A Fluorescence-Based Neurotransmitter Transporter Uptake Assay

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Abstract

Here we present a homogeneous assay of norepinephrine, dopamine, and serotonin transporter activity based on cellular uptake of a fluorescent dye coupled with a proprietary masking dye. In this no-wash kit recently introduced by Molecular Devices, cells expressing the transporter of interest are incubated with the dye/reagent mix and transferred to a plate reader for evaluation. The assay can be performed in 96- or 384-well microtiter plates and read on any fluorescence microplate reader in bottom-read mode. Either an endpoint or kinetic assay can be performed. Both methods provide K_i's that are comparable to literature values. Existing detection methods use a radioactive filter binding assay that detects assay end points only, and have complicated disposal requirements. The proprietary masking dye ensures that fluorescence interference from compound libraries or media is minimized. This method of measuring transporter activity provides a new approach to screening for drug therapies addressing ailments such as depression, bipolar disorder, Parkinson's, and Alzheimer's disease.

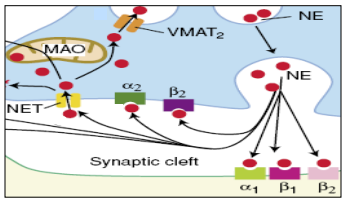


Figure 1: Example: Role of transporters in neurotransmission of norepinephrine (NE).

Introduction

Norepinephrine, serotonin and dopamine are released from vesicles into the synaptic cleft, where they can diffuse and bind to pre- and post-synaptic receptors. Neurotransmitter transporters allow reuptake of these neurotransmitters for recycling by active co-transport down a Na+/Cl- gradient. Thus neurotransmitter transporters regulate neuronal signaling by modulating neurotransmitter concentration in the synaptic cleft and are important targets for neuroscience drug discovery.

The assay described exploits the similarity of the norepinephrine, serotonin and dopamine reuptake transporters (NET, SERT and DAT) by using one fluorescent substrate mimetic for all three transporters.

Assay Principle

A proprietary fluorescent dye (Ex:440 nm; Em:520 nm) serves as substrate mimetic and is taken up into the cell specifically through DAT, SERT and NET. This dye, in combination with a proprietary masking dye, results in a homogenous no-wash assay that can be read in real-time kinetic or endpoint mode, enabling mechanistic studies as well as HTS applications. Suitable instruments are fluorescence plate readers like Spectramax M5, Analyst GT, Flexstation, and FLIPR Tetra. **Figure 2** shows the simple assay protocol.

Figure 2: Basic assay protocol

Kinetic read example raw data: HEK-hSERT cells

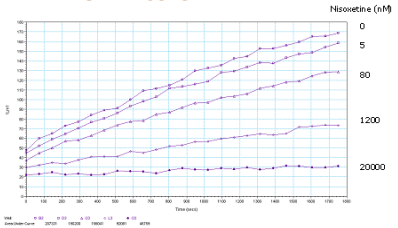


Figure 3 HEK cells stably expressing human SERT were plated O/N at 10,000 cells per well in poly D-lysine-coated 384 well plates. Medium was removed and nisoxetine in HBSS-0.1% BSA was incubated with cells for 10 minutes at 37°C prior to dye addition. The assay was read on Molecular Devices' Flexstation I instrument in kinetic mode for 30 minutes.

Plating options

Cells for the fluorescent neurotransmitter assay can be plated the day before or on the same day of the experiment. This increases the flexibility of assay planning, especially in an HTS setting. **Figure 4** shows an inhibition curve with Nomifensine on HEK-NET cells.

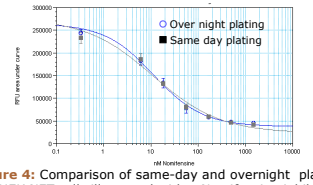


Figure 4: Comparison of same-day and overnight plating for HEK NET cells illustrated with a Nomifensine inhibition curve. Response is expressed as area under the curve.

K_m determination of fluorescent dye on SERT, DAT and NET

K_m's for the fluorescent substrate mimetic used were determined for DAT, SERT and NET to be 1.85µM, 0.96µM and 0.63µM respectively **(Figure 5).**

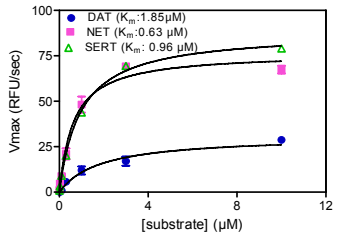
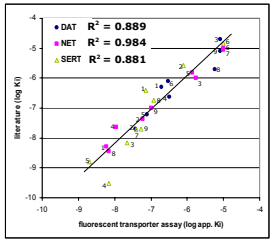


Figure 5: K_m determination of fluorescent substrate mimetic for SERT, DAT, and NET.

K_i determination for known inhibitors on hSERT, hDAT and hNET

The fluorescence based assay kit results in K_i's that correlate very well with literature values for all three transporter targets as shown in **figure 6**. The K_i's were calculated from IC_{50}'s using the K_m's shown in **Figure 5** and a fluorescent substrate concentration of 2µM using the Cheng-Prusoff equation. The correlation factor R^2 over all compounds was greater than 0.9.



1 = nisoxetine
2 = nomifensine
3 = fluoxetine
4 = duloxetine
5 = sertraline
6 = bupropion
7 = citalopram
8 = desipramine
9 = imipramine

Figure 6: Correlation between inhibition values obtained with the Neurotransmitter Transporter Uptake Assay and those found in literature for nine known inhibitors.

z' factor determination for NET DAT and SERT

We evaluated the relationship of cell number to assay window and z' factor. **Table 1** shows that the z' factor is > 0.6 at all cell number conditions tested. However, optimization of cell number can further improve the assay quality for each cell line used.

		10K cells/well (signal ± s.d.)	15K cells/well (signal ± s.d.)	20K cells/well (signal ± s.d.)
SERT	max. signal	136 ± 12	158 ± 7	189 ± 9
	min. signal (max. inhib.)	23 ± 2	23 ± 1	23 ± 1
	z' factor (n=24)	0.6	0.8	0.8
DAT	max. signal	134 ± 6	163 ± 10	206 ± 34
	min. signal (max. inhib.)	25 ± 1	27 ± 1	34 ±2
	z' factor (n=24)	0.8	0.8	0.6
NET	max. signal	184 ± 9		252 ± 8
	min. signal (max. inhib.)	25 ± 2		27 ± 2
	z' factor (n=24)	0.8		0.9

Table1: z' factors for HEK cells overexpressing DAT or NET or SERT assessed in 384 well format and read on a Flexstation III.

NET LOPAC screen: Analysis

In order to assess the suitability of the technology for HTS applications, we ran the Library of pharmacologically active compounds (LOPAC) at 3µM against the HEK-NET cells. A scatter plot with the results of all 4 plates is shown in **Figure 7**. All compounds that are classified in the LOPAC documentation under "uptake" in combination with depression or "re-uptake" are labeled pink. We were able to identify the majority of these as hits. Compounds not showing significant inhibition could be shown to have K_i's in the micromolar range for NET greater than the compound concentration used. All compounds labeled yellow were described as agonists or antagonists for the neurotransmitter transporter receptors. Only 8 compounds showing inhibition greater than 60% were not described as being related to neurotransmitters.

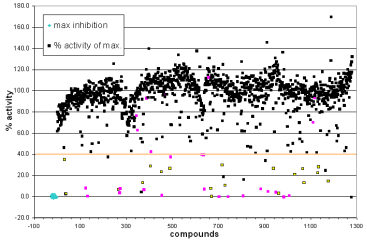


Figure 7: Scatter plot for LOPAC screen at 3µM compound on HEK-NET cells.

NET LOPAC screen: cherry pick

Six inhibitors were selected from the library screen and their IC_{50} for NET was determined. The resulting inhibition curves are shown in **figure 8**. The corresponding K_i's were calculated using a K_m of 0.96µM determined for this particular NET cell line (data not shown) and a fluorescent substrate concentration of 2 µM. Inhibitors tested showed good correlation (R^2>0.9) with the literature as shown in **figure 9**.

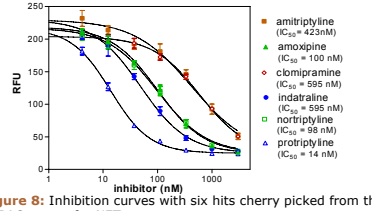


Figure 8: Inhibition curves with six hits cherry picked from the LOPAC screen for NET.

inhibitor	literature source
amitriptyline, nortriptyline	**Owens MJ** et al, J Pharmacol Exp Ther. 1997 Dec;283(3):1305-22
amoxapine, protripyline	**Tatsumi M** et al. Eur J Pharmacol. 1997 Dec 11;340(2-3):249-581997
clomipramine	**Millan MJ** et al, J Pharmacol Exp Ther. 2001 Aug;298(2):565-80
indatraline	**Zhou J**, Drugs Future. 2004 December; 29(12): 1235–1244

Table 3: literature sources for inhibitors tested.

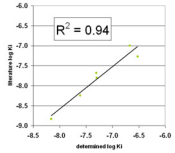


Figure 9: correlation graph of observed K_i's vs. literature values.

Conclusions

A novel fluorescence-based transporter activity assay has been developed that enables real-time analysis of DAT, NET or SERT uptake in a homogenous, nonradioactive format.

- Either kinetic or endpoint mode can be used; the assay is scalable to 96 or 384 well plates.
- IC_{50}'s obtained are in good agreement with published K_i values for DAT, NET and SERT antagonists.
- Due to the brightness of the fluorescent dye and its lack of non-specific uptake, z' factors for this assay are robust using any of the FlexStation family of instruments or the M5 multimode reader from Molecular Devices.

Molecular Devices