InCELL Hunter™: A Novel Cellular Assay Platform for the Direct Measurement of Compound Binding to Intracellular Targets in Intact Mammalian Cells

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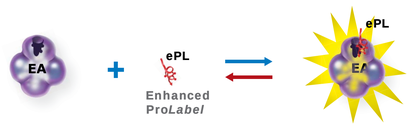
Abstract

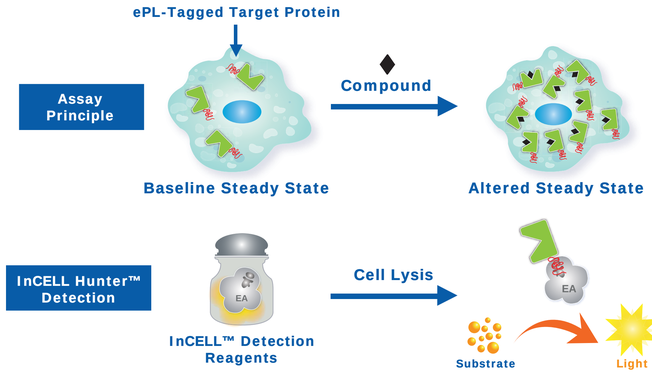
Assays that directly detect compound binding inside cells can be of great utility in translating *in vitro* data to more biologically relevant models. However, these types of intracellular binding assays have not been possible so far. Cellular assays for these targets are limited to down-stream activity-based 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 for the detection of compound binding to intracellular proteins termed InCELL Hunter™. This assay bypasses the need for knowledge of downstream substrates or catalytic activity of the target, relieving many of the constraints of current cell-based assay technologies. We show the generic application of this technology to a number of challenging target classes including methyltransferases, bromodomains, and kinases. As demonstrated here, the assay reports cellular perme-ability and appropriately reports compound binding activity making this a promising platform for bridging the gap between *in vitro* assays and functional cellular assays.

EFC Technology



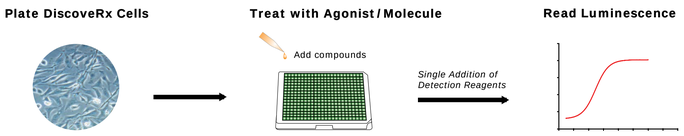
PathHunter® assays utilize the well established Enzyme Fragment Complementation (EFC) technology pioneered by DiscoveRx®. This robust technology is based on the complementation of two inactive β-galactosidase enzyme fragments, EA which encodes the majority of the enzyme, and a small peptide termed ProLabel. 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 better complementation with EA than the traditional ProLabel.

Assay Principle



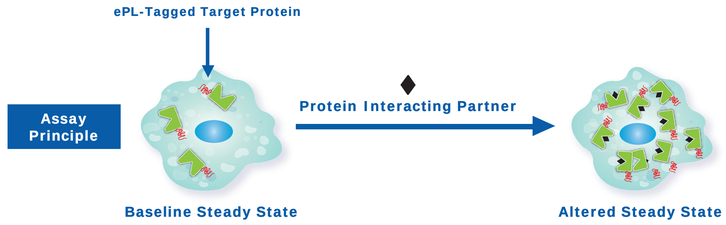
The target protein is fused to an enhanced ProLabel (ePL) tag. In the absence of a binding molecule, the target-ePL fusion reaches a steady state inside the cell. When a molecule binds the target, it changes the stability of the target protein and alters the cellular steady-state protein levels. The 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 sub-strate to a luminescent signal.

Protocol



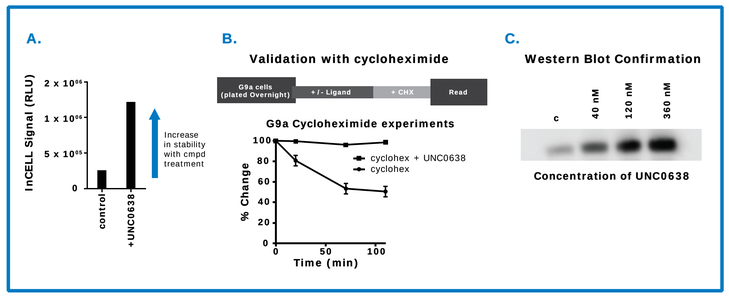
The cells are seeded in 384-well plates and incubated overnight in assay media. The following day the cells are treated with the indicated compounds. After compound incubation which ranges from 30 minutes to 6 hours, the detection reagents are added. The detection reagents, containing lysis buffer, the 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.

Protein Binding Induced Stabilization in InCELL Hunter™ Assay

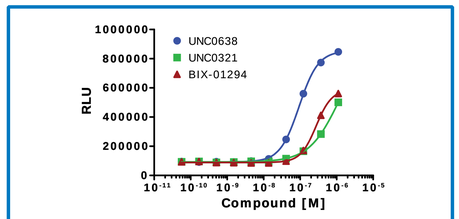


Target–ePL can be stabilized by binding of a protein partner as well as by compound binding. Bcl2 and Bim are members of the apoptosis pathway. Bim, tagged with ePL, was rapidly degraded resulting in low protein signal. However, Bcl2 can bind Bim and stabilize the fusion resulting in an increase in luminescent signal. This increase in signal was specific to the protein interaction as the use of a compound, ABT737, known to specifically dis-rupt Bcl2-Bim interaction gave a dose dependent decrease in the assay.

InCELL Methyltransferase Assay



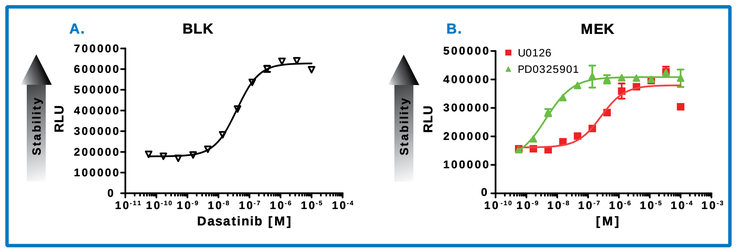
(A) Treatment of G9a-ePL cells with UNC0638 results in robust signal compared to baseline levels of G9a-ePL detected in untreated cells. **(B)** Pretreatment with UNC0638 stabilizes G9a-ePL in cells incubated with cycloheximide. Decreasing amounts of target protein are observed in untreated cells with longer cycloheximide incubation times. **(C)** Increas-ing G9a protein levels in cells treated with increasing doses of UNC0638 was verified by Western blot.



	BIX01294	UNC0321	UNC0638
Biochemical IC_{50}	180 nM	15 nM	15 nM
Published Cellular IC_{50}	700 nM	NA	210 nM
InCELL Hunter Cellular EC_{50}	950 nM	1183 nM	215 nM

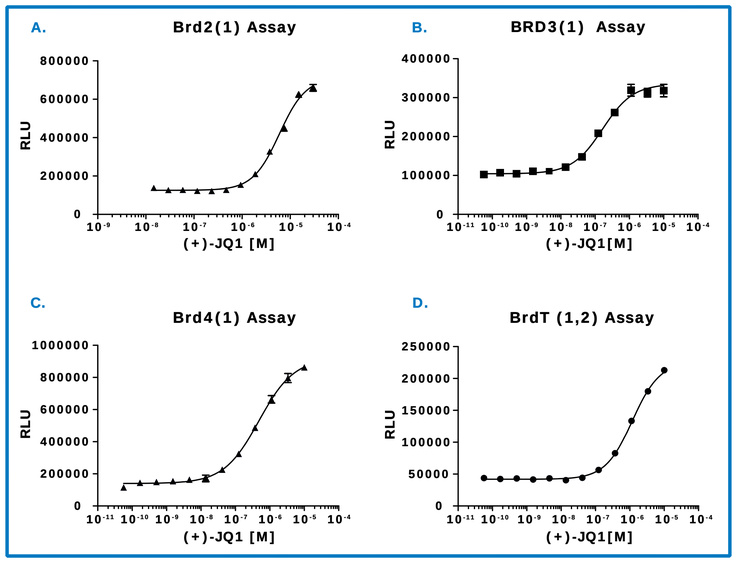
The published biochemical IC_{50} of these compounds indicated that these compounds bind the protein with defined affinities outside the cell. However, as the InCELL Hunter™ assay indicates, compounds with similar biochemical IC_{50} may not have the same binding affinity to the target inside a cell. This data suggests that the UNC0321 compound is not as cell permeable as UNC0638 or not as good at binding the target in an intracel-lular environment.

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.

InCELL Bromodomain Assays



Single or tandem bromodomains from 4 different family members were tested with the bromodomain inhibitor (+)-JQ1 in InCELL assays. Increasing JQ1 concentrations resulted in increased signal for each of the bromodo-mains demonstrating compound binding to the target.

Summary

We have applied the concept of binding-enhanced stability to intracellular target proteins to create cell-based assays, with a demonstrated application in drug discovery across a broad range of target classes. This new platform is made possible due to the unique properties of the ePL tag–an inert tag with enzyme-amplified signal, enabling sensitive and quantitative detection of the natural protein life cycle. Taken together these results indicate that InCELL Hunter™ is a powerful tool to create novel cell-based assays, to investigate compound-target binding and to determine cellular potency of difficult targets in intact cells.

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