EGFR signaling and pharmacology in oncology revealed with an innovative RTK biosensor technology

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Introduction

Receptor Tyrosine Kinases (RTKs) regulate many critical biological processes such as cell growth, differentiation, and survival through the recruitment of intracellular signaling molecules. The binding of growth factors to RTKs promotes their dimerization and leads to the recruitment of intracellular signaling proteins containing Src homology-2 (SH2) domains, which then engage downstream effectors involved in initiating multiple cellular signaling events.

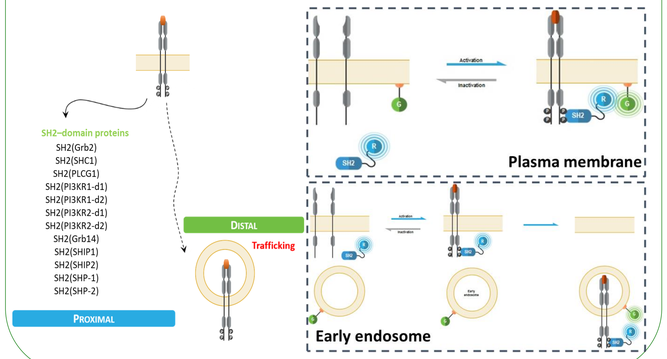
Dysregulated RTK activity aberrantly affects many cellular functions, often culminating in cancer. Consequently, RTKs serve as prime targets for new anti-cancer agents. However, the efficacy of several current drugs is limited by the development of drug-induced adverse events. Furthermore, the emergence of somatic and acquired RTK mutations allows tumors to develop resistance to certain drugs which may alter receptor activity and/or prevent drug binding through various mechanisms (i.e., alteration of receptor subcellular localization, signaling/trafficking, and kinetics signaling bias). Development of tools providing new insight into RTK complex mechanisms is crucial to develop more effective RTK-targeting drugs.

The RTK bioSensAll™ platform is a quantitative, live-cell, enhanced bystander bioluminescence resonance energy transfer (ebBRET)-based biosensor platform, that allows for real-time mapping and monitoring of the signal transduction pathways engaged upon activation of unmodified receptors.

Unlike conventional BRET assays, ebBRET exploits the ability of luciferase from *Renilla luciferase* (RlucII) and green fluorescent protein from *Renilla reniformis* (rGFP) to self-associate with mild affinity and to optimally transfer energy, resulting in enhanced assay windows and sensitivity. Consequently, these naturally interacting chromophores were exploited to develop new, highly dynamic ebBRET-based trafficking sensors designed to monitor the trafficking of various SH2-domain containing proteins, specifically interacting with ligand-activated RTKs. Events can be detected at both the plasma membrane and early endosomes compartments.

We have developed biosensors that monitor the activation of 12 distinct SH2-domain containing proteins (Grb2, SHC1, PLCG1, PI3KR1-d1, PI3KR1-d2, PI3KR2-d1, PI3KR2-d2, Grb14, SHIP1, SHIP2, SHP-1, SHP-2). The proximal biosensors forming the basis of the RTK biosensor platform contain a specific SH2 domain of the previously named proteins fused to RlucII. The recruitment of biosensors to the plasma membrane upon RTK activation translates into an increased BRET efficiency with a plasma membrane-anchored rGFP. The same translocation principle is used to measure the internalization of RTKs with an early endosome-anchored rGFP.

Using EGFR as a model receptor, we demonstrate here the applicability of our biosensor platform to perform in-depth characterization of RTK biology and pharmacology.



Characterization of EGFR

SH2(PLCG1)

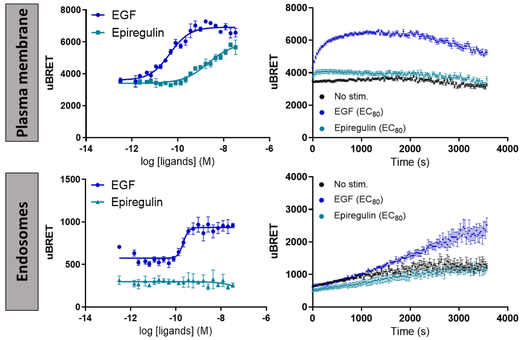


Figure 1: Profiling of EGFR ligands at two different cellular compartments – Dose-responses and real-time kinetics. EGF-induced SH2(PLCG1) effector recruitment to the plasma membrane was more potent and efficacious compared to Epiregulin, but Epiregulin-induced SH2(PLCG1) effector recruitment displayed faster kinetics relative to that observed with EGF. Furthermore, EGF, but not Epiregulin, promoted a gradual time-dependent increase in SH2(PLCG1) levels in the early endosome compartment. **The RTK biosensor platform enables to discriminate the effects of different agonists and assess signaling kinetics on timescales ranging from milliseconds to hours, as well as to clearly depict the unique signaling signature induced by different EGFR ligands.**

EGFR endogenous signaling – A431

SH2(Grb2)

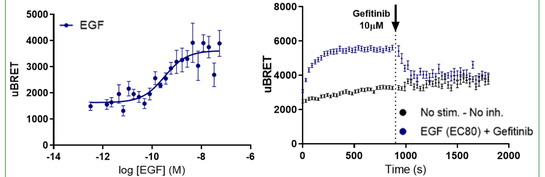


Figure 2: Endogenous measurement of EGFR signaling and real-time kinetics at the plasma membrane. The recruitment of SH2(Grb2) effector to the plasma membrane was measured following stimulation of endogenous EGFR with EGF. 1st generation Tyrosine Kinase Inhibitor (TKI) Gefitinib (Iressa™) rapidly (within 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.**

BRET-based imaging of EGFR

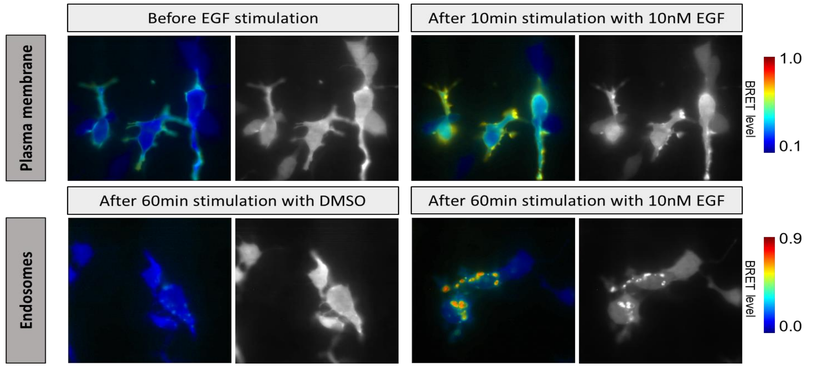
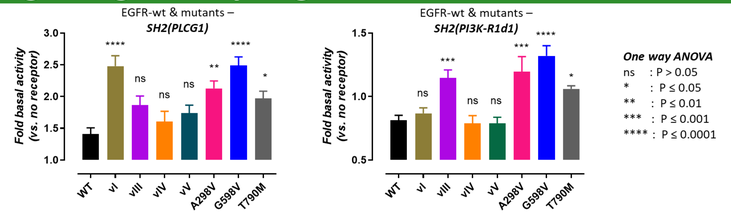


Figure 3: BRET-based imaging of the recruitment of SH2(Grb2) biosensor in two different cellular compartments. These BRET imaging results visually support the data described earlier, at the plasma membrane and at the early endosomes, and further confirmed the proper localization of the measured BRET signals. **These observations highlight the applicability of the biosensor platform to microscopy.**

Signaling activity of glioblastoma EGFR mutants



	SH2(PLCG1)	SH2(PI3K-R1d1)	Constitutive activity:
vI	✓	-	biased
vIII	-	✓	
vIV	-	-	devoid
vV	-	-	
A289V	✓	✓	on 2 pathways
G598V	✓	✓	
T790M	✓	✓	

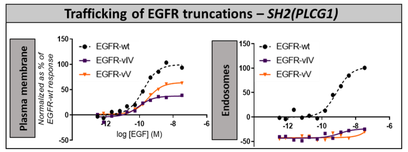


Figure 4: Analysis of constitutive activities of EGFR mutations at the plasma membrane and trafficking of EGFR truncations in two different cellular compartments. The outcome of diverse EGFR mutations on receptor constitutive activity were determined for PKC and Akt pathways. EGFR-vIV and -vV mutants demonstrated no constitutive activity on both pathways and had a reduced capacity to internalize in response to EGF compared to EGFR-wt, despite being able to recruit SH2(PLCG1) at the plasma membrane. **The RTK biosensor platform allows to differentiate the effects of various mutations on RTK signaling and 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.**

Activity of TKI on EGFR mutants

SH2(PLCG1)

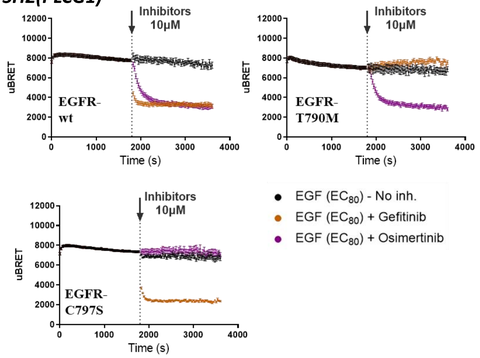


Figure 5: Real-time kinetics of inhibition by Gefitinib & Osimertinib on EGFR-wt and EGFR-T790M and -C797S mutants. 1st generation TKI Gefitinib (Iressa™) reverses the activity of EGFR-wt and -C797S, but this TKI is ineffective on -T790M, at the level of the plasma membrane. 2nd generation TKI Osimertinib (Tagrisso™) retains activity on EGFR-wt and -T790M, but not on -C797S mutant. **The RTK biosensor platform can be exploited as a tool to develop next generation of TKIs effective against different RTK variants.**

Conclusion

The ebBRET-based biosensor technology displayed new insights in RTK biology and revealed different modes of action of ligands and inhibitors, extensive RTK signal profiling of EGFR mutants, and trafficking of RTK effectors. The technology represents a powerful tool for the analysis of RTK mutations and for the identification of next generation TKIs and antibodies directed against RTKs.

The RTK platform presents qualitative and quantitative functional readouts using the SH2-domain containing biosensors that allow real-time spatiotemporal monitoring of ligand activity across various effector proteins/pathways. Furthermore, these biosensors are applicable for the identification of biased signaling in response to different ligands. Such technology enabled us to identify unique signaling signatures triggered by different RTK ligands using unmodified receptors. Moreover, the RTK biosensor platform allowed us to differentiate the impact of different TKIs on various RTK mutations highlighting the platform's capacity to be exploited as a tool to develop next generation TKIs effective against RTK variants.

The bioSensAll™ platform offers a unique combination of RTK signal transduction profiling capability, is HTS compatible and allows real-time kinetic measurements across multiple effector pathways to better understand RTK complex biology. This deeper level of understanding will enable the identification of safer and more effective RTK-targeting drugs against various mutations identified in different cancers.