Exploring Kinase Inhibitor Selectivity and Affinity in Live Cells Using NanoBRET

INTRODUCTION

The selectivity and affinity of kinase inhibitors for their intracellular targets underlie their potential as therapeutic agents. The target binding characteristics of kinase inhibitors are often quantified using isolated enzymes. Cell-free enzymatic assays are often robust and scalable, providing ideal platforms for HTS and lead prioritization. However, when performing mechanistic studies in cells and tissues, the binding characteristics of isolated enzymes may diverge from their behaviors in a disease-relevant context[1]. This discordance can arise due to the complexity of the complex milieu where kinases reside. For example, the high concentration (1 – 10 mM) of competing intracellular ATP can dramatically impact engagement potency for kinase inhibitors. Cell-free kinase assays are generally performed near ATP Km using kinase fragments, and these key discrepancies often result in offset in IC50 values between cellular and acellular formats. Furthermore, kinases may function as members of intracellular multiprotein complexes, which may be difficult to simulate with isolated proteins. Finally, cell-free approaches cannot query compound permeability or partitioning, which may further shift engagement potency. To achieve more accurate mechanistic analysis of compound pharmacology, efforts have been increasingly directed to query kinase engagement in a native cellular context.

To characterize intracellular compound pharmacology, indirect pathway analysis tools are often used as proxies for a biophysical analysis of compound engagement in cells and tissues. For example, intracellular substrate phosphorylation assays can query kinase pathway activity in cells. However, the molecular targets of a kinase inhibitor are often ambiguous in the context of a complex signaling pathway. Furthermore, some kinases lack well characterized cellular substrates or suitable antibodies for phosphorylation analysis. Consequently, direct and unambiguous analysis of inhibitor engagement at select kinase targets has represented a challenging task, where only a small set of technologies have proven useful.

A QUANTITATIVE APPROACH FOR KINASE PROFILING IN LIVE CELLS

NanoBRET enables the first biophysical approach to directly quantify compound binding to full length kinases in live cells. This technique utilizes Bioluminescence Energy Transfer (BRET) between a kinase labeled with a luminescent reporter (NanoLuc) and a fluorescent kinase probe introduced to the cell culture medium[2,3]. Binding of the test compound to the kinase is evident as competitive displacement of the fluorescent probe and loss of NanoBRET in live cells (Figure 1). Quantitation and specificity are key attributes of the NanoBRET system. As the BRET signal is highly sensitive to molecular proximity, only the tagged kinase is interrogated. When the tracer is introduced at an appropriate concentration, the resulting IC50 from the unlabeled test molecule is a constant value (Kd-apparent) in live cells[2]. With only a small set (<10) of fluorescent drug tracers and a library of kinase/NanoLuc fusion plasmids, nearly 200 kinases can be evaluated in live cells using NanoBRET.

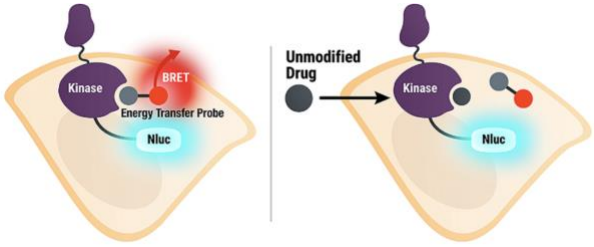

Figure 1. Illustration of NanoBRET Target Engagement Assay in Live Cells, as described in Vasta et al. 2018[2]. Bioluminescence Energy transfer (BRET) enables quantitative analysis of compound engagement in cells. NanoBRET occurs when a fluorescent tracer reversibly binds to a NanoLuc®-tagged kinase. When an unmodified test compound is added to the cells, it competes for the tracer resulting in a loss of BRET that can be easily quantified on a microplate-based luminometer.

REACTION BIOLOGY CORP.

NANOBRET IS HTS COMPATIBLE

Through a collaborative effort between scientists at Reaction Biology Corporation and Promega, this approach has been successfully scaled to 384-well plates, enabling HTS-based profiling of kinase selectivity and affinity. For each assay, HEK293 cells were transiently transfected with kinase/NanoLuc fusion plasmids and seeded into 384-well plates. Using a simple add/mix read protocol, NanoBRET was successfully scaled to the 384 format. The Z' value is typically ranged from 0.6 to 0.8, and the signal/background ratio is ranged 2 to 5-fold.

NanoBRET can enable a quantitative analysis of compound affinity in live cells. Representative target engagement profiling data is shown in Figure 2 for therapeutic kinase targets using panels of reference inhibitors. Unlike cell-free kinase assays, NanoBRET allows for interrogation of full-length kinases in their native intracellular setting. Such kinases include the integral membrane target cMET. Figure 2 demonstrates the results of compound profiling against full-length MET in live cells. Each compound potently engages MET in live cells, with the exception of tivatinib serving as a negative control. As shown in Figure 2, a panel of clinically-relevant inhibitors engages abl kinase, as well as the collateral target DDR1. Such analysis can be valuable to characterize inhibitor selectivity for related kinases. While the rank order of compound inhibition was in general agreement with intracellular engagement, the potency values varied between formats. These shifts in cellular potency may be due to use of full-length kinases as well as a variety of intracellular factors that may be difficult to simulate in a cell-free context. These examples serve to illustrate the complementary value of biochemical and cellular target engagement studies for broad spectrum kinase profiling, and underscore the value of live cell studies as a standard practice for accurate analysis of intracellular occupancy for lead clinical candidates.

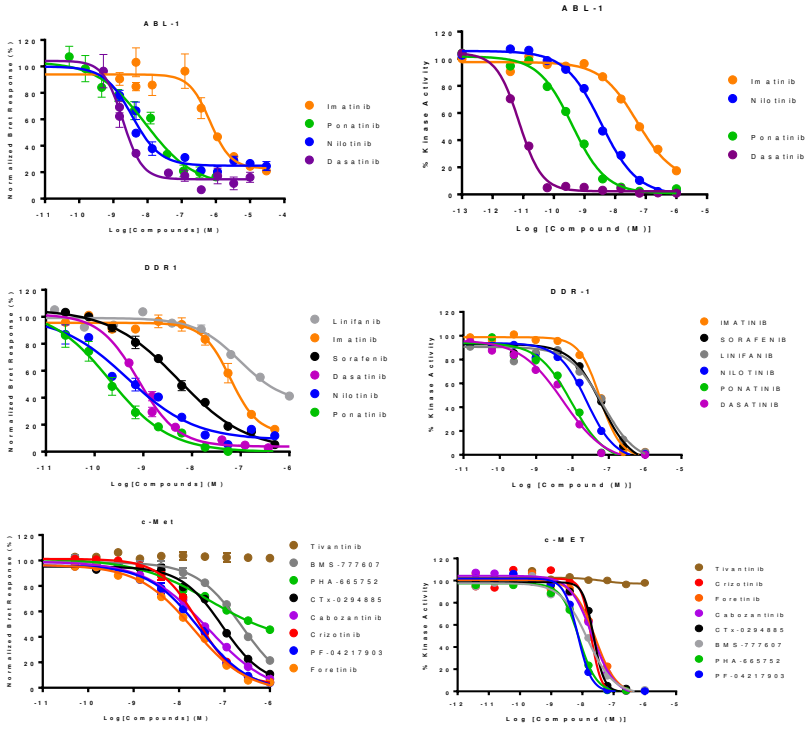

Figure 2. Profiling of clinically approved drugs and clinical candidates using NanoBRET (left panels) vs HotSpot (right panels). While the rank-order potencies of the inhibitor panels were in good agreement between formats, potencies were generally right-shifted in cells.

1 Knight ZA & Shokat KM. 2005, Features of selective kinase inhibitors. *Chemistry & biology* **12**, 621-637.

2 Vasta JD *et al.* 2018, Quantitative, Wide-Spectrum Kinase Profiling in Live Cells for Assessing the Effect of Cellular ATP on Target Engagement. *Cell Chem Biol* **25**, 206-214.

3 Robers MB *et al.* 2015, Target engagement and drug residence time can be observed in living cells with BRET. *Nature communications* **6**, 10091.