Application Note

Molecular Biology and Biochemical

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Receptor-Mediated Stimulation of AKT pan-kinase in NIH3T3 Cells

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

Author

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

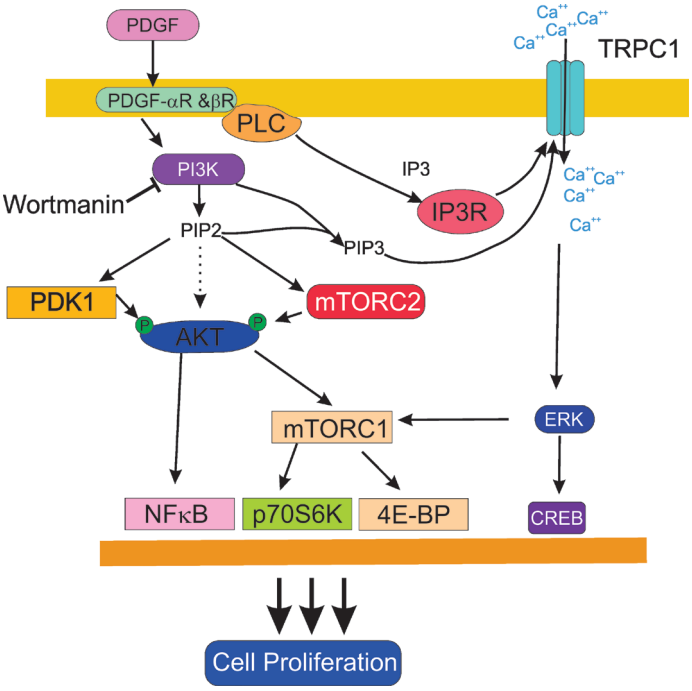

Figure 1. Schematic of the PDGF receptor pathway for cell proliferation.

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

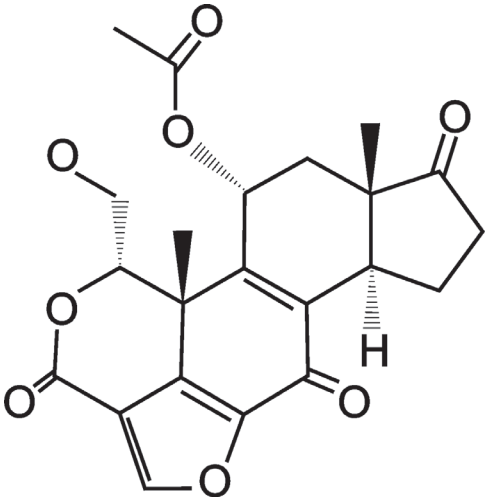

Figure 2. Structure of wortmannin.

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.

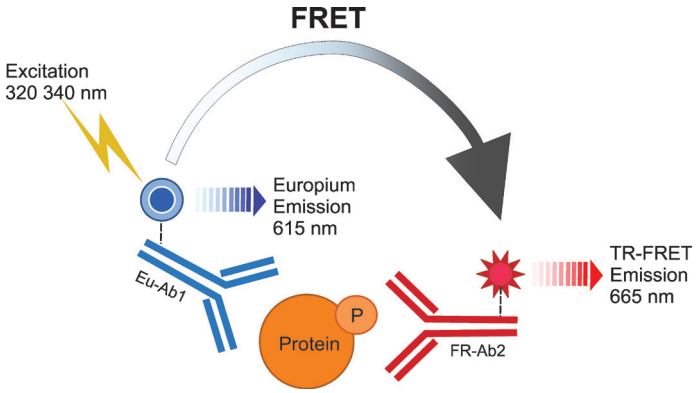

Figure 3. Schematic of the TR-FRET reaction of THUNDER assays.

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.

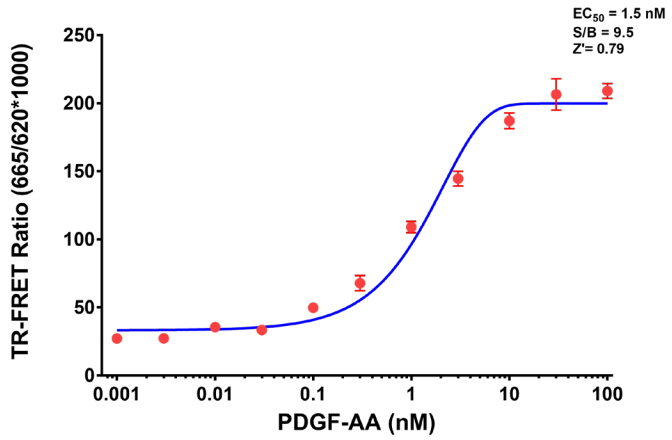

Figure 4. Stimulation of AKT pan (S473) phosphorylation by PDGF. NIH3T3 cells (10,000 cells/ well) were incubated with serial dilutions of PDGF-AA for 15 minutes at 37 °C and then lysed.

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

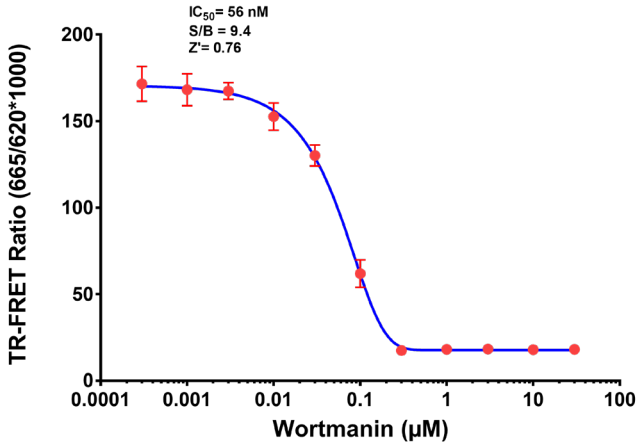

Figure 5. Wortmannin inhibition of phosphorylation of AKT pan (S473). NIH3T3 cells (10,000 cells/well) were incubated with serial dilutions of Wortmannin for 30 minutes at 37 °C. Cells were then stimulated with 10 nM PDGF-AA for 15 minutes at 37 °C and then lysed.

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.

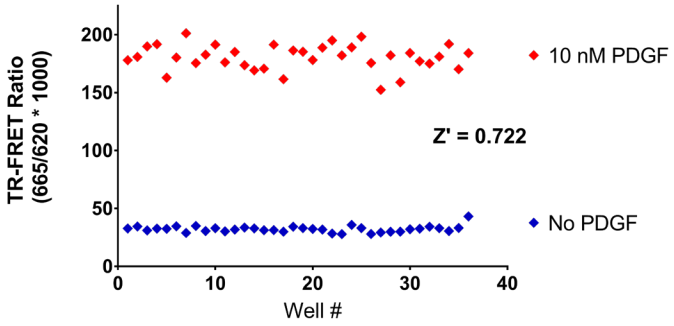

Figure 6. Z'-factor determination in NIH3T3 cells. NIH3T3 cells (10,000 cells/well) were incubated with 10 nM PDGF-AA for 15 minutes at 37 °C and then lysed. The Z'-factor was determined using a total number of 36 wells for each treatment group.

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.

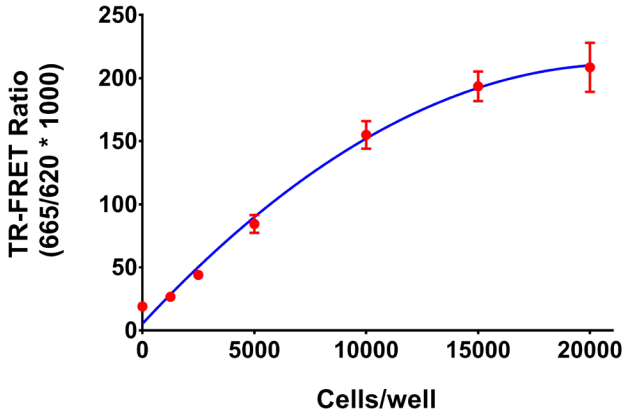

Figure 7. NIH3T3 cell number titration. NIH3T3 cells were seeded into 96-well plates at different seeding densities. After an overnight attachment and 24 hours of serum starvation, cells were stimulated with 10 nM PDGF for 15 minutes at 37 °C and then lysed.

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.

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