NanoBRET assays to assess cellular target engagement of compounds

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1. Rationale/Aim

The NanoBRET method is used for monitoring small molecule-binding to a target in cellular setting using an nLuc-tagged intracellular protein of interest and a cell-permeable fluorescent dye-ligand conjugate. Binding of small molecules of interest to a target in cells is monitored through the displacement of the fluorescent ligand. This method allows quantitative assessment of the interaction between small molecule inhibitors and their targets in cells, providing valuable information essentially the on-target cellular efficacies of the inhibitors. (Robers et al., 2015, DOI: 10.1038/ncomms10091; Vasta et al., 2018, DOI: 10.1016/j.chembiol.2017.10.010).

2. Experimental conditions

2.1 Key Requirement:

Preparation of target DNA linked N-terminal or C-terminal to nLuc (luciferase)

Plasmids carrying an insert DNA encoding gene for full-length protein, obtained from Promega, are prepared using a MidiPrep Kit (Qiagen) and are stored at a concentration of 200 ng/µl in TE buffer (TRIS, EDTA) at 4°C.

Transfection and recombinant expression of protein in HEK293T cells

The plasmid DNA (final concentration of 1 ng/µl) is mixed with transfection carrier DNA (final concentration 9 ng/µl; Promega) in OptiMEM prior to an addition of the transfection reagent FuGene HD (Promega) at a volume ratio of 1:30. The mixture is incubated for 20 min at RT to allow the formation of DNA-vesicles. The mixture is then added to the cells at $2x10^5$ cells/mL in DMEM +10% FBS and 1% PenStrep at a volume ratio of 1:20. Cells are cultured for 18h to allow expression the protein target prior to harvesting.

Addition of test compound (titration)

Titration of test compound typically in a range of 20 µM – 1 nM using three input concentrations (10 mM, 100 µM, 1.667 µM) with the Echo Accoustic dispenser into a Greiner 781 207 white assay plate. Total volume is 40 µl. Maximal volume of compound is 100 nl, and hence the total added DMSO concentration is 0.25%.

Addition of tracer molecule

A suitable tracer molecule is added to the plate in a concentration determined by its individual $K_{D,app}$ that has been determined via a previous tracer titration experiment. Maximal final tracer concentration is 1 µM because of solubility problems of the tracer molecules and subsequent cell toxicity. Maximal volume of tracer added is therefore 100 nl, and hence the total added DMSO concentration is 0.25%. Using the tracer at its individual $K_{D,app}$ makes assays comparable and resulting test compound IC_{50}s would not be shifted more than 2-fold according to the Cheng-Prusoff-Relationship.

Addition of cells expressing the target of interest

Cells are treated with trypsin, resuspended in DMEM and subsequent harvested by centrifugation at 200 x*g* for 5 min. DMEM is removed and cells are then resuspended in OptiMEM at a concentration of $2x10^5$ cells/ml. 38 µL of cells are added to the assay plate using Multidrop Dispenser (standard tube cassette, Thermo). This makes up to a final volume of 40 µl. The assay plate is equilibrated for 2 h at 37 °C with 5 % CO_2 prior to assay readout.

Addition of substrate/extracellular nLuc inhibitor and readout

20 µl of substrate solution is added to the assay plate including 1:500 (v/v) Extracellular nLuc inhibitor (Promega) and 1:166 (v/v) Substrate (Promega) in OptiMEM. The assay plate is read with a PheraSTAR Platereader (BMG Labtech) within 20 min at 450 nm (Emission nLuc luminescence) and 610 nm (Emission BRET to tracer BODIPY fluorophore) and the BRET ratio is calculated as Em620/Em450.

Protocol Step	Reagent	Volume per Well [µl]	Stock concentration	Final concentration
Transfection Mix	nLuc-tagged DNA	0.2	200 ng/µl	1 ng/µl
	Transfection Carrier DNA	0.36	1000 ng/µl	9 ng/µl
	FuGene HD	0.066	100%	3%
	OptiMEM	1.374	100%	~100%
Transient transfection of HEK293T cells	HEK293T	38	$2x10^5$ cells/ml	~ $2x10^5$ cells/ml =7500 cells/well
	Transfection Mix	2	20x	1x
Compound Titration	Test Compound	Max 0.1	10 mM, 0.1 mM and 0.0001667 mM	20 µM – 1 nM (11-point)
Tracer addition	Tracer Molecule K3, K4, K5, K8, K9, K10, K11, K12, K14, K16, 7297, 7394	Max 0.1	400 µM	Concentration at IC50 determined

				previously, max. 1 µM
Substrate addition	Furimazine Substrate	0.12	100%	0.6%
	Extracellular nLuc inhibitor	0.04	100%	0.2%

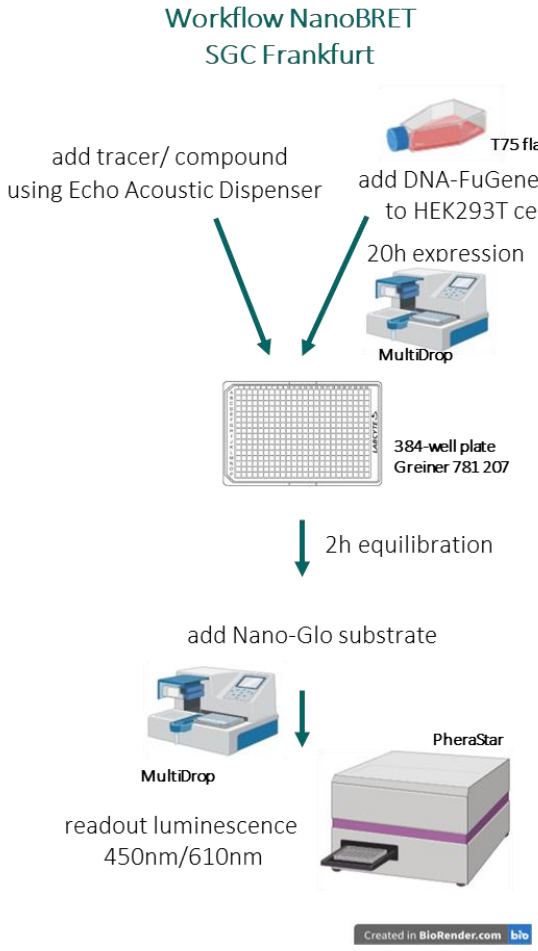
2.2 Key resources table:

Reagent	***Supplier***	***Code***
HEK293T cells	ATCC	CRL-3216
384 well plates, white	Greiner	781 207
DNA-nLuc construct	Promega	Custom[§]
Transfection Carrier DNA	Promega	E4881
FuGene HD	Promega	E2312
NanoBRET-Glo substrate	Promega	N2161
Extracellular NanoBRET inhibitor	Promega	N2161
Tracer K3	Promega	N2600
Tracer K4	Promega	N2520
Tracer K5	Promega	N2500
Tracer K8	Promega	N2620
Tracer K9	Promega	N2630
Tracer K10	Promega	N2640
Tracer K11	Promega	N2650
Opti-MEM (w/o phenol red)	Gibco, Life Technologies	11058-021
DMEM	Gibco, Life Technologies	41966-029
PenStrep	Gibco, Life Technologies	15140-122
Trypsin-EDTA	Gibco, Life Technologies	15400-054
FBS	Gibco, Life Technologies	A4766801
DBPS	Gibco, Life Technologies	14190-094

[§] see https://www.promega.de/resources/guides/kinase-target-engagement-assay-selection-table/#sort=%40fkinasez32xname41824%20ascending

3. Protocol

3.1 Workflow



3.2 Protocol

The assay is performed as described in (Robers et al., 2015, DOI: 10.1038/ncomms10091; Vasta et al., 2018, DOI: 10.1016/j.chembiol.2017.10.010) and the manual provided by the manufacturer (Promega) https://www.promega.de/-/media/files/resources/protocols/technical-manuals/500/nanobret-target-engagement-intracellular-kinase-assay-nonbinding-surface-format-protocol.pdf?la=en .

1. Full-length kinases were obtained as plasmids cloned in frame with a terminal NanoLuc-fusion (Promega).
2. Plasmids were transfected into HEK293T cells using FuGene HD (Promega, E2312) and proteins were allowed to express for 20h.
3. Serially diluted inhibitor and NanoBRET Kinase Tracer K10 (Promega) at a concentration determined previously as the Tracer K10 $K_{D,app}$ were pipetted into white 384-well plates (Greiner 781207) using an Echo acoustic dispenser (Labcyte).
4. The corresponding protein-transfected cells were added using a Multidrop Dispenser (Thermo) and reseeded at a density of 2 x 10^5 cells/mL after trypsinization and resuspending in Opti-MEM without phenol red (Life Technologies).
5. The system was allowed to equilibrate for 2 hours at 37°C/5% CO_2 prior to BRET measurements.
6. To measure BRET, NanoBRET NanoGlo Substrate and Extracellular NanoLuc Inhibitor (Promega) was added as per the manufacturer's protocol, and filtered luminescence was measured on a PHERAstar plate reader (BMG Labtech) equipped with a luminescence filter pair (450 nm BP filter (donor) and 610 nm LP filter (acceptor)).
7. Competitive displacement data were then graphed using GraphPad Prism 8 software using a normalized 3-parameter curve fit with the following equation: Y=100/(1+10^(X-LogIC50))