Supporting Information for:

A Fluorescence Displacement Assay for Antidepressant Drug Discovery Based on Ligand Conjugated Quantum Dots

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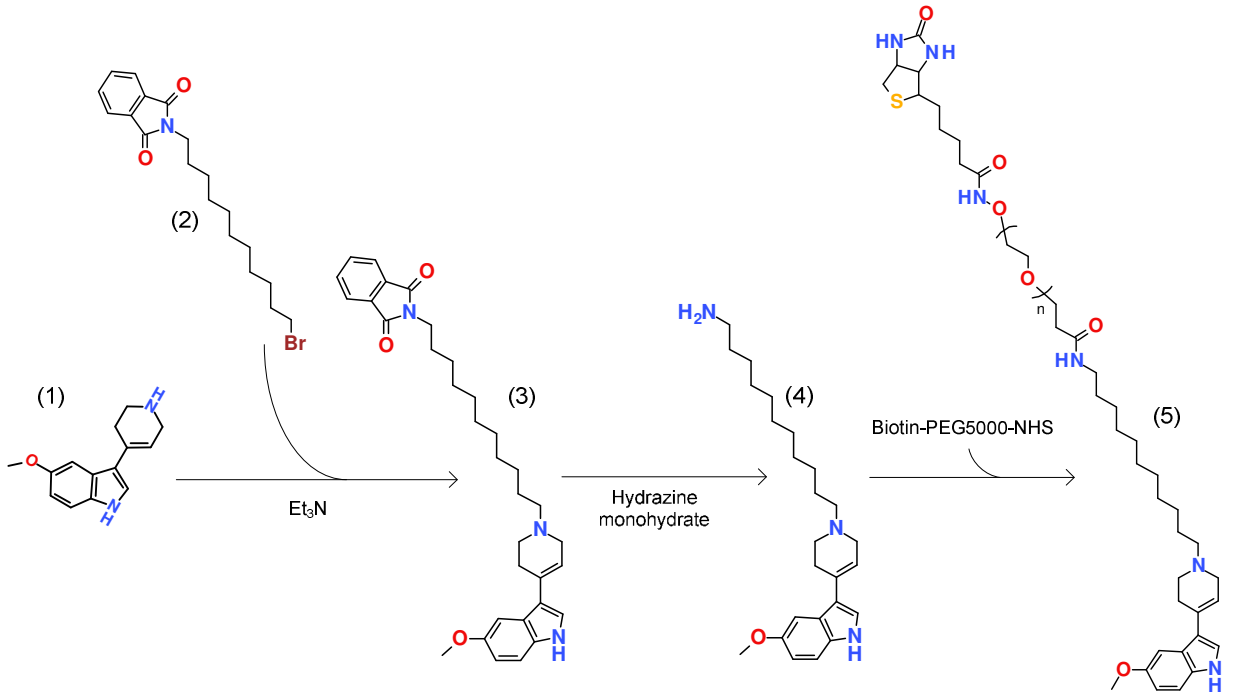
Experimental Section

IDT318 Ligand synthesis

The synthesis details and spectroscopic characterization have been previously described in our recent publications.[1,2] Brief outline is shown in the scheme below. Initially, the parent drug 5-methoxy-3-(1,2,5,6-tetrahydro-4-pyridinyl)-1*H*-indole **(1)** was synthesized and coupled to 11-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-undecyl bromide **(2)** to yield 2-(11-(4-(5-methoxy-1*H*-indol-3yl)-5,6-dihydropyridin-(2*H*)-yl)undecyl) isoindoline-1,3-dione **(3)** in the presence of triethylamine (Et_3N). The phthalimide protecting group was removed using hydrazine monohydrate to give 11-(4-(5-methoxy-1*H*-indol-3-yl)-5,6-dihydropyridin-1(2*H*)-yl)undecan-1-amine **(4).** This is then coupled to biotin-polyethylene glycol-*N*-hydroxysuccinimide ester to give the SERT ligand IDT318 **(5)** for the study.



hSERT-espressing oocytes prepapation

Xenopus oocytes and hSERT cRNA were prepared and isolated as previously described.[3,4] In brief, stage V-VI oocytes were harvested from *Xenopus laevis* (Nasco, Medesto, CA). After harvesting, the follicle cell layer was removed by incubation with 2 mg/ml collagenase in Ringer's buffer (in mM, 96 NaCl, 2 KCl, 5 $MgCl_2$, 5 HEPES, pH 7.4) for an hour. cRNA injections were performed on the day of harvest. hSERT cRNA was transcribed from NotI (New England BioLabs, Beverly, MA)-digested cDNA in pOTV vector (a gift of Dr. Mark Sonders,

Columbia University) using Ambion mMessage Machine T7 kit (Ambion, Austin, TX). The cRNA concentrations were confirmed by UV spectroscopy and gel electrophoresis. Each oocyte was injected with 3 ng cRNA and incubated at 18 C° for 3-6 days in Ringer's buffer supplemented with 550 μM/ml sodium pyruvate, 100 μg/ml streptomycin, 50 μg/ml tetracycline, and 5 % dialyzed horse serum. Healthy oocytes for subsequent electrophysiological and fluorescence assays were selected by visual inspection.

Quantum dot-hSERT labeling and displacement in oocytes

For two-step QD-SERT labeling, oocytes were first incubated with 1 μM biotinylated ligand in PBS for 60 minutes prior to 5 minutes incubation of 2.5 nM SA-QD (Qdot® 655 streptavidin conjugate, Invitrogen, Carlsbad, CA).

The pre-incubation affinity assay (Fig. 2 column 3 and 4).was demonstrated using paroxetine and 5-HT as testing drugs. In pre-incubation affinity assay, oocytes were first incubated with testing drug (1 μM for paroxetine; 1 mM for 5-HT) in PBS for 60 minutes, followed by a ligand/drug mixture incubation (1 μM/1 μM for paroxetine; 1 μM/1 mM for 5-HT) in PBS for another 60 minutes, and then finally incubated with 2.5 nM SA-QD for 5 minutes. After QD labeling, single oocytes were transferred to 8-well Lab-Tek chamber slides (NUNC, Roskilde, Denmark) and imaged in PBS. Excess ligand, drug, and QD were removed by two washes with PBS at each step.

For time course displacement assay demonstrated in Fig. 3, selected single hSERT expressing oocyte was transferred to one well of Lab-Tek 8-well chamber slide after processing two-step QD-SERT labeling, each well contain either 0 μM (control), 10 μM or 20 μM of paroxetine in 400 μL PBS. A time series of fluorescent images was immediately acquired at a 1 minute interval over a 30 minute period.

Microscopy

Confocal images were obtained on a Zeiss LSM 510META confocal imaging system (Carl Zeiss Microimaging, Inc., Thornwood, NY). Images were collected using a Zeiss Plan-Apochromat 5×/0.16 numerical aperture (NA) objective lens and excited by an argon laser at 458 nm with 25% transmission rate and pinhole was set to 2 Airy units. All images were 512×512 pixels in size and had an 8-bit pixel depth. For single ligand labeling experiments, fluorescence signal was collected on photomultiplier-tube (PMT) detector after passing through a 650 nm cutoff filter to ensure the transmission of only the Qdot 655 signal.

Wide-field fluorescent images were acquired using a Zeiss Axiovert 200 M inverted fluorescence microscope equipped with a Photometrics Cool-Snap™ HQ2 electrically cooled CCD camera (Intelligent Imaging Innovations, Denver, CO), a Zeiss Plan-Neofluar 20×/0.4 numerical aperture (NA) objective lens and QD655 filter set (XF 1002 filter, Omega Optical, Brattleboro, VT). Exposure time was set at 200 ms for all fluorescent imaging. Image acquisition and analysis was processed using Metamorph® 7 imaging software (Molecular Devices Corp.; Downingtown, PA).

Two-electrode voltage-clamp electrophysiological analysis

Whole-cell currents were measured with two electrode voltage clamp techniques using a GeneClamp 500 (Molecular Devices, Palo Alto, CA). Microelectrodes were pulled using a programmable puller (Model P-87, Sutter Instrument, Novato, CA) and filled with 3 M KCl (0.5-5 MΩ resistance). A 16-bit A/D converter (Digidata 1322A, Molecular Devices) interfaced

to a PC computer running Clampex 9 software (Molecular Devices) was used to control membrane voltage and to acquire data. To induce hSERT-associated current, serotonin was dissolved (typically 10 μM) in a buffer solution (in mM, 120 NaCl, 5.4 potassium gluconate, 1.2 calcium gluconate, 7.2 HEPES, pH 7.4) and applied to oocytes using a gravity-flow perfusion system (4-5 ml/min flow rate). Serotonin-induced current is defined by subtraction of current in the presence of serotonin from current in the absence of serotonin. For recordings, data were low-pass filtered at 10 Hz and digitized at 20 Hz. Analyses were performed using Origin 7 (Origin Lab, Northampton, MA).

Data Analysis

For displacement assays, each time-series of wide-field fluorescence images was analyzed using Metamorph's active region measurement program. Briefly, an initial active region was selected from the membrane halo region of the fluorescent image, and this same region was applied to each fluorescence image for subsequent time flame. The correlation of background signal was performed by subtracting the result from the background image on a pixel-by-pixel basis. Background signal of the CCD detector was measured by taking the fluorescence image under the same experimental setup without sample loading. Data were measured as relative fluorescent units (RFUs) and normalized to the following equation:

$$normalized\ response = \frac{F_t}{F_0} \qquad \text{(Eq. 1)}$$

where F_t is the fluorescent signal generated in the presence of paroxetine at time t; F_0 is the initial fluorescent intensity generated in the absence of paroxetine.

Data were evaluated by fitting to either a linear or single exponential equation for control experiment and Paroxetine displacing experiment, respectively. For the control experiment, each time course data points were fit into a linear curve regression equation:

$$\frac{F_t}{F_0} = \frac{F_\infty}{F_0} + A \cdot t \qquad \text{(Eq. 2)}$$

where F_t is the fluorescent signal generated in the presence of paroxetine at time t; F_0 is the initial fluorescent intensity generated in the absence of paroxetine, F_∞ is the fluorescent intensity at infinite time, and A represents amplitude. For the paroxetine displacing experiment, the time courses were fit into a single-exponential equation:

$$\frac{F_t}{F_0} = \frac{F_\infty}{F_0} + B \cdot e^{-k_{app} \cdot t} \qquad \text{(Eq. 3)}$$

where F_t is the fluorescent signal generated in the presence of paroxetine at time t; F_0, the initial fluorescent intensity generated in the absence of paroxetine; F_∞, the fluorescent intensity at infinite time; k_{app}, the apparent rate constant; B, the amplitude. All the fitting curves were generated by using Sigmaplot™ software (Version 11.2, Systat Software Inc., San Jose, CA).

Measurements of each reaction kinetics for QD-based displacement assays were performed by taking the natural logarithm of each time courses fluorescent data point generated from the paroxetine displacing experiments and then plotted as a function of time. First-order dissociation was evaluated by fitting the 0-10 minute data points to the linear curve regression using Sigmaplot software.

Note that the SA-QDs we used in this study have potentially 4-10 streptavidins/QD, suggesting a possible 16-40 binding sites/QD.[5] A recent study reported that this particular kind of SA-QDs has ~5 biotin binding sites/QD.[6] As can be seen in Fig. 3B, the dissociation of

QD-ligand from hSERT proteins can be categorized into roughly two stages where the first 10 minutes seems significantly faster. Further analysis in Fig. 3C indicated that a plot of ln(Ft/F0) as a function of time at the initial 10 minutes appear to be linear, a character of first-order dissociation, suggesting the binding valency is monovalent. As illustrated in the figure below, we recognize that it is possible that the displacement in our platform may be indicative of a two-stage dissociation process in which the initial monovalent dissociation is followed by a multivalent dissociation. A solution to resolve this issue is to introduce the monovalent streptavidin conjugated QDs developed by Ting and coworkers.[5]

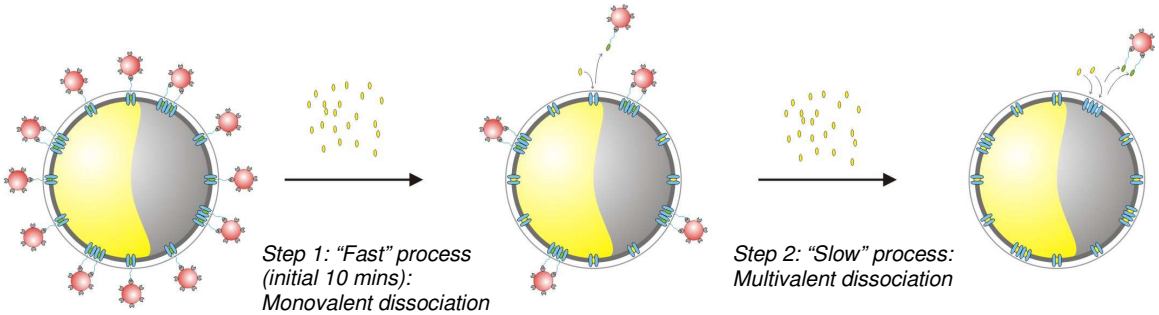


Figure S1

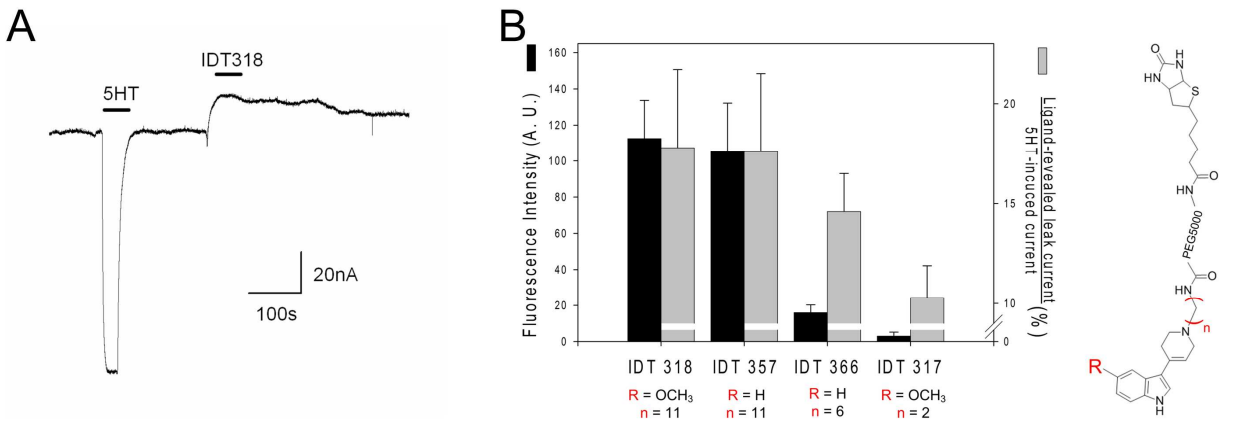


Figure S1. (A) Current response induced by IDT318 ligand incubation. Figure shown here is a representative recording from a single hSERT expressing oocyte voltage-clamped at -60 mV. The leakage current profile after incubating with IDT318 displays a typical SERT antagonist behavior.[7] (5HT: 5-Hydroxytryptamine, serotonin) (B) Comparison of current responses with QD labeling results for the indication of alkyl space participated in ligand binding. We recently published the current responses of the IDT318 and its structure-related ligands (left scale) using hSERT expression oocytes. Here we compared the current responses with QD labeling results (right scale). As can be seen, results generated by both methods show a similar trend as the length of alkyl spacer increases. However, the QD labeling result shows higher sensitivity as evident by a smaller standard deviation. (Fluorescent results: n = 6 oocytes/group; Current results: n = 4 oocytes/group)

Figure S2

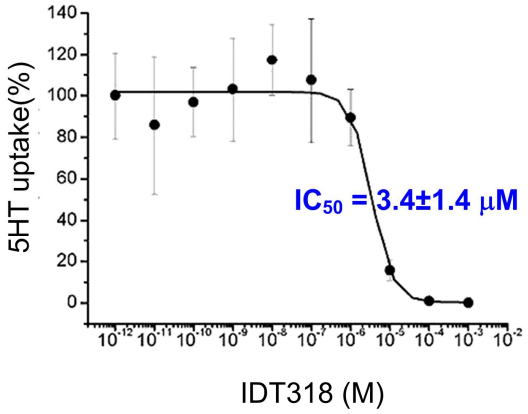


Figure S2. IC_{50} measurement of IDT318 ligand. The ability of IDT318 to inhibit the uptake of serotonin was measured by incubating hSERT expressing oocytes in the presence of 50 nM tritiated serotonin and increasing concentrations of IDT318. The accumulated radioactivity was plotted against concentration of IDT318. The IC_{50} of IDT318 was found to be 3.4 ± 1.4 μM (mean ± SD).

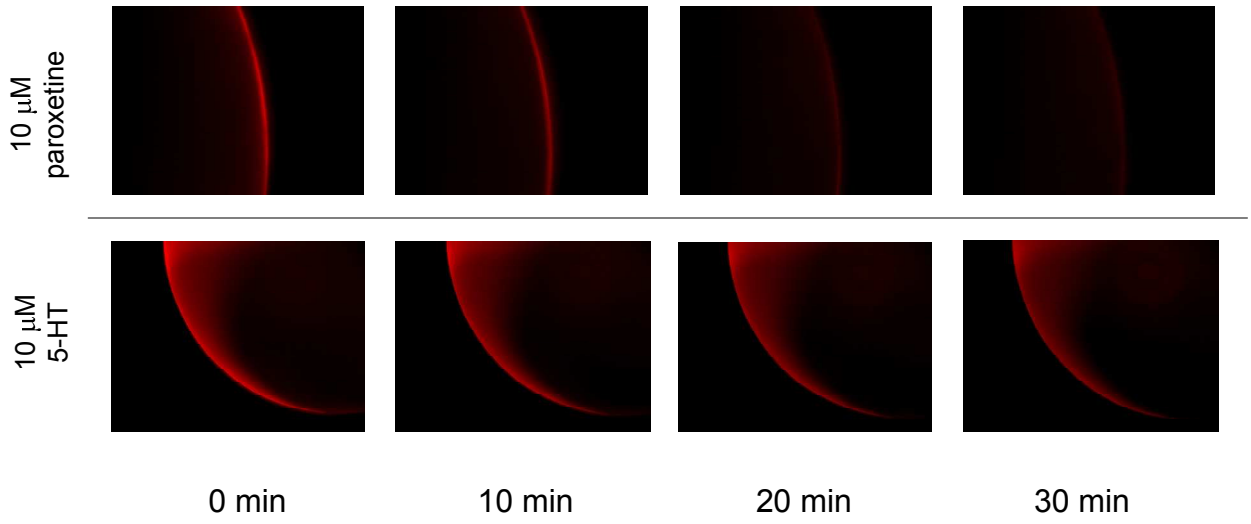


Figure S3. Displacement of the IDT318 conjugated QD with 10 μM paroxetine and 5-HT. Results are representative micrographs from at least 3 independent experiments.

References:

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