High-throughput evolution of near-infrared serotonin nanosensors

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Imaging neuromodulation with synthetic probes is an emerging technology for studying neurotransmission. However, most synthetic probes are developed through conjugation of fluorescent signal transducers to pre-existing recognition moieties such as antibodies or receptors. We introduce a generic platform to evolve synthetic molecular recognition on the surface of near-infrared fluorescent single-wall carbon nanotube (SWCNT) signal transducers. We demonstrate evolution of molecular recognition toward neuromodulator serotonin generated from large libraries of ~6.9 x 10^16 unique ssDNA sequences conjugated to SWCNTs. This probe is reversible and produces a ~200% fluorescence enhancement upon exposure to serotonin with a K_d = 6.3 μM, and shows selective responsivity over serotonin analogs, metabolites, and receptor-targeting drugs. Furthermore, this probe remains responsive and reversible upon repeat exposure to exogenous serotonin in the extracellular space of acute brain slices. Our results suggest that evolution of nanosensors could be generically implemented to develop other neuromodulator probes with synthetic molecular recognition.

INTRODUCTION
Optical probes are of fundamental importance for advancing our understanding of biological processes such as neurotransmission. Specifically, serotonin, or 5-hydroxytryptamine (5-HT), is a monoamine neuromodulator that plays diverse roles in the central nervous system and is critically implicated in the etiology and treatment of mood and psychiatric disorders (1). 5-HT also plays critical roles in modulating memory and learning (2, 3) and in the regulation of mood (4), sleep (5), and appetite (5). As such, aberrations in 5-HT signaling are thought to underlie multiple mental health disorders including major depressive disorder (6), bipolar disorder (7), autism (8), schizophrenia (9), anxiety (10), and addiction (11). Classical neurotransmission occurs through rapid activation of ligand-gated ion channels in which neurotransmitter signaling is confined at the synaptic cleft and thus to singular synaptic partners (12). Conversely, 5-HT acts primarily as a neuromodulator and may undergo signaling through extrasynaptic diffusion, enabling a few neurons to affect broader networks of neuronal activity through 5-HT volume transmission. As such, there is great interest in monitoring the spatial component of 5-HT modulation in addition to its temporal dynamics. Current methods for measuring 5-HT and other neuromodulators include tools adapted from analytical chemistry to measure 5-HT in the brain extracellular space with varying levels of selectivity and spatiotemporal resolution: Fast-scan cyclic voltammetry leverages redox chemistry to enable rapid temporal measurement of 5-HT by inserting a carbon electrode into brain tissue (13). Microdialysis is an analytical chemistry method in which fluid samples from brain tissue are subject to downstream analysis with chromatography (14). Recently, a field-effect transistor sensor modified with a 5-HT aptamer was reported to exhibit a highly sensitive and selective electrochemical response to 5-HT under physiological conditions (15). However, previous methods suffer from limited spatial resolution, and microdialysis sampling occurs on the order of minutes, which precludes assaying fast 5-HT modulatory transients in the brain. While a few optical probes for 5-HT of varying selectivity have been developed, to date, none have been implemented for monitoring 5-HT modulation on spatiotemporal scales relevant to monitoring endogenous 5-HT kinetics (16–21). This lack of tools to probe the dynamics of neuromodulators such as 5-HT stems from the difficulty in developing synthetic optical probes for extracellular analytes.

To address the challenge of reliable generation of synthetic neuromodulator probes, we describe a generic platform for evolution of molecular selectivity and demonstrate its utility by developing a nanosensor for neuromodulator 5-HT. Our approach entails systematic evolution of ligands by exponential enrichment implemented on single-wall carbon nanotube (SWCNT) surfaces, a process we have termed SELEC. Specifically, our near-infrared 5-HT probe (nIRHT) is developed through nanosensor evolution in which large libraries of more than 10^10 polynucleotide-SWCNT conjugates are evolved for 5-HT selectivity through iterative enzymatic amplification of 5-HT–selective polymers and deep sequencing to identify unique polynucleotide sequences that confer 5-HT selectivity when pinned to SWCNT surfaces. Molecular recognition and selectivity for 5-HT over its metabolic precursors, 5-HT metabolites, and 5-HT receptor drugs are conferred through surface adsorption of single-stranded polynucleotides to the SWCNT surface. We show that six rounds of polynucleotide–SWCNT evolution enable generation of multiple candidate optical sensors, whereby the best-responding construct from the evolution cohort, nIRHT, is next characterized as a 5-HT optical probe.

We further assess nIRHT for potential use as a 5-HT imaging probe with appropriate spatiotemporal resolution for imaging endogenous 5-HT modulation. nIRHT responds to 5-HT with a change in fluorescence, ΔF/F_0, of up to 194 ± 6% in the nIR fluorescence emission window of 1000 to 1300 nm and is shown to bind 5-HT with a K_d of 6.3 μM (R^2 > 0.99). We further demonstrate that nIRHT is reversible and exhibits unaltered performance in artificial...
cerebrospinal fluid (aCSF). Of importance to understanding pharmacology in the context of 5-HT signaling, we show that nIRHT does not respond to 5-HT metabolites of 5-hydroxyindoleacetic acid (HIAA), 5-hydroxytryptophan (HTP), and 5-methoxytryptophan (MTP) and 5-HT receptor-targeting drugs, such as fluoxetine, 3,4-methylenedioxymethamphetamine (MDMA), 25I-NMOMe, and quetiapine. Last, we demonstrate that nIRHT can be introduced into the brain extracellular space in acute striatal brain slices and can be implemented to image exogenous 5-HT. While we have implemented SELEC to develop an optical probe for 5-HT here, the platform is fundamentally generic for generating other optical probes of neurological relevance.

RESULTS

SELEC enables high-throughput screening of single-stranded DNA–SWCNT constructs for 5-HT neuromodulator sensitivity

Previous work has demonstrated that semiconducting nIR fluorescent SWCNT, when noncovalently functionalized by amphiphilic polymers, such as single-stranded DNA (ssDNA), can generate synthetic molecular recognition elements on the SWCNT surface. The resulting ssDNA-SWCNT moiety can then selectively bind a molecular analyte for its optical detection (22–25). The process of generating ssDNA-SWCNT nanosensor candidates, and their screening against target analytes, has since revealed optical nanosensors for neurotransmitters including a (GT)₆-SWCNT nanosensor for dopamine (24), which was subsequently used to image dopamine dynamics in the brain striatum and capture the influence of dopamine receptor drugs on dopamine dynamics at the level of individual synapses (26). Despite the nascent utility of SWCNT-based nanosensor technology, the nanosensor screening process involves a heuristic approach in which each nanosensor candidate must be synthesized individually before screening, limiting candidate nanosensor libraries to a few dozen, thus restricting the throughput of nanosensor discovery. Here, we develop and validate an evolution-based approach for producing ssDNA-SWCNT nanosensors in which upward of 6.9 × 10¹⁰ unique ssDNA sequences are assessed for their ability to bind to a molecular analyte and produce an optical response. This strategy is inspired by SELEX protocols for aptamer generation (27) and entails systematic evolution of ssDNA ligands by exponential enrichment following adsorption to carbon nanotubes (SELEC), which we implement to identify ssDNA-SWCNT constructs that optically respond to neurotransmitter 5-HT.

For both the experimental and control libraries, we found that a total of six consecutive rounds of competitive ssDNA SWCNT binding, desorption, recovery, and amplification of high-affinity ssDNA binders was sufficient to generate several promising 5-HT candidate nanosensors.

The 50 most numerous, thus the putatively highest-affinity, sequences in the sixth round of the experimental group were identified, and their corresponding frequencies in the total rounds were plotted in Fig. 1B (see tables S1 and S2 for sequences of the top 10 binders). We observed a clear enrichment in the 50 highest-affinity sequences following the sixth round of SELEC, in which each sequence has a mean frequency value of 19 × 10⁻⁶. Conversely, those sequences have mean frequency values of 1.6 × 10⁻⁶ and 3.8 × 10⁻⁶, respectively, in rounds 4 and 5 and negligible or zero frequency preceding round 4. Next, the identity of each nucleotide base was evaluated with respect to position within the top 200 ranked sequences in each library. The round 2 experimental library showed a near random distribution of nucleotides at each position, with sequence identities near the ~25% expected value at each position (Fig. 1C). Following six rounds of SELEC, base identity preference trends emerged in the experimental (Fig. 1D) and control (fig. S1) libraries. For instance, nucleotide A was preferred with a frequency of 86 and 65% in the experimental and control libraries at position 7, the first nucleotide following the 5‘ (C)₆ spacer. The experimental library round 6 sequences showed 65% C and 62% T identities at positions 23 and 24, respectively, indicating a preference toward a 3‘ CT terminus within the random region of the sequence. This preference was not observed in control library round 6, which instead showed enrichment of a GNG motif (fig. S1). Libraries were analyzed further by counting the incidences of nucleotide trimers with respect to position in the top 200 ranked sequences of each library. This analysis produced a trimer frequency table per nucleotide position for each control and experimental libraries. Classical multidimensional scaling (MDS) was performed on these frequency tables to evaluate the statistical difference between libraries. MDS reduces the dimensionality of library frequency tables into a two-dimensional (2D) projection, preserving the distances between trimer counts across libraries. A 2D MDS plot on first and second principal coordinates showed that the experimental and control libraries at round 2 could not be distinguished, but the divergence between the experimental and control libraries became considerable by round 6 (Fig. 1E). Round 6 divergence was driven, in part, by differences in abundance of repeating patterns such as AC and GT repeats. These differences were quantified by plotting counts of palindromic trimers such as ACA and CAC, and GTG and TGT in the top 200 ranking sequences with respect to position (fig. S2 and Fig. 1, F and G). The repeating AC motif was preferred in both experimental and control SELEC groups and predominately located near the 5‘ end. The repeating GT motif showed the largest contrast of motif population between experimental and control groups, which was selectively enriched at the 3‘ end of the control group and not in the experimental group. We also performed direct principal components analysis of the top 200 ranked sequences in each SELEC
library. The first and second principal component axes do not distinguish between the experimental and control libraries in the first four rounds; however, divergence is observed in round 5, and distinct clustering of the experimental from control libraries can be observed by round 6 (fig. S3). Together, these results suggest that SELEC can evolve to select ssDNA sequences with high affinity for SWCNT in the presence and absence of an analyte and that the sequences evolved in each are nearly mutually exclusive, with only one common sequence in the top 1000 sequences of both libraries at round 6. We therefore hypothesize that the experimental library

Fig. 1. SELEC evolves ssDNA-SWCNT nanosensors for 5-HT molecular recognition. (A) Schematic illustration of the SELEC evolution process. SWCNTs are mixed with a >10-fold mass excess of ssDNA with 100 μM 5-HT (experimental library) or alone (control library). (1) The mixture is sonicated to generate the ssDNA-SWCNT complex of either 5-HT–bound ssDNA-SWCNT (blue) or ssDNA-SWCNT (red). (2) Unbound ssDNAs are removed, and (3) bound ssDNAs are isolated from SWCNT by thermal desorption. (4) Isolated ssDNAs are amplified by PCR, and the ssDNA pool is prepared for the next selection round and (5) characterized by high-throughput sequencing. (B) Sequence frequency of the top 50 unique sequences in the sixth round of the experimental library, compared to their sequence prevalence in rounds 2 to 5. (C and D) Probability for each nucleotide at each position in the experimental library calculated from the top 200 sequences from (C) round 2 and (D) round 6. (E) MDS plot of trimer frequency table for the top 200 sequences in the experimental and control SELEC libraries at each round. Data are plotted on the first and second coordinates of MDS analysis. Contour lines are included for better visualization of mutual divergence between experimental and control SELEC libraries for the same round. Divergence at round 2 may be too small to be distinguished. (F and G) Palindromic trimer incidences of (GTG)/(TGT) at each nucleotide position for (F) experimental and (G) control libraries.
will evolve ssDNA sequences that are selective for 5-HT when bound to SWCNT, whereas the control library will evolve ssDNA sequences that exhibit highest affinity for the SWCNT surface.

**In vitro characterization of nIRHT optical nanosensor identified from SELEC evolution**

We next analyzed the sensitivity of SELEC-evolved sequences to 5-HT when adsorbed to the surface of SWCNT. We denote sequences in experimental group as EN#M (Mth place sequence of round N in the order of frequency) and sequences from the control group as CN#M. ssDNA-SWCNT nanosensor candidates were synthesized with the C₆ anchor domains, but without the primers used for PCR-based amplification. Omission of primer regions from testing ssDNA-SWCNT fluorescence responses was implemented to increase the mass density of the evolved N-18 library on the SWCNT surface. This omission was performed following confirmation that both C₆-N₁₈-C₆ and primer-C₆-N₁₈-C₆-primer SELEC-evolved sequences from the experimental cohort showed enhanced sensitivity toward 5-HT, albeit with a reduced sensitivity for the latter, as predicted (fig. S4).

We prepared C₆-N₁₈-C₆ ssDNA-SWCNT constructs from the top 10 most abundant sequences from the experimental and control groups for SELEC rounds 3 to 6 and tested the ΔF/F₀ fluorescence response of each ssDNA-SWCNT construct to addition of 100 μM 5-HT in phosphate-buffered saline (PBS) (figs. S5 and S6). ΔF/F₀ is calculated as (F − F₀)/F₀ based on baseline fluorescence intensity before analyte addition (F₀) and fluorescence intensity 10 s after analyte addition (F) at 1195-nm emission. Multiple peaks in the fluorescence and absorption spectra of the nIRHT nanosensor showed the presence of different (n,m) SWCNT chiralities in ensemble mixture (Fig. 2C and fig. S7) (30), whereby the magnitude of the nIRHT nanosensor response to 5-HT is dependent on the (n,m) SWCNT chirality (fig. S8). Therefore, for consistency in nanosensor analysis, we use the optical response of the ~1195-nm nanosensor emission peak corresponding to the (8,6) SWCNT chirality, unless otherwise noted. As shown in Fig. 2A, we find that there is a statistically insignificant (P = 0.055) but qualitatively noticeable increase in ssDNA-SWCNT sensitivity for 5-HT at the final SELEC round, relative to the baseline fluorescence modulation of ΔF/F₀ = 0.73 for the “unevolved” ssDNA-SWCNT sample prepared with the starting ssDNA library. Although the sensitivity difference is statistically insignificant (P = 0.184) between the experimental and control SELEC groups at round 6, enhanced sensitivity toward 5-HT is most evident for the experimental library in which highly 5-HT sensitive constructs (ΔF/F₀ > 1.2) are only found in the experimental SELEC group and not in the control group (Fig. 2B). We identified the most 5-HT sensitive construct from both libraries and all SELEC rounds as E6#9 from the experimental library, which exhibited a ΔF/F₀ of 1.94 ± 0.06 upon exposure to 100 μM 5-HT and could thus be used as a nIRHT nanosensor. We prepared E6#9 ssDNA-SWCNT with different mass ratios of ssDNA and SWCNT and found that the mass ratio does not affect the nanosensor response to 100 μM 5-HT (fig. S9). We observed the turn-on response of nIRHT to 5-HT to be nearly instantaneous followed by a low fluorescence decay with τ₁/₂ > 100 s, which we confirmed is not a result of 5-HT structural or chemical change (figs. S10 and S11).

We next characterized nIRHT for use as a 5-HT brain imaging probe. We assessed the dynamic range of nIRHT to a 100 nM to 100 μM range of 5-HT concentrations and showed nIRHT sensitivity for 5-HT over a 100 nM to 50 μM dynamic range (Fig. 2D), largely suitable for measuring endogenous 5-HT dynamics, which are predicted to fall in the broad ~100 pM to ~1 μM concentration range (31–35). nIRHT showed a ΔF/F₀ = 0.20 ± 0.03 upon exposure to 1 μM 5-HT with a high signal-to-noise ratio of 12.4. Fitting nIRHT responsivity toward 5-HT to a Hill equation shows a micro-molar dissociation constant (Kₘ) of 6.3 μM (R² > 0.99) for 5-HT (Fig. 2D), a Kₘ previously identified as optimal for imaging neuromodulation to balance nanosensor reversibility for capturing fast transients (36). We further confirmed that the ssDNA sequence for nIRHT has selectivity for 5-HT through a solvatochromic shift assay with sodium cholate (SC) (fig. S12) and also confirmed that nIRHT shows a stable and reproducible fluorescence response to 5-HT for over a week after synthesis (figs. S13 and S14). We next compared the sensitivity of nIRHT to an ssDNA-SWCNT construct from the control SELEC group, C₆#8, chosen for its strong affinity to the SWCNT surface (fig. S12). When bound to SWCNT, C₆#8 showed reduced affinity (Kₘ = 41 μM) and a suppressed optical response (ΔF/F₀ = 0.53 ± 0.01 at 100 μM 5-HT) compared to nIRHT (Fig. 2D). These results suggest that SELEC can be implemented to evolve ssDNA-SWCNT selectivity for an analyte such as 5-HT and that nIRHT can serve as an optical reporter for 5-HT imaging.

**Characterization of nIRHT as a nanosensor for 5-HT imaging in brain tissue**

We next explored the utility of nIRHT for use as an optical probe to image extracellular 5-HT signaling in brain tissue. The response of nIRHT for 5-HT was assessed in aCSF, a common medium for imaging experiments in brain slice preparations. In aCSF, nIRHT demonstrated marginally superior performance than in PBS, with ΔF/F₀ = 2.45 ± 0.07 upon exposure to 100 μM 5-HT and Kₘ = 10 μM (Fig. 3A). We next examined the selectivity of nIRHT for 5-HT over other neurotransmitters. nIRHT nanosensor exhibited negligible ΔF/F₀ response upon exposure to 100 μM acetylcholine, γ-aminobutyric acid, glutamate, tyrosine, and octopamine (Fig. 3B). Dopamine and norepinephrine, catecholamine neurotransmitters, induced a moderate response of ΔF/F₀ = 1.40 ± 0.03 and 1.06 ± 0.03 upon addition of 100 μM dopamine and norepinephrine, respectively. Notably, nIRHT exhibited a fivefold higher affinity for 5-HT over dopamine (Kₘ = 6.3 μM for 5-HT and Kₘ = 33 μM for dopamine) (fig. S15). We next measured nIRHT selectivity against 5-HT metabolites HTP, HIAC, and MTP, considering their high structural similarities to 5-HT. Addition of 100 μM HTP, HIAC, and MTP to nIRHT induced minimal fluorescence modulation of ΔF/F₀ = 0.02 ± 0.02, 0.17 ± 0.10, and −0.14 ± 0.03, respectively. We also studied the ability of nIRHT to measure 5-HT in the presence of interfering molecules. nIRHT preincubated with 100 μM dopamine, norepinephrine, or HIAC exhibited attenuated fluorescence response to 100 μM 5-HT with ΔF/F₀ = 0.09 ± 0.01, 0.12 ± 0.03, and 0.92 ± 0.12, respectively (fig. S16). Last, given the relevance of 5-HT receptor drugs on the study of 5-HT modulation and pharmacology, we assessed selectivity of nIRHT against non-selective agonists fluoxetine and MDMA, 5-HT₂ agonist 2S1-NMOMe, and 5-HT₁A agonist quetiapine. Exposure of nIRHT to 100 μM fluoxetine, MDMA, 2S1-NMOME, and quetiapine induced negligible fluorescence modulation, and we additionally confirmed that 5-HT could be detected without attenuation even if nIRHT is preincubated with, and remains in the presence of, 1 μM each of these drugs (Fig. 3C and fig. S17).
Toward endogenous 5-HT imaging, we next investigated the reversibility of the nIRHT nanosensor by immobilizing nIRHT in a microfluidic slide and imaging individual nIRHT nanosensors upon successive infusions of 100 μM 5-HT. nIRHT nanosensors were immobilized onto a 3-aminopropyltriethoxysilane (APTES)–treated glass slide and placed in a flow chamber with a syringe pump. The nanosensor chamber was exposed to three cycles of 100 μM 5-HT in PBS subsequently rinsed with PBS only, where the nIRHT nIR fluorescence response was imaged on an inverted microscope through a 900-nm longpass filter to obtain nIR fluorescence from 900 to 1700 nm, as previously described (Fig. 3, D to F, and movie S1) (26). We observed nIRHT fluorescence increase and decrease as predicted by the influx and washing of 5-HT, respectively. Specifically, the time-dependent nIR fluorescence of three individual nanosensors was measured across three cycles and were found to be ∆F/F₀ = 1.55 ± 0.01, 1.71 ± 0.08, and 1.10 ± 0.14 (means ± SD for three cycles), which indicates that nIRHT nanosensors are reversible (Fig. 3G). We suggest that the variation in ∆F/F₀ among nanosensors is a result of the chirality-dependent variability in SWCNT response as observed in bulk measurements. We further demonstrated that surface-immobilized SWCNT, without the ssDNA coating, do not respond to 5-HT, confirming the molecular recognition role of the ssDNA polymer when adsorbed to the SWCNT surface (fig. S18). Previous studies of ssDNA-SWCNT nanosensors have confirmed millisecond-scale nanosensor reversibility kinetics (24, 26), the absence of photobleaching (37), and in vivo compatibility (38), suggesting that nIRHT could serve as an optical probe for long-term endogenous 5-HT imaging in the brain.

Last, we tested nIRHT nanosensor compatibility for 5-HT imaging in acute brain slices. Coronal mouse brain slices of 300 μm thickness were incubated with a nIRHT suspension (2 μg/ml) for 15 min and subsequently rinsed with aCSF to remove excess nIRHT. This labeling procedure was previously found to label acute slices with nanosensor localization in the brain extracellular space (26). nIRHT-labeled acute brain slices were coincubated with a cell-penetrating calcein-AM (acetoxymethyl) fluorescent dye to stain the live cell bodies, and we observed negligible colocalization (Pearson’s correlation coefficient Rₚ = −0.150) between the fluorescence signal from calcein-AM and nIRHT (fig. S19), confirming that nIRHT nanosensors remain extracellular. We next imaged nIRHT-labeled slices in aCSF on a custom-built nIR fluorescence upright epifluorescence microscope equipped with a 900-nm longpass filter to obtain nIR fluorescence from 900 to 1700 nm (Fig. 3H). Time-dependent fluorescence images were recorded to confirm stable nIR fluorescence of nanosensors when no external stimuli were present (movie S2). We then investigated the ability of nIRHT nanosensors to respond to 5-HT in slice. Following acquisition of 1-min fluorescence baseline, bath application of 100 μM 5-HT resulted in a nIRHT fluorescence increase of ∆F/F₀ = 0.35 ± 0.26 (means ± SD) (Fig. 3, I and J, fig. S20, and movie S3). While nanosensor labeling in slice shows a heterogeneous distribution in the tissue, previous work has shown that nanosensor labeling density does not correlate with endogenous
neuromodulator $\Delta F/F_0$ response (26). Rinsing of the slice imaging chamber with aCSF confirmed that nIRHT fluorescence when in slice returns to baseline in the absence of 5-HT (Fig. 3K). Furthermore, sequential 5-HT perfusion and aCSF rinsing of the same nIRHT-labeled brain slice over the course of 1 hour generated consistent and reproducible nIRHT fluorescence response without noticeable attenuation of the nanosensor response (fig. S21). We further confirmed that nIRHT response to exogenous 5-HT can be observed for multiple biological replicates with modest variation ($\Delta F/F_0 = 0.35 \pm 0.26$ to $0.67 \pm 0.14$) in fluorescent response to addition of 100 $\mu$M 5-HT. We attribute the variability in nIRHT response across animals and brain slices to variable diffusive characteristics of 5-HT through the heterogeneous extracellular microstructures of brain tissue (fig. S22). As a control, an acute striatal brain slice labeled with a minimally 5-HT–responsive construct from our control library, C6#8-SWCNT, showed minimal $\Delta F/F_0 = 0.18 \pm 0.03$ (means $\pm$ SD fluorescence modulation in slice upon bath application of 100 $\mu$M 5-HT) (fig. S23). We also labeled an acute slice with SWCNT coated...
with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG-SWCNT), which has near-zero response upon exposure to 100 μM 5-HT, and this slice did not exhibit observable fluorescence response upon exogenous perfusion of 100 μM 5-HT (fig. S24). Last, we tested the robustness of the nIRHT nanosensor against endonuclease degradation by incubating nanosensor with S1 nuclease. We used the highest suggested concentration of endonuclease at 1000 U/ml, but note that nuclease concentrations in the brain extracellular space are unlikely to reach these levels. While free ssDNA degrades fully by 1 hour, ssDNA on SWCNT remains 78% intact at this time point (fig. S25), suggesting that surface adsorption promotes ssDNA biostability and motivates nIRHT use in tissue.

**DISCUSSION**

High spatiotemporal imaging of neuromodulatory signaling is essential to understand the dynamics of neuromodulator release and uptake in neural circuits and to elucidate the function of chemical neuromodulation in animal behavior (39). Synthetic probes are an attractive approach to imaging neuromodulators such as 5-HT owing to their photostability, compatibility with pharmacology, and feasibility for use in genetically tractable organisms. However, their generation depends either on trial-and-error screening for synthetic molecular recognition or conjugation of preexisting molecular recognition elements to nanomaterial signal transducers. Of note, the process of tethering the recognition moiety to a surface, particularly with nanomaterial signal transducers, often compromises the fidelity, specificity, and sensitivity of recognition elements due to surface steric effects, further limiting advances in synthetic optical probes. Here, we develop an approach to generate nIR optical probes through evolution of ssDNA-SWCNT selectivity toward 5-HT. ssDNA-SWCNT constructs whose polymer corona phase recognizes 5-HT were evolved from an initial 18-mer nucleotide library of >10^10 unique sequences by inducing competitive complexation between ssDNAs and SWCNTs in the presence of 5-HT through a process we termed SELEC. Previous studies have shown that analytes that induce selective fluorescence modulation of SWCNTs also stabilize the ssDNA corona (24); thus, we reason that evolved 5-HT-recognizing sequences may preferentially populate the evolved ssDNA pool via stabilization of the 5-HT-ssDNA-SWCNT constructs. Conversely, the control SELEC library evolved in the absence of 5-HT should contain ssDNA sequences with high affinity for the SWCNT surface but not specifically for 5-HT. We find that pE6#9-SWCNT (the 5-HT–responsive nIRHT nanosensor with PCR primer regions) is stabilized by the presence of 5-HT substantially more so than pR0-SWCNT (the initial unevolved ssDNA library) (fig. S9). This result further indicates the ability of SELEC to evolve ssDNA-SWCNT nanosensors for selective recognition of analytes such as 5-HT. Together, these data demonstrate that SELEC can be implemented to rapidly screen large libraries of ssDNA-SWCNT constructs to evolve selectivity for small biomolecule analytes, circumventing low-throughput screening of these constructs previously used for SWCNT-based nanosensor development. It is feasible that SELEC could be implemented for other ssDNA-SWCNT technologies such as SWCNT-based chirality separation (30, 40, 41), which is strongly dependent on SWCNT surface adsorption of specific ssDNA sequences. Furthermore, because SELEC is fundamentally agnostic to the biomolecule analyze(s), SELEC may be implemented to develop nIR optical probes for other small-molecule targets of interest.

By implementing SELEC to evolve molecular recognition for neuromodulator 5-HT, we identified a nanosensor, nIRHT, with ΔF/F₀ of 1.94 ± 0.06 in PBS and ΔF/F₀ of up to 2.45 ± 0.07 in aCSF. Furthermore, we demonstrated that nIRHT provides a selective optical response for 5-HT against 5-HT metabolites, other neuromodulators, and pharmacology of relevance to 5-HT neuromodulation. While neurotransmitter dopamine also induced a moderate ΔF/F₀ = 1.36 fluorescence increase of nIRHT (Fig. 3B), nIRHT exhibits a fivefold greater affinity for 5-HT over dopamine with K_d,5-HT = 6.3 μM and K_d,dopamine = 33 μM (fig. S11). In the future, implementing an additional “positive” selection library could help identify sequences with domains uniquely responsive to interfering molecules and eliminate those motifs from positive hits for the target ligand. It is noteworthy that the synthetically evolved nIRHT nanosensor shows negligible cross-responsivity with 5-HT precursors and metabolites and 5-HT receptor agonists and antagonists. Many optical probes for neurochemicals are based on proteins engineered from endogenous receptors and thus show cross-reactivity with receptor-targeting drugs. However, because the molecular recognition sites of nIRHT nanosensors are synthetic in nature, we demonstrate that nIRHT nanosensors do not show fluorescence modulation upon exposure to 5-HT receptor drugs fluoxetine, MDMA, 25I-NMOMe, and quetiapine (Fig. 3C). Thus, nIRHT optical probes and others of its class could enable insights into synaptic-level effects of 5-HT receptor agonists and antagonists to further our understanding of pharmacology of relevance to major depressive disorder (6), bipolar disorder (7), autism (8), schizophrenia (9), anxiety (10), and addiction (11), among others.

In comparison to the reported methodologies for 5-HT dynamic imaging such as FSCV (fast-scan cyclic voltammetry) (13), microdialysis (14), and paramagnetic 5-HT binding proteins for magnetic resonance imaging (42), nIRHT nanosensors are promising high spatiotemporal fluorescence imaging probes for studying 5-HT neuromodulation, which provide submicrometer spatial resolution, reversibility, photostability, and selectivity against 5-HT metabolites and receptor-targeting drugs. Furthermore, SWCNT fluoresce in a unique nIR window (1000 to 1300 nm), which reduces photon scattering and enhances imaging penetration depths, enabling through-cranium (43) and in vivo imaging (38). This optical window could enable the through-cranium fluorescence imaging of cortical 5-HT neuromodulation without a cranial window for chronic 5-HT imaging in animal models, although it is necessary to note that probe delivery may require cranial surgeries or injections similar to the status quo for delivery of adeno-associated viruses (43, 44). Furthermore, previous work has demonstrated that SWCNT-based neuromodulator probes are tractable for use in nonmodel species (26), further expanding the repertoire of neurological studies to be undertaken with nIRHT and other probes of its class.

**MATERIALS AND METHODS**

**Materials**

HiPCo SWCNTs were purchased from NanoIntegris (batch #27-104). Serotonin hydrochloride (hydroxytryptamine hydrochloride), dopamine hydrochloride, acetylcholine, γ-aminobutyric acid (GABA), tyrosine, glutamate, octopamine, HTP, HIAA, MTP, fluoxetine, 3,4-MDMA, 25-NMOMe, and quetiapine were purchased from Sigma-Aldrich. All ssDNA sequences were purchased from Integrated DNA Technologies (IDT; USA).
Optical characterization of SWCNT

nIR fluorescence spectra were measured with a 20× objective on an inverted Zeiss microscope (Axio Observer.D1) coupled to a spectrograph (SCT 320, Princeton Instruments) and liquid nitrogen-cooled InGaAs linear array detector (PyLoN-IR, Princeton Instruments). A 721-nm laser (OptoEngine LLC) was used as the excitation light source for all spectroscopy and imaging studies. The absorption spectra were measured with a UV-3600 Plus absorption spectrophotometer (Shimadzu). Our custom nIR spectrometer and microscope have been described in detail in previous works (45, 46).

SELEC experimental protocol

The initial ssDNA random library was purchased from IDT (USA), which consisted of 18 random nucleotides flanked by two fixed 6-mer (C)₆ and two 18-mer primer regions for PCR amplification: AGCGTCAATACACTAC-CCCCCC-N18-CCCCCCC-GAC-CACGAGCTCCATTAG. For the first round, 100 nM of the ssDNA library was dissolved in PBS buffer, and the mixture was denatured by heating at 95°C for 5 min and subsequently cooled to room temperature. For the experimental SELEC group to evolve 5-HT molecular recognition, the ssDNA library was incubated with 100 μM 5-HT for 10 min in 0.99 ml of PBS buffer. Next, 10 μg of SWCNT was added to the ssDNA and 5-HT mixture. A separate SELEC control group was prepared as described above but without the addition of 5-HT. The resulting mixtures were bath-sonicated for 2 min and next probe tip-sonicated (Cole-Parmer Ultrasonic Processor, 3-mm tip) for 10 min at 5-W power in an ice bath. After sonication, the black SWCNT suspension was centrifuged for 60 min at 16,100g to precipitate any unsuspended SWCNT, and the supernatant containing the ssDNA-SWCNT construct solution was collected. The supernatant was spin-filtered using a 100-kDa molecular weight cutoff (MWCO) centrifugal filter (Amicon Ultra-0.5, Millipore) at 6000 rpm for 5 min with deoxyribonuclease (DNase)–free water to remove unbound ssDNAs and 5-HT, and the remaining solution was collected. The spin filtration was repeated five times. Next, the purified ssDNA-SWCNT suspension was heated at 95°C for 1 h to detach surface-bound ssDNAs from SWCNT. Following heating, black SWCNT aggregates were observed, as expected when the ssDNA corona desorbs from the SWCNT surface. The ssDNA solution with aggregated SWCNT was centrifuged for 10 min at 16,100g to further pellet undissolved SWCNT, and the supernatant containing ssDNA was collected. The collected ssDNA-SWCNT library was amplified by PCR using a FAM-modified forward primer (FAM-AGCGTCAATACACACTAC) and biotinylated backward primer (biotin-CTAATGGAGCTGTCGTTGCT), following a previously described protocol (27). Standard solution conditions for PCR amplification were used as follows to maximize double-stranded DNA (dsDNA) yield while reducing by-products after amplification: 2.5 U of Hot Start Taq DNA polymerase (New England BioLabs), 1× Hot Start Taq reaction buffer, 1 μM forward primer, 1 μM backward primer, 500 μM deoxynucleotide triphosphate, and ssDNA library template (~100 ng/ml) in a total 10-ml volume for each of the 100-μl volume 96-well reaction plates. A negative control well was prepared without ssDNA library template.

A preparative PCR was performed with the following standard cycling conditions: initial denaturation for 150 s at 95°C, N cycles of denaturation for 30 s at 95°C and annealing for 30 s at 50°C and extension for 30 s at 72°C, and final extension for 180 s at 72°C. The cycle number N was determined before preparative PCR, which yielded maximal ssDNA and negligible PCR by-product formation (usually between 20 and 30 cycles). Next, 100 μl of the PCR products from each of the experimental and the control SELEC libraries was separately collected and purified with a GeneJET PCR Purification kit (Thermo Fisher Scientific) for preparation of high-throughput sequencing libraries. PCR products were confirmed by gel electrophoresis in a 4% agarose gel (low-range ultra agarose, Bio-Rad Laboratories) in 1× tris-borate-EDTA buffer (run for 18 min at 110 V). After electrophoresis, the gel was stained with SYBR Gold, and DNA bands were observed under ultraviolet light. To generate ssDNA from PCR products, 2.5 ml of streptavidin-coated beads (Streptavidin Sepharose High Performance, GE Healthcare) was placed on a sintered glass Buchner funnel (pore size, <10 μm) and washed with 10 ml of PBS buffer. The PCR product solution was incubated with the beads for 30 min and passed through the funnel. The incubation step was repeated three times to bind dsDNA onto the beads, and the beads were washed again with 10 ml of water. To elute the FAM-labeled ssDNAs, 8 ml of 0.2 M NaOH aqueous solution was added slowly to the beads, and the eluate was collected containing the FAM-labeled ssDNAs. The eluted solution was desalted with a NAP-10 desalting column (GE Healthcare) and concentrated using a DNA SpeedVac dryer. The amount of ssDNA was quantified by absorbance at 260 nm, and usually 2 to 5 nmol of ssDNA were obtained and used for the next round of SELEC. It usually takes 3 to 4 days to accomplish one round of SELEC. For the control SELEC library evolved with only ssDNA and SWCNT in the absence of 5-HT, the steps above were performed without 5-HT coincubation.

High-throughput sequencing and analysis

Sequencing libraries were prepared by using two sequential PCR steps (47) to add Illumina TruSeq universal adapter sequences and sequencing indices for multiplexed sequencing of rounds 2 to 6 from experimental and control SELEC groups. The sequencing libraries were sequenced with an Illumina HiSeq 4000 at the Genomic Sequencing Laboratory at the University of California, Berkeley, which generated about 20 million raw sequences from each round. We filtered out sequences that did not contain correct fixed regions (18-mer from 5′ primer + 6-mer cytosine block + 18-mer random region + 6-mer cytosine block + 3-mer from 3′ primer). A FASTAptamer toolkit was used to filter out and determine sequence frequencies (48). In the manuscript, all experiments used ssDNA sequences comprising only the 18-mer random region flanked by 6-mer cytosine blocks at each end (without PCR primer regions), unless otherwise noted. For classical MDS, the frequencies of trimers (e.g., ACA) at each position in the top 200 ranking sequences from the experimental or control SELEC libraries at each round were calculated and used as input parameters. 2D MDS analysis was performed by using the cmdscale() function of the stats package in R.

ssDNA-SWCNT construct preparation for nanosensor characterization

To characterize nanosensor properties such as fluorescence sensitivity and selectivity to 5-HT, ssDNA-functionalized SWCNT constructs were generated with the following protocol: 1 mg of HiPCo SWCNT was added to 0.9 ml of PBS buffer, and the solution was mixed with 100 μl of 1 mM ssDNA. The resulting mixture was bath-sonicated for 2 min and tip-sonicated for 10 min at 5-W power in an ice bath. After sonication, the black ssDNA-SWCNT suspension was centrifuged for 30 min at 16,100g to precipitate nondispersed SWCNT, and the supernatant containing solubilized ssDNA-SWCNT was...
collected. The supernatant was spin-filtered with 100-kDa MWCO centrifugal filters at 6000 rpm for 5 min with DNase-free water to remove unbound ssDNAs, and the purified solution at the top of the filter was collected. This spin filtration to remove unbound ssDNAs was repeated three times. The ssDNA-SWCNT suspension was diluted with PBS buffer and stored at 4°C until use. The concentration of the ssDNA-SWCNT suspension was calculated by measuring its absorbance at 632 nm with an extinction coefficient for SWCNT (25) of 0.036 (mg/liter)−1 cm−1.

**Spectral solvatochromic shift of ssDNA-SWCNT induced by SC**

For SC-induced spectral shift assays without 5-HT, ssDNA-SWCNT samples were prepared by creating a 100-μl PBS solution with ssDNA-SWCNT (20 mg/liter) generated with the following ssDNAs: pRO, p6#9, and pC6#8 containing the PCR primer regions (table S3). These solutions were loaded onto a custom-built nIR fluorescence spectrometer, and the time-dependent nIR fluorescence spectra were measured before and after the addition of 100 μl of 0.5 weight % (wt %) SC in PBS. The spectral blueshift was observed for all the fluorescence peaks, and we quantified the time-dependent spectral shift of the (8,6) SWCNT chirality peak (~1195 nm). For the SC-induced spectral shift assay with 5-HT preincubation, a 100-μl PBS solution containing ssDNA-SWCNT (20 mg/liter) of the abovementioned sequences was incubated with 100 μM 5-HT for 1 min. The fluorescence spectra of the ssDNA-SWCNT constructs were subsequently measured every 2 s for a total of ~6 min, with 0.5 wt % SC added to each sample at a time point marked by a solid black arrow (fig. S9).

**Screening the fluorescence response of ssDNA-SWCNT constructs to 5-HT and other analytes**

Fluorescence spectra of 198-μl ssDNA-SWCNT suspensions (10 mg/liter) in PBS were measured before and 10 s after the addition of 2 μl of 10 mM 5-HT solution for a final 5-HT concentration of 100 μM. We analyzed the nIR fluorescence change of the (8,6) SWCNT chirality peak (~1195 nm) in this study. \( \Delta F/F_0 \) was calculated as \( \Delta F/F_0 = (F - F_0)/F_0 \) based on the baseline fluorescence intensity before analyte addition \( F_0 \) and the fluorescence intensity 10 s after analyte addition for the (8,6) SWCNT chirality (~1195 nm), \( F \). Most of the analyte solutions were prepared with dissolution in deionized (DI) water as 10 mM concentration stocks, except tyrosine, which was dissolved in dimethyl sulfoxide. Serotonin hydrochloride and dopamine hydrochloride were used to prepare the analyte solutions to prevent oxidation of the neuromodulators under ambient conditions, and the hydrochloride salt did not affect the pH of resulting mixture because the PBS buffer capacity (~10 mM) was substantially higher than the amount of hydrochloride in the neuromodulator stocks (0.1 mM). All \( \Delta F/F_0 \) measurements were performed in triplicate, and the mean and SD of triplicate measurements were plotted. The assessment of 5-HT metabolites on nIRHT fluorescence was tested by preparing a 198-μl nIRHT suspension (10 mg/liter) in PBS and taking the nIR fluorescence spectra before and 10 s after the addition of 2 μl of 10 mM 5-HT metabolites (HTP, HIAA, and MTP). The assessment of 5-HT receptor drugs on nIRHT fluorescence was tested by preincubating a 198-μl nIRHT suspension (10 mg/liter) in 1 μM drug (fluoxetine, MDMA, 25I-NMMe, and quetiapine) in PBS and taking the nIR fluorescence spectra before and after the addition of 2 μl of 10 mM 5-HT. All concentration-dependent \( \Delta F/F_0 \) response curves were fitted to the Hill equation (23) to determine \( K_d \)’s.

To measure the optical \( \Delta F/F_0 \) response of nIRHT in brain-relevant environments, we prepared a nIRHT stock solution (10 mg/liter) in aCSF composed of 119 mM NaCl, 26.2 mM NaHCO3, 2.5 mM KCl, 1 mM Na2HPO4, 1.3 mM MgCl2, 10 mM glucose, and 2 mM CaCl2 (pH 7.4). In a separate assay, 100 μl of nIRHTs (100 mg/l) was incubated in 900 μl of stock human cerebrospinal fluid (from Lee Biosolutions).

**NMR analysis of 5-HT after coincubation with nIRHT**

For nuclear magnetic resonance (NMR) analysis, PBS buffer was prepared in D2O (PBS-d), nIRHT (396 μl; 10 mg/liter) in PBS-d was mixed with 4 μl of 10 mM 5-HT in D2O. The mixture was excited with a 721-nm laser for 10 min on the same nIR fluorescence spectrometer used for nano sensor screening and fluorescence measurements. The solution was spin-filtered using a 100-kDa MWCO centrifugal filter at 6000 rpm for 5 min with D2O, and the flow-through solution was collected. The 1H NMR spectra of the flow-through solution were measured with a 400-MHz Avance AVB-400 NMR (Bruker). The 1H NMR spectra of a 0.1 mM 5-HT solution without laser excitation in D2O were also measured as a control.

**Reversibility of nIRHT nanosensors immobilized in a microfluidic chamber**

A glass coverslip (#1.5; thickness, 0.17 mm; HS159879H, Heathrow Scientific) was functionalized with APTES (Sigma-Aldrich) by soaking in 10% (v/v) APTES in anhydrous ethanol for 5 min, following a previously reported protocol (26). The coverslip was then rinsed with DI water and left to dry. The coverslip was then fixed onto an ibidi μSlide VT1-2 forming six microfluidic channels. Next, 100 μl of PBS was pipetted through a microfluidic channel. The channel was then filled with 50 μl of a nIRHT solution (10 mg/liter) and left to incubate at room temperature for 5 min. The channel was rinsed using three successive washes of 50-μl PBS. The surface-immobilized nIRHT in PBS was then imaged on an inverted light microscope with 721-nm excitation and a Ninox VIS-SWIR 640 camera (Raptor Photonics). Fluorescence signal was collected through a 900-nm longpass filter. One end of the flow channel was connected to a syringe pump (Harvard Apparatus) with Luer lock fittings. Before the start of image acquisition, the opposite flow reservoir was filled with PBS, and the pump was set to refill mode at a volumetric flow rate of 100 μl/min. Once the liquid in the reservoir was depleted, 50 μl of 100 μM 5-HT in PBS was added. The process was repeated using alternating additions of 200 μl of PBS and 50 μl of 5-HT solution.

To test the optical response of bare SWCNT immobilized onto a glass substrate, an SDS-coated SWCNT suspension was prepared: 1 mg of HiPCo SWCNT was added to 1 ml of 2 wt % SDS in PBS buffer. The resulting mixture was bath-sonicated for 2 min and tip-sonicated for 30 min at 5-W power in an ice bath. After sonication, the black suspension was centrifuged for 60 min at 16,100g to precipitate undissolved SWCNT, and the supernatant solution was collected. Fifty microliters of SDS-SWCNT (5 mg/liter) was incubated in a microfluidic slide as described above, and the SDS coating was gradually removed by introducing a continuous flow of PBS buffer into the channel for 1 hour at a flow rate of 50 μl/min. Following this surfactant stripping step, 50 μl of 10 μM 5-HT in PBS was added to the microfluidic channel.

**nIRHT nanosensor imaging in an acute brain slice**

Sixty-day-old C57Bl/6 strain mice were used for nIRHT brain slice imaging experiments. Mice were group-housed after weaning at P21.
and kept with nesting material on a 12:12 light/dark cycle. All animal procedures were approved by the University of California Berkeley Animal Care and Use Committee. Acute brain slices were prepared using established protocols (49). Briefly, mice were deeply anesthetized via intraperitoneal injection of ketamine/xylazine cocktail, and transcardial perfusion was subsequently performed using ice-cold cutting buffer (119 mM NaCl, 26.2 mM NaHCO₃, 2.5 mM KCl, 1 mM NaH₂PO₄, 3.5 mM MgCl₂, 10 mM glucose, and 0 mM CaCl₂), after which the brain was rapidly extracted. The cerebellum and other connective tissues were trimmed using a razor blade, and the brain was mounted onto the cutting stage of a vibratome (Leica VT1200 S). Slices were incubated at 37°C for 60 min in oxygen-saturated aCSF (119 mM NaCl, 26.2 mM NaHCO₃, 2.5 mM KCl, 1 mM NaH₂PO₄, 1.3 mM MgCl₂, 10 mM glucose, and 2 mM CaCl₂) before use. Slices were then transferred to room temperature for 30 min before starting imaging experiments and were maintained at room temperature for the remainder of experimentation. For nanosensor labeling, slices were transferred into a small-volume brain slice incubation chamber (Scientific Systems Design Inc., AutoMate Scientific) and kept under oxygen-saturated aCSF (total of 5-ml volume). One hundred microliters of nIRHT nanosensor (100 mg/liter) was added to the 5-ml volume, and the slice was incubated in this solution for 15 min. The slice was subsequently recovered and rinsed in oxygen-saturated aCSF to wash off nIRHT that did not localize into the brain tissue. The rinsing step was performed by transferring the slice through three wells of the aCSF in a 24-well plate (5 s in each well), followed by labeled slice transfer to the recording chamber with aCSF perfusion for a 15-min equilibration period before starting the imaging experimentation. All imaging experiments were performed at 32°C with continuous perfusion of oxygen-saturated aCSF to maintain brain slice viability. Ex vivo slice imaging was performed with a modified upright epifluorescence microscope mounted onto a motorized stage as we previously described (26). The brain slice labeled with nIRHT was illuminated with 785-nm light, and the fluorescence image was taken in a field of view within the brain dorsomedial striatum with an InGaAs array detector (Ninox 640, Raptor Photonics) with 100-ms exposure and a 60x objective. Fluorescence signal was collected through a 900-nm longpass filter. To observe nIRHT response to 5-HT influx, the brain slice was immersed in 3 ml of oxygen-saturated aCSF in the flow chamber, and 0.3 ml of 1 mM 5-HT in aCSF was added into the system without further mixing. After the fluorescence reached a plateau, the 5-HT in the chamber solution was slowly depleted by continuous flow of oxygen-saturated aCSF (washing). Time-dependent ΔF/ΔF₀ was calculated from multiple regions of interest identified by a custom image analysis program (available for download at https://github.com/jtdsbod/Nanosensor-Brain-Imaging).

**SUPPLEMENTARY MATERIALS**

Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/12/eaay3771/DC1

Table S1. Ten most frequent ssDNA sequences from rounds 3 to 6 in experimental SELEC groups (E3 to E6). Table S2. Ten most frequent ssDNA sequences from rounds 3 to 6 in control SELEC groups (C3 to C6). Table S3. ssDNA sequences used in experiments: p80 is the unevolved initial ssDNA library, p6b#p and p6c#q are sequences used for investigating the effect of the PCR primer region to the ssDNA-SWCNT 5-HT response and solvatochromatic shift assay by SC, and R0 is the PCR primer–truncated initial ssDNA library. Fig. S1. Evolution of nucleotide identity prevalence in the control SELEC library.

**REFERENCES AND NOTES**


**Fig. S2.** Superscalar spectral shift indicates that SELEC for 5-HT nanosensors selects for ssDNA sequences that have molecular recognition for 5-HT when adsorbed to SWCNT. **Fig. S3.** Stability of nIRHT nanosensors against endonuclease degradation. **Fig. S4.** Dopamine concentration dependence of nIRHT in PBS. **Fig. S5.** nIRHT nanosensor response in the presence of interfering agents. **Fig. S6.** The mass proportion of ssDNA and SWCNT for nIRHT synthesis does not affect nanosensor response upon exposure to 100 μM 5-HT. **Fig. S7.** Time-dependent nIR fluorescent response of nIRHT nanosensors following 5-HT addition is not due to 5-HT oxidation. **Fig. S8.** Deconvolution of nIRHT fluorescence spectrum. **Fig. S9.** Fluorescence intensity profile of nIRHT nanosensors following 5-HT addition is not due to 5-HT oxidation. **Fig. S10.** Deconvolution of nIRHT fluorescence spectrum. **Fig. S11.** Fluorescence intensity profile of nIRHT nanosensors following 5-HT addition is not due to 5-HT oxidation. **Fig. S12.** Deconvolution of nIRHT fluorescence spectrum. **Fig. S13.** Fluorescence intensity profile of nIRHT nanosensors following 5-HT addition is not due to 5-HT oxidation.

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High-throughput evolution of near-infrared serotonin nanosensors
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