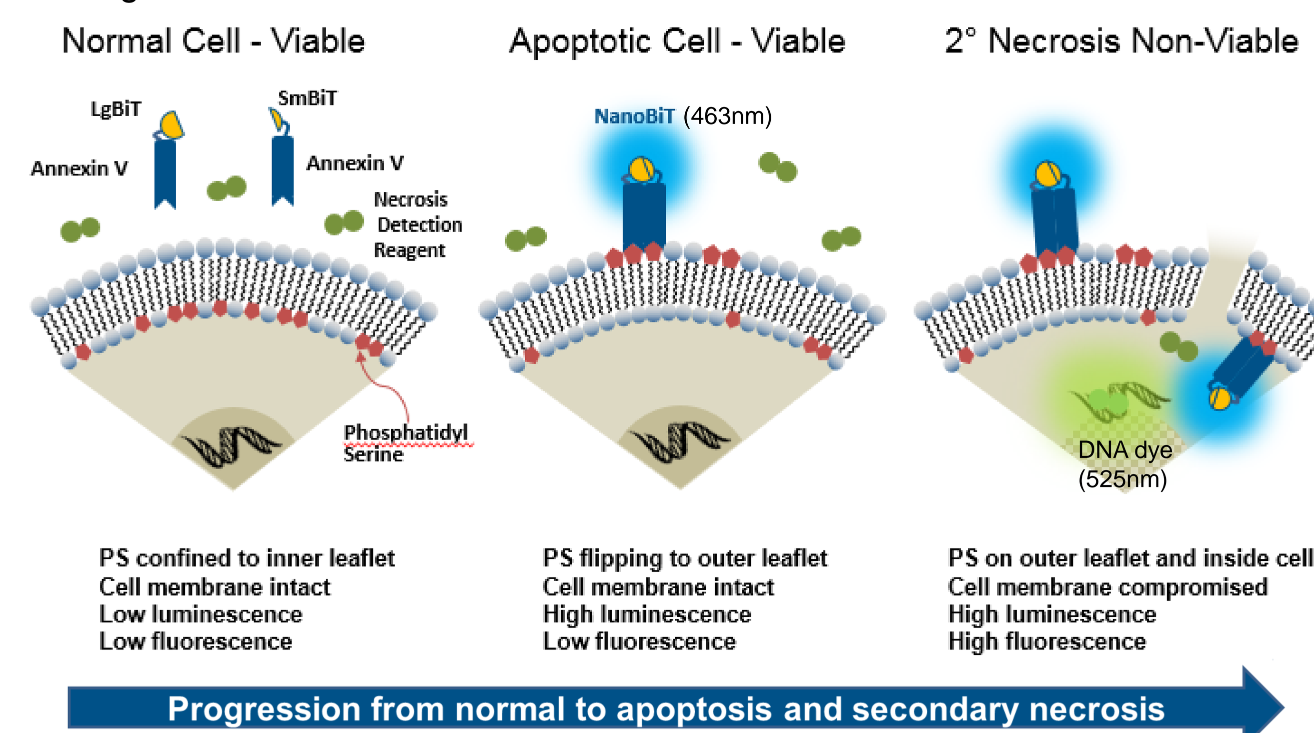
2. Real-Time Live Cell Detection of Caspase Activity

We created a stable U2OS cell line that expresses Luc2 luciferase (Promega). To detect caspase-3/7 activity, the aminoluciferin incorporating the DEVD (Asp-Glu-Val-Asp) motif recognized by the caspase-3/7 (VivoGlo™ Caspase-3/7 substrate, Promega) was added into the culture medium. When caspase-3/7 is activated, liberated luciferin reacts with Luc2 and generates bioluminescent light (609nm).



3. Real-Time Detection of Apoptosis using Annexin V Enzyme Complementation Assay

To detect externalization of PS and secondary necrosis (late apoptosis), we applied RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay Kit (Promega corp.) to the U2OS stable cell line in usage as prescribed in the kit protocol. This kit contains equal ratios of two annexin V molecules expressed as fusions with large or small subunits of NanoBiT luciferase (Promega corp.) and time-released substrate. When PS is externalized on the cell membrane, annexin V binds to PS as a dimmer on the cell surface and NanoBiT Luciferase is reconstructed, and bioluminescence light (λmax=463 nm) is generated. This kit also contains a cell-impermeant, profluorescent DNA dye, which detects necrosis by fluorescent light (λmax=525nm) with the dye binding to nuclear DNA.



4. Real-Time Imaging of Apoptotic to Necrotic Process in the Same Cells by Microscopy

We observed apoptosis and necrosis of the U2OS cells by sequential bioluminescence and fluorescence imaging using a microscope, LV200 (OLYMPUS) after induction of apoptosis by 100nM staurosporine (STS).

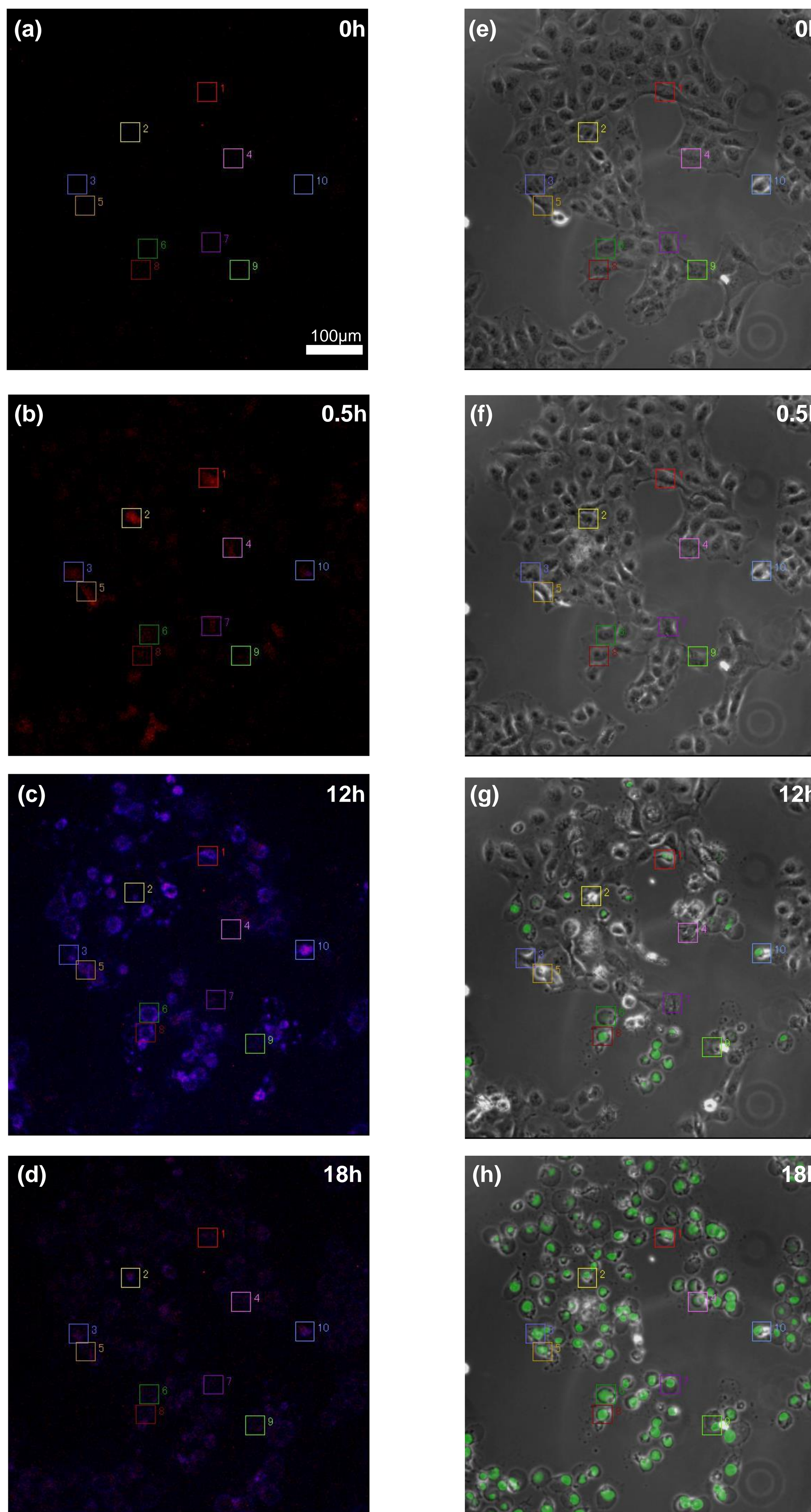


Fig. 1 (a)-(d) Bioluminescence images of caspase-3/7 activity (red) and annexin V dimmerization (blue) indicating apoptotic process from 0 to 18h.
(e)-(h) Fluorescence images of nucleus (green) indicating necrosis merged with phase contrast image from 0 to 18h.

0h: (a) No bioluminescence signals.
(e) No morphological changes of the cells.

0.5h: (b) Caspase-3/7 activity increased as apoptotic initiation.
(f) Cells were shrunk slightly.

12h: (c) Annexin V-NanoBiT dimmerization occurred.
(g) Nucleus of penetrated cells were stained as 2nd necrosis.

18h: (d) Annexin V dimmerization signal decreased as cell membrane compromise.
(h) Nucleus of all cells were stained as 2nd necrosis.

Measurement conditions:
The U2OS stable cells were seeded on a 35mm glass bottom dish. Luminescence images were acquired by the luminescence imaging system LUMINOVIEW (LV200, Olympus) attached with an electron multiplier charge-coupled device camera (ImagEM, Hamamatsu Photonics) binning 1x1 and EM-gain 1200.
Each images were taken by 40x phase contrast objective lens [numerical aperture (NA) 0.75] at 100msec exposure (Phase contrast), 100msec exposure (fluorescence, Ex: BP490-500, Em: BP515-560), 3min exposure (Annexin V-NanoBiT, BP460-510 filter), 5min exposure (luc2-caspase-3/7, 610ALP filter), 10min interval. Duration time of observation was 24 hours.
The dish was kept at 37ºC under 5% CO_2 in the humidified chamber during observation.

5. Time-Course Analysis of Apoptotic to Necrotic Process

Fluorescence and bioluminescence signals from single cells were measured as an average value in a region of interest (ROI) enclosed for each cells of Fig.1 by Time-lapse Imaging Analysis software (TiLIA, Olympus). Similarly, signals in the whole visual field were measured as a plate reader-like analysis.

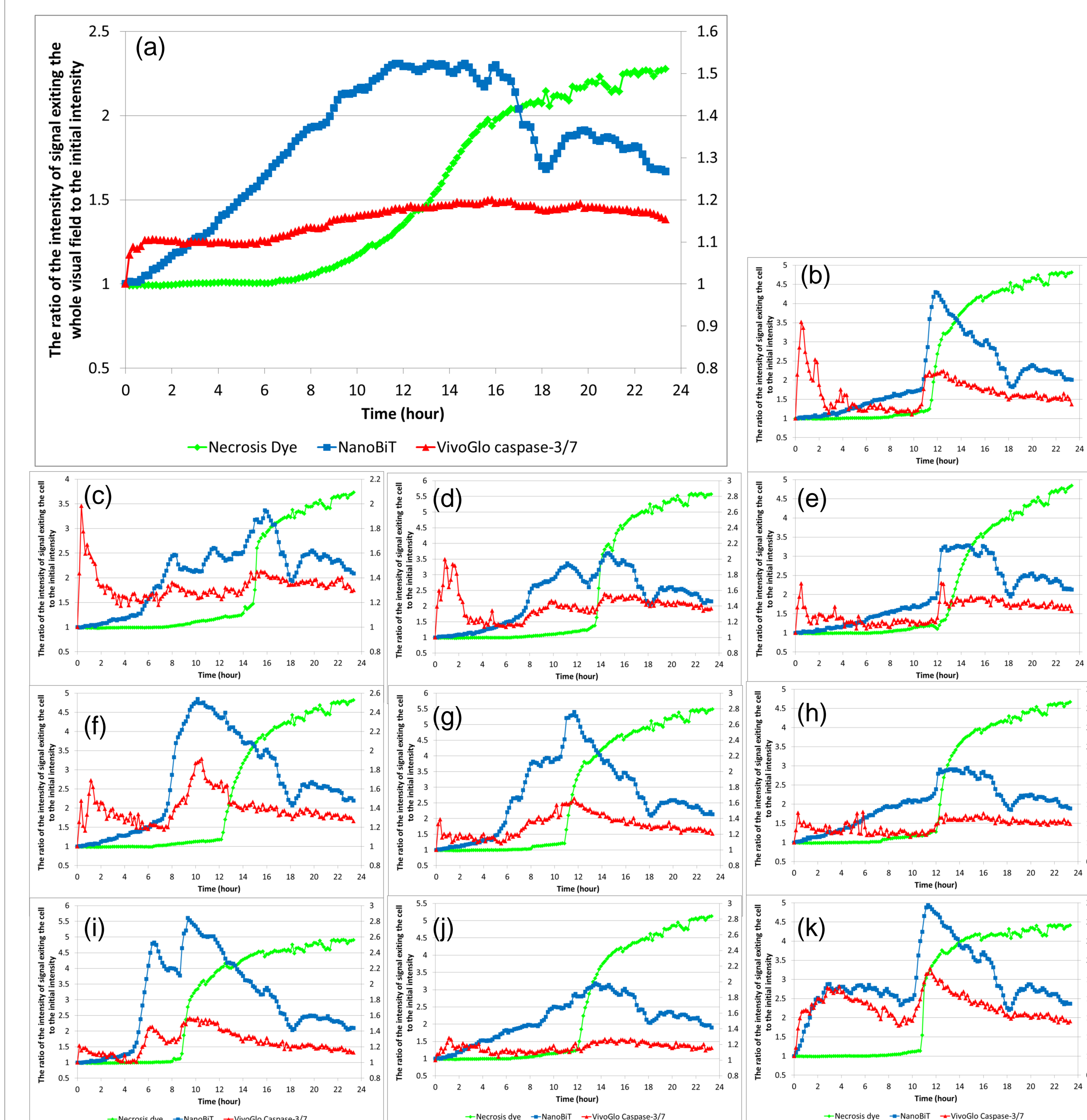


Fig. 2 (a) Time-course analysis of bioluminescence and fluorescence signals in the whole area of the image (from total cells). Necrosis Dye and NanoBiT signals were plotted in the primary axis, VivoGlo signals were shown in the secondary axis. Caspase-3/7 activity reached the first peak within 1 h after STS addition. Annexin V dimmerization signal rose in 1 h and reached the maximum in 12 h, and then decreased. Secondary necrosis signal rose in 7 h and increased for 24 h.

(b)-(k) Time-course analysis for each cell in Fig. 1 (ROI 1 to 10). Caspase-3/7 activity reached the first peak within 1 h, and annexin V dimmerization signal rose in 1 h as same as total cells analysis (a). However, signal profile of annexin V dimmerization varied among cells and the maximum peak time from 9 to 14 h. Secondary necrosis signal rose after the maximum peak time of annexin V dimmerization.

6. Conclusions

- Our imaging method makes possible real-time analysis of the apoptotic to necrotic process by visualizing the same cell samples using VivoGlo™ Caspase-3/7 Assay and RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay.
- This method revealed heterogeneous responses of apoptotic to necrotic process in individual cells, and that suggests importance of single cell analysis.
- However, this result does not conflict with total cell analysis, and that would also strengthen plate-reader based screening results. Namely, before and after screening, reliability of the assay conditions and efficacy of the candidates can be confirmed by image analysis.

February 2018

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1212-C

Bioluminescent High-Throughput Succinate Detection Method for Monitoring the Activity of JMJC Histone Demethylases and Fe(II)/2-Oxoglutarate-Dependent Dioxygenases

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1. Introduction

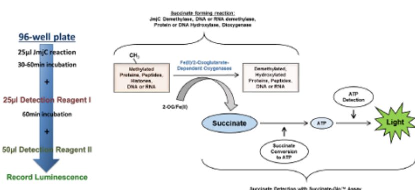
The covalent modification of histone proteins, DNA and RNA by Fe(II)/2-oxoglutarate-dependent dioxygenases are key to the modulation of biological processes such as epigenetics, hypoxic signaling and DNA/RNA repair. Of these, JumonjiC domain-containing histone lysine demethylases (JMJCs), the ten-eleven-translocation (TET) DNA dioxygenases, the ALKB DNA/RNA hydroxylases and the prolyl hydroxylases EGLN1-3 have generated increased interest as potential drug targets for the treatment of a number of pathological conditions, including cancer. Since succinate is a common product to all Fe(II)/2-oxoglutarate-dependent dioxygenases, we developed a novel bioluminescent and homogenous activity detection assay for JmjC histone demethylases and Fe(II)/2-oxoglutarate-dependent dioxygenases based on succinate measurement. The assay was used successfully in the following applications:

- Determine substrate specificities for JmJC enzymes.
- Measure apparent kinetic constants for several JMJCs and members of the dioxygenage superfamily
- Screen a compound library for inhibitors of JmJC demethylase
- Study inhibition mode of action of reported inhibitors.
- Determine selectivity profiles for several compounds against JMJD2A and FTO enzymes.

Our results demonstrate that succinate detection is a useful strategy for the characterization of multiple Fe(II)/2-oxoglutarate-dependent dioxygenases with distinct substrate requirements, enabling the investigation of a large number of enzymes that cannot be evaluated in a miniaturized or high-throughput manner with the methods currently available.

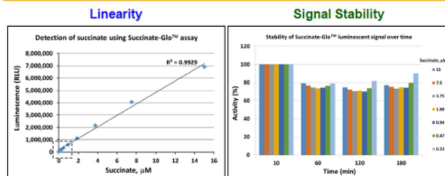
2. Succinate Detection Assay Principle

Principle: Succinate is converted into ATP that is detected via a luciferase/luciferin reaction



- Two Step Detection: After the demethylase reaction, the Succinate Detection Reagents are added in 1:1:2 ratio
- Luminescence signal is proportional to the succinate produced and to the demethylase activity
- Simple "Add and Read": No radioisotopes. No product separation. No antibodies

3. High Sensitivity, Linearity and Signal Stability

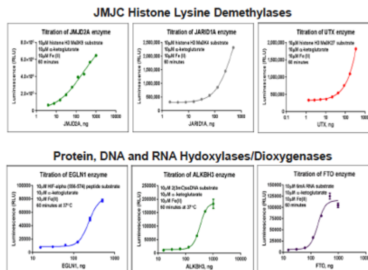


Sensitivity

Signal to Background ratios at different succinate concentrations									
Succinate, µM	15	7.5	3.75	1.88	0.9375	0.47	0.234	0.12	0
S/B values	118	69	37	19	10	5	3	2	1

- Succinate detection is linear up to 15µM and it has a high dynamic range
- It can detect as low as 200nM with a signal/background (SB) of 2
- The signal is stable for up to 3 hours with ~80% remaining signal

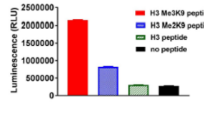
4. Detection of Different Fe(II)-2-oxoglutarate-Dependent Dioxygenases Activities

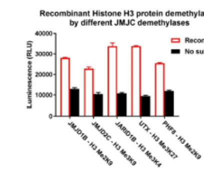


Succinate detection is suitable for measuring the activity of:

- JMJC demethylases regardless of substrate methylation state and position
- 2-oxoglutarate dependent dioxygenases and hydroxylases

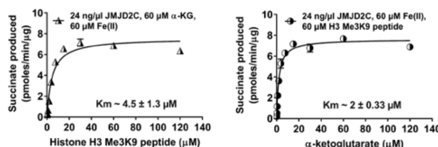
5. JMJC Demethylase Substrate Specificity Studies





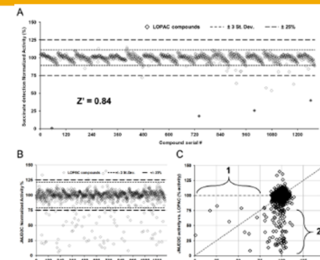
Succinate detection can be used to study JMJC demethylase specificity toward diverse methylated peptide or protein substrates

6. Evaluating Enzyme Kinetic Parameters Using Succinate Detection



Km values for substrates and cofactors detected with Succinate-Glo assay are similar to the ones reported in literature.

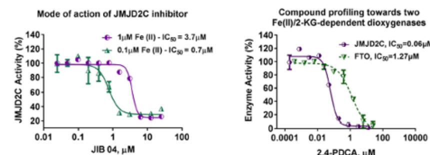
7. JMJC Demethylase Inhibitor Studies



(A) Screening for potential inhibitors of Succinate-Glo reagents performance using LOPAC compound library. (B) Screening for demethylase inhibitors (C) Inhibition correlation graph showing the effect of each compound on the assay reagents at the same time as on the JMJD2C enzyme. All the inhibitors are inhibiting the enzyme (section 2) but not the assay reagents (section 1).

- Small molecule inhibitors do not interfere with succinate assay detection.
- Succinate-Glo assay is robust with high Z' factor.

8. Inhibitor Profiling and Mode of Action Studies



Bioluminescent succinate assay can be used to:

- Study 2-oxoglutarate dependent dioxygenase inhibitors
- Evaluate competitive inhibitors toward different substrates.
- Create selectivity profiles of different inhibitors

9. Conclusions

Universality:

- Bioluminescent succinate assay can be used with the majority of 2-oxoglutarate dependent dioxygenases.
- One assay for diverse JMJC demethylase-substrate combinations, regardless of substrate methylation state and position.

Versatility:

- Easy to use assay. 2-step addition and read
- Suitable for studying substrate specificity, kinetic parameters and mode of action of JMJC demethylase inhibitors

HTS friendly:

- Sensitive in low volume format
- Signal is stable for batch processing
- Resistant to chemical interference

Publication: Alves et al,. SLAS Discovery, Online First 2017.
https://doi.org/10.1177/2472555217745657

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1257-C

Improving your data with Internally Cooled Multifunctional Reader

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1. Introduction

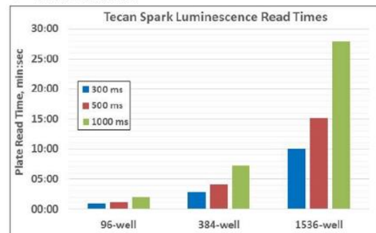
Temperature is considered a major factor that affects the rate of chemical reactions. The average speed of a reaction is related to the square root of the absolute temperature. Arrhenius' equation describes this dependence: $k = Ae^{-E_a/(k_B T)}$
For most biological systems, the rate of change as a consequence of increasing the temperature by 10°C (Q_{10} value) is ~ 2 to 3.

All instruments, including multimode readers, emit heat from mechanical and electronic components. This heat can affect the reaction kinetics of samples being investigated inside the reader. The Tecan Spark® plate reader with Te-Cool™ module allows adjustment of the instrument's internal working temperature from 18°C to 42°C.
Having the ability to investigate samples of interest at ambient temperature, can limit heating effects on reaction dynamics. In this work we present the benefits of an actively cooled multimode reader in investigations employing bioluminescence and biofluorescence reactions, kinetic profiling and high throughput screening.
The kinetic profile of enzymatic reactions can appear very different when acquired on readers that are equipped with an internal cooling capability versus instruments that do not have this functionality. This effect can be mitigated by pre-warming assay plates before reading.

2. Luminescence Read Times per Plate and Consequential Plate's Warming up can be Significant

Luminescence read Time per plate depends on:

- ✓ # of wells / plate
- ✓ Read Time / well



Internal Temperature of Multifunctional readers that are not actively cooled is 2 - 10°C warmer than ambient laboratory temperature.

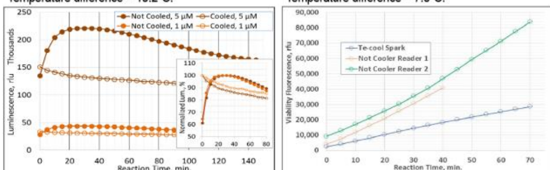
So, plate inside the reader warms up by up to 10°C causing acceleration of the chemical processes.

In the work presented 384 well plates were read for 300ms/well, so all wells were read just in under 3min.

3. Both Bioluminescent and Biofluorescent Reaction Rates are Affected by Temperature

Succinate-Glo™ assay at 5µM or 1µM of Succinate in Actively Cooled vs. Non-Cooled Readers.
Room Temperature = 22.6°C;
Non-Cooled reader internal Temperature = 32.8°C;
Temperature difference = 10.2°C.

ApoLive-Glo™ assay, Fluorescence Viability signal of K562 cells in Actively Cooled vs. Non-Cooled Readers.
Room Temperature = 21.0°C;
Non-Cooled Reader 1 inside Temperature = 28.0°C;
Temperature difference = 7.0°C.



- The internal temperature of a reader affects both bioluminescent and biofluorescent reaction rates.
- Reaction rate is significantly faster at elevated temperatures inside a non-cooled reader.
- Temperature effect on bioluminescence reaction rate is concentration independent.

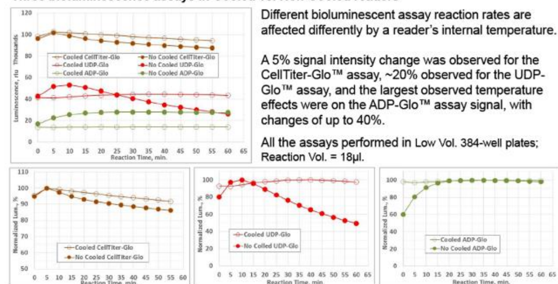
4. Different Bioluminescent Reactions are Affected Differently by Reader Internal Temperature

Three bioluminescence assays in Cooled vs. Non-Cooled readers

Different bioluminescent assay reaction rates are affected differently by a reader's internal temperature.

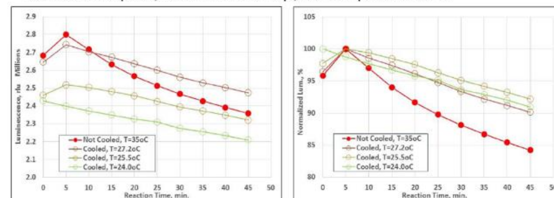
A 5% signal intensity change was observed for the CellTiter-Glo™ assay, ~20% observed for the UDP-Glo™ assay, and the largest observed temperature effects were on the ADP-Glo™ assay signal, with changes of up to 40%.

All the assays performed in Low Vol. 384-well plates; Reaction Vol. = 18µl.



5. Internal Te-cool™ Spark® Temperature Can Be Fine-Tuned to Match Room Temperature

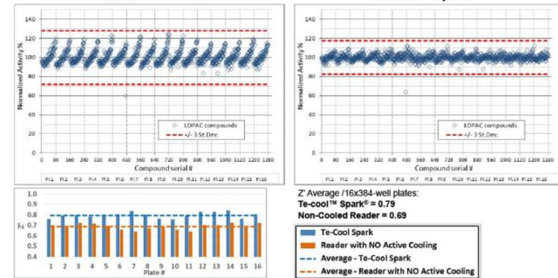
Kinase-Glo™ Assay Luminescence signal in Non-Cooled and Te-cool™ Spark® readers; Low Volume 384-well plates; Reaction Volume = 18µl; Room temperature = 24 °C.



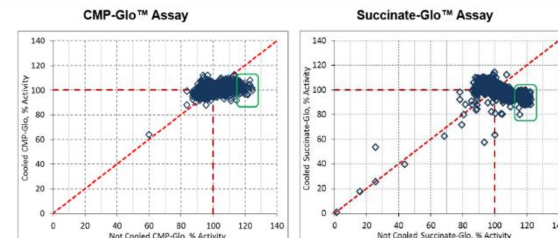
Internal Te-cool™ Spark® temperature can be adjusted to precisely match room temperature. When reaction temperature remains steady, bioluminescent signal shows a steady decrease. In Non-Cooled readers we usually observed a bioluminescence reaction signal increase during the first few reads. This is attributed to the reaction solution equilibration to the internal temperature of the reader and concurrent reaction rate increase.

6. LOPAC Library Screens: Te-cool™ Spark® and Non Actively Cooled Reader

CMP-Glo™ Assay screen vs. LOPAC library; 0.5µM CMP; Low Vol. 384-well plates; Total Vol. =10µl.

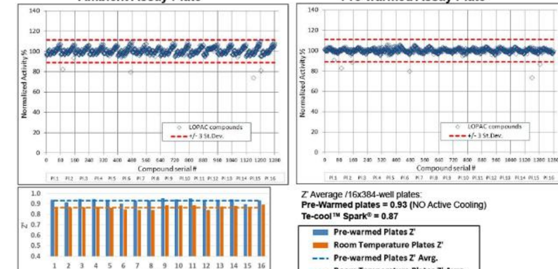


7. LOPAC Screening Results Correlation Graphs: Te-cool™ Spark® vs. Non-Cooled Reader



Temperature equilibration within a Non-Cooled reader leads to greater assay variability and can obscure "Hits"

8. Plate Pre-warming Mitigates the Problem on Non-Cooled Readers



9. Conclusions

Temperature effects on chemical reactions

- Chemical reaction rates are significantly impacted by a reader's internal temperature.
- Both bioluminescence and biofluorescence reaction rates are affected by a reader's internal temperature.
- The extent of the temperature effect can vary based on the specific bioluminescent assay.
- When reaction temperature remains constant, bioluminescent reaction rate remains steady and luminescence signal decreases steadily.
- Bioluminescent assay-based small compound library screen results can be distorted due to the plate reader's internal heat effect on bioluminescent assay.
- Pre-warming assay plates prior to a read can successfully mitigate the effects of a reader's internal temperature.

Benefits of Actively Cooled reader

- With the internal Te-cool™ module the temperature inside the Spark® reader can be adjusted precisely to match the reaction temperature of the plate.
- Constant reaction temperature outside and inside the reader:
  - Allows detection of the true reaction rate and signal intensity.
  - Tightens assay windows to expose more "Hits".

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1358-D

Quantitative Cell-Based Bioassays to Advance Immunotherapy Programs Targeting Immune Checkpoint Receptors

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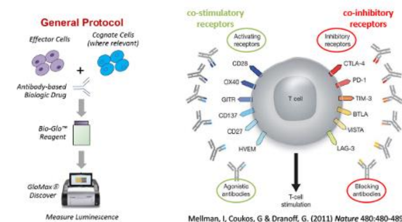
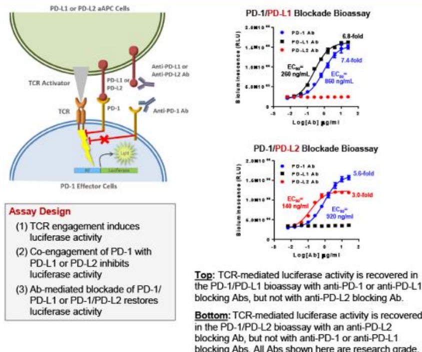
1. Abstract

A major challenge in the development of antibody-based biologics drugs is access to quantitative and reproducible functional bioassays. Existing methods rely on primary cells and measurement of complex functional drug discovery, development, potency and stability studies. endpoints that are cumbersome, variable, and often fail to yield data quality required for drug development in a quality-controlled environment. We have developed a portfolio of functional cell-based reporter bioassays to measure the activity of biologics drugs designed to target immune checkpoint receptors including co-inhibitory (e.g., PD-1, CTLA-4, LAG-3) and co-stimulatory (e.g., 4-1BB, GITR, OX40) receptors. These bioassays consist of stable cell lines that express luciferase under the precise control of receptor-mediated intracellular signals. Here we describe the application of these MOA-based bioassays for biologics..



Mellman, I, Coukos, G & Dranoff, G. (2011) *Nature* 480:480-489.

2. PD-1 Blockade Bioassays



Assay Design

(1) TCR engagement induces luciferase activity

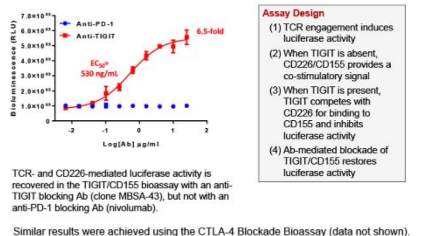
(2) Co-engagement of PD-1 with PD-L1 or PD-L2 inhibits luciferase activity

(3) Ab-mediated blockade of PD-1/ PD-L1 or PD-1/PD-L2 restores luciferase activity

Top: TCR-mediated luciferase activity is recovered in the PD-1/PD-L1 bioassay with anti-PD-1 or anti-PD-L1 blocking Abs, but not with anti-PD-L2 blocking Ab.

Bottom: TCR-mediated luciferase activity is recovered in the PD-1/PD-L2 bioassay with an anti-PD-L2 blocking Ab, but not with anti-PD-1 or anti-PD-L1 blocking Abs. All Abs shown here are research grade.

3. TIGIT Blockade Bioassay



Assay Design

(1) TCR engagement induces luciferase activity

(2) When TIGIT is absent, CD226/CD155 provides a co-stimulatory signal

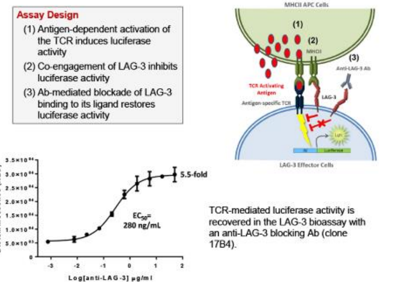
(3) When TIGIT is present, TIGIT competes with CD226 for binding to CD155 and inhibits luciferase activity

(4) Ab-mediated blockade of TIGIT/CD155 restores luciferase activity

TCR- and CD226-mediated luciferase activity is recovered in the TIGIT/CD155 bioassay with an anti-TIGIT blocking Ab (clone MBSA-43), but not with an anti-PD-1 blocking Ab (nivolumab).

Similar results were achieved using the CTLA-4 Blockade Bioassay (data not shown).

4. LAG-3 Blockade Bioassay



Assay Design

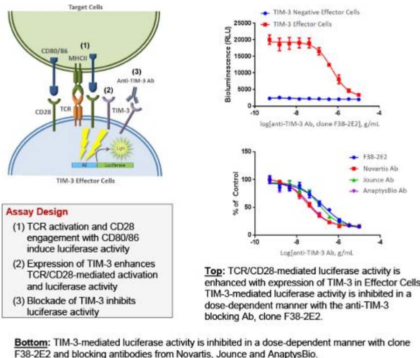
(1) Antigen-dependent activation of the TCR induces luciferase activity

(2) Co-engagement of LAG-3 inhibits luciferase activity

(3) Ab-mediated blockade of LAG-3 binding to its ligand restores luciferase activity

TCR-mediated luciferase activity is recovered in the LAG-3 bioassay with an anti-LAG-3 blocking Ab (clone 17B4).

5. TIM-3 Bioassay



Assay Design

(1) TCR activation and CD28 engagement with CD80/86 induce luciferase activity

(2) Expression of TIM-3 enhances TCR/CD28-mediated activation and luciferase activity

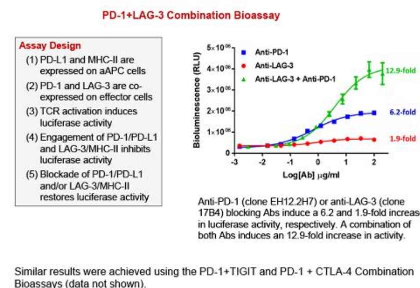
(3) Blockade of TIM-3 inhibits luciferase activity

Top: TCR/CD28-mediated luciferase activity is enhanced with expression of TIM-3 in Effector Cells. TIM-3-mediated luciferase activity is inhibited in a dose-dependent manner with the anti-TIM-3 blocking Ab, clone F38-2E2.

Bottom: TIM-3-mediated luciferase activity is inhibited in a dose-dependent manner with clone F38-2E2 and blocking antibodies from Novartis, Jounce and AnaptysBio.

6. Combination Bioassays

PD-1+LAG-3 Combination Bioassay



Assay Design

(1) PD-L1 and MHC-II are expressed on aAPC cells

(2) PD-1 and LAG-3 are co-expressed on effector cells

(3) TCR activation induces luciferase activity

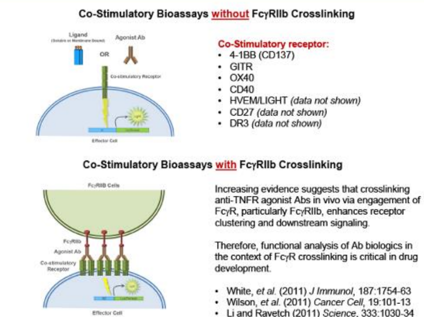
(4) Engagement of PD-1/PD-L1 and LAG-3/MHC-II inhibits luciferase activity

(5) Blockade of PD-1/PD-L1 and/or LAG-3/MHC-II restores luciferase activity

Anti-PD-1 (clone EH12.2H7) or anti-LAG-3 (clone 17B4) blocking Abs induce a 6.2 and 1.9-fold increase in luciferase activity, respectively. A combination of both Abs induces an 12.9-fold increase in activity.

Similar results were achieved using the PD-1+TIGIT and PD-1 + CTLA-4 Combination Bioassays (data not shown).

7. Co-Stimulatory Bioassays



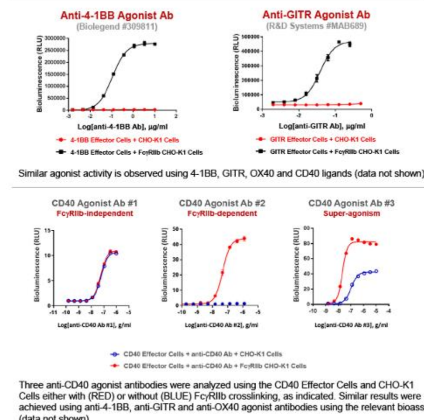
Co-Stimulatory receptor:

- 4-1BB (CD137)
- GITR
- OX40
- CD40
- HVEM/LIGHT *(data not shown)*
- CD27 *(data not shown)*
- DR3 *(data not shown)*

Increasing evidence suggests that crosslinking anti-TNFR agonist Abs in vivo via engagement of FcγR, particularly FcγRIIb, enhances receptor clustering and downstream signaling.

Therefore, functional analysis of Ab biologics in the context of FcγR crosslinking is critical in drug development.

- White, *et al.* (2011) *J Immunol*, 187:1754-63
- Wilson, *et al.* (2011) *Cancer Cell*, 19:101-13
- Li and Ravetch (2011) *Science*, 333:1030-34



Similar agonist activity is observed using 4-1BB, GITR, OX40 and CD40 ligands (data not shown).

Three anti-CD40 agonist antibodies were analyzed using the CD40 Effector Cells and CHO-K1 Cells either with (RED) or without (BLUE) FcγRIIb crosslinking, as indicated. Similar results were achieved using anti-4-1BB, anti-GITR and anti-OX40 agonist antibodies using the relevant bioassay (data not shown).

8. Conclusions

Cell-based reporter bioassays overcome the limitations of primary cell-based assays for functional characterization of antibody and other biologics drugs targeting individual or combination immune checkpoint receptors. Here we show a portfolio of MOA-based bioassays for co-inhibitory and co-stimulatory immune checkpoint receptors that can be used for antibody screening, characterization, potency and stability studies.

Biologically relevant measurement of antibody MOA

- Specific immune checkpoint regulated expression of luciferase that reflects the native biology of T cell activation.
- Demonstrated ability to measure the potencies of immune checkpoint-targeted antibodies

Consistent and reliable measure of antibody activity

- Demonstrated precision, accuracy, reproducibility, robustness
- All assays can be used as "Thaw-and-use" cell format, no cell culture required
- Functional performance suitable for development into potency, stability, and NAb assays

Easy-to-implement

- Rapid and convenient workflow
- Amenable to standard 96-well and 384-well plate formats

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