Decrypting EGFR signaling with BRET biosensors: A novel approach to study RTK mutations and the effects of inhibitors

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Receptor Tyrosine Kinases (RTKs) bind to a variety of signaling molecules and regulate many critical processes such as cell growth, differentiation and survival. Dysregulated RTK activity can affect many cellular functions and lead to the development of cancer, making RTKs prime targets for new anti-cancer agents. However, various somatic and acquired RTK mutations have been shown to confer resistance to RTK-targeting drugs and thus limit their therapeutic efficacy. Indeed, RTK mutations may alter receptor activity or prevent drug binding.

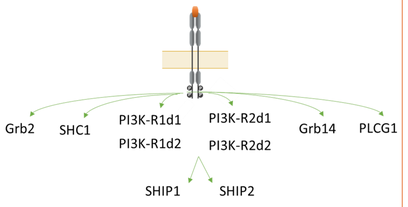
Additionally, mutations may act via more complex and understudied mechanisms (i.e., alteration of receptor subcellular localization, dimerization, signaling bias, and signaling / trafficking kinetics). The development of tools providing new insight into these complex mechanisms is crucial to better understanding RTK biology and to developing more effective RTK-targeting drugs.

We present herein a live-cell BRET-based biosensor platform allowing for real-time spatiotemporal monitoring of RTK signaling across 10 effector proteins/pathways.

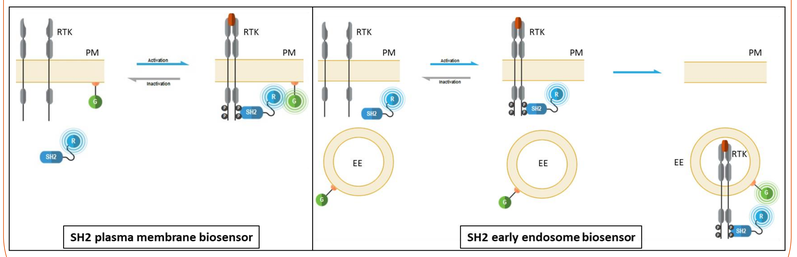
Using the naturally interacting chromophores Renilla luciferase (RLuc) and Renilla green fluorescent protein (rGFP), we developed biosensors that allow for quantitative live-cell monitoring of RTK-mediated and -specific SH2 domain-containing protein trafficking to the plasma membrane and early endosomes following receptor activation.

We use EGFR as a model receptor to demonstrate the spectrum of applications of our biosensor platform in studying RTK biology and pharmacology.

SH2–domain biosensors



bioSensAll™ RTK assays principle



: RlucII : rGFP : phosphorylation sites : ligand PM: plasma membrane EE: early endosomes

Conclusion : technology advantages

This study highlights the applicability of our biosensor platform to perform in depth characterization of RTK biology and pharmacology. The bioSensAll™ RTK platform represents a tool for the development and characterization of novel TKIs, effective against various mutations involved in drug resistance.

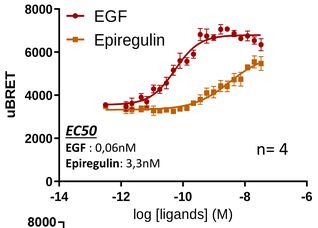
Capacity to:

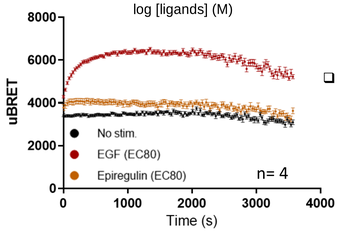
- Identify signaling and trafficking bias
- Analyze trafficking to cellular compartments
- Detect mutation-induced variations in the signaling of RTKs
- Study untagged receptors
- Adapt assays to a HTS compatible format
- Characterize small molecules and biologics-based therapeutics

Characterization of EGFR

Plasma membrane

Figure 1: Profiling of EGFR ligands – recruitment of **PLCG1**
Dose response curves and real-time kinetics upon EGF and Epiregulin stimulation

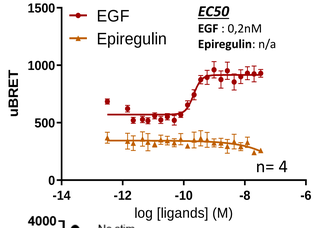


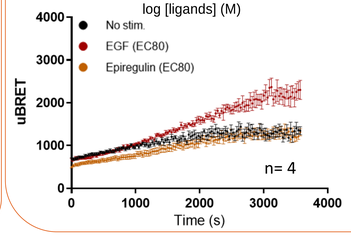


- Compared to EGF, Epiregulin is less potent in promoting the recruitment of PLCG1 effector at the plasma membrane.
- EGF-induced PLCG1 effector recruitment was more efficacious compared to Epiregulin, but Epiregulin-induced PLCG1 effector recruitment displayed faster kinetics relative to that observed with EGF.
- **The RTK biosensor platform enables to discriminate the effects of different agonists and to assess signaling kinetics on timescales ranging from milliseconds to hours.**

Endosomes

Figure 2: Ligand-biased trafficking of the recruitment of **PLCG1** to EGFR
Dose response curves and real-time kinetics upon EGF and Epiregulin stimulation

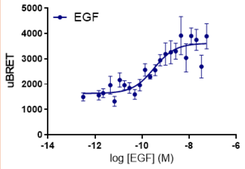


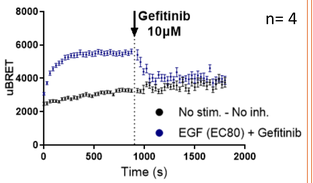


- EGF, but not Epiregulin, promoted a gradual time-dependent increase in PLCG1 levels in the early endosome compartment.
- **The RTK biosensor platform permits to clearly depict the unique signaling signature induced by different EGFR ligands.**

Endogenous EGFR signaling

Figure 3: Endogenous measurement of EGFR signaling & Kinetics of inhibition by Gefitinib in **A431 cells** – recruitment of **Grb2**

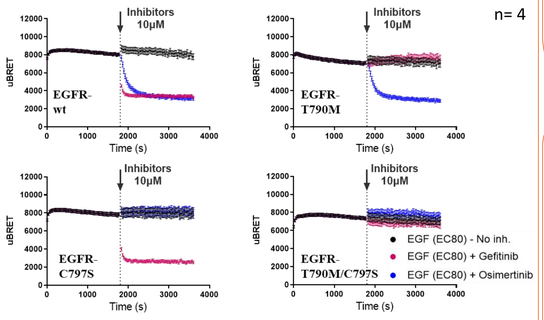




- The recruitment of Grb2 effector to the plasma membrane was measured following stimulation of endogenous EGFR with EGF.
- 1st generation Tyrosine Kinase Inhibitor (TKI) Gefitinib (Iressa™) rapidly (within 120s) reverses the activity of endogenous EGFR following stimulation with EGF.
- **These data demonstrate the ability of our biosensors to detect signaling from endogenously expressed RTKs, thus highlighting the capacity of the RTK platform to be transposed to different, pathophysiologically-relevant cellular models.**

Real-time characterization of Tyrosine Kinase Inhibitors on EGFR mutants

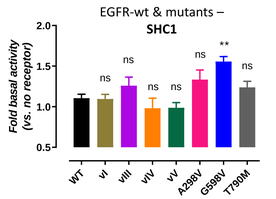
Figure 4: Kinetics of inhibition by Gefitinib & Osimertinib on EGFR-T790M, -C797S and -T790M/C797S mutants – recruitment of **PLCG1**

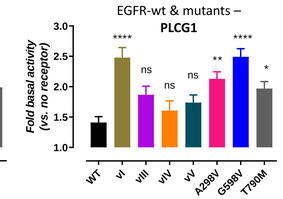


- 1st generation TKI Gefitinib (Iressa™) reverses the activity of EGFR-wt and -C797S, but this TKI is ineffective on -T790M, at the level of the plasma membrane.
- 2nd generation TKI Osimertinib (Tagrisso™) retains activity on EGFR-wt and -T790M, but not on -C797S mutant.
- Both TKIs are ineffective on the -T790M / C797S double mutant.
- **The RTK biosensor platform can be exploited as a tool to develop next generation of TKIs effective against different RTK variants.**

Constitutive activity of EGFR mutations

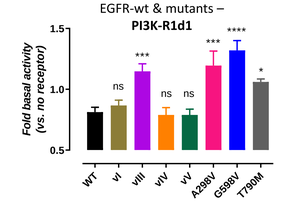
Figure 5: Analysis of constitutive activity of EGFR mutations on 3 pathways : MAPK (**SHC1**), PKC (**PLCG1**) and Akt (**PI3K-R1d1**) at the **plasma membrane**





n= 4 - 6

One way ANOVA
ns : P > 0.05
• : P ≤ 0.05
** : P ≤ 0.01
*** : P ≤ 0.001
**** : P ≤ 0.0001



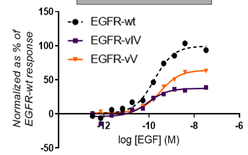
	SHC1	PLCG1	PI3K-R1d1	Constitutive activity:
vI	-	✓	-	biased
vIII	-	-	✓	
vIV	-	-	-	devoid
vV	-	-	-	
A289V	-	✓	✓	on > 2 pathways
G598V	✓	✓	✓	
T790M	-	✓	✓	

- The outcome of diverse EGFR mutations on receptor constitutive activity were determined for MAPK, PKC and Akt pathways :
  - Point mutations found in glioblastoma (EGFR-A289V and -G598V) → constitutive activity on multiple pathways;
  - Intra-kinase deletion (EGFR-vIV and -vV) → no constitutive activity;
  - Binding site deletion (EGFR-vI and –vIII) → pathway specific constitutive activity.
- **The RTK biosensor platform allows to differentiate the effects of various mutations on RTK signaling.**

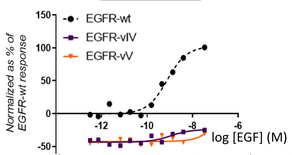
Trafficking of EGFR truncations

Figure 6: Dose response curves on EGFR-vIV and –vV: EGF activation of **PLCG1**

Plasma membrane



Endosomes



- EGFR-vIV and -vV mutants had a reduced capacity to internalize in comparison to EGFR-wt, despite being able to recruit PLCG1 at the plasma membrane.
- This observation may be linked to the absence of **Y1045** in those EGFR mutants (c-CBL phosphorylation site involved in receptor ubiquitination and internalization).
- **The RTK biosensor platform can identify mutants impacting receptor trafficking from the plasma membrane to the endosomes and may help unmask mutation-induced mechanisms of drug resistance and oncogenesis.**