Application of BRET Technology to Quantitatively Determine Kinase Inhibitor Potency in Live Cells

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Abstract

NanobRET™ target engagement (TE) is a first biophysical technique that broadly enables the quantitative determination of kinase inhibitor occupancy in live cells, without disruption of cellular membrane integrity. This live-cell quantitative capability is achieved in live cells via BRET with an optimized set of cell-permeable kinase tracers. As the specificity of the BRET signal is dictated by the placement of Nanoluc on the chosen kinase target, a diverse set of broad-coverage tracers supports a method to profile kinase-specific affinity over a large portion of the kinase NanoBRET™ TE has enabled the development of live cell quantitative compound binding assays for >200 individual full-length protein kinases, including a key panel of integral membrane kinase receptors. The assays were scaled to 384-well plate format and 2° analysis demonstrated the assays to be HTS-compatible. Enabled by this new HTS capability, a systematic profiling of dasatinib against kinases was performed in both NanoBRET™ cellular and HotSpot™ biochemical assays. The comparative analysis revealed a surprising intracellular selectivity profile for this drug. IC50 profiling of reference compound panels against ABL, DRR1 and c-Met were further tested in both assay formats for potency comparison. These results demonstrate high concordance between in vitro biochemical assay (HotSpot™) and cell-based assay (NanoBRET™). This data strongly suggest the value of assessing live-cell kinase target engagement in parallel with the biochemical tests in kinase lead optimization programs, as the cellular environment may influence potency and selectivity profiles.

Introduction

The NanoBRET™ TE Assay analyzes the apparent binding affinity and permeability of test compounds by competitive displacement of a NanobRET™ tracer reversibly bound to a Nanoluc fusion protein in cells. Compound engagement is measured in a competitive format-using cell-permeable NanoBRET™ tracer binding of the test compound results in a loss of NanoBRET™ signal between the target protein and the tracer in intact cells.

Experimental Procedures

Materials

HEK293 cells were from ATCC. FuGENE HD Transfection Reagent, Kinase-Nanoluc Fusion Plasmids, Transfection Carrier DNA, NanoBRET™ Tracer and Dilution Buffer, NanoBRET Nano-Glo® Substrate, Extracellular Nanoluc® Inhibitor from Promega. All compounds were purchased from Selleckchem.com.

Cellular kinase NanoBRET™ assay

Assays were conducted by following the Promega’s assay protocol with some modifications. HEK293 Cells were transiently transfected with Kinase-Nanoluc® Fusion Vector DNA by FuGENE HD Transfection Reagent. Testing compounds were delivered into 384 well assay plate by Echo 550 (Labcyte Inc, Sunnyvale, CA). Transfected cells were harvested and mixed with NanoBRET™ Tracer Reagent and dispensed into 384 well plates and incubated the plates at 37°C in 5% CO2 cell culture incubator for 1 hour. The NanoBRET™ Nano-Glo® Substrate plus Extracellular Nanoluc® Inhibitor Solution were added into the wells of the assay plate and incubated for 2–3 minutes at room temperature. The donor emission wavelength (660nm) and acceptor emission wavelength (600nm) were measured in the EnVision plate reader. The BRET Ratio was calculated: BRET Ratio = [Acceptor sample - Donor sample] / [Acceptor no-tracer control - Donor no-tracer control]. The IC50 values of compounds were estimated using Prism (GraphPad Software).

Biochemical HotSpot™ Kinase assay

The radioligand filtration binding assays were performed as previously published (Anastassiadis T et al, 2011, Nature Biotechnol. 29, 1039-1045); Dong-Li KC et al, 2016, Cell Rep. 24, 773-781). Briefly, the 20 nM Protein substrate and the recombinant human kinase protein were prepared in the reaction buffer (20 mM Hapes (pH 7.5), 10 mM MgCl2, 1 mM EDTA, 0.02% Brij-35, 0.02 mg/ml BSA, 0.1 mM NaVO4, 2 mM DTT, 1% DMSO). Compounds were delivered into the reaction using an Echo 550 (Labcyte Inc, Sunnyvale, CA). After 30 minute incubation at room temperature, 20 nM [3H]-ATP was added to the mixture to initiate reaction. Reactions were carried out at room temperature for 120 minutes. The IC50 values of compounds were estimated using Prism (GraphPad Software).

1. Assay Window and Z-factors of NanoBRET™ assays in 384 well plate format

2. Comparison of Reference Compounds in cellular NanoBRET™ and biochemical HotSpot™ assays

3. IC50s of Dasatinib in HotSpot™ and NanoBRET™ assays

4. Over 50 kinases have been validated in NanoBRET™

Conclusion

Cellular kinase NanoBRET™ technology has been successfully validated and optimized into a 384 well plate format in intact cells.

The assay is suitable for high throughput screening to identify specific kinase inhibitors in intact cell, with Z-factor >0.5; Assay S/N >2.4M.

A systematic profiling and comparative analysis of dasatinib against a spectrum of kinases in both NanoBRET™ cellular and HotSpot™ biochemical assays revealed a high concordance between two assay platforms.

More than 50 kinases have been validated at RBC. Our major goal is to validate >200 kinases using NanoBRET™.

Reaction Biology is now offering target engagement assay service in collaboration with Promega using the NanoBRET™ technology.