An Enabling Technology to Compare Kinase Compound Residency Time with Potency Within Living Cells in a HTS Format

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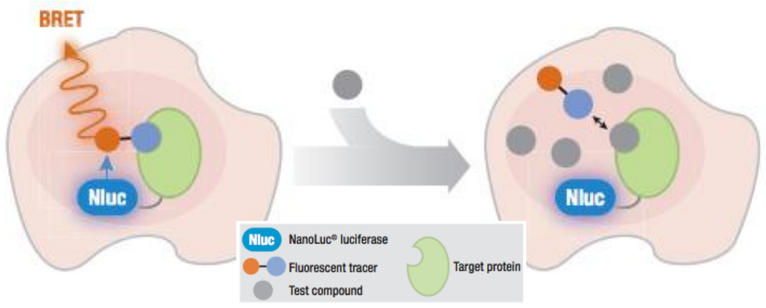
Introduction

As a Pharmacologist screening compounds for your kinase target what parameters are important? Binding affinity and selectivity are used for Structure Activity Relationships (SAR) progression but what about the kinetics of compound binding? The association (K_{on}) and dissociation (K_{off}) rates of compounds are important consideration. Is 1nM binding and 60 minute occupancy 'better' than 100nM binding and 8 hour occupancy in living cells? How would occupancy time influence off-target effects and could these be removed by studying binding kinetics? When choosing which compounds to progress this may influence your SAR decisions. Using Promega Target Engagement reagents we have developed assays designed to study the interaction (K_{on} and K_{off}) of compounds on kinase targets in living cells at physiological ATP concentrations, studying the rate of association and dissociation of compounds for kinase in living cells.

How the technology works



The technology uses Bioluminescence Energy Transfer (BRET) to measure compound engagement with the kinase target. The kinase cDNA plus the Nanoluc enzyme cDNA are transiently transfected (24hrs) on a single plasmid into cells. When expressed, the kinase protein is connected to the luminescence enzyme (NanoLuc). In the presence of a cell permeable Nanoluc substrate, photons are generated and emitted at 460nm. When cells are treated with a cell-permeable fluorescently tagged tracer, binding of the tracer to the kinase brings the tag into close proximity with NanoLuc and the photons excite the fluorescent tag resulting in fluorescence emission at 600+nm and generating BRET. Compound engagement is measured in a competitive format of tracer versus compound. Binding of the test compound results in a loss of NanoBRET™ signal between the target protein and the tracer inside living intact cells. For analysis of target engagement by a test compound, cells are treated with a fixed concentration of NanoBRET™ tracer that is near the EC_{50} value of the NanoBRET™ tracer dose response curve. To determine test compound affinity, cells are titrated with varying concentrations of the test compound in the presence of a fixed concentration (EC_{50}–EC_{80}) of tracer.

Kinase Tracer Affinity Determination

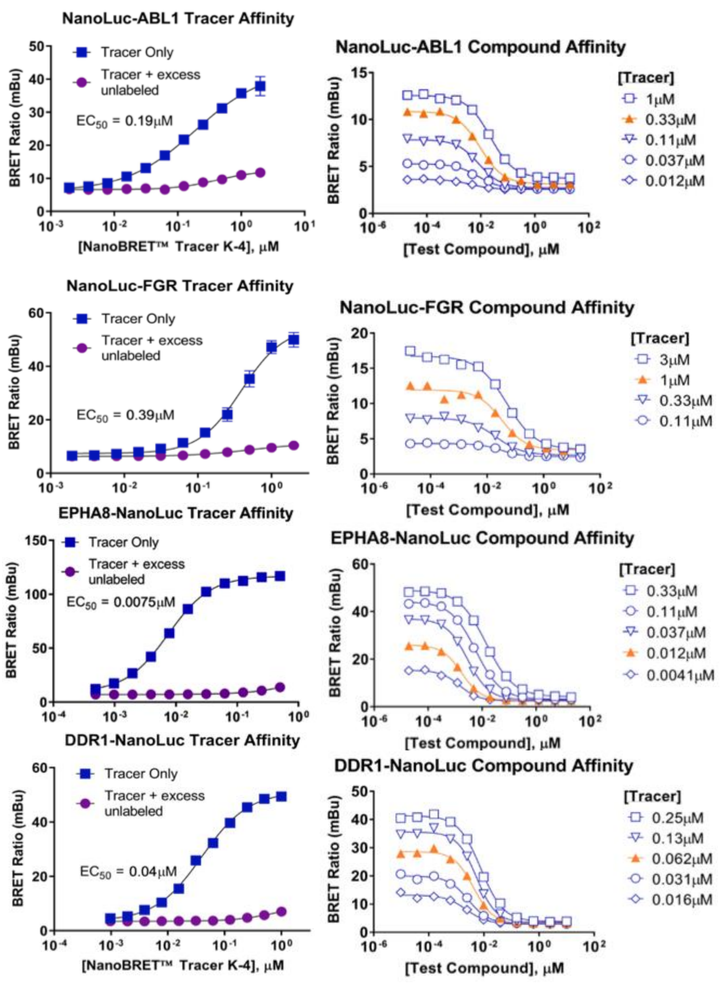
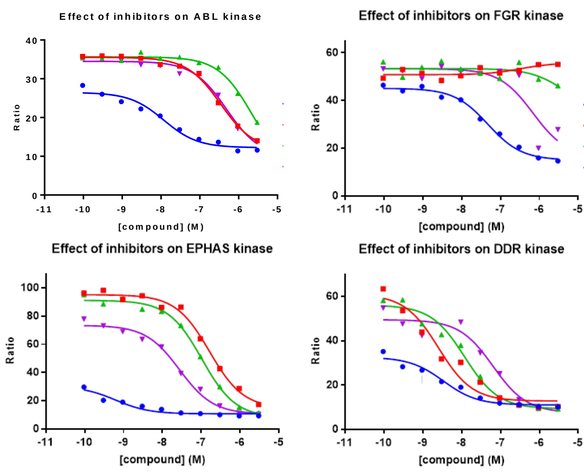


Table. 1. IC_{50} values (nM) of unlabelled compound measured at multiple fixed concentrations of tracer (uM)

Kinase	Tracer Concentration (uM)						
	3	1	0.33	0.11	0.037	0.012	0.0041
ABL		22	10	8	8	4	
FGR	49	33	20	31			
EPHA8			14	6	3	2	2
DDR-1			8	5	4	3	2

A pan-agonist compound (K4) was fluorescently labelled and used as the competing tracer for evaluation of binding on four kinases; Abl, FGR, EPAH8 and DDR-1. Initially the affinity of the tracer for each kinase was determined (left panel), subsequently the tracer was used at fixed concentrations (right panel) to examine competition of the unlabelled compound to determine the IC_{50} for the compound on each kinase (Table. 1.)



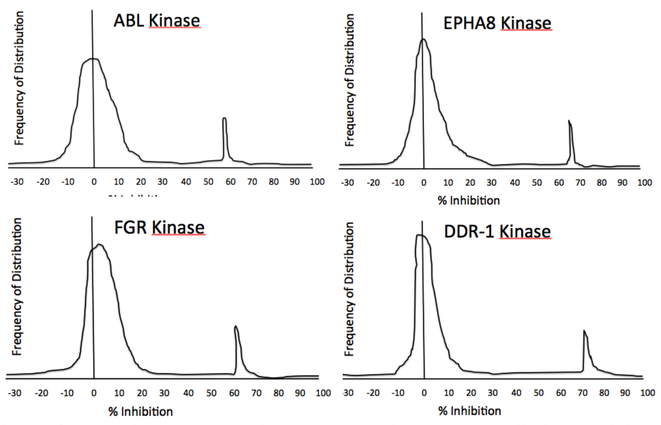
	ABL	EPHA8	FGR	DDR-1
Dasatinib	11	<1	45	4
Nilotinib	340	164	IA	3
Foretinib	~2,000	107	IA	12
Ponatinib	480	27	~600	67

Table. 2. IC50 values (nM) for the binding of Dasatinib, Nilotinib, Foretinib and Ponatinib to ABL, FGR, EPHA8 and DDR-1

Left Panel: Tracer affinity was measured by treating transfected cells with increasing concentrations of tracer in the presence or absence of molar excess of unlabelled compound. Right Panel: Affinity of unlabelled compound was measured at multiple fixed concentrations of tracer, where the IC_{50} (shown in the table) at the recommended tracer concentration (also in table) is depicted in orange.

Right Panel: Binding activity of each compound was determined in living cells. Cells were transfected with each of four kinase; ABL, FGR, EPHA8 and DDR-1. Cells were treated with exemplar kinase compounds including dasatinib, nilotinib, foretinib and ponatinib as a dose response for each compound competed against a fixed concentration of fluorescent tracer K4 at the concentration depicted in orange (left panel).

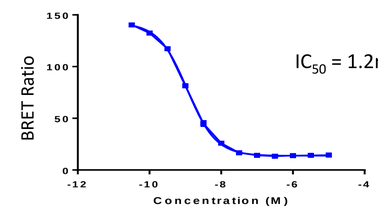
Screening – HTS on one Kinase and Selectivity Screening against many Kinases



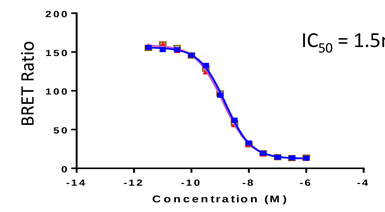
A subset of 80 random compounds were arrayed onto a 96 well plate and discrete wells were spiked with an active compound. All were tested at 10uM on each of the four kinase in the target engagement assay. Most compounds as expected showed a distribution centred around zero percent inhibition (no binding) and all spiked active compounds were detected in each plate tested. The Z factor for each plate was above 0.7. Data suggests that all four kinase assays could be used as a high throughput screen to detect compounds that binding to the kinase target in living cells

HEK-293 cells were transiently transfected with a construct containing DDR-1 Nanoluc and cultured for 24hrs. Cells were then divided into two aliquots, one was cryo-preserved while other cells was cultured for an additional 24 hrs then evaluated using dasatinib and tracer K4. Frozen cells were defrosted, cultured for 24 hrs then assayed using dasatinib and tracer K4. The IC_{50} for dasatinib between fresh and frozen cells was very similar.

Dasatinib binding to DDR-1 in non-cryo preserved cells



Dasatinib binding to DDR-1 in cryo preserved cells

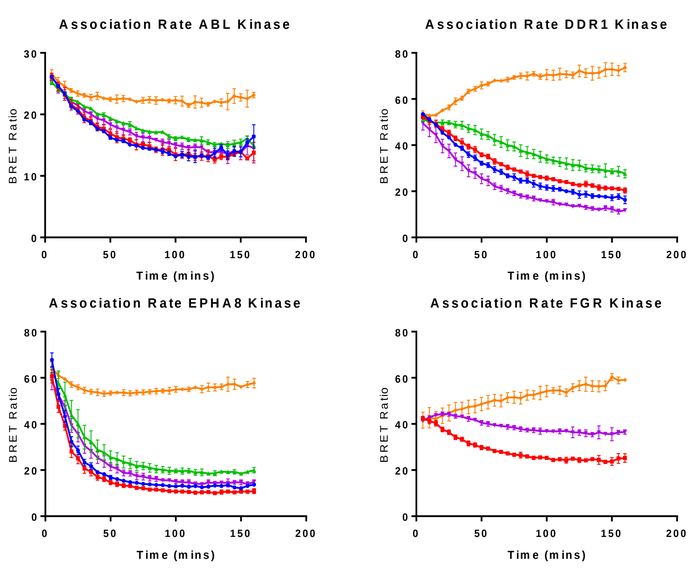


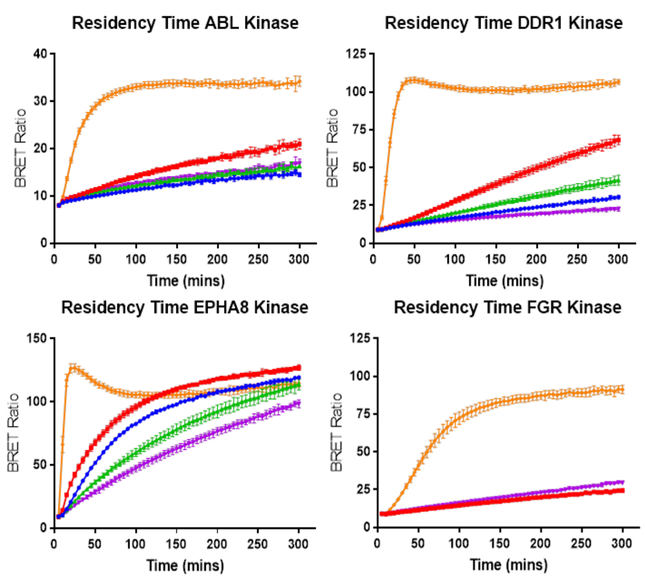
Data suggests it is possible to transfect and cryo-preserve cells allowing these to be rapidly defrosted and used, improving the efficiency of selectivity testing of a single compounds against many kinase targets

Kinetics of Binding – On rates and Off rates of compounds inside cells

HEK-293 cells transiently expressing ABL, FGR, EPHA8 or DDR-1 were incubated for 2hrs at 37ºC with a nominal tracer K4 concentration of 0.33uM, 1uM, 0.012uM and 0.062uM respectively (see figure above in orange) to allow the tracer to bind to the kinase. Simultaneously both Nanoluc substrate and 10X IC_{50} concentrations of dasatinib (red), nilotinib (green), foretinib (blue), ponatinib (purple) or DMSO (orange) were added to the media surrounding the cells and the plate incubated in the reader with readings taken every 5 mins. Compounds compete for binding to the kinase by displacing the tracer, as such the rate of association (K_{on}) can be determined.

HEK-293 cells transiently expressing ABL, FGR, EPHA8 or DDR-1 were incubated for 2hrs with a 10X IC_{50} concentrations of dasatinib (red), nilotinib (green), foretinib (blue), ponatinib (purple) or DMSO (orange), washed to remove the compounds from media surrounding the cells then treated with a fixed concentration of tracer K4 in the presence of the Nanoluc substrate and the plate incubated in the reader with readings taken every 5 mins. Data suggest that tracer K4 competes for binding to the kinase targets and therefore the rate of dissociation (K_{off}) of each compound can be determined.





By studying both the association and dissociation rate of compounds from each of the target kinase it is possible to consider introducing kinetic binding parameter evaluation during compound SAR development.

Summary and Conclusion

- We have developed high throughput screening assays in living cells looking at the competition of compounds to displace the fluorescent tracer with good assay quality and reproducibility (Z factor = 0.7 and above)
- By using cryo-preserved cells we can simply and efficiently perform selectivity screening taking a single compound and testing for binding against many kinase targets stored in the cryostore
- We can determine the binding (as IC_{50}'s) of exemplar compounds such as dasatinib, nilotibin, foretinib and ponatinib to ABL, FGR, EPHA8 and DDR-1 kinases.
- We can measure both the association constant (K_{on}) and dissociation (K_{off}) for compounds within living cells at physiological ATP concentrations
- These assays can be used to examine binding and kinetics for individual kinase targets and off-target kinase toxicity testing
- The cDNA construct can be designed to work within a CRISPR Cas9 system to replace the endogenous kinase in different cell backgrounds
- This technology represents a significant step forward for SAR screening as compound kinetics of binding can, in addition to potency, be used to rank compounds in projects