A Novel Assay Platform for the Detection of Kinase-Inhibitor Binding in Intact Mammalian Cells

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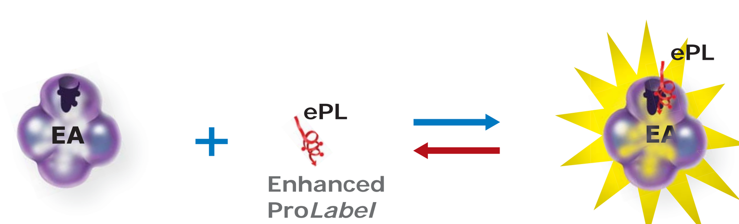
Abstract

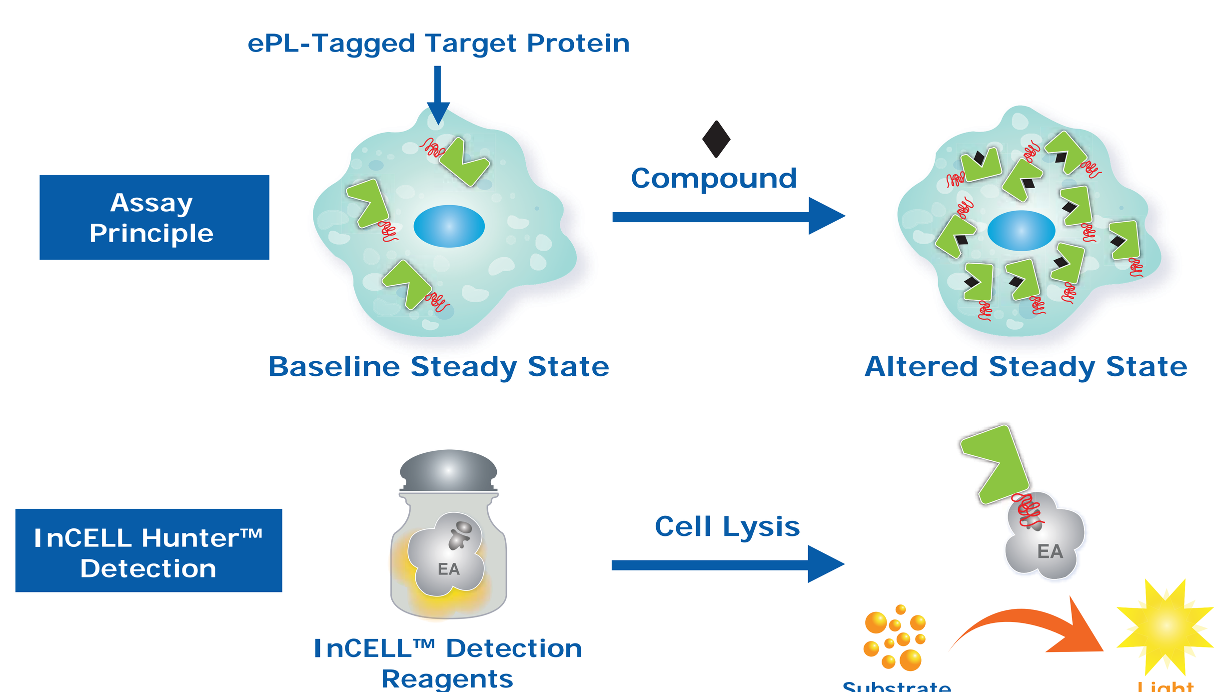
Assays that directly detect compound binding inside cells can be of great utility in translating *in vitro* data to more biologically relevant models. However, these types of intracellular binding assays have not been possible so far. Cellular assays for serveral targets are limited to downstream activity-based readouts which can be difficult to develop, may not be proximal to the target, or may require reagents or knowledge that does not currently exist. Here, we describe the development of a novel cell-based assay platform for the detection of compound binding to intracellular proteins termed InCELL Hunter™. This assay platform detects the binding of a small molecule to a protein target in intact mammalian cells by monitoring changes in protein structure and stability. Using this assay, we have profiled a number of kinase targets and show that the system can detect allosteric as well as ATP-competitive inhibitors and can appropriately rank-order compounds for cellular potency. Since the system is activity-independent, HTS-friendly cell-based assays can be developed for kinases where the substrates are unknown or antibodies have not been developed, greatly expanding the number of immediately tractable kinase targets.

EFC Technology



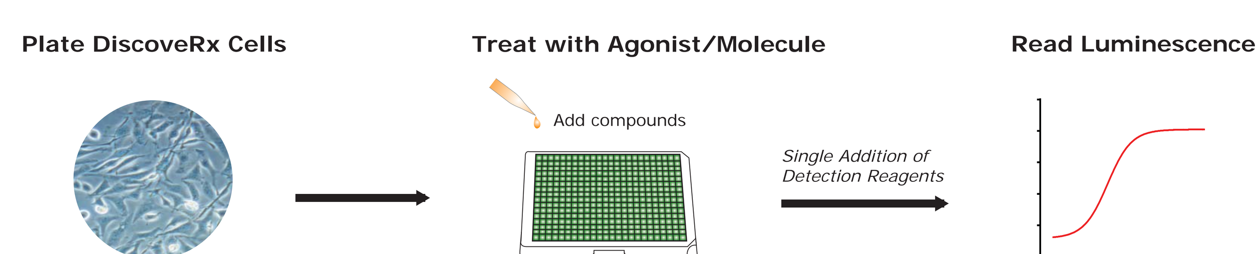
PathHunter® assays utilize the well established Enzyme Fragment Complementation (EFC) technology pioneered by DiscoveRx. This robust technology is based on the complementation of two inactive enzyme fragments, EA which encodes the majority of the enzyme, and a small peptide termed ProLabel®. The ProLabel® tag has a high affinity for EA. Here we introduce a new tag referred to as enhanced ProLabel that is smaller and has more complementation activity than the traditional ProLabel®.

Assay Principle



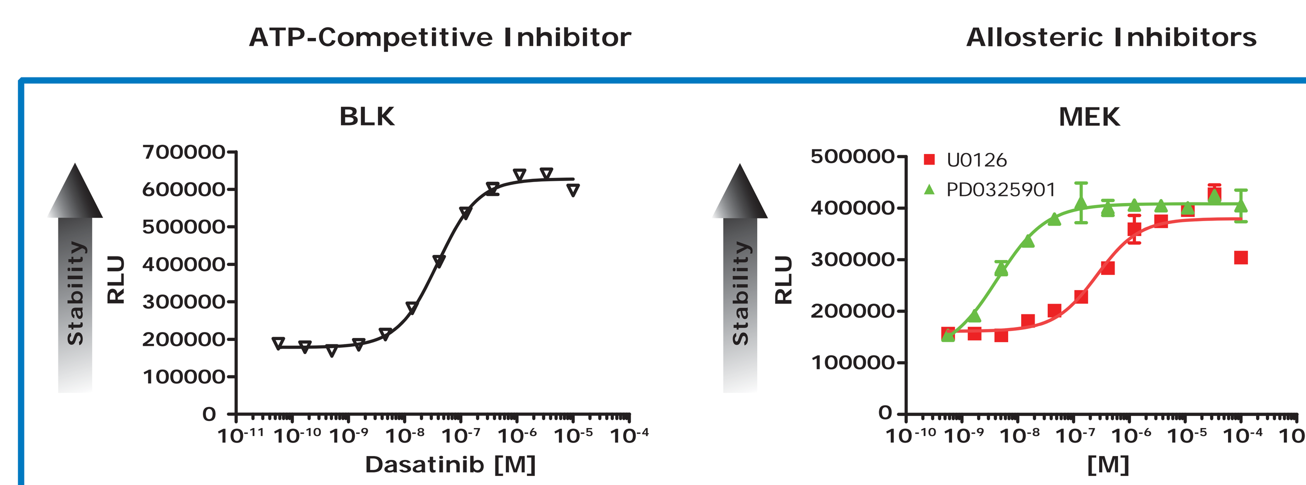
The target protein is fused to an enhanced ProLabel (ePL) tag. In the absence of a binding molecule, the target-ePL fusion reaches a steady state inside the cell. When a molecule binds the target, it changes the stability of the target protein and alters the cellular steady-state protein levels. The protein level is detected with InCELL Hunter™ detection reagents. The EA fragment in the reagent naturally combines with the ePL epitope on the target protein to create active β-gal enzyme, which converts substrate to a luminescent signal.

Protocol



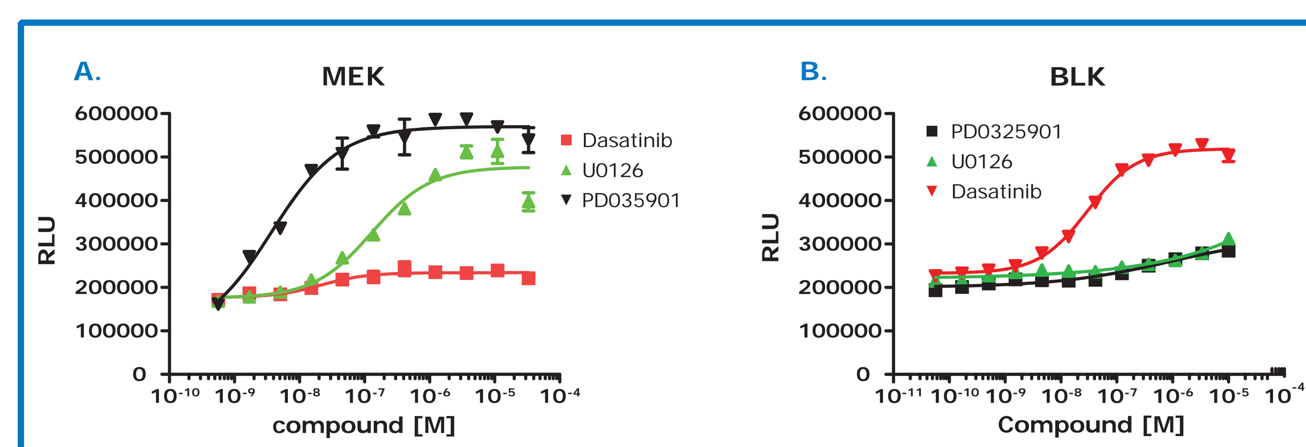
The cells are seeded in 384-well plates and incubated overnight in assay media. The following day the cells are treated with the indicated compounds. After compound incubation which ranges from 30 minutes to 6 hours, the detection reagents are added. The detection reagents, containing lysis buffer, the EA fragment and substrate, are optimized for enzyme complementation for this assay format. After a 30 minute incubation, the plate is read on a standard luminometer.

Application of the InCELL Technology to Kinase Inhibitors



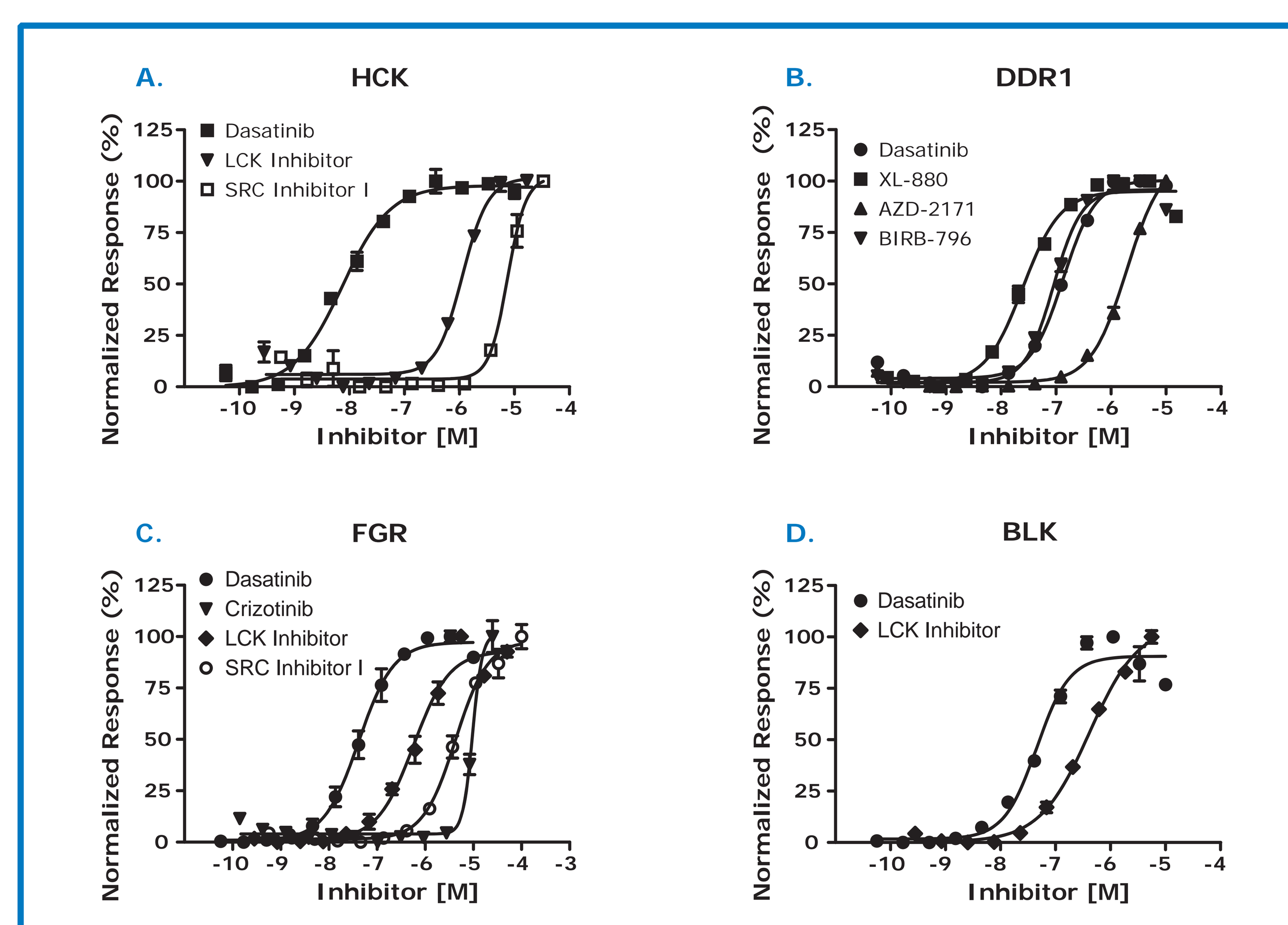
The PathHunter® InCELL Kinase binding assay is capable of detecting binding of ATP-competitive and allosteric inhibitors to kinase targets of interest. Increasing inhibitor concentrations led to increases in levels of stable kinase. Cells expressing the indicated Kinase domain fused to the ePL tag were incubated with test compound for 3 hours prior to detection.

High Signal Specificity of Assay



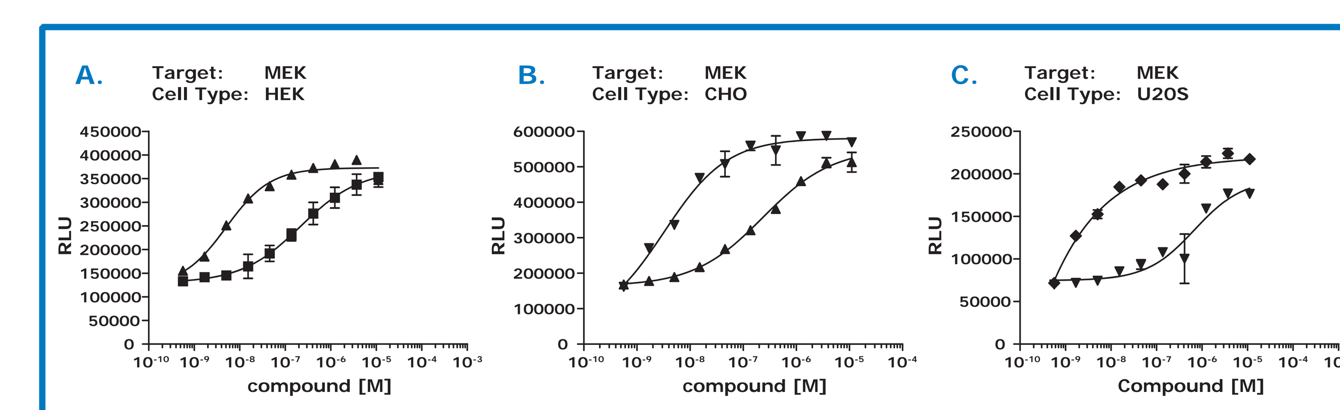
Protein stabilization results from specific binding of an inhibitor to a target of interest. Two kinase targets MEK and BLK, are profiled with three compounds to examine compound-target specificity. PD 0325901 and U0126 are known allosteric inhibitors to MEK, but do not bind BLK. Dasatinib is an ATP-competitive inhibitor known to bind BLK, but not MEK. Exposure of the MEK-ePL and PLK-ePL cell lines to the three compounds induced the expected inhibitor binding profiles based on known binding.

Compound Profiling Using InCELL Binding Assays



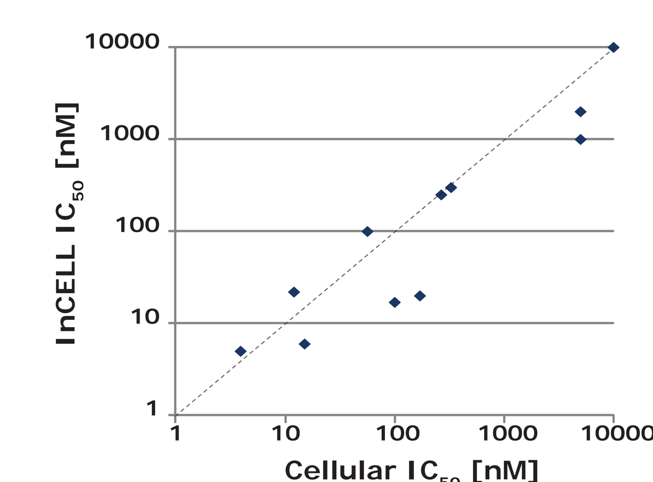
InCELL Hunter™ technology is suitable for profiling kinase binding compounds in a cell based assay format. Here, potencies of known kinase inhibitors are determined in four cell lines expressing protein tyrosine kinases labeled with ePL. Cells were plated in a 384-well assay plate. The next day, cells were exposed to titrations of compounds for 6 hours at 37°C. InCELL Hunter™ Kinase Detection reagent was added and, after a 1 hour incubation, the assay plates were read on a luminometer. Raw RLU were normalized to 100% and the data were plotted using GraphPad Prizm software.

InCELL Binding Assays are Cell-Type Independent



Binding assays can be developed in a variety of mammalian cell backgrounds. In this example, a MEK-ePL fusion is expressed in three mammalian cell backgrounds. The three cell lines are all exposed to PD 0325901 and U0126. MEK-ePL stabilization and consistent compound rank orders are observed in all three cell backgrounds, demonstrating that target stabilization is independent of cell type.

InCELL Data Matches Published Reference Data



Target	Compound	InCELL IC50 [nM]	Phospho IC50 [nM]	Source
MEK	PD 0325901	3.9	5	Henderson et al. Mol Cancer Ther. 2010
MEK	U0126	327.3	300	Granas et al. JBS 2006
MEK	MEK Inhibitor I	56.3	100	Wilkinson et al. Assay D&D 2008
MEK	PD 184352	266.3	250	Solit et al. Nature 2006
HCK	SKI-606	5000	1000	PathHunter® - DiscoveRx
HCK	Dasatinib	15	6	PathHunter® - DiscoveRx
FGR	Dasatinib	100	17	PathHunter® - DiscoveRx
FGR	LCK Inhibitor	170	20	PathHunter® - DiscoveRx
LYN	Dasatinib	12	22	PathHunter® - DiscoveRx
LYN	XL-880	5000	2000	PathHunter® - DiscoveRx

The potencies obtained in the InCELL Hunter™ assay were compared to available cellular assay data. Reference data was obtained from the literature where available, or generated in-house using the PathHunter® tyrosine phosphorylation assays. The IC_{50} values of the InCELL data are on the Y-axis, and the reference data is plotted on the X-axis. Although only a limited data set is available at this time, these data suggest the InCELL potency matches the expected cellular potency.

Summary

In this work we have designed a novel method for interrogating compound target engagement in a cellular format. This system utilizes changes in protein half-life measured with the enahanced ProLabel tag. Compound binding to the Target-ePL fusion alters the half-life of the fusion protein resulting in an alteration in signal. The enhanced ProLabel system is uniquely suited for this application due to its small size and lack of tertiary structure that makes its impact on the fusion protein negligible. Thus, the natural life-cycle of the protein is preserved while providing a straightforward, homogeneous, HTS-friendly read-out of protein-compound binding in intact cells. The system has the potential to enable the development of cell-based-assays for difficult kinase targets including low turnover enzymes and targets whose substrates have not yet been identified.

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