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

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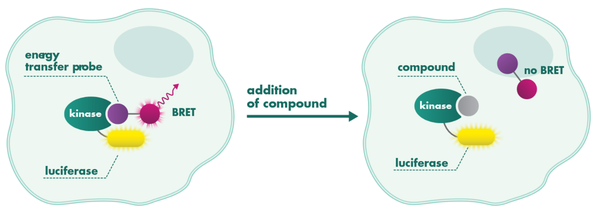
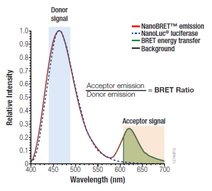
Abstract

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 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 kinome. 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 Z' analysis demonstrated the assays to be HTS-compatible. Enabled by this new HTS capability, we performed a systematic profiling of dasatinib against kinases in both NanoBRET cell-based and HotSpot™ biochemical assays. The comparative analysis revealed a surprising intracellular selectivity profile for this drug. IC50 profiling of reference compound panels against Abl1, DDR1 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). Our 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 a 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 purchased from ATCC. FuGENE HD Transfection Reagent, Kinase-NanoLuc fusion plasmids, Transfection Carrier DNA, NanoBRET Tracers and dilution buffer, NanoBRET Nano-Glo Substrate, Extracellular NanoLuc Inhibitor were obtained from Promega. All compounds were purchased from Selleckchem.

Cellular kinase NanoBRET™ Assay

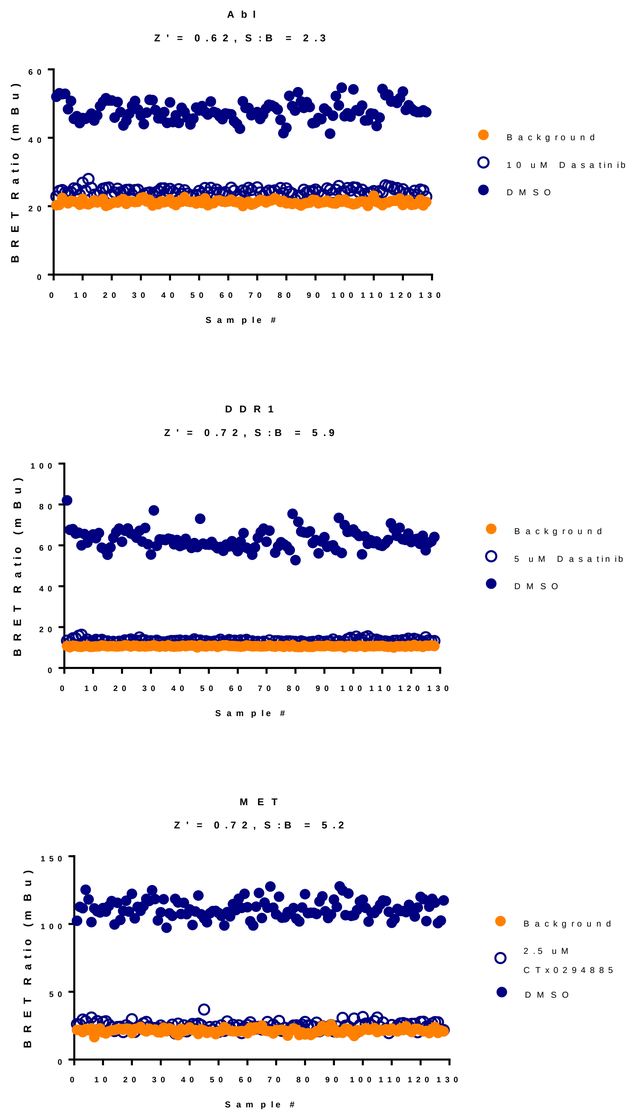
Assays were conducted following Promega assay protocol with some modifications. HEK293 Cells were transiently transfected with Kinase-NanoLuc Fusion Vector DNA by FuGENE HD Transfection Reagent. Testing compounds were delivered into 384 well assay plate by Echo 550 (Labcyte Inc, Sunnyvale, CA). Transfected cells were harvested and mixed with NanoBRET Tracer Reagent and dispensed into 384 well plates and incubated the plates at 37C 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 (460nm) and acceptor emission wavelength (600nm) were measured in the EnVision plate reader. The BRET Ratio were calculated. BRET Ratio = [(Acceptor sample ÷ Donor sample) – (Acceptor no-tracer control ÷ Donor no-tracer control)]. The IC50 values of compounds were calculated with Prism GraphPad program.

Biochemical HotSpot™ Kinase assay

The radioisotope filtration binding assays were performed as previously published (Anastassiadis T et al, 2011, Nature Biotechnol. 29, 1039-1045; Duong-Ly KC et al, 2016, Cell Rep. 14, 772-781). Briefly, 20 uM of protein substrate and recombinant human kinase protein were prepared in the reaction buffer (20 mM Hepes (pH 7.5), 10 mM MgCl2, 1 mM EGTA, 0.02% Brij35, 0.02 mg/ml BSA, 0.1 mM Na3VO4, 2 mM DTT, 1% DMSO). Compounds were delivered into the reaction using an Echo 550 (Labcyte Inc, Sunnyvale, CA). After ~20 minute incubation at room temperature, 10 uM 33P-ATP was added to the mixture to initiate reactions. Reactions were carried out at room temperature for 120 minutes. The IC50 values of compounds were calculated with Prism GraphPad program.

Results

01 Assay window and Z'-factors of NanoBRET™ assays in 384-well plate format



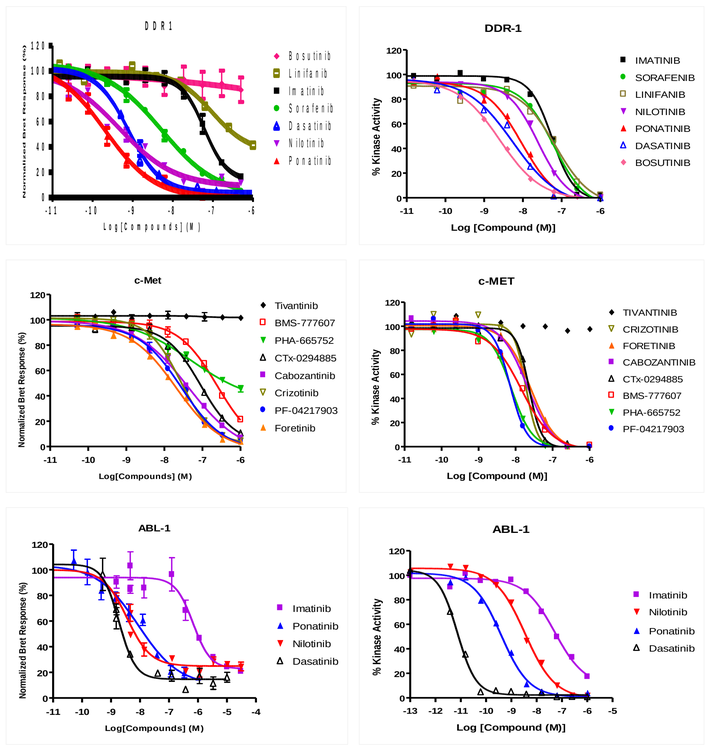
*4000 cells /well in 384-well assay plate

*Compound treatment time: 1 hour

*Tracer concentration: DDR1: 0.0625 mM K4; ABL1: 0.33 mM K4; c-MET: 1 mM K5

*The Z-factor is defined in terms of four parameters: $\text{Z-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$

02 Comparison test of reference compounds in cellular NanoBRET and biochemical HotSpot® assays



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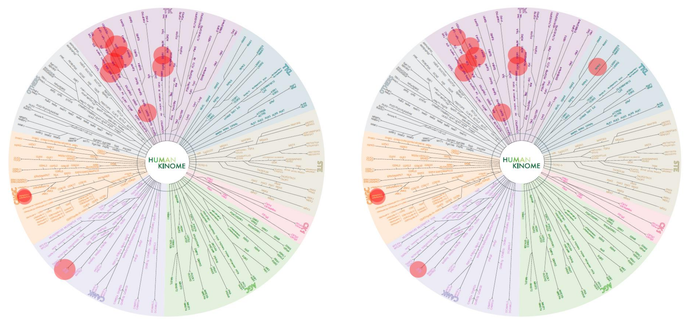
*Compound treatment time: 1 hour

Results

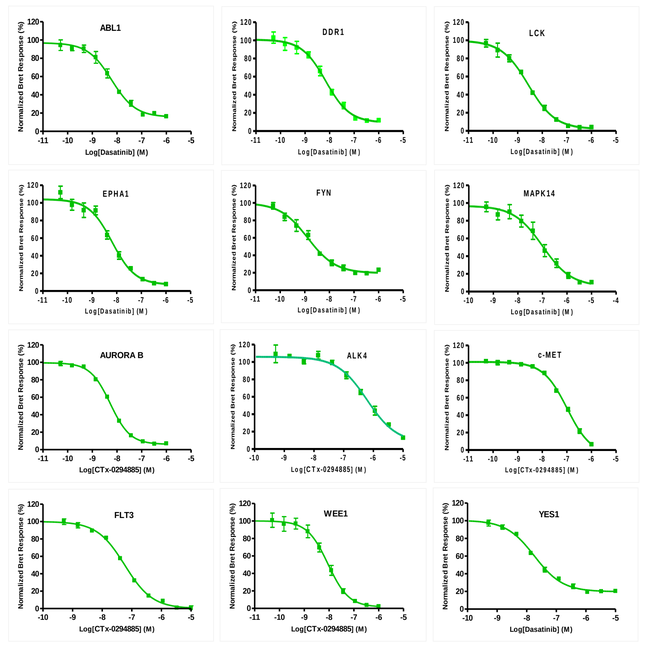
03 IC50s of dasatinib in HotSpot™ and NanoBRET™ assays

Kinases	HotSpot™, M	NanoBRET™, M
ABL1	3.336E-11	6.08E-09
c-Src	1.635E-10	1.765E-08
DDR1	7.297E-09	6.991E-09
DDR2	2.516E-10	2.267E-09
EPHA1	4.819E-09	6.49E-09
FYN	1.49E-10	1.332E-09
LCK	2.357E-10	2.707E-09
LYN	1.462E-10	1.283E-08
P38s	2.167E-07	1.008E-07
P38b	5.972E-07	4.286E-07
RIPK2	No activity	6.991E-09
SIK1	3.58E-10	3.188E-09
SIK3	5.343E-09	3.507E-08



HotSpot™ NanoBRET™

04 Representative data with NanoBRET TE cellular kinase assays



Conclusions

NanoBRET target engagement cellular kinase assay has been successfully validated and optimized into a 384-well plate format in intact live cells.

The assay is suitable for high throughput screenings to identify specific kinase inhibitors in live cells, with Z-factor > 0.5; Assay S/N ratio >2 .3 folds.

IC50 profiling of compound panels against Abl1, DDR1 and c-Met demonstrates the value of using live cell kinase target engagement assay platform with biochemical assay platform for profiling compounds.

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

More than 82 kinases have been validated at RBC. Our future goal is to validate >200 cellular kinases with NanoBRET TE assay.

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