Cell-based biosensor transport assay

Version 1.0

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1. Rationale/Aim

Biosensors are a rapidly growing toolset of fluorescence-based genetically encoded proteins for monitoring specific conditions within cells. This includes detecting the transport of small molecules or ions into the cell, or monitoring changes in pH due to proton exchange. Therefore, these tools are quite powerful for characterizing SLC transporters and their interactions with substrates and other small-molecules. In a 96-well plate format, this allows for relatively high throughput assaying of transport kinetics, as well as screening the effects of compounds on target function.

Essentially, substrates or inhibitors will induce changes in the fluorescence of cells co-transfected with a specific biosensor and the target transporter. Depending on the sensor design and substrate concentration, this may yield a change in fluorescence intensity, excitation or emission wavelengths, or fluorescence-resonance energy transfer efficiency.

Using cells in suspension culture makes this a relatively flexible and straightforward assay that can be iterated quite quickly for the screening of compounds as well as the effects of mutations on transport parameters. This fluorescence can be followed using a plate reader in a time course or end-point assays. After measuring transport activity, GDN is added to permeabilize the cell's membranes, allowing direct access of substrate to sensor and therefore an internal calibration of the sensor's response.

2. Experimental conditions

2.1 Key Requirement:

- Expi293F cells in suspension at $2x10^6$/ml

    - Cell type is flexible, but should not have endogenous expression of the protein of interest or other transporters of the same substrate.
- Microbiological safety cabinet
- Biosensor and target plasmid DNA
- Shaking incubator with humidity and CO_2 control
- Plate reader with fluorescence and FRET capabilities and filters in the appropriate excitation and emission ranges (Hidex or Pherastar type plate reader)
- Linear PEI at 1 mg/ml.
    - Add 20 mg to 18 ml ddH_2O then, with stirring, bring the pH down to below 2 using 1M HCl. Once dissolved, return the pH to 7 by dropwise addition of 3 M NaOH dropwise. In a microbiological safety cabinet, filter-sterilized the solution through a 0.22 µm syringe filter.
- 1M Sodium butyrate in PBS
    - Filter-sterilize through a 0.22 µm syringe filter in a microbiological safety cabinet.
- GDN in water at 10 % (w/v)
- Relevant substrates/compounds at 10 x the desired final concentration required. Concentrations will depend on the affinity of the target for the substrate/compound.
    - The sensitivity of cells to the compound solvent will vary by cell type, and must be optimized by cell type and solvent. Generally, the final DMSO concentration should to be greater than 0.25-0.5 %. Appropriate solvent controls, without substrate, should be included to account for solvent effects.

2.2 Key Resources Table:

A list of key materials (in table format). Must include vendors and Cat. No. For in-house materials, provide internal IDs.

Reagents (items)	*Suppliers*	*Cat. No.*

Linear Polyethylene imine (PEI) 25000	Polysciences Europe GmbH	23966-1
Expi293F cells	Life Technologies Ltd.	A14528
FreeStyle 293 Expression Medium	Gibco	12338018
Recombinant plasmid with mammalian expression promoter (CMV) (1 µg/ml of culture)	In-house	
Recombinant biosensor plasmid with mammalian CMV promoter (1 µg/ml of culture)	AddGene	
Optimem reduced serum medium	Gibco	1850613
Black clear bottomed 96 well plates	VWR	736-0230
Clear v-bottomed 96 well plate	Greiner	651161
Deep-well 96 well block	Greiner	780270
Sterile 24-well cell culture block	Whatman	GE Healthcare, 7701-5110
Sodium butyrate	Sigma-Aldrich	8202360250
Fetal bovine serum (FBS)	Gibco	10082147
Sterile HBSS	Merck	55037C-1000ML
Sterile HEPES	Life Technologies Ltd.	15630080
Glyco-diosgenin (GDN)	Anatrace	GDN101
Substrates/compounds	Various	N/A
50 ml sterile falcon tubes	Thermo Scientific	339653

*20 mM sterile HEPES was added to the HBSS before use, or HEPES-buffered HBSS can be purchased

3. Protocol

3.1 Protocol:

3.1.1 Cell culture

1) Culture Expi293F in Freestyle 293 medium at 37 C, 8 % CO_2, 75 % humidity until they reach a density of $2x10^6$/ml

2) Aliquot cells into a 24-well tissue culture block (3 ml/well), and leave in incubator shaking at 200-220 rpm while preparing DNA/PEI mixture. (Note: it is not necessary to transfect an entire 24-well block, but it is useful to have several technical replicates to ensure reproducibility).

3) In a 96-well, 200 µl V-bottom microplate, aliquot 150 µl of Opti-MEM, mix with 3 µg of DNA and leave it for 5 min. As negative controls, include wells without any DNA or with only the biosensor DNA. The remaining wells should include 3 µg for both the biosensor and target DNA.

4) In a 1.8 ml 96-deep-well block, mix 141 µl Opti-MEM and 9 µl of Linear PEI, for each reaction and leave for up to 5 min.

5) Add the mixture from step 3 to the one in step 4, and incubate for 10 minutes, then add this mixture to the cells in the tissue culture block.

6) Add 10 mM sodium butyrate (from a 1 M stock solution) to each well of cells.

7) Cover the block with a porous plate seal and incubate at 30°C for 72 h with shaking at 200 rpm, or 37°C for 48 h with the same shaking speed.

3.1.2 Transport assay

1) In a microbiological safety cabinet, remove a small volume of untransfected and transfected cells and use a cell counter to determine density. Also check viability with Trypan blue.

2) Calculate how much to dilute the cells to achieve approximately $5x10^4$-$1x10^5$cells/well, to a final volume sufficient for 2 rows of 100 µl/well (untransfected cells) and 6 rows of 100 µl/well (target plus biosensor).

Note: The number of cells will need to be optimized, depending on quantum efficiency and substrate response of the biosensor, and the cell's transfection efficiency.

3) Transfer the required volume of cells from the relevant wells in the 24-well block to a 50 ml falcon tube. Return the block to the incubator while doing the assay.

4) Spin the cells at 200 g for 5 minutes at 20°C.

Note: The following steps can be performed outside of the microbiological safety cabinet.

5) Gently aspirate the media off the centrifuged cells and carefully resuspend them in HEPES-supplemented HBSS to the target cell density.

6) Pour into a sterile reservoir and aliquot 100 µl untransfected and transfected cells into a clear-bottomed 96-well plate.

7) Set up the plate reader with the correct excitation and emission wavelengths for the biosensor in use. Collect a baseline time course for about 10 min prior to adding substrate.

Note: The instrument fluorescence data collection parameters will need to be optimized for each biosensor.

8) Add substrates/compounds to desired concentration into each well using a multichannel pipette. Return the plate to the plate reader and collect for a further 30 min.

Note: Include substrate-free controls to account for the effects of the substrate solubilization buffer (i.e. DMSO).

9) Add GDN to a final concentration of 0.5 % in each well. Return the plate to the plate reader and collect for a further 10-20 min.

Note: The data collection times will need to be optimized for each biosensor and transporter.

10) Within data processing package, convert raw fluorescence signals to signal specific to the biosensor. For example, if using a FRET-based

biosensor the donor- acceptor fluorescence ratio can be calculated by using Fa/(Fd + Fa).

11) Using the final point after the addition GDN, calculate a calibration curve for converting the biosensor-specific signal to substrate concentration. Recalculate the time course's biosensor-specific signal as substrate concentration.

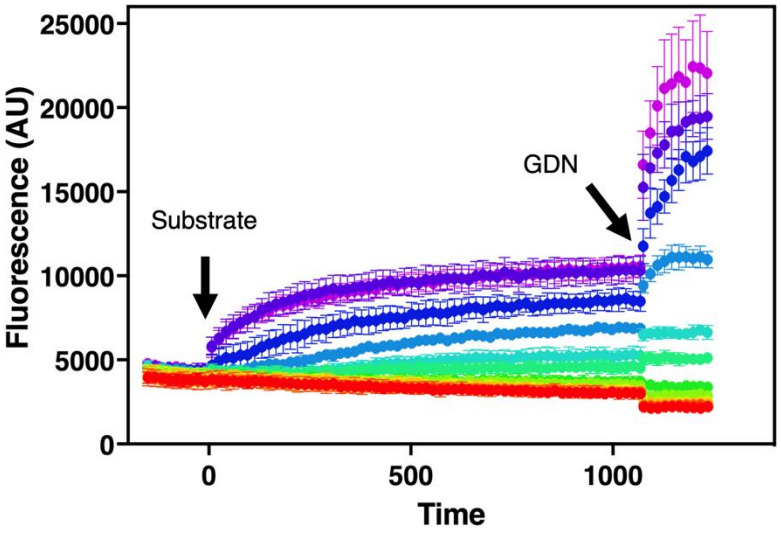

Fig 1. Raw Fluorescence traces. Substrate and GDN additions are indicated with arrows.

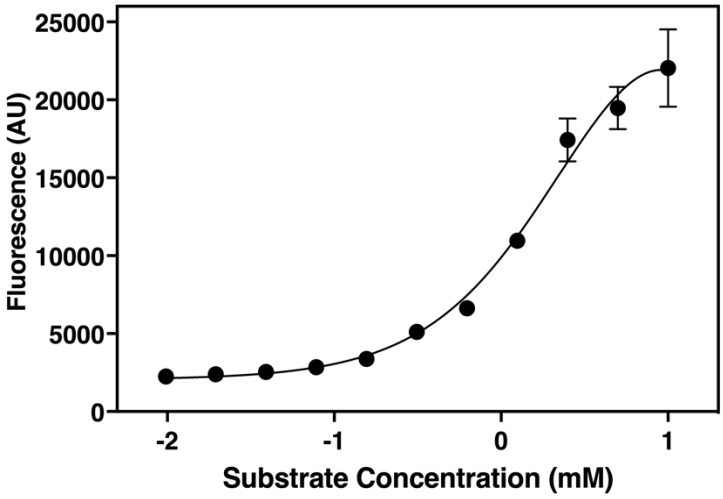

Fig 2. Biosensor calibration curve of substrate concentration versus biosensor fluorescence after addition of GDN.

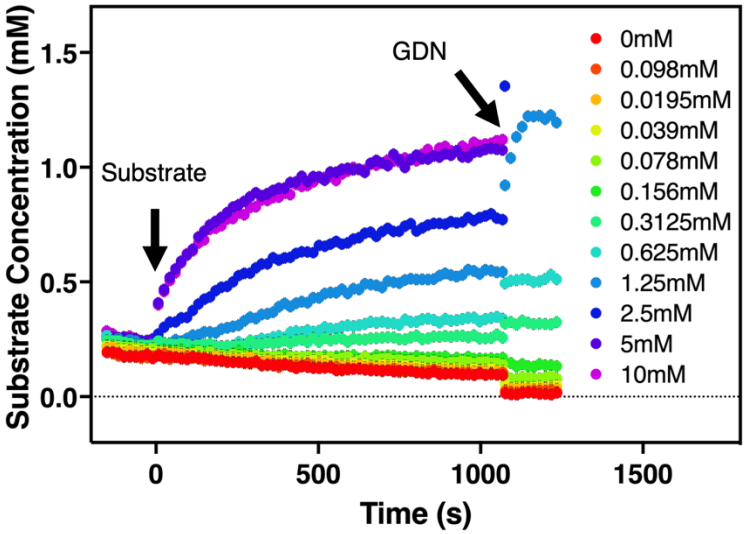

Fig 3. Time-dependent transport of substrate by an SLC transporter. Absolute substrate concentrations are calculated from the calibration curve. Substrate and GDN additions are indicated with arrows.