Detection of Cytokine and Growth Factor Receptor Dimerization Using Enzyme Fragment Complementation

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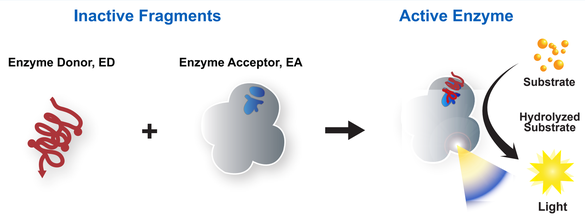
Abstract

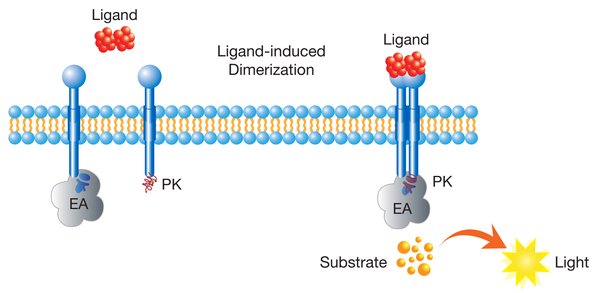
Ligand-induced receptor dimerization is the first functional step in receptor activation, representing the most proximal, functional read-out for receptor activation. For the majority of Receptor Tyrosine Kinases these are homomultimer events whereas for cytokine receptor and other cell surface kinases these are typically heteromultimeric events. Standard cellular protein interaction techniques have not been able to faithfully monitor these interactions in a drug discovery setting. Here we present a novel application of the ProLink and EA complementation system to monitor native receptor-receptor interactions at the cell surface. We show that the technology is applicable across diverse receptor types such as the IL-17, TGFβ, IL-5, Epo, ErbB4, and BMP family receptors. Further, we present quantitative data demonstrating ligand-dependent homo- and heterodimer formation for receptors previously thought to exist in pre-formed complexes, such as the EPO and IL-17 receptors. The high signal to noise, serum tolerance, and low CV's make these assays applicable to small molecule discovery as well as a diverse range of antibody applications including functional characterization, QC and neutralizing antibody studies.

Enzyme Fragment Complementation



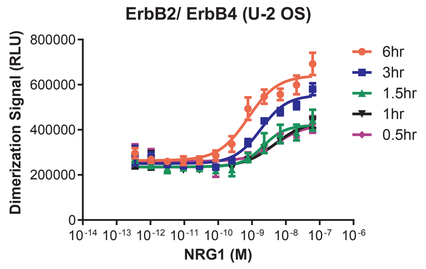
Enzyme Fragment Complementation (EFC) technology consists of the β-galactosidase (β-gal) enzyme, split into two inactive components, the enzyme donor peptide (ED) and enzyme acceptor (EA). When brought together in close proximity, ED complements with EA forming active β-gal. The active enzyme will give a chemiluminescent signal in the presence of substrate providing a highly amplified signal, and therefore, a very sensitive assay.

How PathHunter Dimerization Assays Work

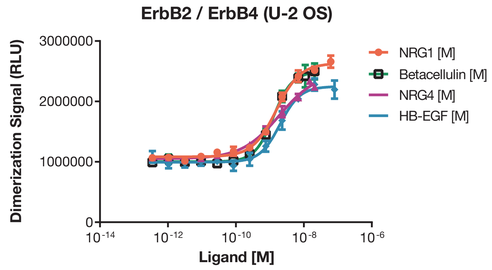


Using EFC, assays have been created to measure receptor heterodimerization, homodimerization or co-receptor recruitment. The two target receptors are tagged with ProLink™ (PK) or Enzyme Acceptor (EA). Upon ligand-induced activation, the receptors naturally dimerize forcing the two β-gal components to complement and create an active enzyme. Active β-gal generates a chemiluminescent signal in the presence of substrate, signaling the formation of receptor dimers.

ErbB2/ErbB4 heterodimerization & ErbB4 homodimerization assays

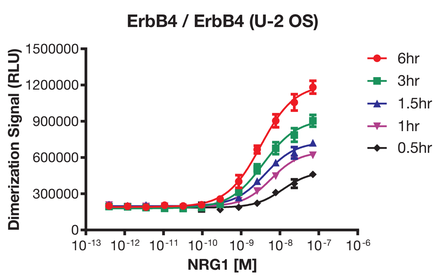


Time point, hr	0.5	1	1.5	3	6
EC_{50} (nM)	5.5	4.7	2.2	1.8	0.77
S/B	1.4	1.6	1.6	2.0	2.3

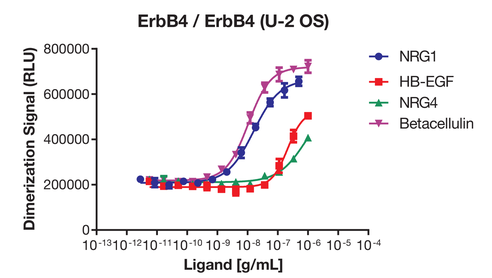


Ligand	NRG1β	Betacellulin	HB-EGF	NRG4
EC_{50} (nM)	1.56	1.28	2	1.9
S/B	2.4	2.4	2.0	2.3

Ligand-induced dimerization of ErbB2 and ErbB4 receptors. (Left Panel) Time course of NRG1-induced ErbB2/ErbB4 heterodimerization. U-2 OS cells stably expressing the extracellular domains of the ErbB2 receptor fused to the PK tag and ErbB4 fused to the EA tag were seeded 24hr before treatment with NRG1b for 0.5- 6hr in dose response format. Robust dimerization is detected within 3hr of ligand incubation, with EC_{50} plateauing by 6hr. (Right Panel) Ligand profile for ErbB2/ErbB4 heterodimer assay. Note al lligands are essentially equipotent in the ErbB2/ErbB4 assay (compare to ErbB4 homodimer), demonstrating that ErbB2 can enhance the binding of NRG4 and HB-EGF to ErbB4 (relative to ErbB4 homodimer). These data are consistent with reports in the literature that NRG1b and NRG4 are ~equipotent towards ErbB4 in cells over-expressing ErbB2.



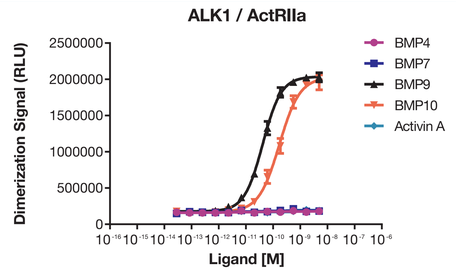
Time point, hr	0.5	1	1.5	3	6
EC_{50} (nM)	11.8	5.9	4.2	3.7	3.2
S/B	2.4	3.0	3.4	4.7	5.9



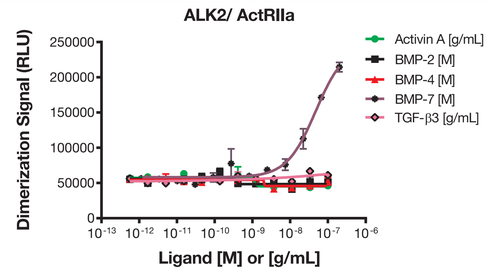
Ligand	NRG1β	Betacellulin	HB-EGF	NRG4
EC_{50} (nM)	2	1.4	28	123
S/B	3.0	3.3	2.3	1.8

Ligand-induced dimerization of ErbB4 receptors. (Left Panel) Time course of NRG1-induced ErbB4 homodimerization. U-2 OS cells stably expressing the extracellular domain of the ErbB4 receptor fused to the PK or EA tags were seeded 24hr before treatment with NRG1 for 0.5- 6hr in dose response format. Robust dimerization is detected within 1hr of ligand incubation, with EC_{50} plateauing by 6hr. (Right Panel) Ligand profile for ErbB4/ErbB4 homodimer assay. ErbB4/ErbB4 –expressing cells were treated with the indicated ErbB4 ligands for 6hr at 37C. Observed EC_{50}s for NRG1 and betacellulin are in good agreement with cell-based ligand binding affinities described in the literature (1.5-5nM).

TGFβ superfamily Type I/Type II receptor heterodimer assays display expected ligand specificity



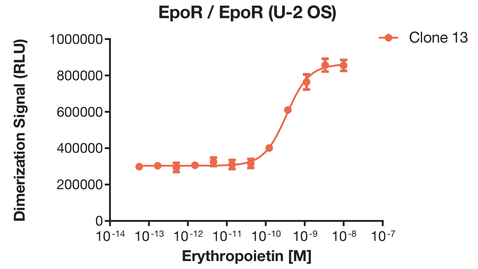
Ligand	BMP7	BMP9	BMP10	Activin A
EC_{50} (pM)	–	42	171	–
S/B	1	12	10	1



Ligand	BMP2	BMP4	BMP7	Activin A
EC_{50} (nM)	–	–	45	–
S/B	1	1	16	1

BMP receptors belong to the TGFβ superfamily of type I receptor serine/threonine kinases, which heteromultimerize with type II receptors to transduce extracellular signals. Ligand specificity of a given heteromeric complex is conferred by the type I receptor. Shown are the BMP receptors ALK1 (Left Panel) and ALK2 (Right Panel) in complex with the type II receptor ActRIIa (activin receptor IIa). ALK1, an important angiogenesis target, binds BMP9 and 10 with high affinity, but not BMP4, 7 or activin A. ALK2 binds BMP7 with high affinity, but none of the other ligands tested, demonstrating the excellent specificity of the ALK1 and ALK2 heterodimer assays.

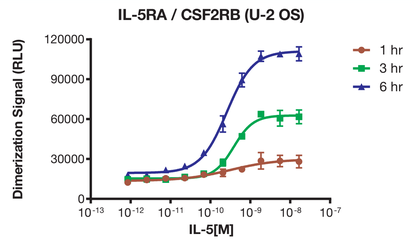
Class I cytokine receptor homodimerization: EpoR Receptor



Ligand	Epo
EC_{50} (nM)	0.341
S/B	2.9

A major fraction of cellular EpoR (a class I cytokine receptor) is believed to exist as preformed dimers/oligomers. Transmembrane (TM) domain-induced dimerization is thought to maintain the unliganded EpoR in an inactive state that can readily be switched to an active state by stimulation with Epo. As shown above, stimulation of U-2 OS cells stably expressing the extracellular regions (including the TM domain) of the EpoR as PK and EA-tagged proteins results in robust dimerization, with an EC_{50} of 341pM. This suggests that other receptors that exist in 'preformed' complexes may also be accessible with this technology.

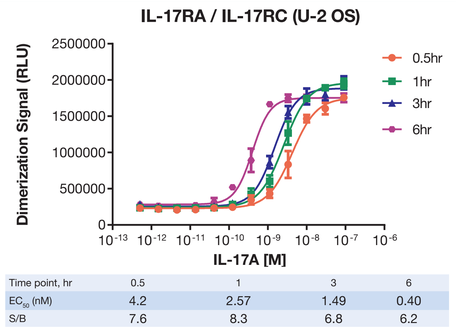
Class I cytokine receptor heterodimerization: IL-5 Receptor



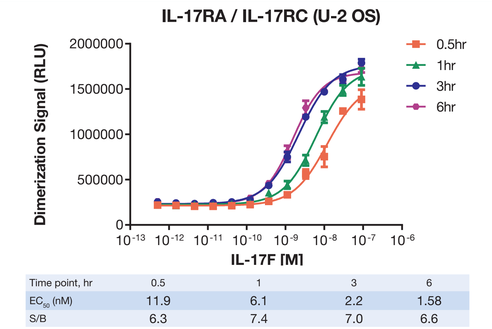
Time point, hr	1	3	6
EC_{50} (pM)	316	385	259
S/B	1.9	4.3	6.1

The IL-5 receptor is a heteromeric class I cytokine receptor comprised of the cytokine recognition subunit IL-5RA and the common cytokine b-chain, also known as CSF2RB, which is required for signal transduction. U-2 OS cells stably expressing full length receptors fused to either the PK or the EA EFC tag were plated 24hr prior to stimulation with increasing concentrations of IL-5 for 1, 3 or 6hr. The optimal condition of 6h yields an EC_{50} in good agreement with that of IL-5 in other cell-based functional assays.

Cytokine receptor heterodimerization: IL-17RA/RC



Time point, hr	0.5	1	3	6
EC_{50} (nM)	4.2	2.57	1.49	0.40
S/B	7.6	8.3	6.8	6.2



Time point, hr	0.5	1	3	6
EC_{50} (nM)	11.9	6.1	2.2	1.58
S/B	6.3	7.4	7.0	6.6

The IL-17 cytokine family is produced by a subset of CD4+ T helper (Th) cells known as "Th17" cells. Th17 effector function results from the IL-17 cytokine / IL-17 receptor signaling axis, which is initiated by binding of specific members of the IL-17 cytokine family to a specific cell surface receptor complex. The best characterized of these is the heterodimeric IL-17RA/RC complex, which is activated by homodimeric IL-17A and IL-17F or the heterodimeric IL-17A/F. Shown above, U-2 OS cells stably expressing the full length IL-17RA fused to PK and IL-17RC fused to EA stimulated with either IL-17A or IL-17F produce robust dimerization. Intriguingly, the complex appears to become more stable with increasing incubation time with both ligands, a phenomenon not observed for any of the other receptor dimers tested thus far.

Summary & Conclusions

We present here a novel cell-based assay platform for the quantitation of ligand-induced receptor dimerization utilizing our well established Enzyme Fragment Complementation system. We demonstrate that the technology harnesses the native biology of receptor dimerization and is applicable across diverse receptor types. Further, we have shown that the pharmacology of ligand binding is consistent with reports in the literature. Intriguingly, our success with receptors widely reported to exist in pre-formed complexes (eg. EpoR; based on biophysical studies) suggests that other pre-formed receptor complexes may also be accessible as assay targets. This platform represents a unique tool to address a wide variety of applications for the development of biologics, including mechanism of action studies, QC lot release and neutralizing anti-drug antibody assays as well as bispecific antibodies. These robust assays have also been used to identify small molecules that block ligand-receptor interactions.

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