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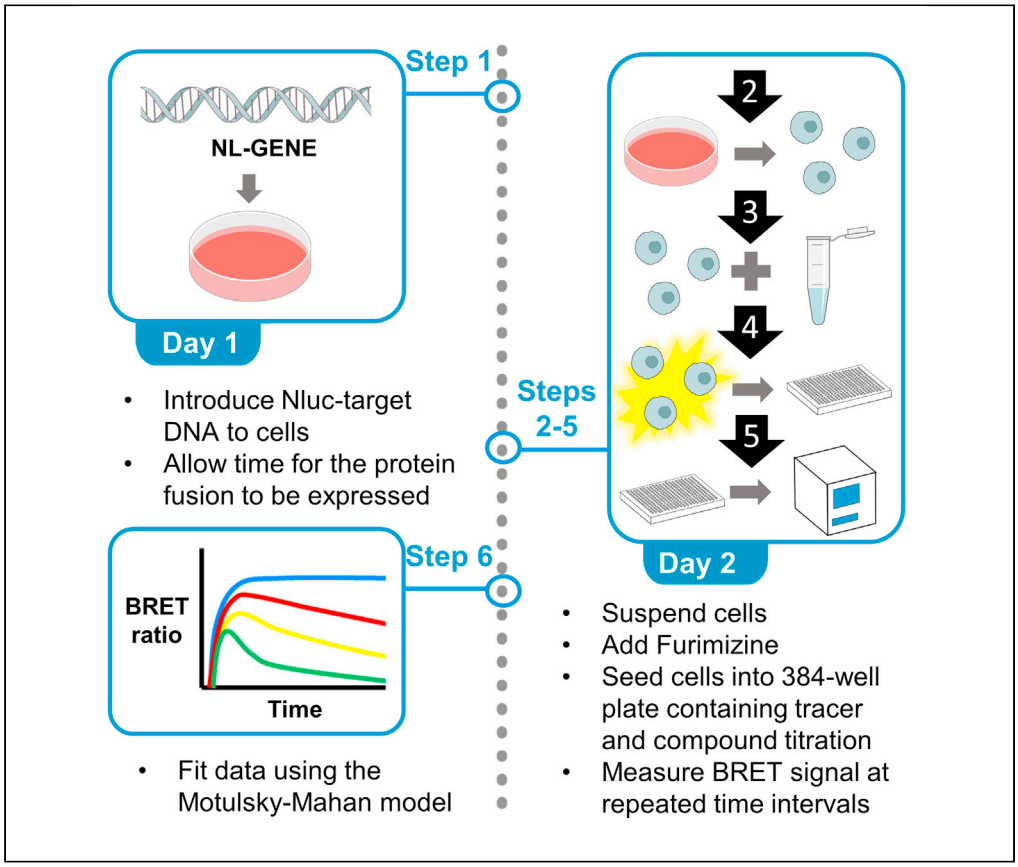
Protocol

A kinetic intra-cellular assay (KICA) to measure quantitative compound binding kinetics within living cells



The Kinetic Intra-Cellular Assay (KICA) is a recombinant cell-based technique that utilizes NanoBRET technology. KICA enables the measurement of intracellular binding kinetics. This protocol describes steps for cellular transfection and expression, followed by addition of a target specific fluorophore conjugated probe and a range of concentrations of competitor compounds, followed by the measurement of BRET in a 384 well format. Fitting the BRET data allows measurement of forward and reverse binding rates and the determination of K_D.

Charles S. Lay, Daniel A. Thomas, John P. Evans, Emma J. Jones, Kelly M. Gatfield, Peter D. Craggs

peter.d.craggs@gsk.com

Highlights

The step-by-step protocol for the Kinetic Intra-Cellular Assay (KICA) is outlined

KICA is a recombinant cellular technique that utilizes NanoBRET technology

Protocol enables the measurement of intra-cellular compound binding kinetics

The KICA technique is quantitative, scalable, and reproducible

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A kinetic intra-cellular assay (KICA) to measure quantitative compound binding kinetics within living cells

Charles S. Lay,[1,2,3] Daniel A. Thomas,[3,4] John P. Evans,[3] Emma J. Jones,[5] Kelly M. Gatfield,[3] and Peter D. Craggs[3,6,7,8,*]

[1]Division of Physiology, Pharmacology and Neuroscience, School of Life Sciences, University of Nottingham, Nottingham NG7 2UH, UK

[2]Centre of Membrane Proteins and Receptors, University of Birmingham and Nottingham, The Midlands, UK

[3]Medicine Design, Medicinal Science and Technology, GlaxoSmithKline, Stevenage SG1 2NY, UK

[4]Arctoris, Oxford OX14 4SA, UK

[5]Protein and Cellular Sciences, Medicinal Science and Technology, GlaxoSmithKline, Stevenage SG1 2NY, UK

[6]GSK-Francis Crick Institute Linklabs, Medicinal Science and Technology, GlaxoSmithKline, Stevenage SG1 2NY, UK

[7]Technical contact

[8]Lead contact

*Correspondence: peter.d.craggs@gsk.com
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SUMMARY

The Kinetic Intra-Cellular Assay (KICA) is a recombinant cell-based technique that utilizes NanoBRET technology. KICA enables the measurement of intracellular binding kinetics. This protocol describes steps for cellular transfection and expression, followed by addition of a target specific fluorophore conjugated probe and a range of concentrations of competitor compounds, followed by the measurement of BRET in a 384 well format. Fitting the BRET data allows measurement of forward and reverse binding rates and the determination of K_D. For complete details on the use and execution of this profile, please refer to Lay et al. (2021).

BEFORE YOU BEGIN

Generation and characterization of reagents

After the KICA experimental approach has been selected, reagents must be procured and characterized. The two key reagents required are the expression construct for the NanoLuciferase (NL) fused macromolecule (hereon referred to as the target) and a fluorescently labeled probe molecule for the target of interest (hereon referred to as the tracer). There are many ways to introduce NL-target DNA into the chosen cell line, including viral transduction, stable transfection and CRISPR-Cas9 knock-in. The protocol below will detail how to introduce NL-BRD4 expression plasmids into HEK293 cells via transient transfection, however an alternative CRISPR-Cas9 knock-in approach to insert a HiBit tag (part of a split NL) onto the *N*-terminus of BRD4 in HEK293 cells has also been successful for this target (Phillipou et al., 2019). It is advisable to test that the addition of the NL tag has not affected the expected localization of your protein, which can be done by immune-cytochemical or luminescence imaging.

Transient transfection of model cell lines such as HEK293s is often the quickest route to optimize an assay, however this requires repetitive transfection of cell lines prior to the experiment. To reduce reagent use or if working with a difficult to transfect cell line, a stable transfection can be carried out in which a transiently transfected cell population is exposed to antibiotic selection. Whilst this

may improve reproducibility, we chose not to carry out this step so that comparable experiments could be carried out on BRD4, 3, 2 and T using the same transiently transfected population of HEK293 cells. The creation of viral vectors for protein expression (e.g., BacMam) is a useful tool for introducing genes into difficult to transfect cell lines, however production of vectors is more complicated than that of expression plasmids which are commercially available for many targets including the BET proteins. CRISPR-Cas9 addition of HiBit followed by introduction of LgBit enables the use of physiological expression levels and can remove artifacts from overexpression (for example an affinity limit caused by titration of the target rather than the compound). However, the cell line used must express the target at a high enough level to enable the measurement of a luminescent signal. Overexpression artifacts can also be combated by using weaker promoters in expression vectors (for example, a Herpes Simplex Virus thymidine kinase promoter; Ali et al., 2018). For the BRD4 KICA developed in this study, a CMV promoter was utilized but overexpression was combated by using a low concentration of DNA in the transfection.

This protocol utilizes FuGENE HD mediated transient transfection of cells pre-seeded into 6 well microplates. Alternative transfection reagents (e.g., lipofectamine) and protocols (e.g., seeding and transfecting cells simultaneously) are available and optimization should be considered especially when using different cell lines or constructs to those outlined here.

Once a tracer has been selected for the target it is advisable to characterize the binding kinetics to ensure these are suitable for KICA. KICA tracers should be cell permeable and have rapid association and dissociation rates for the target. The binding kinetics of the tracer will determine the fastest test compound rates that can be monitored in the subsequent KICA. The accurate measurement of the forward and reverse rates of the tracer binding to the target are essential for the configuration of a KICA. An approach is presented in this protocol to determine these rates, using NL-BRD4 and the BET tracer BSP-590 as an example. A commercially available alternative to BSP-590 is available from Promega (cat. # N234A).

Culture of cells

HEK293 cells frozen in Fetal Bovine Serum (FBS) with 10% DMSO were slowly thawed before warm supplemented DMEM was added dropwise. The cells were then pelleted and the media replaced with fresh supplemented DMEM to remove any residual DMSO, cells were then added to tissue culture flasks and incubated at 37°C and 5% CO_2. Cells were passaged several times to enable full recovery from this process before beginning the assay. During maintenance of HEK293 cell lines, cells were grown in supplemented DMEM in T175 flasks and regularly split to avoid over-confluence.

On the experimental day

Ensure cell confluence is between 70%–90% before seeding for transfection. Prepare enough of each buffer and pre-warm to the correct temperature. Trypsin mix and PBS should be used at 18°C–25°C, supplemented DMEM should be warmed to 37°C prior to use and OptiMEM can be used at either 18°C–25°C (for transfections) or 37°C (for KICA experiments). It is advisable to practice kinetic assay steps to avoid unexpected delays after cells have been added to microplates.

Transient transfection of HEK293 cells with NL-BRD4

Timing: 4–6 h

This step facilitates the introduction of NL-Target DNA into HEK293 cells. There are several alternative strategies to accomplish this (see section above "before you begin").

1. Remove media from the flask containing HEK293 cells and wash cells with PBS to remove dead cells and excess FBS from the medium.
2. Add 3 mL of Trypsin solution to each T175 flask and incubate for 4 min.

3. Resuspend the cells in 10 mL of supplemented DMEM media to inactivate Trypsin, and homogenize the cells by aspiration.
4. Determine the cell concentration using a cell counter (a Vi-CELL XR Cell Viability Analyzer; Beckman-Coulter, was used) and adjust the concentration to 3×10^5 cells/mL.
5. Dispense cells into 6-well tissue culture microplates and incubate for 4–6 h.
6. Prepare a transfection mix with the below constituents (per well of the 6-well microplate) in the following order:
   a. OptiMEM without phenol red to a final volume of 100 μL minus the volume of DNA and FuGENE to be added (1:20 ratio with total well volume)
   b. 2.2 μg DNA (0.2 μg of NL-BRD4 Fusion Vector DNA and 2 μg of carrier DNA; 1:10 ratio)
   c. 8 μL FuGene HD transfection reagent (1:3.6 ratio to total DNA mass)
7. Mix this 10-times by inversion, or gentle pipetting and incubate for 15 min.
8. Add 100 μL of the transfection mix to each well of the 6 well tissue culture microplate containing
9. The pre-seeded HEK293 cells and incubate for 20–24 h to allow sufficient protein expression.

⚠ CRITICAL: Pipette FuGENE directly into the OptiMEM/DNA mix ensuring that the reagent does not touch the sides of the tube. In addition, make sure to use vessels made of polypropylene or glass (polystyrene should be avoided). These steps prevent FuGENE from sticking to the sides of the container, leading to a failed transfection.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals and recombinant proteins		
FuGENE HD	Promega Corporation	Cat. # E2311
Phosphate Buffered Saline (PBS)	Merck	Cat. # D8537
OptiMEM media	Gibco	Cat. # 11058021
Dulbecco's modified eagle medium (DMEM)/F12	Merck	Cat. # D6421
Glutamax	Gibco	Cat. # 45050
Hanks Balanced Salts Powder	Merck	Cat. # H2387
EDTA	Invitrogen	Cat. # 15575-038
Sodium Bicarbonate	Merck	Cat. # S5761
TrypLE Express	Gibco	Cat. # 12604021
Fetal Bovine Serum (FBS)	Gibco	Cat. # 1009
BET inhibitors	GlaxoSmithKline	Custom Synthesis
BSP-590	Promega Corporation/ GlaxoSmithKline	Custom Synthesis (Phillipou et al., 2019; referred to as BSP-BODIPY)
Polyethyleneglycol (PEG)	Merck	Cat. # 91893-1L-F
4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)	Merck	Cat. # 7365-45-9/H0887
Digitonin	Merck	Cat. # 11024-24-1
Critical commercial assays		
Nano-Glo luciferase assay system (Furimizine)	Promega Corporation	Cat. # N1130
Experimental models: Cell lines		
HEK293 Cells	ATCC	Cat. # CRL-1573
Recombinant DNA		
NL-BRD4	Promega Corporation	Cat. # N169A
Transfection Carrier DNA	Promega Corporation	Cat. # E4881
Software and algorithms		
GraphPad Prism v. 7	GraphPad Software	https://www.graphpad.com/scientific-software/prism/

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Excel	Microsoft	https://www.microsoft.com/en-gb/microsoft-365/excel
Other		
Non-binding surface, 384 well white micromicroplates	Corning	Cat. # 3574BC
6-well tissue culture microplates	Greiner Bio-one	Cat. # 657165

MATERIALS AND EQUIPMENT

Supplemented DMEM		
Reagent	Final concentration	Amount
Glutamax	1×	5 mL
Dulbecco's Modified Eagle Medium (DMEM)/F12	1×	500 mL
Fetal Bovine Serum (FBS)	10%	50 mL
Total	**N/A**	**555 mL**
Stored at 4°C and warmed to 37°C. Typically used within one month once opened.		

Trypsin mix		
Reagent	Final concentration	Amount
Hanks Balanced Salts powder	1×	9.8 g
EDTA	54 μM	1.08 mL
Sodium Bicarbonate	7.62 mM	0.64 g
TrypLE Express	0.1×	100 mL
MQ H_2O	N/A	899 mL
Total	**N/A**	**1 L**
Stored at 4°C and warmed to 37°C. Typically used within one month once opened.		

Tracer dilution buffer		
Reagent	Final concentration	Amount
HEPES solution (pH 7–7.6)	12.5 mM	125 μL
Poly-Ethylene Glycol (PEG)	30 %	3 mL
OptiMEM	N/A	6.85 mL
Total	**N/A**	**10 mL**
Stored at 4°C and warmed to 37°C. Typically used within one month once opened.		

⚠ CRITICAL: Active pharmaceutical ingredients (APIs) including BET inhibitors may be highly toxic if accidentally ingested. Ensure personal protective equipment (PPE) including gloves, safety glasses and lab coats are worn during the handling of APIs. Avoid handling solid samples of BET compounds but, if necessary, ensure a suitable containment cabinet is used.

Alternatives: The trypsin mix used here could also be purchased pre-prepared (for example Gibco Cat# 15400054).

An alternative tracer dilution buffer is available from Promega (Cat. # N2191).

The KICA protocol is relatively simple and can be performed in a laboratory with standard cell culture facilities and screening capabilities. The most important step in the protocol is the rapid addition of cells to 384-wellmicroplates, containing both compound and tracer, quickly followed by the measurement of Bioluminescence Resonance Energy Transfer (BRET) signals. The protocol below was carried out utilizing a Multidrop combi reagent dispenser (ThermoFisher) and an Envision microplate reader (Perkin Elmer). The same process can be accomplished with higher temporal consistency and frequency by using a microplate reader equipped with reagent injectors, such as a PHERAstar or CLARIOstar (BMG Labtech).

STEP-BY-STEP METHOD DETAILS

Characterization of tracer

Timing: 1–2 h

This step enables the measurement of the forward and reverse binding rates of the Tracer. These must be used later in the analysis of KICA data to determine the forward and reverse rates of competitor ligands.

1. Prepare a concentration titration of BSP-590 in 100% DMSO in a white 384-well micromicroplate.

   Note: If the expected affinity of a new tracer is not known it is advisable to carry out an initial experiment to identify a suitable concentration range for the kinetic study. To remove the complexity of kinetics from this initial study, a single endpoint read is recommended at a time point where the experiment is likely at equilibrium. For instance, for the BRD4 KICA experiments, equilibrium binding was achieved for most concentrations by 60 min. When configuring kinetic experiments, using concentrations where binding to the target can be observed is advisable and will lead to greater accuracy in the measurement of rates. Tracer binding rates may be limited by solubility so make sure that higher concentrations are fully dissolved in the buffer before adding cells.

2. Normalize DMSO volume across wells by ensuring the same concentration is present in each test well.
3. Apply 5 μL of tracer dilution buffer per well.
4. Apply 5 μL of OptiMEM per well and place on a microplate shaker for 15 min at 550 RPM, in the dark.

   Note: The BRD4 KICA exemplar experiment (Figure 1; Table 1) utilized a logarithmic 15-point titration of BSP-590 between 2000 and 5 nM, achieved using an HP D300 digital compound dispenser from a stock prepared in 100% DMSO. The amount of DMSO per well for this titration was then normalized using the same dispenser. The addition of tracer dilution buffer and OptiMEM are to aid the transfer of the titration into solution, which can be difficult, especially if pre-dispensed microplates have been stored at −20°C. If nanoliter dispensing is not available, a DMSO normalized serial dilution of tracer could be made up at 10× final concentration and added in 5 μL tracer dilution buffer. If the chosen tracer is easily solubilized then the extra addition of 5 μL OptiMEM can be removed and the tracer instead solubilized in a 10 μL 0.5× tracer dilution buffer.

5. Wash pre-transfected HEK293 cells with PBS, apply 0.5 mL of Trypsin solution per well and incubate for 4 min.
6. Suspend and combine the cells by adding 2 mL of supplemented DMEM per well.

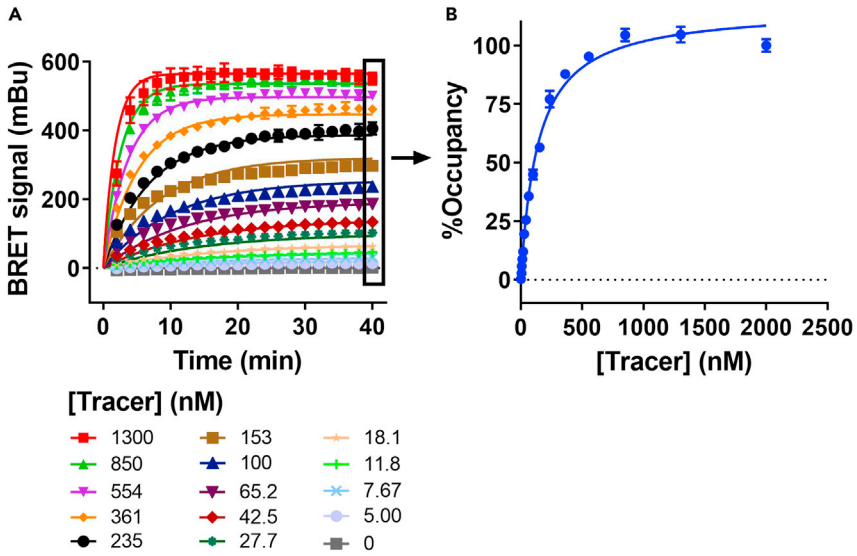

Figure 1. Measuring BSP-590 forward and reverse binding rates and equilibrium affinity to NL-BRD4

(A) Association traces of BSP-590 to NL tagged BRD4 fitted with Equation (2). Values are the mean of a triplicate experiment and the error bars are the standard deviation (SD).

(B) The microplateau values from (A) were normalized to a percentage of the microplateau (top concentration), and then plotted against the Tracer concentration and fitted with Equation (6) to enable calculation of an equilibrium-derived $K_{D,app}$ value. Error bars are the SD calculated for the microplateau mean values.

7. Transfer cells to a 50 mL falcon tube and spin the cells at 125 g for 5 min in a centrifuge.
8. Resuspend the resulting cell pellet in an equivalent volume of OptiMEM (2.5 mL per well of the 6 well microplate in which cells were transfected).
9. Count the cells and adjust the concentration to 2.5×10^5 cells/mL.
10. Add NanoGlo Substrate (Furimizine) to the cells at a 1 in 500 final concentration, then mix by inversion and incubate for 2 min.

Note: NanoGlo substrate was used at a final concentration of 1 in 500 (7.7 μM). This will need to be optimized for the plate reading length and final assay configuration used. In the case of the BRD4 KICA method, a 1 in 500 dilution of NanoGlo substrate gave a stable luminescence signal for at least 2 h of continuous reading. Higher concentrations of substrate may lead to a 'flash' effect in which a brighter but shorter luciferase signal is observed. Changes in luminescence signal should not lead to differences in the BRET signal as this is a ratiometric output, however low luminescence counts often lead to higher error in the BRET readings.

11. Seed 40 μL of the cell suspension to each well of the pre-prepared white 384-well microplate containing the BSP-590 titration and quickly initiate repetitive readings of luminescence and fluorescence for at least one hour using a microplate reader equipped with 450 ± 20 nm bandpass and 610 nm longpass filters.

⚠ CRITICAL: It is important to quickly begin reads after the addition of cells to the BSP-590, however, unless a reader equipped with injectors is used, this is unlikely to be instantaneous. In instances where there is a gap between the addition of cells and the first read, measure the exact time and add this to the first reading time later during analysis.

Note: The time taken for the tracer to reach equilibrium will vary according to the tracer and target used. It is advisable to use a sufficiently long read time in the first instance until the time to reach equilibrium has been defined.

Table 1. Cellular kinetic values for BSP-590 and commercially available reference compounds

Compound	$k_{forward}$ (M^{-1} s^{-1})	$k_{reverse}$ (s^{-1})	Kinetic pK_D	Equilibrium pK_D
BSP-590 (Figure 1)	1.01 ± 0.12 × 10^4	1.14 ± 0.10 × 10^{-3}	6.90 ± 0.03	6.84 ± 0.04
JQ1 (Figure 2A)	1.54 ± 0.18 × 10^5	1.79 ± 0.17 × 10^{-3}	7.92 ± 0.02	7.80 ± 0.03
iBET-151	1.64 ± 0.46 × 10^5	2.86 ± 0.33 × 10^{-3}	7.66 ± 0.05	7.61 ± 0.05
Mivebresib	2.94 ± 0.42 × 10^5	4.88 ± 1.05 × 10^{-4}	8.82 ± 0.04	8.64 ± 0.03

KICA

Timing: 3–4 h

Carrying out a KICA experiment enables the measurement of forward and reverse binding rates ($k_{forward}$ and $k_{reverse}$, respectively) of unlabeled test compounds through perturbations in the binding of the tracer to the target.

12. Prepare a concentration titration of competitor compounds in a 384-well white microplate in 100% DMSO and normalize total DMSO volume across wells.

Note: It is advisable to include control columns of fully inhibited (for example, 7 μM JQ1) and uninhibited (DMSO alone) tracer binding on each test microplate. These control populations can be used to generate signal to background ratios (S/B) and z' values to ensure assay quality is maintained over time.

13. Apply 5 μL of tracer dilution buffer per well containing 1.5 μM BSP-590 (10× the previously determined $K_{D,\ app}$).

Note: The concentration of BSP-590 used for this experiment (150 nM) was selected as it was approximately the concentration equivalent to the cellular $K_{D,\ app}$ measured in the tracer characterization experiments. Utilization of a concentration of tracer equivalent to the $K_{D,\ app}$ of the macromolecule of interest is advisable to avoid excessive target saturation, while still enabling a usable assay signal.

14. Add 5 μL of OptiMEM to each well and place the microplate on a shaker at 550 RPM for 15 min in the dark.
15. Wash pre-transfected HEK293 cells with PBS, apply 0.5 mL per well of Trypsin solution and incubate for 4 min.
16. Suspend and combine the cells by adding 2 mL per well of supplemented DMEM.
17. Transfer to a 50 mL falcon tube and spin the cells down at 125 g for 5 min in a centrifuge.
18. Resuspend the cell pellet in an equivalent volume of OptiMEM (2.5 mL per well of the 6 well microplate in which cells were transfected).
19. Count the cells and adjust the concentration to 2.5 × 10^5 cells/mL.
20. Add NanoGlo Substrate (Furimizine) to the cells at a 1 in 500 final concentration, mix by inversion and incubate for 2 min.
21. Seed 40 μL of the cell suspension to each well of the pre-prepared white 384-well microplate containing the competitor titration and quickly initiate repetitive readings of luminescence and fluorescence for at least 2 h using a microplate reader equipped with 450 ± 20 nm bandpass and 610 nm longpass filters. Troubleshooting 1.

EXPECTED OUTCOMES

Tracer characterization

The results of the tracer characterization experiment should be a rapid rise in BRET signal, followed by a steady, equilibrium signal. Higher concentrations of tracer will achieve equilibrium at a faster

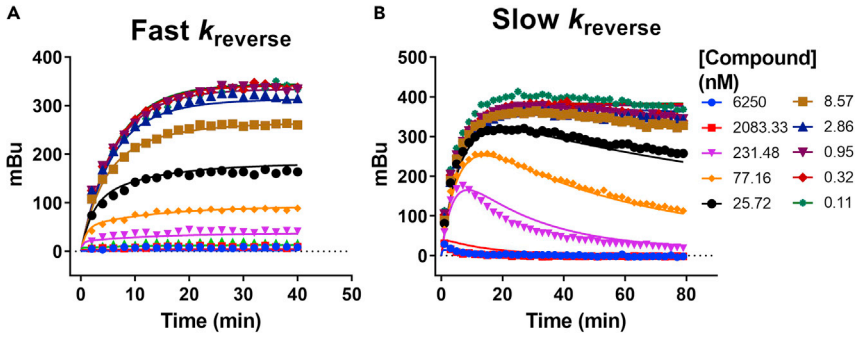

Figure 2. Measuring the effective forward and reverse rates of unlabeled competitor ligands using KICA

KICA traces for an exemplar fast (A; JQ1) and slow (B; GSK propriety compound) $k_{reverse}$ compound (within the context of the compound set used in the study) fit using the Motulsky-Mahan equation (Equation 7).

rate, which is due to the concentration dependence of association rates. An example of BSP-590 associating with NL-BRD4 is shown in Figure 1A.

KICA

The KICA methodology should result in an increase in BRET signal with different concentrations of inhibitor leading to different amplitudes of the progress curves (with higher concentrations giving lower amplitudes). Compounds with faster forward and reverse rates should exhibit rapid approaches to equilibrium similar in profile to those observed in the tracer association experiments. Compounds with slower rates will most likely exhibit an 'overshoot' profile with a rapid increase in signal followed by a decline formed as the inhibitor's slower approach to equilibrium competes the tracer from the target. Examples of both 'fast' and 'slow' (within the context of the initial BRD4 KICA study) reverse rate compound profiles are shown in Figure 2 (cellular kinetic values for the fast $k_{reverse}$ compound are presented in Table 1).

QUANTIFICATION AND STATISTICAL ANALYSIS

Initial data formatting can be carried out in Microsoft Excel. Data is recorded in the form of the luminescence donor signal at 450 nm and the fluorescent acceptor signal at 610 nm. The luminescence donor signal originates from the NL-fused target and as such is a useful control value. Large variations in the luminescence signal for wells on a microplate could be indicative of misdispensing of transfected cells. Widespread variation in luminescence signal, in comparison to previous experiments, could be indicative of variation in transfection efficiency or cell health. A specific dose-dependent increase in luminescence with decreasing competitor concentration, observed after incubation with inhibitor, may be indicative of competitor cell toxicity. The first step of the analysis is to convert these values to a BRET ratio. In the BRD4 KICA study the BRET ratio was expressed in milliBRET units (mBu), which were calculated using Equation (1):

$$mBu = \left(\frac{Acceptor\ (610\ nm)}{Donor\ (450\ nm)}\right)1000 \qquad \text{(Equation 1)}$$

BRET values can then be normalized to the background signal by the subtraction of the BRET ratio from wells containing no tracer in tracer characterization experiments or to wells with an excess concentration of competitor in KICA experiments.

Once the BRET values are normalized, further analysis can be carried out in Graphpad Prism (or an equivalent data analysis software), whereby the BRET values for each experiment condition (y-axis, typically for varying concentrations of either tracer or test compound concentration, respectively) are plotted against time (x-axis).

Normalized data from tracer characterization experiments can be fitted with a global equation named in prism as 'Association kinetics - Two or more conc. of hot' which uses Equation (2):

$$K_D = \frac{k_r}{k_f} \quad \text{(Equation 2)}$$

$$k_{obs} = k_f[L] + k_r \quad \text{(Equation 3)}$$

$$a = \frac{[L]}{([L] + K_d)} \quad \text{(Equation 4)}$$

$$b = ac \quad \text{(Equation 5)}$$

$$y = b(1 - exp^{-1k_{obs}x}) \quad \text{(Equation 6)}$$

where K_D is the dissociation constant, [L] is the concentration of tracer, a is the plateau of the individual curve, c is the maximum plateau of any of the curves, k_r is the reverse rate of the tracer and k_f is the forward rate of the tracer.

These values can also be determined by fitting the same data with Prism's 'One-phase association' (single exponential) fit with Equation (3):

$$y = a + (b - a)(1 - exp^{-k_{obs}x}) \quad \text{(Equation 7)}$$

where a is Y at time 0, b is the microplateau of the curve and k_{obs} is the rate of association.

The resulting k_{obs} values can then be plotted against the tracer concentration, the data fitted with linear regression and the forward and reverse rates be determined from the gradient and the y-intercept using Equation (4):

$$k_{obs} = k_f[Tracer] + k_r \quad \text{(Equation 8)}$$

Note: the resulting plot should be linear. If k_{obs} does not increase with concentration, it is a sign that the association rate of the tracer is being limited by another factor (e.g. solubility or permeability). If this is the case, exclude these concentrations from kinetic analysis (as was the case for 2 μM in Figure 1).

In addition, the affinity of the tracer for the target (important for selecting a concentration for subsequent KICA experiments) can be calculated kinetically from the forward and reverse rates using Equation (5):

$$K_D = \frac{k_r}{k_f} \quad \text{(Equation 9)}$$

This is especially useful if the compound does not reach equilibrium over the course of the experiment. The values can also be compared with the equilibrium values to check that the kinetics rates are generating realistic predictions of affinity. If the assay is working well these values should correlate.

The equilibrium affinity of the tracer can be calculated by plotting the BRET values from tracer equilibrium, against the concentration of tracer used and subsequent fitting with the Prism fit 'One site – specific binding' which uses Equation (6):

$$y = \frac{ax}{(K_D + x)} \quad \text{(Equation 10)}$$

where a is the plateau of the curve.

Note: For the BRD4 KICA study, similar BRD4 $K_{D,\ BSP\text{-}590}$ values were measured in both the cellular (KICA) and lysed (KLA) assays, despite the likely higher local concentration of competing histones in the KICA version of the assay. However, when probing other cellular targets high concentrations of endogenously produced competing binders may lead to K_D

values that are weaker than the true value. Therefore, if any disparity is observed between cellular and non-cellular tracer affinity values for the target of interest, or the non-cellular K_D for the interaction is not known, the use of an apparent suffix is recommended ($K_{D,\ app}$).

Normalized KICA data can be analyzed using the Prism fit 'Kinetics of competitive binding' (Motulsky-Mahan equation) which uses Equation (7) (Motulsky and Mahan, 1984):

$$K_A = k_1[L] + k_2 \quad \text{(Equation 11)}$$

$$K_B = k_3[I] + k_4 \quad \text{(Equation 12)}$$

$$S = \sqrt{(K_A - K_B)^2 + 4k_1k_3[L][I]} \quad \text{(Equation 13)}$$

$$K_F = 0.5(K_A + K_B + S) \quad \text{(Equation 14)}$$

$$K_S = 0.5(K_A + K_B - S) \quad \text{(Equation 15)}$$

$$Q = \frac{B_{max}k_1[L]}{K_F - K_S} \quad \text{(Equation 16)}$$

$$y = Q\left(\frac{k_4(K_F - K_S)}{K_FK_S} + \frac{k_4 - K_F}{K_F}\exp(-K_Fx) - \frac{k_4 - K_S}{K_S}\exp(-K_Sx)\right) \quad \text{(Equation 17)}$$

where [L] is concentration of Tracer, [I] is concentration of the unlabeled competitor, B_{max} is the total binding in mBu, k_1 is the forward rate of the tracer, k_2 is the reverse rate of the tracer, k_3 is the forward rate of the competitor and k_4 is the reverse rate of the competitor.

The concentration of tracer used and the pre-measured tracer forward and reverse rates are used as input for the equation to calculate the competitor rates. The competitor's kinetically calculated affinity can be determined using Equation (5) and the equilibrium affinity can be determined by plotting the inhibition of tracer binding at equilibrium against the respective competitor's concentration and determining the IC_{50} with Equation (8):

$$y = a + \frac{b - a}{1 + 10^{(LogIC_{50} - x)c}} \quad \text{(Equation 18)}$$

where a is the maximum inhibition, b is the minimum inhibition, $LogIC_{50}$ is the log of the compound's 50% inhibitory concentration and c is the Hill slope.

If the affinity values appear limited, refer to Troubleshooting 2.

The competitive affinity can then be determined using the Cheng-Prusoff equation below (Cheng and Prusoff, 1973):

$$K_I = \frac{IC_{50}}{1 + ([L]/K_D)} \quad \text{(Equation 19)}$$

where [L] is the concentration of the labeled tracer and K_D is the dissociation constant of the labeled tracer.

LIMITATIONS

KICA measures forward and reverse rates of intracellular compound binding. These rates are influenced both by permeability and the association and dissociation rates of the compound. As such KICA alone cannot distinguish between poorly permeable or slowly dissociating and associating compounds. It is advisable to run a parallel permeability assay such as Caco-2 (Hidalgo et al., 1989), AMP (Zhu et al., 2002), or an LC-MS based cell concentration assay (Gordon et al., 2016; Mateus et al., 2013) to flag poorly permeable compounds. In addition, the rates can be simplified to association and dissociation alone by

first incubating the suspended cells in OptiMEM with 50 μg/mL of Digitonin in an Erlenmeyer flask at 110 RPM for 1 h at 37°C before running the assay. If this protocol leads to a degradation of the luminescence signal, refer to Troubleshooting 3. This style of assay is known as a Kinetic Lysate Assay (KLA) and is equivalent to the established kinetic Probe competition assay (kPCA) (Schiele et al., 2015) which utilizes TR-FRET between fluorescently labeled purified proteins. KICA rates are substantially slower than KLA rates although the rank order of rates is comparable for permeable compounds. If a compound goes from having slow rates in KICA to fast rates in KLA relative to the other compounds tested, then this may indicate poor cell permeability.

TROUBLESHOOTING

Problem 1

Regarding KICA: In the BRD4 KICA study the KICA rates measured intracellular binding rates for test compounds were elongated when compared to those measured in cell-free systems. This was an advantage as most compounds tested were very quick to reach equilibrium in lysate and purified protein kinetic assays. However, when the intracellular binding rates of test compounds are slow, the read time may need to be extended to capture the full process. In these situations, the breakdown of the furimizine substrate may reduce luminescent signal overtime and thereby increase experimental error.

Potential solution

Longer-lived NL substrates, such as Vivazine (medium lived) and Endurazine (long lived) are available from Promega and can be used to extend luminescence signal stability (Cat. numbers N2580 and N2570 respectively).

Problem 2

Regarding Quantification and statistical analysis: If the measurable affinity values in a KICA experiment appear restricted to no stronger than a specific affinity, then it is likely that the concentration of the NL-fused target is a limiting factor (this would be the so called 'assay wall' or 'tight binding limit'). This can happen if the NL-fused target is expressed at a high concentration or the test compounds are highly potent (or a combination of both factors).

Potential solution

Expression of the NL-fused target can be reduced by decreasing the amount of DNA used in a transfection, switching to a less efficient promoter or by using a CRISPR-Cas9 system to insert NL or HiBit onto one of the termini of the gene of interest. A quick means of ascertaining the concentration of the NL-fused target is to compare the luminescence measured under the KICA conditions to a standard curve, which can be prepared by titrating purified NL (available from Promega). The estimated concentration of NL present in the KICA can then be compared with published values for endogenous protein expression.

Problem 3

Regarding Limitations: If the luminescence signal in your lysate assay is weaker or gives more error prone BRET values than your cellular assay.

Potential solution

This may be a sign that the lysis protocol is leading to degradation of your luciferase or target protein. Try adding protease inhibitors into the lysis mix. The lysis protocol could also be optimized using different temperatures, concentrations of digitonin and lengths of incubation, however it is important to ensure that the cells are fully lysed to avoid sampling intermediate rates from a population of both lysed and intact cells. The fraction of intact cells can be sampled on a cell counter.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Peter Craggs (peter.d.craggs@gsk.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

There are restrictions to the availability of the small molecule test-set kinetic data due to the dataset being generated with anonymized molecules from the GSK compound collection.

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AUTHOR CONTRIBUTIONS

K.M.G. supervised the project and conceived the kinetic and NanoBRET aspects of the study. P.D.C. conceived and facilitated the use of BET proteins as an exemplar and acted in a supervisory role at later stages of the study. E.J.J. generated reagents. K.M.G., P.D.C., and C.S.L. participated in the design and interpretation of experiments. C.S.L. conducted experiments. K.M.G., P.D.C., C.S.L., D.A.T., and J.P.E. conceived of and participated in the creation of the original manuscript on which this piece is based. C.S.L. wrote the current manuscript with reviewing and editing from P.D.C.

DECLARATION OF INTERESTS

J.P.E., E.J.J., K.M.G., and P.D.C. are employees and shareholders of GSK.

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