A high value pharmacological platform dedicated to the real time study of Stimulatory immune checkpoint signaling pathways

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INTRODUCTION

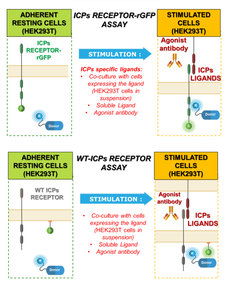
- **bioSensAll™** : a Domain Therapeutics powerful and proprietary pharmacology BRET-based platform that enables the analysis and monitoring in real-time of signal transduction downstream of GPCRs and RTKs activation (1).
- As an Immuno and Oncology focused Biotech we are engaged in the creation and development of an innovative pharmacological **BRET platform dedicated to either Inhibitory or Stimulatory Immune Checkpoint (ICP).**
  - **Inhibitory ICPs (i.e. PD-1 or CTLA-4):** front-runner targets in immuno-oncology field (2) lead to immune cells exhaustion and the blockade of the anti-tumor response.
  - **Stimulatory ICPS (i.e. 4-1BB):** promotes immune response and tumor clearance (3).
- Blocking strategies targeting the PD-1/PD-L1 or CTLA-4 axis, or stimulating 4-1BB pathway are:
  - reliable and very promising therapies to strike cancer.
  - more and more molecules such as blocking/agonist antibodies (Abs) or small molecule entities (SMEs) are in development. Some of them are now in Clinical trial or drug market.

Drug Discovery : new robust and reliable cell-based assays to screen and characterize these therapies are urgently needed.

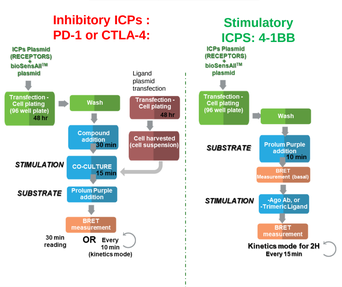
METHODS

BRET ASSAY DEV	INHIBITORY ICPs: PD-1/PD-L1- PD-L2, CTLA-4/CD80-CD86	STIMULATORY ICP: 4-1BB/4/1BB Ligand
Type of ICPs receptors	Wild type forms of ICP receptors, or fused with rGFP	ICP receptors fused with rGFP
Stimulation strategies	Co-culture with cells expressing transiently PD-L1/PD-L2 or CD80-CD86 to activate effector cells. Co-culture with cells PD-L1+ stable in cancer cells (U2OS) or in HEK293T cell lines.	Stimulation with 4-1BB trimeric ligand, agonist antibody or co-culture with cells expressing 4-1BB ligand.
Assays validation	Inhibitory activity of know ICPs inhibitors (SMEs or blocking antibodies.)	Antagonist activity of an antagonist antibody (Clone BBK-2)
Miniaturisation	PD-1-rGFP assays miniaturised in 384 format	

BRET Assays – PRINCIPLE



BRET Assays - WORKFLOW



OBJECTIVES

The objectives is to generate HTS and SAR compatible assays dedicated to:

- **Inhibitory/Stimulatory ICPs** :
  - Monitor the activation of PD-1, CTLA-4 or 4-1BB
  - Assess pharmacological profiles of ICPs inhibitors (PD-1/PD-L1, and CTLA-4/CD80-CD86) or Agonists (4-1BB/4-1BB Ligand)
  - Miniaturize in 384 format

Cell-based assays contribute to reveal the power and activity of ICP agonists or antagonists and can be used to characterize new developed molecules and contribute to the success of new immune therapies.

Activation of PD-1 or CTLA-4 pathways in BRET assays

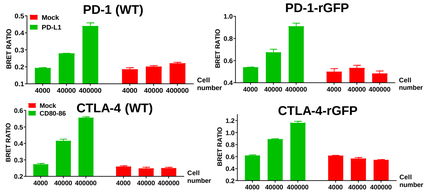


Figure 1. PD-1 and CTLA-4 BRET assays respond to ligand stimulation.

For both targets and both assays, a BRET ratio is observed in presence of ligand expressing cells. This activation of the pathway is dependent of the ligand quantity.

Monitoring of pathways activation in real time (PD-1, CTLA-4)

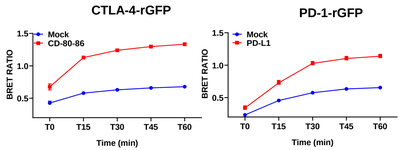


Figure 2. Kinetic of the pathway activation in PD-1 and CTLA-4 assays.
The kinetic activation of PD-1 and CTLA-4 pathways can be monitored in real-time. The maximal BRET signal is reached at 1H of incubation.

PD-1 and CTLA-4 assays have been assessed and validated in both –rGFP fused or in the WT receptor version

CTLA-4 BRET assays validation using CTLA-4 blocking antibodies

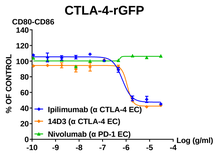
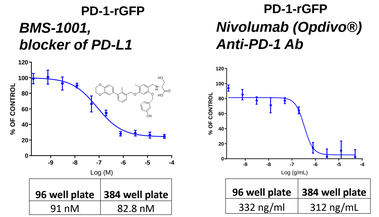


Figure 3. Pharmacology profile of CTLA-4 neutralizing antibodies into CTLA-4 assays.
The CTLA-4-rGFP assay highlight the inhibitory effect of either Ipilimumab or clone 14D3 antibodies. As a specific control, Nivolumab doesn't inhibit CTLA-4 pathway.

Miniaturization and Validation of the PD-1 BRET assays 384 format



96 well plate	384 well plate
91 nM	82.8 nM

96 well plate	384 well plate
332 ng/ml	312 ng/mL

Figure 4. Pharmacology profile of PD-1/PD-L1 inhibitors into PD-1-rGFP assay miniaturized (384 format.)
PD-1-rGFP assay was miniaturized in 384 format and is able to reveal the inhibitory capacity of SMEs or Antibody described to block PD-1 pathway: BMS-1001, and Nivolumab respectively.
IC50 are similar compared to 96 format assay

Activation of the 4-1BB pathway in BRET assay

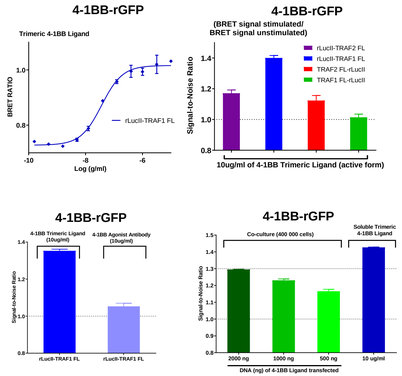


Figure 5. Determination of the best conditions to activate 4-1BB-rGFP assay .

Among 4 rLucII fused 4-1BB effectors, the rLucII-TRAF1 biosensors was optimal to activate the 4-1BB-rGFP assay (stimulated with 10ug/ml, or a CRC of Trimeric 4-1BB ligand)

Stimulation using 4-1BB Trimeric Ligand (active form) is the optimum way to activate the 4-1BB pathway. The use of 4-1BB agonist antibody or a co-culture of cell expressing 4-1BB don't improve the Signal to Noise

Real time monitoring of 4-1BB pathway activation

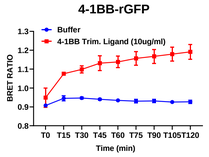


Figure 6. Activation of 4-1BB pathway.
The assay dedicated to 4-1BB allows to monitor the activation of the pathway in real time.

Stimulatory ICPs assays for 4-1BB, validation with mutant

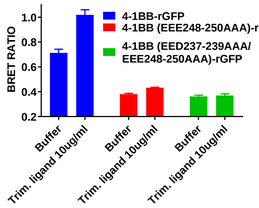


Figure 7. The 4-1BB mutant versions lacking either one or two binding domains to TRAF1 inhibit the BRET signal into 4-1BB-rGFP assay.
As previously characterized (5), the EEE248-250AAA and the EED237-239AAA/EEE248-250AAA mutations are not able to recruit TRAF1 anymore. This is perfectly translated into our 4-1BB-rGFP assay. A complete loss of BRET signal is observed

4-1BB BRET assay validation using blocking 4-1BB antibody

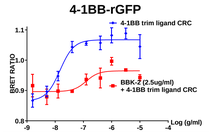


Figure 8. Pharmacology profile of blocking antibody (Clone BBK-2) into 4-1BB-rGFP assay.
A fixed dose of the antagonist antibody (clone BBK-2) is able to block the 4-1BB signalization. The BRET ratio in the highest dose of BBK-2 is dramatically reduced.

CONCLUSION

bioSensAll™ is a powerful pharmacological platform:

- A **versatile pharmacological platform** able to be highly adaptable for hot topic targets such as **Immune Checkpoint targets (Inhibitory and Stimulatory ICPs):**
  - Assays are able to **reveal the Stimulatory or Inhibitory activities of therapeutic molecules (SMEs, mAbs, soluble ligands)**
- **Adaptably of the platform across several biosensors,**
- Capable to **monitor signaling pathways in real time kinetics for inhibitory ICPs, and will be assessed for Stimulatory ICPs,**
- Can be companion tests to enhance drug discovery and also can be used as **quality control/batch release testing** for therapeutic molecules.

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