Agilent

BioTek is
now Agilent!

ADVANCING PROTEOMIC RESEARCH

with Proximity Assays

Contents

4 **Receptor-Mediated Stimulation of AKT Pan-Kinase in NIH3T3 Cells**

Using the Synergy Neo2 multimode reader to measure THUNDER TR-FRET cell signaling assays.

10 **A Homogeneous FRET-Based HTS Assay for Quantification of pRb in Cancer Cell Lines to Monitor Inhibition of G_0/G_1 Cell Phase Transition**

Novel HTRF cell-based assay quantifies endogenous phosphorylated retinoblastoma protein as a readout.

16 **Investigation of Protein: Protein Interactions (PPI) Using a Novel Bioluminescence Resonance Energy Transfer (BRET) Proximity-Based, High Throughput Screening Assay**

Synergy Neo2 provides rapid detection of multiple signals by simultaneously detecting BRET donor and acceptor emission signals.

22 **Automation of a Homogeneous Proximity Assay for Detection of ERK1/2 or SMAD3 Phosphorylation**

Unique combination of assay and instrumentation provides an ideal solution for high-throughput detection of phosphorylation events.

Agilent

Trusted Answers

The Fastest, Highest-Performing Agilent BioTek Multimode Reader

Agilent BioTek Synergy Neo2 hybrid multimode reader

Synergy Neo2 is our most advanced, high-performance, high-speed plate reader, designed to meet the sophisticated needs of laboratories. The fully featured and flexible Synergy Neo2 offers uncompromising performance for cell-based and biochemical assays.

www.agilent.com/lifesciences/synergyneo2

Agilent BioTek Synergy Neo2 hybrid multimode reader

DE38119968

Receptor-Mediated Stimulation of AKT Pan-Kinase in NIH3T3 Cells

Using the Synergy Neo2 multimode reader to measure THUNDER TR-FRET cell signaling assays.

Paul Held, Ph.D.

Abstract

Cell proliferation is usually initiated though cell surface receptors, which interact with specific ligands. This interaction elicits a signal cascade that transmits from the cell surface to the nucleus. The signal cascade involves the activation of proteins by phosphorylation as a result of specific protein kinases. AKT pan is a key protein in this signaling pathway that is activated by phosphorylation.

Thus, the ability to monitor the phosphorylation status of this protein can provide insight to the growth status of cells in culture. This application note describes the quantitation of phospho-AKT pan (S473) as a result PDGF stimulation of NIH3T3 fibroblast cells using THUNDER TR-FRET assay kits in conjunction with the Agilent BioTek Synergy Neo2.

Introduction

Platelet-derived growth factor (PDGF) stimulates proliferation, migration and survival of mesenchymal cells and plays a pivotal role during embryonic development and wound healing.[1] The binding of the bivalent ligand induces dimerization and activation of PDGF receptors, leading to autophosphorylation of tyrosine residues in the intracellular region.[2] As a result, several signal transduction pathways are initiated, including phosphatidylinositol 3-kinase (PI3K), the Src tyrosine kinase,

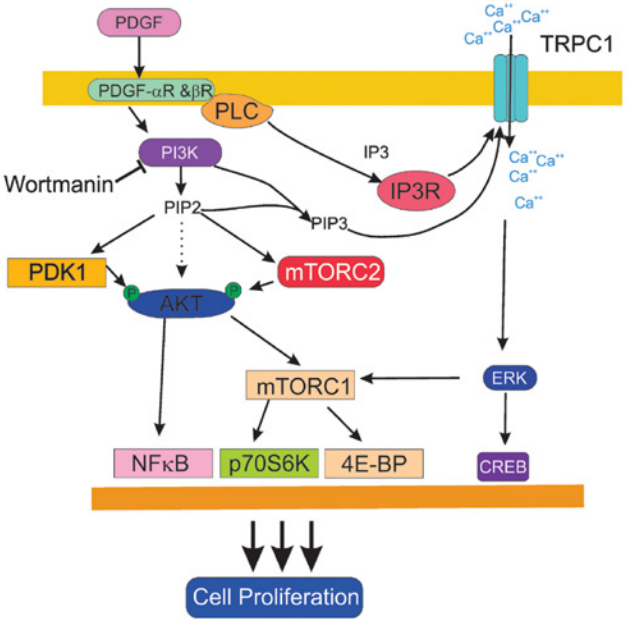

Figure 1. Schematic of the PDGF receptor pathway for cell proliferation.

phospholipase Cγ (PLC), and several mitogen-activated protein (MAP) kinase cascades (Figure 1).

AKT, also known as protein kinase B, is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription, and cell migration.[3] AKT is involved in cellular survival pathways, by inhibiting apoptotic processes. AKT is also able to induce protein synthesis pathways, and is therefore a key signaling protein in the cellular pathways that lead to general tissue growth. Since it can block apoptosis and promote cell survival, AKT has been implicated as a major factor in many types of cancer.

The Pleckstrin homology domain of AKT binds directly to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and phosphatidylinositol (3,4,)-diphosphate (PIP2), which are produced by activated PI3Ks.[4] Because both of these compounds are restricted to the plasma membrane, AKT binding results in its translocation to the plasma membrane.

Once correctly positioned at the membrane, AKT can then be phosphorylated by its activating kinases, the mammalian target of rapamycin complex 2 (mTORC2) at serine 473 followed by phosphoinositide dependent kinase 1 (PDPK1) at threonine 308.[5] Activated AKT can then go on to activate or deactivate its myriad substrates (e.g. mTORC1) via its kinase activity (Figure 1).

Wortmannin is a nonspecific, irreversible inhibitor of phosphoinositide 3-kinases (PI3Ks). Wortmannin is a steroid metabolite of the fungi *Penicillium funiculosum* and *Talaromyces wortmanni*i (Figure 2). The compound has a highly reactive C20 carbon that covalently binds phosphoinositol 3-kinases (PI3Ks) at their active site and inhibits their activity. By inhibiting the formation of PIP3 and PIP2, AKT

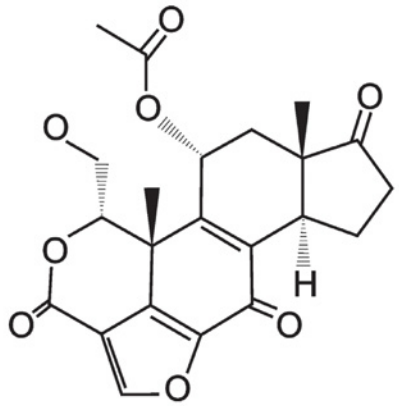

Figure 2. Structure of wortmannin.

cannot translocate to the membrane and be phosphorylated at position S473.

The Phospho-AKT pan (S473) assay is a homogeneous time-resolved Förster resonance energy transfer (TR-FRET) sandwich immunoassay (Figure 3). Following cell treatment, cells are lysed with the specific lysis buffer provided in the kit. Then Phospho-AKT pan (S473) in the cell lysates is detected with a pair of fluorophore-labeled antibodies. One antibody is labeled with a donor fluorophore (Europium chelate; Eu-Ab1) and the second with a far-red acceptor fluorophore (FR-Ab2). The binding of the two labeled antibodies to distinct epitopes on the target protein takes place in solution and brings the two dyes into close proximity. Excitation of the donor Europium chelate molecules triggers a FRET from the donor to the acceptor molecules, which in turn emit a TR-FRET signal at 665 nm. This reaction can only take place in the presence of the specific analyte. Residual energy from the Eu chelate generates light at 615 nm. The signal at 665 nm is proportional to the concentration of Phospho-AKT pan (S473) in the cell lysate. Data can be expressed as either the signal at 665 nm or the 665 nm/615 nm ratio.

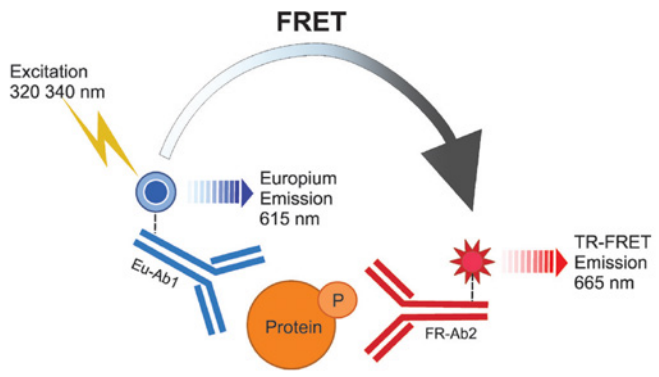

Figure 3. Schematic of the TR-FRET reaction of THUNDER assays.

The Synergy Neo2 is a multimode microplate reader that can be configured with a 337 nm laser specifically designed for the excitation of TR-FRET assays based on the lanthanide Europium. In addition to the laser for excitation wavelength, the filter-based optical system uses dichroic mirrors and deep blocking bandpass filters for emission discrimination. The light signal is captured using direct fiber-less optics with multiple PMTs to capture the dual signal outputs simultaneously.

Materials and methods

Phospho-AKT pan (S473) assay kit, KIT-AKTS473P-500, was from BioAuxillium (QC, Canada). Fetal bovine serum, Advanced-DMEM, and Glutamine-Pen-Strep were purchased from Life Technologies (Carlsbad, CA). Black sided, clear bottom 96-well (3904) and solid white, low volume 384-well (3674) microplates were obtained from Corning (Corning, NY). Lyophilized PDGF-AA (221-AA) was from R&D Systems (Minneapolis, MN) and rehydrated to 100 µg/mL in 4 mM HCl as directed.

Cell culture

NIH3T3 cells were cultured in Advanced DMEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine and penicillin-streptomycin at 37 °C in 5% CO_2. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluency. For experiments, cells were plated into Corning 3904 black sided clear bottom 96-well microplates. Unless otherwise indicated, cells were seeded at a density of 10,000 cells per well in Advanced-DMEM supplemented with 10% FBS 2 mM glutamine and penicillin-streptomycin and allowed to attach overnight. The following day, the medium was changed to Advanced-DMEM supplemented with 0.1% FBS 2 mM glutamine and penicillin-streptomycin. Cells were exposed to low serum for 24 hours prior to experimentation.

Assay process

Assays were run using the two-plate transfer protocol. After stimulation, cells are lysed with 50 µL per well of 1x lysis buffer supplemented with 1 mM sodium fluoride and 2 mM sodium orthovanadate phosphatase inhibitors. Lysis buffer is supplied by the assay kit as a 5x concentration and is diluted with MilliQ water immediately prior to use. Lysis was carried out on an orbital microplate shaker at 400 rpm for 60 minutes. After lysis, 15 µL of each lysate were transferred to a solid white 384-well detection plate followed by 5 µL of a 4x antibody mix. The 4x antibody mix consisted of Eu-labeled Phospho-AKT pan (S473) antibody (Eu-Ab1) and an acceptor-labeled Phospho-AKT pan (S473) antibody (FR-Ab2) mixture in 1x detection buffer, and was prepared immediately prior to use as directed in the assay instructions. The assay plate was sealed with an adhesive plate sealer and incubated in the dark at room temperature for 4 hours.

Detection

The detection plate was read using a Synergy Neo2 configured with a 377 nm laser excitation source.

The emission was detected using a dual emission 620 and 665 nm filter set, with the gain for both PMTs set to 100. Each measurement was the mean of 20 data points. Each data point was used a delay of 50 µsec. after the laser pulse with a collection time of 100 µsec (Table 1).

Table 1. Synergy Neo 2 read parameters for dual emission TR- FRET measurements.

Synergy Neo2 Read Parameters	
Mode	Time-resolved fluorescence
Filter Sets	Dual PMT
Excitation	337 nm (cube 18)
Emission	620 nm and 665 nm (cube 41)
Gain (PMT1, PMT2)	100,100
Read Speed	Normal
Delay After Plate Movement	0
Measurements Per Data Point	20
Read Height	5.00 mm
Dynamic Range	Standard (0 to 99,999)
Light Source	TRF laser (377 nm)
TRF Parameters	
Delay	50 µsec
Data Collection Time	100 µsec

Data reduction

Captured assay data were first blanked by subtracting the mean signal at 620 nm and 665 nm of several empty wells from the experimental wells of the respective wavelength. The TR-FRET ratio is calculated by dividing the blanked 665 nm emission signal by the blanked 620 nm emission and multiplying that value by 1,000. The TR-FRET ratio was then plotted as necessary.

Results and discussion

The data demonstrate that the mouse fibroblast NIH3T3 cell line can be stimulated to phosphorylate the protein AKT at the S473 position. As demonstrated in Figure 4, PDGF produces a concentration dependent increase in phospho-AKT pan (S473) when NIH3T3 cells are stimulated for 15 minutes.

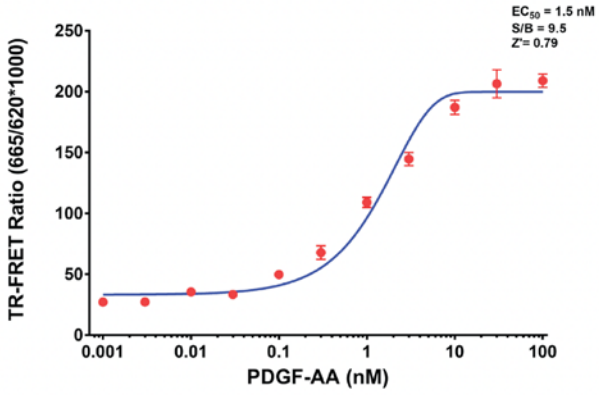

Figure 4. Stimulation of AKT pan (S473) phosphorylation by PDGF. NIH3T3 cells (10,000 cells/ well) were incubated with serial dilutions of PDGF-AA for 15 minutes at 37 °C and then lysed.

The stimulatory response of PDGF can be negated by the compound wortmannin. When NIH3T3 cells are incubated with various concentrations of wortmannin for 30 minutes prior to stimulation with PDGF, a concentration-dependent inhibition of the stimulatory response is observed (Figure 5). Under

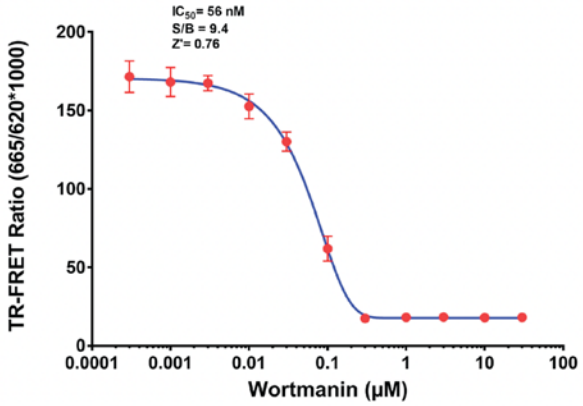

Figure 5. Wortmannin inhibition of phosphorylation of AKT pan (S473). NIH3T3 cells (10,000 cells/well) were incubated with serial dilutions of Wortmannin for 30 minutes at 37 °C. Cells were then stimulated with 10 nM PDGF-AA for 15 minutes at 37 °C and then lysed.

these conditions, the IC_{50} for wortmannin was determined to be 56 nM.

A comparison of wells treated with 10 nM PDGF and untreated wells indicates that the assay is quite robust. The assay quantitation window for the phospho-AKT pan (S473) assay was 6x over basal levels (Figure 6). The mean TR-FRET ratio of cells stimulated with 10 nM PDGF was determined to be 180, while untreated wells had an observed ratio of 30. The calculated Z'-factor for this experiment was 0.722, indicating that the signal change is significantly different from unstimulated control cells.

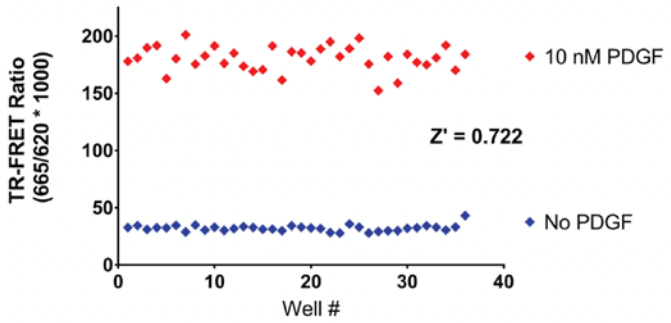

Figure 6. Z'-factor determination in NIH3T3 cells. NIH3T3 cells (10,000 cells/well) were incubated with 10 nM PDGF-AA for 15 minutes at 37 °C and then lysed. The Z'-factor was determined using a total number of 36 wells for each treatment group.

The assay is quantitative in regards to cell number. As demonstrated in Figure 7, when different number of NIH3T3 cells were seeded into 96-well microplates and stimulated with PDGF a linear relationship between seeding number at TR-FRET ratio is observed. As few as 2,500 cells can be distinguished against a no-cell control, albeit with a small assay window. At high cell concentrations (20,000 cells/well), adequate cell lysis and cell over growth become problematic, leading to more sample variability. For NIH3T3 cells, a seeding density of 10,000 to 15,000 cells per well was found to provide an adequate assay window and consistent lysis. These data are corroborated by the S/B and Z'-factor analysis depicted in Table 2. When 10 to 15K cells per well are seeded, the S/B ratio was found to be between 8 to 10 and had a Z'-factor approximately 0.75.

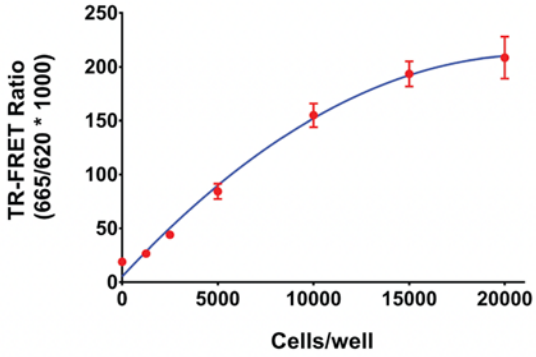

Figure 7. NIH3T3 cell number titration. NIH3T3 cells were seeded into 96-well plates at different seeding densities. After an overnight attachment and 24 hours of serum starvation, cells were stimulated with 10 nM PDGF for 15 minutes at 37 °C and then lysed.

Table 2. Signal-to-background ratio and Z'-factor for different cell seeding densities. NIH3T3 cells were seeded into 96-well plates at several different seeding densities. After an overnight attachment and 24 hours of serum starvation, cells were stimulated with 10 nM PDGF for 15 minutes at 37 °C and then lysed. Data represent the statistics for 12 data points each.

Cells/Well	TR-FRET Ratio	S/B	Z'
0	19	–	–
1,250	27	1.40	–0.122
2,500	44	2.31	0.472
5,000	84	4.44	0.642
10,000	155	8.15	0.742
15,000	193	10.17	0.785
20,000	208	10.96	0.680

Platelet-derived growth factor (PDGF) is one among numerous growth factors that regulate cell growth and division. In particular, PDGF plays a significant role in blood vessel formation, the growth of blood vessels from already-existing blood vessel

tissue, mitogenesis, i.e. proliferation, of mesenchymal cells such as fibroblasts, osteoblasts, tenocytes, vascular smooth muscle cells. These data demonstrate that the stimulation of NIH3T3 cells with PDGH promotes the phosphorylation of AKT pan at S473. In addition, the stimulatory affect can be negated by the addition of the PKI3K inhibitor wortmannin. The THUNDER TR-FRET cell signaling assay for phospho-AKT pan (S473) used in these experiments is a robust and easily performed assay, as demonstrated by high Z' values.

The cellular signaling biology is complex and involves numerous proteins that are regulated by a number of dynamic post translational modifications. The THUNDER assay portfolio provides a series of assays all run in using the same add-incubate-measure format. These homogeneous assays do not require any wash steps and can be performed in a single day. Cell lysates from experiments can be kept frozen at -80° and assayed as a group in order to improve efficiencies with assay reagents. This study used one 96-well plate for the cellular experiment and a separate low-volume 384-well assay plate for the detection step (two-plate assay protocol), protocols are provided for single plate assays in the assay kit instructions. The THUNDER assay kits provide all of the necessary reagents in order to carry out the assay with the exception of phosphatase inhibitors. While absolutely necessary for the assay, the correct cocktail mix of phosphatase inhibitors will be dependent on the analyte in question and need to be freshly prepared.

The Agilent BioTek Synergy Neo2 hybrid multimode reader is an ideal platform to measure TR-FRET reactions such as the THUNDER assays from BioAuxillium. The Synergy Neo2 can be configured with TRF laser and up to 4 PMTs, allowing for high-throughput assay development and screening. This allows for simultaneous capture both the 620 and 665 nm emissions. This fiber-less design provides direct illumination for very strong sample excitation which guarantees the highest levels of sensitivity. The optical system uses barcoded filter cubes containing dichroic mirrors and deep blocking bandpass filters for error free determination of excitation and emission. The reader is controlled and the data reduction performed by Agilent BioTek Gen5 data analysis software. This combination of assay technology, software and instrumentation provides an ideal solution for high-throughput detection for a variety of applications.

References

1. Andrae, J.; Gallini, R.; Betsholtz, C. Role of Platelet-Derived Growth Factors in Physiology and Medicine. Genes Dev. 2008, 22, 1276–1312.

2. Heldin C. H.; Westermark, B. Mechanism of Action and in vivo Role of Platelet-Derived Growth Factor. Physiol. Rev. 1999, 79, 1283–1316.

3. Chen, W. S. et al. Growth Retardation And Increased Apoptosis in Mice with Homozygous Disruption of the Akt1 Gene. Genes & Development, September 2001, 15(17), 2203–8. doi:10.1101/gad.913901. PMC 312770. PMID 11544177.

4. Franke, T. F. et al. Direct Regulation of the Akt Proto-Oncogene Product by Phosphatidylinositol-3,4-Bisphosphate. Science January 1997, 275(5300), 665–8. doi:10.1126/science.275.5300.665. PMID 9005852.

5. Sarbassov, D. D. et al. Phosphorylation and Regulation of Akt/PKB by the Rictor-mTOR Complex. Science February 2005, 307(5712), 1098–101. doi:10.1126/science.1106148. PMID 15718470.

About the author

Paul Held, Ph.D., is with Agilent Technologies.

A Homogeneous FRET-Based HTS Assay for Quantification of pRb in Cancer Cell Lines to Monitor Inhibition of G_0/G_1 Cell Phase Transition

Novel HTRF cell-based assay quantifies endogenous phosphorylated retinoblastoma protein as a readout.

Peter J. Brescia

Abstract

The field of cancer biology remains one of the most rapidly expanding areas of investigation in both industry and academia. Understanding disruptions that control pathways and checkpoints leading to uncontrolled tumor progression are key to understating disease progression. Presented here is a novel HTRF cell-based assay that quantifies endogenous phosphorylated retinoblastoma protein as a readout of the G_0/ G_1 cell phase transition.

Introduction

Cancer remains a global concern given the significant number of new cases reported annually. Recent advances in understanding the underlying biology of disease progression at the molecular level have proved beneficial for identification of new targets and agents against tumor cell growth.[1] A key factor of cancer progression in general is a disruption of cell cycle control leading to unobstructed cell proliferation. The ability to identify control pathways and checkpoints to target may provide points of intervention through novel therapeutics.[2]

During normal cell cycle control the distinct phases of the cell cycle are conserved progressing from G_0 (quiescence) followed by G_1 (pre-DNA synthesis), S (DNA synthesis), G_2 (pre-division), and M (cell division). Of those, the progression from G_1 to S provides a sentry point that restricts cell proliferation via the interaction between the cyclin-dependent kinases (CDKs) and cyclin proteins.[3] One key role of a subgroup of CDKs, serine/threonine kinases,

Agilent

Trusted Answers

The Bench-Size Microplate Imaging and Analysis Workhorse

Agilent BioTek Cytation C10 confocal imaging reader

From confocal, widefield, and live cell imaging to multimode reading, the Agilent BioTek Cytation C10 confocal imaging reader delivers crisp, high-resolution images and the ability to image and analyze 3D samples. Plus, without having to schedule time at a core lab, you'll have more control over your workflow. Small and powerful – a perfect fit in any lab.

www.agilent.com/lifesciences/CytationC10

The new Agilent BioTek Cytation C10 confocal imaging reader

RA.44158.3299537037

is the hyperphosphorylation of the retinoblastoma (Rb) gene product, pRb, in early G_1 by CDK4 and CDK6 interacting with cyclin D1 resulting in inactivation and the subsequent release of a number of transcription factors necessary for passage into S phase.[4] Previous observations suggest that CDK4/6 inhibition may prevent tumor growth and may help return cells to a near normal phenotype.[2]

Demonstrated here is a novel HTRF cell-based assay that simply and accurately quantifies endogenous phosphorylated retinoblastoma protein at Ser807/811 as a readout of the G_0/ G_1 cell phase transition. The dose response and IC_{50} concentration was determined for a representative kinase inhibitor in a high throughput 384-well, homogeneous cell-based assay format using a representative cancer cell line.

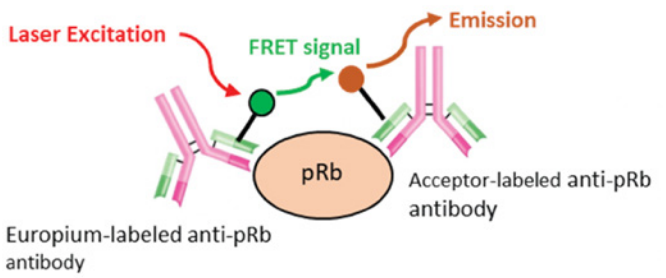

Figure 1. The HTRF® assay. The HTRF proximity assay relies on two different specific antibodies targeting phosphorylated Rb. One antibody is labeled with a europium cryptate (donor) and the second with an acceptor. When the labeled antibodies come into close proximity, a FRET signal is detectable.

Materials and Methods

HTRF Reader Control Kit (PN: 62RCLPEA), Hu- man TNF Alpha Kit (PN:62HTNFAPET) and Phos- ph-Rb (Ser807/811) Cellular Kit (63ADK105PEG) were from Cisbio (Bedford, MA, USA). HCT116 (PN: ATCC® CCL-247™, ATCC, Manassas, MD, USA) cells were cultured using standard tissue culture methods as per the manufacturers' recommendations. The phospho Rb assay was performed using the One-plate Assay Protocol. Briefly, the cells were harvested at 80-90% confluence using TrypLE dissociation reagent (PN:12605036, Thermo Fisher, Waltham, MA, USA) with gentle handling. The cells were collected by centrifugation and resuspended at the desired cell density in the appropriate growth medium followed by plating 10,000 cells in 8 µL into a small-volume, white, 384-well microplate (PN:3826, Corning, Tewksbury, MA, USA) and allowed to attach during incubation at 37 °C, 5% CO_2 overnight.

Palbociclib (PN: 4786, Tocris, Minneapolis, MN, USA) was prepared as a 3x stock as a half-log dilution series and added in a volume of 4 µL of the appropriate cell growth medium to the cells with mixing and incubated as above for 6 hours. Lysis reagent was prepared as per the manufacturers' recommendation and added in a volume of 4 µL with mixing for 1 hour. Antibody pre-mix was prepared as per the manufacturers' recommendation for the single plate protocol. The antibody pre-mix was added in a volume of 4 µL and incubated at room temperature overnight.

Instrumentation

HTRF measurements for the Reader Control Kit and TNFα assay were taken using optimized parameters determined during reader certification. HTRF measurements for Rb detection were taken using the optimized settings show below (Table 1). Briefly, the Synergy Neo2™ was fitted with TRF laser reader cubes for Europium/Red acceptor (Cubes 18 and 41). The read mode selected was TRF Laser in the top position to take advantage of the dual-PMT

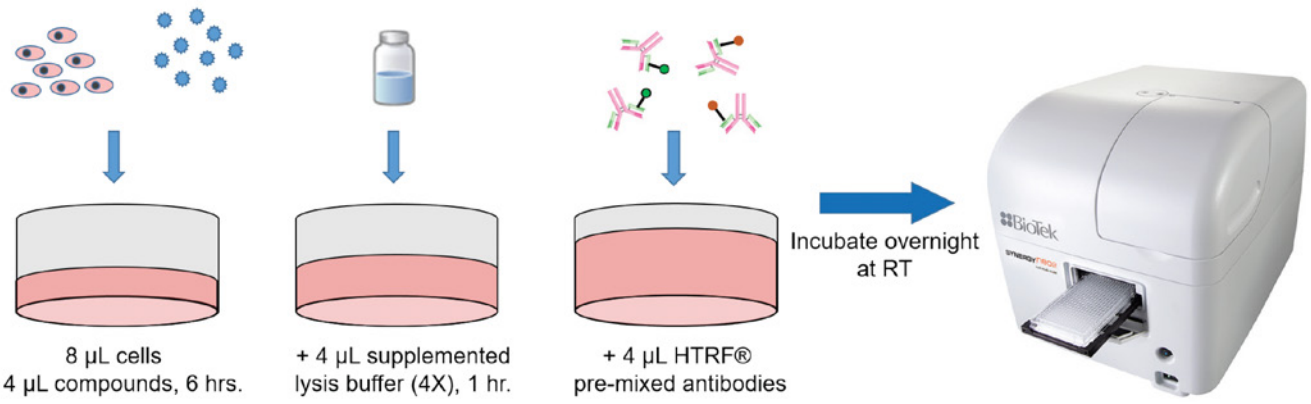

Figure 2. Phosho-Rb Assay workflow.

optical pathway. Dual Photo-Multiplier Tube (PMT) gain settings were optimized using the auto-gain feature. Read height was set to 6.75 mm using auto adjust plate height calibration for use with a 384-well, small-volume plate. The remainder of the settings were based on parameters determined during performance of the HTRF reader certification assays.

Table 1. Synergy Neo2 equipped with TRF laser cubes, with the above settings, was used to rapidly capture dual emission HTRF measurements.

Synergy Neo2 Read Parameters	
Mode	Time-Resolved Fluorescence (Laser)
Filter Sets	Dual PMT
EX/EM	EX337/EM620/665
Gain (PMT1, PMT2)	Auto
Read Speed	Normal
Delay after plate movement	0
Measurements per data point	20
Read Height	6.75 mm
Dynamic Range	Standard
Light Source	TRF Laser
TRF Parameters	
Delay	100 µsec
Data collection time	500 µsec

Results

Data normalization to account for cell plating differences was achieved using a calculated signal ratio by dividing acceptor signal by donor signal and multiplying the value times 10,000 for each well as seen in the following equation:

$$\text{Ratio} = \frac{\text{Signal}_{665\,nm}}{\text{Signal}_{620\,nm}} = \times 10^4$$

The Delta F (DF%) represents the signal-to-background of the assay using internal assay controls to compare day-to-day variability. The DF% is calculated by dividing the difference between the signal and background ratios by the background ratio as seen in the following formula:

$$\frac{\text{Ratio}_{\text{control or sample}} - \text{Ratio}_{\text{negative control}}}{\text{Ratio}_{\text{negtive control}}} = \times 100$$

HTRF Reader Control Kit

The HTRF Reader Control Kit was used to validate laser parameters required to exceed the norms

provided by Cisbio (Table 2). The data collected surpassed the typical norms of assay performance with as little as 1 flash with a standard 0 CV= 6.5%, low calibrator DF = 39%, and high calibrator DF = 1115%, and S/N = 412 (Table 3).

Human TNFα Assay

The Human TNFα Assay Kit was used to validate the laser parameters required to meet or exceed assay norms for LOD seen below (Table 4). The Synergy™ Neo2 was able to surpass the typical assay norms with as little as 10 flashes with ratio CVs ≤2% and LOD = 6.6 pg/mL (Table 5 and Figure 3).

The Z factor was calculated using eight replicate mea- surement of +/- palbociclib (10 uM). The as- say result- ed in Z′ factor of 0.63 indicative of very robust assay performance with low variability in a high-throughput microplate format (Table 6).

Phospho-Rb Detection

Cells were plated as four replicates for each drug concentration in a high throughput 384-well, solid white, small-volume microplate and allowed to at- tach overnight with incubation. An 11-point, half-

Table 2. HTRF Reader Control Kit norms.

Norms	Standard 0 Cv% ≤10%
	Low calibrator ≥15%
	High calibrator ≥550%
	S/N : ≥40

Table 4. Human TNFα Assay norms.

Norm	Detection limit (2SD) <12.5 pg/mL

Table 5. Human TNFα Assay data.

	10 Flashes							
	665 nm		620 nm		Ratio			dF
	mean	cv%	mean	cv%	mean	std dev	cv%	
Calibrator 0	34285	4.4%	46821	3.1%	7320	141.59	1.9%	
Calibrator 1	38768	6.1%	47617	2.2.%	8057	143.91	1.8%	10%
Calibrator 2	41362	3.3.%	46625	2.8%	8871	159.75	1.8%	21%
Calibrator 3	47525	3.1%	46714	2.6%	10173	150.35	1.5%	39%

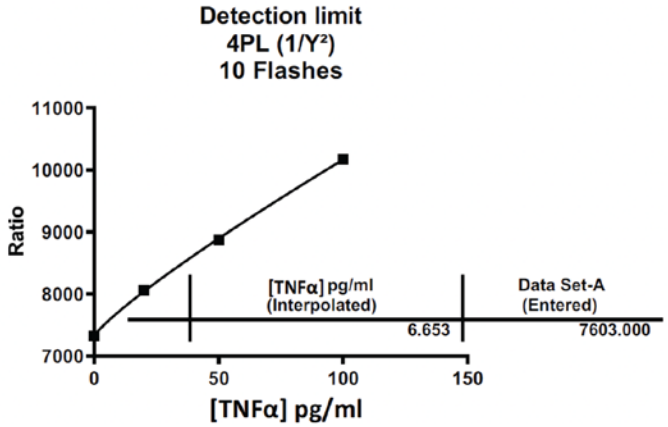

Figure 3. Human TNFα Assay.

Table 6. Z' - Factor.

384-well assay	0.63

Table 3. Reader Control Kit performance data. Synergy Neo2 fitted with laser was used in conjunction with HTRF Reader Control Kit for validation of reader parameters necessary to meet or exceed typical norms of assay performance.

	Reader Control Kit S/N vs. Laser Flashes (Low-Volume, white plate)											
	1 Flash			5 Flashes			10 Flashes			20 Flashes		
Ratio	Mean	CV (%)	DF%	Mean	CV (%)	DF%	Mean	CV (%)	DF%	Mean	CV (%)	DF%
Std 0	2188	6.5		2378	3.33		2378	2.8		2405	2.8	
Low calibrator	3046	5.1	39	3245	2.9	36	3291	1.9	38	3311	1.8	37
High calibrator	26576	3.3	1115	27985	1	1077	27968	0.8	1060	27892	0.8	1052
S/N (620 nm/ blank)	412			378			381			406		

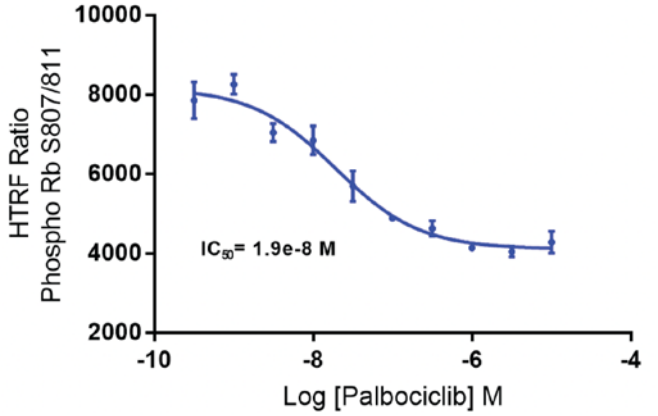

Figure 4. Inhibitor titration. Palbociclib dose response for HCT116 cells generated by the homogeneous, HTS one-plate protocol in a 384-well microplate.

log serial dilution series of the kinase inhibitor palbociclib, including a zero compound addition, was added to each well and allowed to incubate for 6 hours prior to addition of lysis reagent. The HTRF signal was read following a 1 hour incubation period at room temperature. The IC_{50} concentration was determined using a four-parameter dose-response curve fit in Prism software (GraphPad Software, Inc., La Jolla, CA, USA)(Figure 4). The calculated IC_{50} value correlate well with previously reported data.[2]

Discussion

The Synergy™ Neo2 configured with TRF laser and up to 4 PMTs allows for high throughput assay development and screening. A dose response titration of palbociclib against a representative cancer cell line resulted in an IC_{50} value which correlate well with previously reported values.[2] The Synergy Neo2 provides rapid detection which is necessary for high-throughput assay formats. Read time was ~2m42s for 384-wells with 10 flashes per well. The improved sensitivity provided by the laser excitation allows simplified workflows to be performed as indicated by the ability to execute the One-plate protocol in a 384-well assay format. The combination of assay and instrumentation provides an ideal solution for high-throughput detection for a variety of applications.

References

1. Finn, Richard S.; Dering, Judy; Conklin, Dylan; Kalous, Ondrej; Cohen, David J.; Desai, Amrita J. et al. (2009): PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro. In Breast Cancer Research 11 (5), R77. DOI: 10.1186/ bcr2419.

2. Fry, David W.; Harvey, Patricia J.; Keller, Paul R.; Elliott, William L.; Meade, MaryAnne; Trachet, Erin et al. (2004): Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. In Molecular Cancer Therapeutics 3 (11), pp. 1427–1437.

3. Sage, J.; Mulligan, G. J.; Attardi, L. D.; Miller, A.; Chen, S.; Williams, B. et al. (2000): Targeted disruption of the three Rb-related genes leads to loss of G(1) con- trol and immortalization. In Genes & development 14 (23), pp. 3037–3050. DOI: 10.1101/gad.843200.

4. Hsu, Jenny; Arand, Julia; Chaikovsky, Andrea; Mooney, Nancie A.; Demeter, Janos; Brison, Caileen M. et al. (2019): E2F4 regulates transcriptional activation in mouse embryonic stem cells independently of the RB family. In Nature communications 10 (1), p. 2939. DOI: 10.1038/s41467-019-10901-x.

About the author

Peter J. Brescia is with Agilent Technologies.

Investigation of Protein: Protein Interactions (PPI) Using a Novel Bioluminescence Resonance Energy Transfer (BRET) Proximity-Based, High Throughput Screening Assay

Synergy Neo2 provides rapid detection of multiple signals by simultaneously detecting BRET donor and acceptor emission signals.

Peter J. Brescia, Peter Banks, and Amy Landreman

Proteins have been found to play a variety of critical roles in living cells. To better understand how individual proteins function, it is important to be able to elucidate the dynamic interactions occurring within a cellular context. A recently developed reagent platform, similar in configuration and approach to bioluminescence resonance energy transfer (BRET), provides a new tool for investigation of protein:protein interactions (PPI). The system is a proximity-based assay that relies on the measure of energy transfer from a bioluminescent donor protein to a fluorescently tagged acceptor protein. The assay system was used to investigate the PPI of MDM2 and p53 in a cell-based, high throughput screening assay format.

Introduction

While several methods exist to investigate protein:protein interactions (PPI) in living cells, monitoring those interactions remains difficult. Current methods include those that are primarily based on biochemical methods such as co-immunoprecipitation, bimolecular fluorescence complementation, yeast two-hybrid, phage display, chemical cross-linking and proximity ligation assays while physical methods include bio-layer interferometry, dynamic light scattering, surface plasmon resonance, fluorescence polarization/anisotropy and fluorescent energy transfer (FRET). The various methods differ in respect

to sensitivity and specificity in any given system typically requiring multiple approaches for reliable confirmation.

While the use of fluorescent labels plays an important role in many of the methods employed for investigation of PPIs the need for an external illumination source can result in both increased background and/or photobleaching of fluorophores. FRET has been widely accepted as an investigative method to determine the proximity of two labeled molecules of interest. FRET requires the close proximity of donor and acceptor fluorophores for efficient energy transfer from donor to acceptor following excitation from an external light source. A similar methodology, bioluminescent resonance energy transfer (BRET), has gained in popularity by harnessing the bioluminescence energy of luciferase as the excitation source minimizing noise from both background and photobleaching associated with the use of an external light source.

In a manner similar to BRET, NanoBRET™ utilizes a NanoLuc® fusion protein as an energy donor and a fluorescently labeled HaloTag® fusion protein as the acceptor (Figure 1). NanoLuc (Nluc) is an engineered luciferase variant derived from the deep sea shrimp, Oplophorus, and associated substrate, with many of the desirable characteristics mentioned above.[1] The engineered luciferase is a stabilized variant of the smaller catalytic subunit (Oluc-19) from the heteromeric native structure. Nluc, when coupled with substrate, the coelenterazine analogue furimazine, produces a much brighter light than either Fluc or Rluc with a spectral maximum at 454 nm (Figure 2). The optimized blue-shifted donor and red-shifted acceptor pair helps to minimize both assay background due to the biological nature of the sample and from spectral overlap making it amenable to high throughput screening applications.

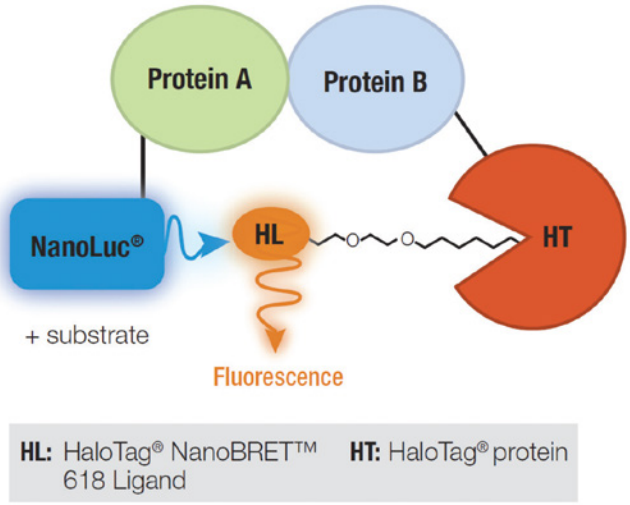

Figure 1. The NanoBRET™ assay. Diagram of energy transfer from NanoLuc-Protein A fusion (donor) to a fluorescently labeled HaloTag-Protein B fusion (acceptor) upon interaction.

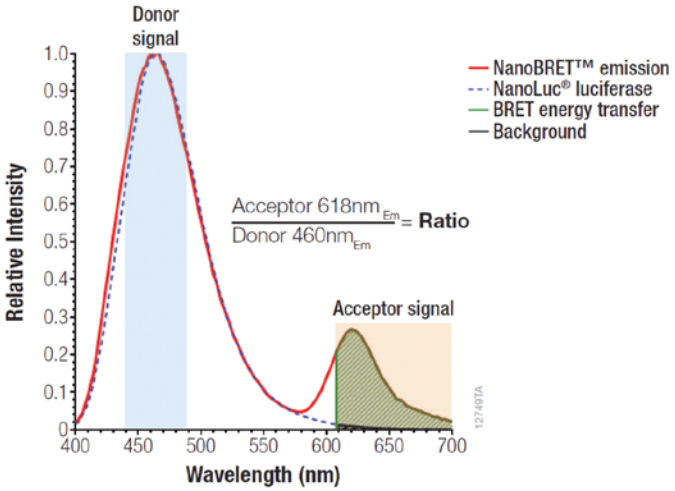

Figure 2. NanoBRET Spectra. Spectra of NanoLuc emission at 460 nm and fluorescent emission at 618 nm of HaloTag NanoBRET ligand. NanoBRET ratio calculation based on donor/acceptor emission.

Preliminary data includes analysis of a donor-acceptor fusion protein for assay and instrumentation optimization as well as investigation of the donor-MDM2 and acceptor-p53 fusion pair in a

cell-based system. The MDM2 and p53 fusion protein pair was transiently transformed into HEK293 cells for analysis including Z'-factor and pharmacological studies.

Materials and Methods

Materials

Plasmid Preparation

NanoBRET™ Protein: Protein Interaction (PPI) System consisting of the NanoLuc®-MDM2 and p-53-Halo- Tag® Fusion Vectors and NanoBRET™ Positive Control consisting of a donor-acceptor fusion protein vector (P/N:N1641 and N1581, Promega Corp., Madison, WI) were used to transform chemically competent *E. coli* strain BL21DE3 using standard methods. Positive transformants were selected and propagated for preparation of plasmid stocks for transfection of HEK293 cells.

Cell Culture, Transient Transfection and Microplate Seeding

HEK293 cells were cultured using standard tissue culture methods. HEK293 cells were harvested at 80-90% confluency and replated into 6-well microplates (P/N:3516, Corning Life Sciences, Tewksbury, MA) at a density of 800,000 cells/well in 2 mL medium and allowed to attach and recover for 4-6 hours. HEK293 cells were transiently transfected with either the NanoBRET™ Protein:Protein Interaction (PPI) System consisting of the NanoLuc®-MDM2 and p-53-HaloTag® Fusion Vectors or NanoBRET™ Positive Control consisting of a donor-acceptor fusion protein vector (P/N:N1641 and N1581, Promega Corp., Madison, WI). Transfections were performed according to the manufacturers recommendations: PPI Control Pair: 2 µg of p-53-HaloTag® Fusion Vector DNA + 0.2 µg NanoLuc®-MDM2 Fusion Vector + 100 µL Opti-MEM® 1 Reduced Serum Medium, no phenol red (P/N: 11058-021,Thermo Fisher Scientific, Waltham, MA)/4% FBS + 8 µL FuGENE® HD Transfection Reagent (P/N: E2311, Promega Corp.). Positive Control: 2 µg of Transfection Carrier DNA + 0.002 µg NanoBRET™ Positive Control Vector diluted in water + 100 µL Opti-MEM® 1 Reduced Serum Medium, no phenol red/4% FBS + 8 µL FuGENE® HD Transfection Reagent. Proteins were allowed to express for ~ 20 hours at 37 ºC, 5% CO_2.

Cells were harvested and replated into either 96- or 384-well microplates (P/N:3917 and 3570, Corning Life Sciences) either with or without HaloTag®

Figure 3. NanoBRET™ Assay Workflow.

NanoBRET™ 618 Ligand. Cells were dispensed in either 100 or 40 µL volumes in 96- or 384-well plates, respectively at a density of 200,000 cells/mL for untreated and 90 or 36 µL at 220,000 cells/mL for treated wells. Treated wells received 10 or 4 µL compound/inhibitor for 96- or 384-well plates, respectively. Plates were incubated at 37 ºC, 5% CO2, for a minimum of 4-6 hours to overnight (18-24 hours). NanoBRET™ Nano-Glo® Substrate in Opti-MEM® I medium was added in a volume of 25 or 10 µL for 96- or 384-well plates, respectively. Donor emission (460 nm) and acceptor emission (618 nm) was measured within 10 min. of substrate addition on a Synergy™ Neo2.

Instrumentation

The Synergy™ Neo2 was fitted with emission filters 450/50 and 610/LP in a dual-emission configuration allowing simultaneous detection of both donor and acceptor signal. Luminescence read mode, using top optics, was selected. Photomultiplier tube gain settings, signal integration time and read height were optimized as discussed below and tabulated (Table 1).

Table 1. Synergy™ Neo2 Read Parameters.

Synergy Neo2 Read Parameters	
Mode	Lum
Light Path	Dual PMT
Optic Position	Top
Gain (PMT1, PMT2)	135, 135
Integration Time	0.5 sec
Read Height	6 or 8 mm (96- or 384-well)
Delay	0 msec

NanoBRET™ Calculations

$$\frac{618\text{nm}_{\text{Em}}}{460\text{nm}_{\text{Em}}} = \text{Raw NanoBRET™ Ratio} = \text{BU}$$

$$\frac{618\text{nm}_{\text{Em}}}{460\text{nm}_{\text{Em}}} = \text{BU} \times 1{,}000 = \text{mBU}$$

$$\text{Mean mBU experimental} - \text{Mean mBU no-ligand control} = \text{Mean corrected mBU}$$

$$\text{Z factor} = 1 - \left[\frac{(3\text{X STDV untreated} + 3\text{X STDV treated})}{(\text{Mean mBU untreated} - \text{Mean mBU treated})}\right]$$

Results

Reader Optimization

Duplicate or quadruplicate wells were plated in either 96- and 384-well format, respectively, with or without (+/-) ligand and were treated with either the specific inhibitor Nutlin-3 at 10 µM or vehicle alone (DMSO). PMT gain settings were varied for each channel as well as investigation of several integration times to determine optimum assay window and read time (representative data shown in Figure 4). The assay window was calculated as the difference between treated and untreated cells. It is apparent from Figure 4 that an integration time of 0.5 seconds, with gain settings of 135 for both

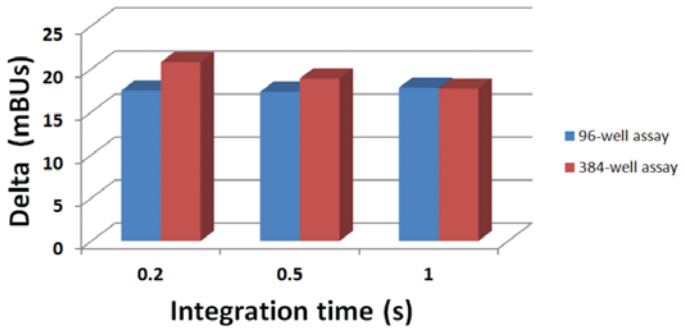

Figure 4. Synergy Neo2 Multi-Mode Reader settings optimization. The delta between mBU response from treated and untreated cells at various integration times and gain settings were compared to determine optimal reader settings.

PMTs, provides an excellent compromise between read speed and response. Testing various read heights found optimums at 6 and 8 mm for 96- or 384-well plates, respectively (data not shown).

Z'-Factor Determination

Following transfection, the cells were replated in the following manner: 96-well assay format: 10 replicates +/- ligand were either treated with 10 µM Nutlin-3 or treated with vehicle alone (DMSO); 384-well assay format: 48 replicates +/- ligand were either treated with 10 µM Nutlin-3 or treated with vehicle alone (DMSO). Following the appropriate incubation period the plate was read on the Synergy Neo2 within 10 mins. of substrate addition. The z' factor was calculated for each assay format as previously described. Both assay formats resulted in z' factors between 0.5 - 1 indicative of robust assay performance with low variability.

Table 2. Z' factor. The z' factor was calculated for each assay format.

	Z Factor
96-well assay	0.83
384-well assay	0.78

Transfected cells were replated as either duplicates or quadruplicates for treatment with each inhibitor concentration in a 96- or 384-well microplate, respectively. A 9-point, 1:3 serial dilution series of the specific inhibitor Nutlin-3, including a zero compound point, was added. Following the appropriate incubation period the plate was read on the Synergy™ Neo2 within 10 minutes of substrate addition. The IC50 concentration was determined using a four-parameter dose-response curve fit in Prism (GraphPad Software, Inc., La Jolla, CA)(Figure 6). IC50 values of 8.4 and 8.2 µM for 96-well and 384-well assay formats, respectively, correlate well with previously published data (Table 3).

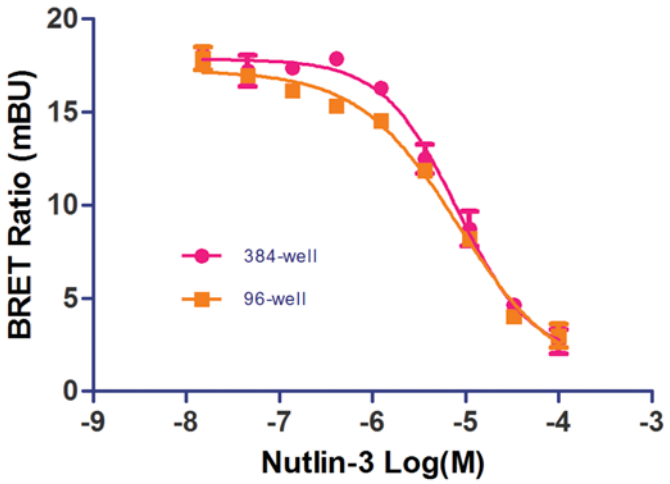

Figure 5. Inhibitor Titration.

Table 3. IC_{50} Values. The IC_{50} values for the inhibitor Nutlin-3 were determined for each assay format.

	Nutlin-3 IC_{50} (µM)
96-well assay	8.4
384-well assay	8.2

Conclusion

The live-cell Bioluminescence Resonance Energy Transfer (BRET) assay allowed for the investigation of dynamic protein:protein interactions within a biologically relevant environment. The blue-shifted donor signal and red-shifted acceptor employed provided excellent signal-to-background within a complex *in vivo* environment. The Synergy™ Neo2 provides rapid detection of multiple signals by simultaneously detecting BRET donor and acceptor emission signals via a dual PMT configuration which is necessary for high-throughput assay formats. Read times were 1min:6sec and 4min:4sec for 96 and 384 wells, respectively.

References

1. Foltman, Magdalena; Sanchez-Diaz, Alberto (2016): Studying Protein-Protein Interactions in Budding Yeast Using Co-immunoprecipitation. In Methods in molecular biology (Clifton, N.J.) 1369, pp. 239–256.

2. Stoddart, Leigh A.; Johnstone, Elizabeth K. M.; Wheal, Amanda J.; Goulding, Joelle; Robers, Matthew B.; Machleidt, Thomas et al. (2015): Application of BRET to monitor ligand binding to GPCRs. In Nat Meth 12 (7), pp. 661–663.

3. Hall, M.P. et al. (2012) Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. ACS Chem. Biol. 7, 1848– 57.

4. Dudgeon, DD., Shinde, SN., Shun, TY., Lazo, JS., Strock, CJ., Giuliano, KA., Taylor, DL., Johnston, PA. and Johnston, PA. (2010) Characterization and optimization of a novel proteinprotein interaction biosensor high- content screening assay to identify disruptors of the interactions between p53 and hDM2. Assay Drug Dev Technol, 8, 437-458.

About the authors

Peter J. Brescia and Peter Banks are with Agilent Technologies. Amy Landreman is with Promega.

Automation of a Homogeneous Proximity Assay for Detection of ERK1/2 or SMAD3 Phosphorylation

Unique combination of assay and instrumentation provides an ideal solution for high-throughput detection of phosphorylation events.

Peter J. Brescia and Peter Banks

Introduction

The transforming growth factor-β (TGF-β) superfamily consists of a range of proteins involved in a wide variety of biological processes such as cell growth, differentiation, development and apoptosis. Members of this superfamily are encoded by 28 genes in the human genome and include TFG-β isoforms as well as activins and bone morphogenetic proteins (BMPs).[1] Cell signaling is initiated by cell surface receptor ligand binding events resulting in the activation and subsequent formation of heterotrimeric complexes of type I and type II serine/threonine receptor kinases. The type II receptors have been shown to bind ligand and activate type I receptors via phosphorylation. TGF-β signaling occurs within the cell through the Smad family of transcriptional activators.[1] Smad family members fall into three subfamilies: receptor-activated Smads (R-smads), common mediator Smads (Co-Smads) and inhibitory Smads (I-Smads). The concomitant phosphorylation of the R-Smads by activated Type 1 receptors initiates association of phosphorylated R-Smad proteins with Co-Smad. Once associated, the R-Smad/Co-Smad complexes translocate to the nucleus where interaction occurs with a range of nuclear protein partners. I-Smads are induced by TGF-β family members exerting a negative feedback loop via competitive inhibition at the receptor level and marking the receptors for degradation.[1] R-Smad 2 and 3, present in the TGF-β/activin Smad pathway, are well studied phosphoproteins for their potential as drug targets for disorders such as cardiovascular, musculoskeletal, fibrosis and cancer.

Protein kinases are components of large signaling networks responsible for propagating extracellular stimuli via cell surface receptors to assist in regulating a wide range of cellular activities. Stimuli including growth factors, cytokines, hormones and heat stress can activate signaling via formation of heteromeric receptor complexes such as receptor tyrosine kinase receptors (RTKs) and G protein-coupled receptors (GPCRs) or epidermal growth factor receptors (EGFRs). Aberrant regulation of a number

of mitogen-activated protein kinase (MAPK) associated pathways have been associated with diseases such as cancer, Alzheimer's and obesity, among others. Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are members of the MAPK superfamily. ERK 1/2 have been shown to be regulated by both RTK and GPCR activation as well as playing a regulatory role in Smad signaling pathways.

Here we investigate the performance of two homogeneous high-throughput screening assays capable of screening both modulators of receptor activation (e.g. agonists and antagonists) as well as intracellularly acting agents, such as inhibitors of upstream events (Figure 1). The assays were coupled to automated processes for increased throughput. Smad3 or ERK1/2 phosphorylation was measured following endogenous receptor activation in HeLa or HEK293 cell lines, respectively. The pharmacology of known inhibitors was also investigated.

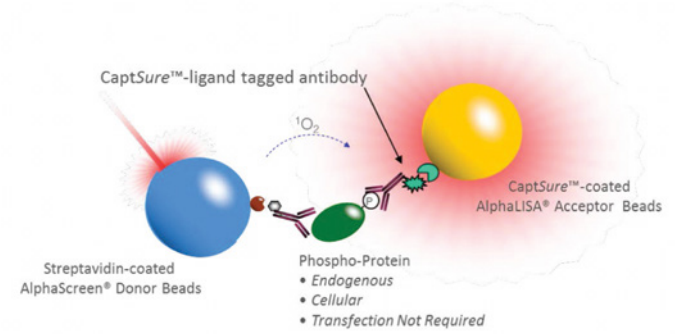

Figure 1. Assay schematic for AlphaLISA® homogeneous proximity assay principle for the detection of phosphorylated proteins. The AlphaLISA® SureFire® Ultra™ assay kits utilize Alpha beads that are each coated to specifically capture the assay antibodies. The Donor bead is coated with streptavidin to capture the biotinylated antibody. The Acceptor bead is coated with a proprietary "CaptSure™" agent that immobilizes the other assay antibody. Upon excitation, the AlphaLISA® donor bead generates singlet oxygen molecules. If the acceptor bead is in close proximity due to the creation of a sandwich immunoassay, the singlet oxygen molecules will trigger a cascade of energy transfer in the acceptor bead, resulting in light emission at 615 nm.

Instrumentation

MultiFlo FX Multi-Mode Dispenser

MultiFlo™ FX is an automated multi-mode reagent dispenser for 6- to 1536-well microplates offering BioTek's unique Parallel Dispense™ technology. Up to four independent reagents can be dispensed in parallel without potential carryover. The instrument was used to dispense assay specific reagents to the 384-well assay plates.

Cytation 5 Cell Imaging Multi-Mode Reader

Cytation 5 combines automated digital microscopy and conventional microplate detection in a configurable, upgradable platform. Cytation 5 includes both filter-based and monochromator-based optics for multi-mode versatility and offers laser-based excitation for Alpha assays.

Materials and Methods

Reagents

AlphaLISA® SureFire™ Ultra phospho-ERK1/2 (No. ALSU-PERK-A500) and phospho-Smad3 Kits (No. ALSU-PSM3-A500) were from PerkinElmer (Waltham, MA, USA)..

Assay Plates

AlphaLISA: CulturPlate™-384 white, opaque 384-well (No.6007680) and AlphaPlate™-384, grey, opaque, 384-well (No. 6005350) microplates were from PerkinElmer (Waltham, MA, USA).

Instrument Settings

The Cytation 5 Cell Imaging Multi-Mode Reader was used with the settings shown in Table 1.

Table 1. Cytation 5 AlphaLISA® reading parameters used in Gen5™ Data Analysis Software.

Mode	Alpha
Gain	120
Delay after plate movement	0 msec
Excitation time	80 msec
Delay after excitation	120 msec
Integration	160 µsec
Read height	8.00 mm

AlphaLISA® Control Lysate Assay

p-ERK1/2 and p-Smad3 control lysates were prepared as 11-pt., 1:3 serial dilutions, including a zero percent control lysate, in lysis buffer that was prepared as per the manufacturers recommendation. Quadruplicate samples were then transferred, 10 µL each, to a 384-well AlphaPlate. Acceptor Mix was prepared as per the manufacturer's recommendation: Activation Buffer was diluted 25-fold in Reaction buffer and Acceptor beads 50- fold into the same Reaction buffer and 5 µL added to each well. For the p-ERK1/2 assay the plate was placed on an orbital shaker for the time needed to prepare the Donor Mix. For the p-Smad3 assay the plate was placed on an orbital shaker and allowed to incubate for 60 minutes at room temperature (RT). Donor Mix was prepared as per the manufacturer's recommendation: Donor beads were diluted 50-fold in Dilution buffer and 5 µL added to each well. For the p-ERK1/2 assay the plate was incubated for a minimum of 2 hours at RT, or up to overnight. For the p-Smad assay the plate was incubated for a minimum of an additional 60 minutes, or up to overnight, following addition of the Donor Mix. Following the final incubation period the plate was read on the microplate reader.

AlphaLISA Cell-based Assay

HEK293 or HeLa cells were cultured using standard tissue culture methods in Advanced DMEM medium supplemented with 10% FBS, 1x P/S/G @ 37 °C, 5% CO_2 in a humidified incubator. Cells were harvested at ~ 80-90% confluency and quadruplicate wells were seeded with 80 µL of cells at the appropriate cell density in 384-well CulturPlates. The cells were allowed to adhere overnight prior to performing the assay. For the p-ERK1/2 assay the cells were serum starved with 80 µL FBS-free media for ~2 hours prior to performing the assay.

Agonist Titrations

Agonist titrations were performed as per the manufacturers' recommendations, with the following modifications, for both p-ERK1/2 and p-Smad3 assays. An 11-pt., 1:3 serial dilution series, including a zero data point, was prepared for the agonists EGF and TGF-β for stimulation of HEK293 and HeLa cells, respectively. A no-cell control was also added for each experiment as well as control lysate prepared at 25%. Briefly, for the p-ERK1/2 assay, following serum starvation, 65 µL of media was removed, leaving 15 µL of residual media, cells were treated with 5 µL of the EGF dilution series prepared at 4x the final concentration (f.c.) and allowed to incubate for 10 minutes @ 37 °C, 5% CO_2 in a humidified incubator. For the p-Smad3 assay 40 µL of media was removed, leaving a 40 µL residual, cells were treated with 20 µL of the TGF-β dilution series prepared at 3x the f.c., and allowed to incubate for 60 minutes @ 37 °C, 5% CO_2 in a humidified incubator. Following incubation, all media was removed and cells were lysed with 10 µL 1x lysis buffer with shaking for 10 min. The AlphaLISA assays were performed

as described above and the Alpha signal was read on a microplate reader.

Inhibitor Titrations

Inhibitor titrations were performed as per the manufacturer's recommendations, with the following modifications, for both p-ERK1/2 and p-Smad3 assays. An 11-pt., 1:3 serial dilution series, including a zero data point, of the inhibitors AG1478 (EGF-R inhibitor) and SB432542 (TGF-β -R inhibitor) was prepared for inhibition of HEK and HeLa stimulation, respectively. A no-cell control was also added for each experiment as well as control lysate prepared at 25%. Briefly, for the p-ERK1/2 assay, following serum starvation, 70 µL of media was removed, leaving 10 µL of residual media, cells were treated with 5 µL of the AG1478 dilution series prepared at 3x the f.c. and allowed to incubate for 60 min. At 37 °C, 5% CO_2 in a humidified incubator. For the p-Smad3 assay 60 µL of media was removed, leaving a 20 µL residual, cells were treated with 20 µL of the SB432542 dilution series prepared at 2x the f.c., and allowed to incubate for 60 minutes @ 37 °C, 5% CO_2 in a humidified incubator. Following incubation with the appropriate inhibitor the EC_{80} of the appropriate agonist was added to all wells as described above for agonist titrations, 5 µL of 4x or 20 µL 3x for EGF or TGF-β, respectively, and incubated for the appropriate time. Following incubation, all media was removed and cells were lysed with 10 µL 1x lysis buffer with shaking for 10 minutes. The AlphaLISA assays were performed as described above and the Alpha signal was read on a microplate reader.

Results and Discussion

AlphaLISA Control Lysate Assay

Positive control lysates for p-ERK1/2 and p-Smad3 provided from the kit manufacturer were used for optimization of Cytation 5 reader parameters (Table 1) and determination of the optimal control concentration for use when performing cell-based assays. As can be seen in figure 4, the data can be fit using a second order polynomial (quadratic) equation. High signal- to-background (S/B) were detected for both control lysates, S/B=1,432 and 2,522 for p-ERK1/2 and p-Smad3, respectively.

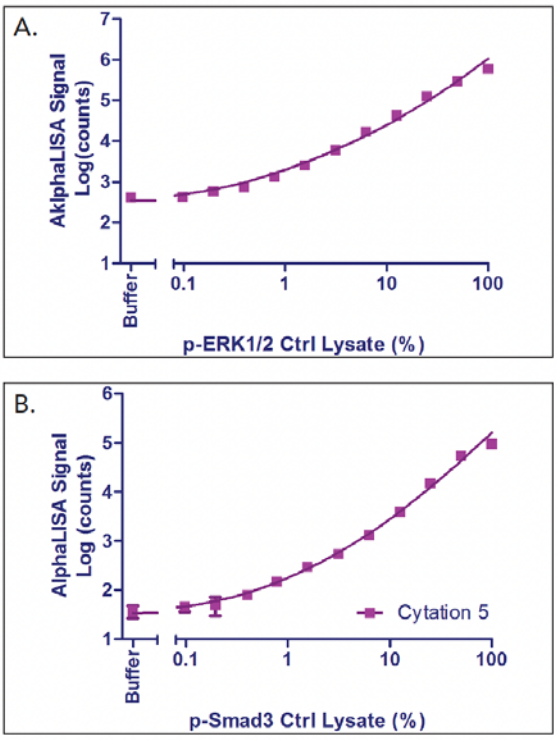

Figure 2. Control Lysate Standard Curves. A) AlphaLISA p-ERK1/2 Assay. B) AlphaLISA p-Smad3 Assay.

AlphaLISA Agonist Titration

Agonist dose response titrations of EGF and TGF-β were prepared for stimulation of HEK293 or HeLa cells, respectively. The cells were stimulated and the production of phosphorylated ERK1/2 and Smad3 was detected and plotted versus Alpha signal. The data can be fit using a Hill Slope model (Figure 3).

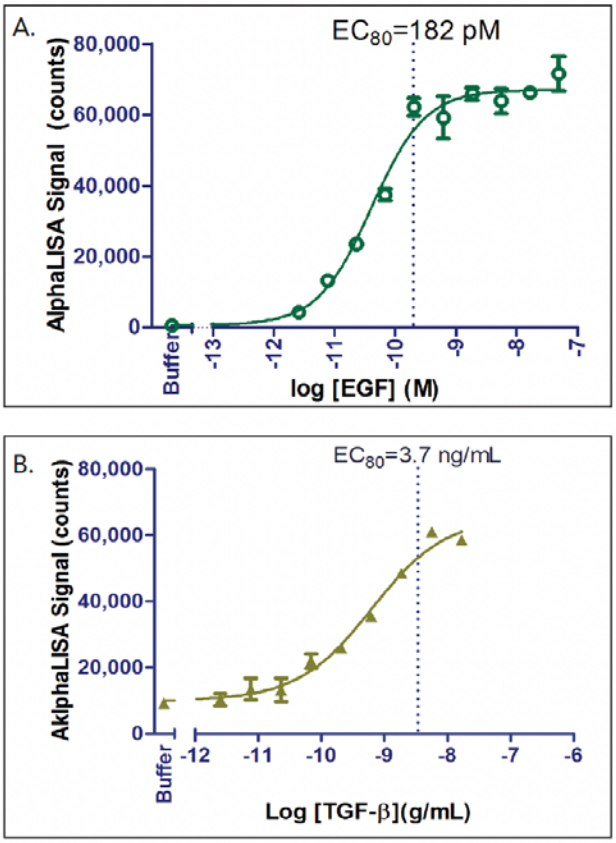

Figure 3. Agonist Titration Curves. A) AlphaLISA p-ERK1/2 Assay. B) AlphaLISA p-Smad3 Assay.

The AlphaLISA assay showed excellent dynamic range, covering nearly 4 decades, and good correlation between replicates for both assays (Figure 3). The agonist dose response curves yielded EC_{80} values of 182 pM and 3.7 ng/mL for p-ERK1/2 and p-Smad3, respectively. The EC_{80} determinants were subsequently used for inhibition studies. The EC50 value of 40 pM EGF for EGFR stimulation of HEK293 cells correlates well with previously generated data provided by the manufacturer (39 pM).

AlphaLISA Inhibitor Titration

Inhibitor dose response titrations of the potent selective kinase inhibitors AG1478 and SB432542 were prepared to evaluate inhibition of p-ERK1/2 and p-Smad3 phosphorylation, respectively. Following incubation with inhibitor, cells were stimulated with the EC_{80} of the appropriate agonist prior to detection of phosphorylated product. The data can be fit using a Hill Slope model as shown in Figure 4.

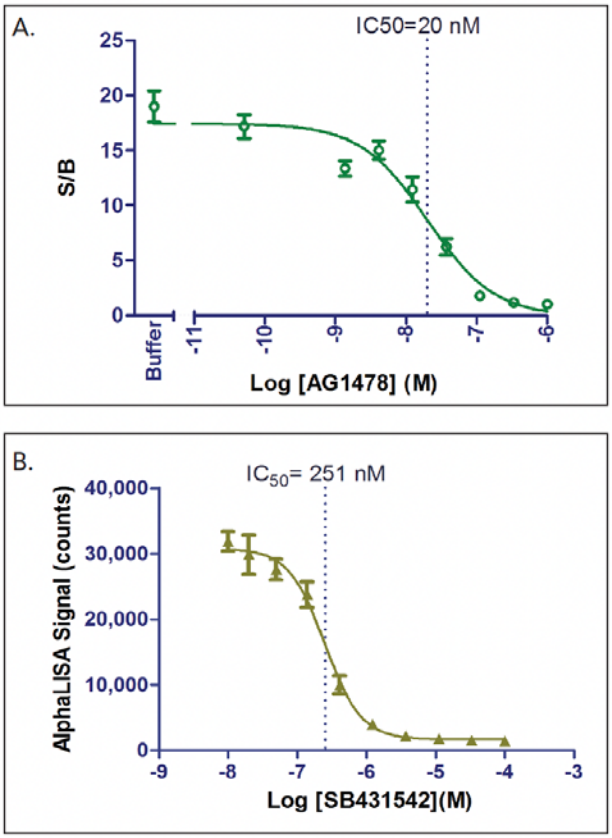

Figure 4. Agonist Titration Curves. A) AlphaLISA p-ERK1/2 Assay. B) AlphaLISA p-Smad3 Assay.

The potent EGFR tyrosine kinase inhibitor AG1478 was determined to have an IC50 of 20 nM, consistent with previously reported values.[1] SB431542 is a potent and selective inhibitor of TGF-β type 1 receptor activin receptor-like kinase ALK5, and its relatives ALK4 and ALK7. The dose response curve and IC50 of 251 nM correlate well with previously reported data.[2]

Conclusion

The assays were performed in their entirety in a HTS compatible 384-well microplate format using automated liquid handling for cell seeding and reagent dispensing. The homogenous assay format allows for improved workflow as compared to the alternative 2-plate protocol requiring culturing and lysis in a 96-well format and transfer of lysate to the higher density 384-well assay plate. Demonstration of both agonist stimulation of kinase phosphorylation and inhibition of receptor signaling were shown for two independent pathways leading to the generation of p-ERK1/2 and p-Smad.[3] The combination of assay and instrumentation provide an ideal solution for high-throughput detection of these phosphorylation events.

References

1. Moustakeas et al (2001). Smad regulation of TGF-β signal transduction. J. Cell Sci. 114 4359-4369.
2. Levitzki, A, and A. Gazit (1995). Tyrosine Kinase Inhibition: An Approach to Drug Development. Science. 267 1782.
3. Inman et al (2002). SB-431542 is a potent and specific inhibitor of transforming growth factor-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol. Pharmacol. 62 65.

About the authors

Peter J. Brescia and Peter Banks are with Agilent Technologies.

DE97322608