Monitoring Protein:Protein Interactions in Living Cells Using a Small, Bright and Reversible Complementation System

Brock F. Binkowski, Ph.D.
Promega’s History in Signal Transduction Research

- Luciferase enzymes used extensively in reporter gene assays to monitor gene expression
- Luciferase-coupled assays to measure the concentration of cellular metabolites (e.g., ATP, ADP, NAD, cAMP, etc.)
- More recently, assays to monitor changes in metabolite concentration, cell health or proteolysis in real-time

A deeper understanding of cell physiology and pathology requires identification of protein:protein interaction networks
Monitoring Protein:Protein Interactions (PPIs) in Living Cells

- Two-hybrid based approaches
  - Yeast two hybrid
  - Split ubiquitin
  - MAPPIT
  - KISS

- Protein complementation assays
  - DHFR
  - Beta-galactosidase
  - Beta-lactamase
  - Split fluorescent proteins (BiFC)
  - Split luciferases (BiLC)

Our goal: develop a system to sensitively monitor intracellular PPIs in real time that minimally perturbs protein function

van den Berg et al. Cell Stem Cell 6-369
NanoLuc® Binary Technology (NanoBiT™)

- Developed by Promega’s Advanced Technology Group
- Divide NanoLuc into two subunits
  - Circularly permute NanoLuc at 91 sites
  - Tether native N- and C-termini with a flexible linker containing a TEV protease cleavage site
  - Screen for sites showing the largest RLU difference +/- TEV protease treatment
- Choose site between 156/157
- **Optimize** subunit properties (not just split!)
Mutagenesis of the Large Subunit

Properties of 1-156
- Insoluble in *E. coli*
- Expressed poorly in mammalian cells
- Rapidly degraded vs. full-length NanoLuc®

Two rounds of mutagenesis
- Screened for increased RLUs in *E. coli* lysates with saturating NP (~15k variants)
- Increased RLUs resulting from increased expression or increased specific activity
- 16 beneficial mutations identified & combined to give 11S
- Increased RLUs in both *E. coli* and HEK293T cells
Improved Structural Stability of the Large Subunit

- Increased thermal stability, approaching that of NanoLuc
- Soluble expression in *E. coli*
- Increased expression in mammalian cells
- Increased specific activity when combined with NP

<table>
<thead>
<tr>
<th></th>
<th>WT fragment</th>
<th>11S</th>
<th>NanoLuc</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_M$ (°C)</td>
<td>45</td>
<td>55</td>
<td>60</td>
</tr>
</tbody>
</table>

**Expression in *E. coli***

<table>
<thead>
<tr>
<th>1-156</th>
<th>11S</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>S</td>
</tr>
<tr>
<td>T</td>
<td>S</td>
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</table>

*T = total (crude) lysate, S = soluble fraction*
Screening a Library of Peptides for an Improved Small Subunit

• Screen peptide library (n = 350) with 11S

Identified peptides over a 5-log affinity range

• PEP86 & PEP114 are the highest and lowest affinity peptides, respectively
• PEP114 chosen for protein interaction assays
• Development underway on applications using higher affinity peptides
**NanoBiT™ for Protein:Protein Interactions**

**Key characteristics**
- Subunits are very small; LgBiT is structurally stable
- Very bright signal, ~1/3 as bright as full-length NanoLuc at saturation
- Low affinity interaction of LgBiT:SmBiT ($K_D = 190 \ \mu M$)
- Reversible interaction of LgBiT:SmBiT ($k_{on} = 500 \ M^{-1} \ sec^{-1}$; $k_{off} = 0.2 \ sec^{-1}$)
NanoBiT™ PPI Assay Workflow

Generate clones encoding LgBiT and SmBiT fusions to proteins A & B (up to 8 possible constructs).

Transient transfection of different plasmid combinations into a cell type of interest (up to 8 possible combinations). In general, plasmids are transfected at a 1:1 mass ratio.
Measure RLUs Using a Live Cell, Non-Lytic Assay Protocol

**Nano-Glo® Live Cell Reagent: 5X**
aqueous stock containing furimazine substrate

Add to living cells

Measure RLUs continuously for up to 2 hours

Furimazine is cell permeable
Orientation Screen Using a Tool Compound

- A tool compound gives an expected response for a known PPI pair
  - Examples: activator of cellular signal transduction pathway or direct PPI inhibitor

- Add tool compound and screen for the orientation giving maximal response
  - Compare to vehicle treatment

- e.g., tool compound as an inducer of the PPI
### Benchmarking vs. Split Fluc

<table>
<thead>
<tr>
<th>Fragment</th>
<th>M.W. (kDa)</th>
</tr>
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<tbody>
<tr>
<td>Fluc 4-398</td>
<td>43.8</td>
</tr>
<tr>
<td>Fluc 394-544</td>
<td>16.6</td>
</tr>
<tr>
<td>LgBiT</td>
<td>17.6</td>
</tr>
<tr>
<td>SmBiT</td>
<td>1.3</td>
</tr>
</tbody>
</table>

- BiTs are much smaller, providing less steric hindrance
- LgBiT provides a structurally stable fusion partner
- Split Fluc fragments likely unstructured

**Goal:** compare NanoBiT™ to split firefly luciferase (4-398 + 394-544) using the FKBP:FRB model system

- Optimal orientation identified for each system
- Identical 10 a.a. Gly/Ser linkers for both systems
NanoBiT™ is Logs Brighter than Split Fluc

- Identical pharmacology for both systems
- Both systems show reversibility with subsequent addition of FK506 (not shown)
- NanoBiT >100 fold brighter at room temperature
- NanoBiT >1,000 fold brighter at 37 °C
- Consistently lower CVs for NanoBiT
NanoBiT™ Applied to GPCRs

**β2-adrenergic receptor (ADRB2)**
- Class A receptor with transient β-arrestin interaction
- Rapid recycling to the plasma membrane

**Arginine vasopressin receptor 2 (AVPR2)**
- Class B receptor with stable β-arrestin interaction
- Slow recycling to the plasma membrane

**Goal:** use NanoBiT to monitor the association & dissociation of β-arrestin-2 (ARRB2) with C-terminally tagged ADRB2 & AVPR2
- **Constructs:**
  - ADRB2-LgBiT:SmBiT-ARRB2
  - AVPR2-SmBiT:LgBiT-ARRB2

*Adapted from Nature Rev Neurosci. 2001; 2:727-733*
NanoBiT™ Reflects Expected Biology for GPCR:ARRB2 Interactions

- ADRB2:ARRB2 signal is more transient (class A receptor)
- AVPR2:ARRB2 signal is more stable (class B receptor)
- NanoBiT can be used to monitor transient PPIs in real-time
NanoBiT™ Minimally Influences Protein Interactions

**Goal:** use the SME-1 β-lactamase (SME1):β-lactamase inhibitory protein (BLIP) interaction to determine the influence of BiT association on equilibrium binding *in vitro*

1) SME1 β-lactamase inhibitory protein (BLIP)
2) SME1 β-lactamase inhibitory protein (BLIP)
3) SME1 β-lactamase inhibitory protein (BLIP)
4) SME1 β-lactamase inhibitory protein (BLIP)

- Measure $K_i$ values for BLIP or BLIP mutants using SME1 substrate (nitrocefin)
  - Compare $K_i$ values between pairs 1-4
  - Compare $K_i$ values to equilibrium association of BiTs for pair 4

Andy Dixon
Minimal Influence Over a Wide Range of Affinity

<table>
<thead>
<tr>
<th>BLIP</th>
<th>$K_i$ (nM)</th>
<th>$K_i$ (nM)</th>
<th>$K_i$ (nM)</th>
<th>$K_i$ (nM)</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2.2 ± 0.2</td>
<td>1.9 ± 0.1</td>
<td>3.6 ± 1.1</td>
<td>3.6 ± 0.9</td>
<td>3.2 ± 1.0</td>
</tr>
<tr>
<td>Y50A</td>
<td>80 ± 4.6</td>
<td>77 ± 4.2</td>
<td>120 ± 6.0</td>
<td>100 ± 27</td>
<td>110 ± 10</td>
</tr>
<tr>
<td>R160A</td>
<td>400 ± 71</td>
<td>530 ± 220</td>
<td>570 ± 220</td>
<td>830 ± 270</td>
<td>970 ± 180</td>
</tr>
</tbody>
</table>

- $K_i$ values were similar for pairs 1-4 for BLIP or BLIP mutants (within ~2-fold)
- Minimal influence of BiTs on equilibrium binding over a wide range of affinity
- $K_D$ values determined using NanoBiT™ were equivalent to $K_i$ values for pair 4
NanoBiT™ Reversibility in Cells

**Goal:** demonstrate reversibility in cells using PKA model system

- NanoBiT fusions: SmBiT-CA:LgBiT-R2A
- Track NanoBiT & changes in intracellular [cAMP] in real-time
  - GloSensor cAMP: firefly luciferase based biosensor for intracellular cAMP
- Transient expression in HEK293, expressing endogenous β2-adrenergic receptor (ADRB2)

**PKA Model**

**GloSensor™ cAMP**

Inactive PKA

Agonist

ATP

cAMP

Active PKA

Firefly luciferase

New N- & C-termini

Fuse wt N- & C-termini
NanoBiT™ Shows Expected Correlation with cAMP Dynamics

Sequential addition of the following:
- Isoproterenol (ISO): ADRB2 agonist (cAMP ↑)
- Propranolol (PRO): ADRB2 antagonist (cAMP ↓)
- Forskolin (FSK): activator of adenylate cyclase (cAMP ↑)

- NanoBiT signal shows good correlation with cAMP biosensor signal
- SmBiT-CA:LgBiT-R2A interaction is reversible
Further Struggles for Split Fluc at 37 °C

**Goal:** compare the reversibility and temperature sensitivity of NanoBiT and split Fluc using the PKA model system.

- Optimal configuration identified for both
- ≥ 10 amino acid Gly/Ser linkers for both
- Transfection of varying amounts of DNA (results shown for optimized conditions for both systems)

Braedy Butler
NanoBiT™ Compared to NanoBRET™

**Goal:** compare response dynamics of NanoBiT & NanoBRET using PKA model system
- BRET relies on energy transfer, not subunit interaction
- Transient expression in HEK293, expressing endogenous β2-adrenergic receptor (ADRB2)
- NanoBiT signal closely approximates NanoBRET
NanoBiT™ Applied to AR Dimerization

**Goal:** determine if NanoBiT can be used to monitor Androgen Receptor dimerization in living cells

- **R1881:** androgen used to induce Androgen Receptor dimerization

*Adapted from Int. J. Mol. Sci. 2013; 14(6):12496-12519*
**NanoBiT™ Applied to CRAF:BRAF**

**Goal:** determine if NanoBiT can be used to monitor RAF dimerization in response to treatment with BRAF inhibitors

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**BRAF^{V600E} Cells**

- In cells expressing BRAF^{V600E}, inhibitors block MEK/ERK activation

**BRAF^{WT} Cells**

- In cells expressing BRAF^{WT}, inhibitors activate MEK/ERK

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**Adapted from Biochemical Society Transactions 2011; 39:472-476**

- EC50 values & rank order potency consistent with literature
- NanoBiT can be used to monitor dimerization of full-length RAF proteins

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Marie Schwinn
**NanoBiT™ Applied to p53:MDM2**

**Goal:** demonstrate that NanoBiT can be used to monitor PPI inhibition using a direct inhibitor

![Diagram showing interactions between MDM2, p53, and Nutlin-3](image)

- Screened all eight possible combinations
- >100 fold S/B with saturating [nutlin-3]
- Robust detection of nutlin-3 inhibition in both 96- and 384-well formats

-Marie Schwinn
NanoBiT™ Scaled to Higher Density Formats

NanoBiT in drug discovery
- NanoBiT can be scaled to 384- and 1536-well formats
- Screen for PPI modulators in a cellular context

AVPR2:ARRB2 in 1536-well format
- 2 μL AVP or vehicle/well
- 8 uL cell suspension (3,000 cells/well) + Nano-Glo Live Cell Reagent
- Measure RLU after 15 minutes
- Z’ = 0.52
**NanoBiT™ Imaging From Single Cells**

**Goal:** determine if bioluminescence imaging (BLI) can be used to monitor GPCR:ARRB2 interactions from isolated cells

- Hela cells expressing optimal orientations for AVPR2:ARRB2 and ADRB2:ARRB2 were treated with 1 µM AVP or 100 µM ISO at t = 0, respectively
- Images acquired every 2-4 sec using an Olympus LV200 microscope (EM gain = 400-600)
NanoBiT™ Using Single Copy Integration

- Single copy integration of a bi-directional CMV promoter expressing BRAF-LgBiT & CRAF-SmBiT
- Treatment with GDC0879 to induce CRAF:BRAF dimerization
- NanoBiT should be favored over related approaches with less sensitivity for genome editing

Mike Slater & Jim Hartnett
NanoBiT™ Entry & Control Vectors

**Cloning vectors**
- Four entry vectors for standard PCR cloning (using MCS)
- Four entry vectors compatible with the Flexi Vector System
  - Facilitate cloning using >9,000 validated ORFs from the Kazusa DNA Research Institute
- All vectors utilize the HSV-TK promoter to minimize non-specific association of LgBiT:SmBiT
  - When needed, expression vectors with stronger promoters will be available via Custom Research Materials

**Positive control constructs:**
- FKBP:FRB for inducible interaction via addition of rapamycin
- PRKACA:PRKAR2A providing a bright, constitutive interaction in many cell types
  - Interaction can also be modulated by adding modulators of intracellular cAMP

**NanoBiT Negative Control Vector**
- Encodes HaloTag®-SmBiT
- Diffuse expression throughout the cell
- Accumulates to high levels
Nano-Glo® Live Cell Assay System

Nano-Glo Live Cell Substrate:
Furimazine + proprietary agent for autoluminescence reduction

Nano-Glo LCS Dilution Buffer:
Aqueous dilution buffer providing enhanced furimazine stability

20X dilution

Nano-Glo Live Cell Reagent
(5X aqueous stock for delivery to cells)

Increased dynamic range by decreasing autoluminescence background

log [Compound] (M)

Luminescence (RLU)

Decreased autoluminescence background
NanoBiT™ Summary

Key characteristics:
• **Very small**, minimizing steric bulk
• **Very bright**, maximizing sensitivity and allowing endogenous levels of expression
• **Reversible**, monitor protein association and dissociation
• **Minimal perturbation**, results with several PPIs indicate minimal influence on equilibrium binding or rates of association/dissociation

Key applications:
• **Real-time measurements** of protein interaction dynamics
• **Drug discovery** in 384- or 1536-well formats
• **Bioluminescence imaging** on isolated cells
• **In vivo imaging** of protein:protein interactions (pending)
• **Mapping** protein:protein interaction networks (pending)
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Martin Rosenberg

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Thank You!
Questions?