DEVELOPMENT OF SELECTIVE HIGH-AFFINITY FLUORESCENT SENSORS FOR
IMAGING NEUROTRANSMITTERS AND APPLICATION IN CELLS AND TISSUE

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LE ZHANG

Dr. Timothy E. Glass, Dissertation Supervisor

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The undersigned, appointed by the dean of the Graduate School, have examined the
dissertation entitled

DEVELOPMENT OF SELECTIVE HIGH-AFFINITY FLUORESCENT SENSORS FOR
IMAGING NEUROTRANSMITTERS AND APPLICATION IN CELLS AND TISSUE

presented by Le Zhang,

a candidate for the degree of doctor of philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

Professor Timothy E. Glass

Professor Michael Harmata

Professor Kent S. Gates

Professor Kevin D. Gillis

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ABSTRACT

Herein, we develop a series of selective fluorescent sensors based on a quinolone core designed to image neurotransmitters catecholamines, serotonin and glutamate. Included are the main design strategy, synthesis, spectroscopic data (UV/Vis and fluorescence) and their applications in cells and tissue.

NeuroSensor 510 (NS510) was designed and synthesized as a selective sensor for imaging norepinephrine. Formation of both an iminium ion and a boronate ester gave high affinity upon binding NS510 to norepinephrine. The sensor was able to label norepinephrine vesicles in chromaffin cells to give punctate staining. Moreover, the sensor was able to monitor norepinephrine exocytosis via correlation of total internal reflective fluorescence (TIRF) microscopy and amperometry.

A NIR fluorescent sensor NS659 was developed based on the structure of NS510. Several different synthetic routes were attempted before the sensor was successfully prepared. Upon binding to serotonin, the sensor gave a relatively high affinity but with quenched fluorescence. Initial tests showed that the sensor could be used to label vesicles.

Finally, a molecular sensor NS560 was developed to image glutamate. Fluorescence titrations showed that the sensor bound to glutamate to give a very large turn-on response. More importantly, the sensor should be able to differentiate glutamate from GABA due to the difference both in the emission wavelength and the fluorescence response. We tested this sensor in photoreceptor cells and observed that vesicles were labeled by this sensor. Destaining of the vesicles was observed via TIRF microscopy. Also, the sensor can label glutamate in cultured glutamatergic neurons, astrocytes and cortex brain

Chapter One

Introduction

1.1 Fluorescent Sensing

1.1.1 Molecular Recognition

Molecular recognition is a broad area that refers to the specific interaction between two or more molecules in chemical or biological systems, using hydrogen bonding, hydrophobic interactions, π - π stacking, metal coordination, van der Waals forces and covalent bonding etc.^{1,2} Sensors and probes are constructs that can measure the interactions between host and guest. In fluorescent sensing, the sensors or probes are always viewed as the host, and analytes are considered as the guest, generating a “visible” change in fluorescence. The fluorescence changes are modulated upon binding of analytes to the sensor.³

In our work, we developed chemosensors based on reversible interaction between host and guest.^{4,5} Because the interactions between host and guest are reversible, there is an equilibrium between bound complex and unbound species. Therefore, the interaction is measured using an associate constant K_a (eq.2). A larger value of binding constant indicates stronger binding between host and guest. The binding constant is defined in terms of concentration.

$$K_a = \frac{[HG]}{[H][G]} \quad (\text{eq. 2})$$

1.2. Fluorescent Imaging Mechanisms

1.2.1 Internal Charge Transfer (ICT)

To obtain efficient internal charge transfer, an electron donating group (donor) and an electron-withdrawing group (acceptor) should be directly conjugated to the extended π system.⁶ Excitation is able to promote electron transfer from donor to acceptor across the extended π system (the fluorophore) and produce an obvious fluorescent response.⁷

Shown below is an example of a fluorescent sensor that applies an internal charge transfer mechanism and makes use of fused BODIPY dye with an extended conjugation system (Figure 1-1).⁸ When there is no analyte (metal ion), the nitrogen atom donates its lone pair electrons to the extended π system of the fluorophore, providing fluorescence at 675 nm, from strong ICT. When the metal ion (cadmium ion) binds to the fluorescent sensor, it coordinates with the three nitrogen atoms of dipicolylamine, the lone pair of electrons of the nitrogen will interact with the d orbital of cadmium ion such that it's not available to donate to the π system. Therefore, this binding leads to reduction of ICT and the emission blue-shifts to 570 nm.

Figure 1-1. Fused BODIPY fluorescent chemosensor that is used to detect cadmium based on ICT mechanism

1.2.2 Förster Resonance Energy Transfer (FRET)

The mechanism of FRET is that energy from an excited fluorophore donor transfers to an acceptor fluorophore which is in the ground state through dipole-dipole interactions (Figure 1-2).⁹ For the general requirement for FRET, the emission of donor fluorophore should be able to have a large overlap with the absorption of acceptor fluorophore.¹⁰

Figure 1-2. FRET mechanism of between donor and acceptor via a linker

To design and synthesize a small-molecule FRET fluorescent probe, a FRET energy transfer platform should be built, including a donor, a linker and an acceptor.¹¹

The distance between donor and acceptor which is called the “Förster distance” must be suitable for FRET. The literature reported the suitable distance range lies between 20 and 60 Å.¹²

Figure 1-3. Several FRET-based fluorescent probes

FRET-based fluorescent probes have been used for measuring distance and detecting the interactions between molecules broadly in the area of both chemistry and biology, such as protein conformation changes, protein-protein interactions, and cellular levels of gas transmitters such as nitric oxide, copper, mercury and some amino acids (cysteine) (Figure 1-3).¹³⁻¹⁶ However, this widely used fluorescent method is associated with some drawbacks, the pairs used in FRET are fluorescent proteins, which are

commonly sensitive to environments, such as pH, temperature and so on. Also, the biggest drawback for FRET is that the signal to noise ratio is relatively low, because some energy is lost during the energy transfer. More work should be done to optimize the linker and maximize the overlap between emission and absorption of FRET pairs.¹⁷

Figure 1-4. FRET-based fluorescent probe for Fe (II)

Since we know FRET-based fluorescent probes are good for bioimaging, a good example described here was done by Chang and coworkers (Figure 1-4).¹⁸ They developed a reactivity-based FRET fluorescent probe for imaging labile iron. The probe was built by incorporating a fluorescein and cyanine 3 through an endoperoxide bridge that is capable of being cleaved by iron (II). Before addition of iron (II), when exciting fluorescein at its maximal absorbance wavelength, transferring the energy in the form of light to the cyanine 3 based on a FRET mechanism. Cyanine 3 was excited and red fluorescence (emission of cyanine 3) was collected. Upon addition of iron (II), which can trigger peroxide cleavage, FRET was decreased due to the cleavage of linker, only green

fluorescence (emission of fluorescein) could be visualized. Due to this change, this FRET based ratiometric probe could be used for detection of labile iron pools with moderate selectivity in cells, and it worked well in a ferroptosis model to investigate the mechanism of cell death.

1.2.3 Photoinduced Electron Transfer (PET)

Photoinduced electron transfer quenching is excitation of a fluorophore that can generate an electron in excited state, which is not able to relax to ground state with fluorescence emitted, because there are available orbitals from electron-donating or electron accepting moiety.³ PET quenching includes two forms, donor-PET (d-PET) and acceptor-PET (a-PET). For d-PET, excitation of a fluorophore (donor) produces an excited electron, but the quencher (acceptor) could provide LUMO which has lower energy barrier than HOMO of fluorophore. Instead of dropping to the ground state and giving fluorescence, the electron transfers to the LUMO of quencher. For a-PET, excitation of fluorophore (acceptor) generates an excited electron (Figure 1-5), the HOMO of the quencher (donor) has lower energy than the HOMO of fluorophore and donates an electron to fill the hole in the ground state. The excited electron can not now drop to the ground state and must transfer to the quencher with no fluorescent output.¹⁹

Figure 1-5. Mechanisms of d-PET and a-PET

PET-based probes are broadly used in detection of analytes in biological system. For instance, a number of PET-based probes were developed for metal ions (Zn^{2+} , Cu^{2+}), which are involved in brain functions.²⁰⁻²⁷ Below is the work done by Spiegel and coworkers. They developed a PET based fluorescent probe for imaging methylglyoxal (Figure 1-6).²⁸

Figure 1-6. Fluorescent probe for methylglyoxal based on a-PET mechanism

Methylglyoxal is an important metabolite of living cells related to several diseases. Upon addition of methylglyoxal, the *o*-phenylenediamine moiety of the probe can trap methylglyoxal by forming 2-methylquinoxaline, turning an electron-rich moiety into an electron-poor moiety. By this method, this removed the a-PET quencher to give strong fluorescence.²⁸

1.3. Imaging Neurotransmitters

Neurotransmission is one of the key processes in living biological systems and is accomplished by interaction between neurons. In this process, endogenous chemicals which are called neurotransmitters are packed in intracellular compartments (synaptic vesicles) at very high concentrations (0.2-1.0 M) in neurons.²⁹⁻³⁴ Those synaptic vesicles with neurotransmitters move towards to axon terminal, after a series of processes (docking, priming) and finally are anchored on membrane proteins. Upon action potential occurring, the vesicles are fused with membrane protein and those neurotransmitters are released into synaptic cleft, which is called exocytosis. Then, some of neurotransmitters are recycled by transporters, other neurotransmitters travel across the synaptic cleft to reach postsynaptic receptor of another neuron and induce a response in the postsynaptic neuron.³⁵

neurotransmitters
epinephrine	norepinephrine	dopamine
glutamate	lysine	glycine
serotonin	GABA	tyrosine
acetylcholine	histamine	adenosine

Figure 1-7. Structures of neurotransmitters

The mechanism for neurotransmitters uptaken into synaptic vesicles is shown below (Figure 1-8). V-ATPase can catalyze ATP to hydrolyze to ADP to provide energy, which is used to pump protons into synaptic vesicles.³⁶ With the accumulation of protons,

the inner environment of synaptic vesicles is acidic ($\text{pH} = 5.5$).^{37,38} The vesicular monoamine transporter 2 (VMAT 2) is able to carry catecholamine neurotransmitters into synaptic vesicles by using proton gradient as energy source to accumulate monoamines against gradient (Figure 1-8).³⁹

Figure 1-8. Mechanism of neurotransmitters uptake by secretory vesicles. Protons are pumped into vesicles by hydrolyze ATP to ADP. Protons are exchanged for uptaking protons by VMAT.

1.3.1. Electrochemistry

From the introduction of neurotransmission, it is easy to see that the neurotransmitters play an important role in this process. Much work has been done to investigate the mechanism of exocytosis. Currently, popular methods used to detect neurotransmitters are indirect and invasive. Among them, electrochemical methods are currently widely used in neurochemistry and neurobiology, including voltammetry, potentiometry, amperometry and coulometry.⁴⁰⁻⁴³ The basic concept behind these methods is that an electrode is used in a charge-transfer process. Since we used

amperometry in this work, I will discuss amperometry in detail.^{44,45} The detection mechanism is shown below (Figure 1-9). An electrode, usually made of carbon fiber, is brought at close proximity to the cells containing neurotransmitters. Upon stimulation, as soon as the cell starts to undergo exocytosis, neurotransmitters come out of the cell, and are readily oxidized and donate two electrons to the electrode, which holds positive potential, producing a current (Figure 1-10). The weak signal can be amplified by an amperometric amplifier. This technique is useful for detection of catecholamine neurotransmitters and serotonin, because these neurotransmitters are very electron-rich, and are readily oxidized. However, for amino acid neurotransmitters, such as glutamate, this is not suitable.

Figure 1-9. Monitoring dopamine release through an electrode using amperometry

Figure 1-10. Working mechanism of amperometry for detection of dopamine

1.3.2. Autofluorescence

Monoamine neurotransmitters bearing conjugated π systems (norepinephrine, dopamine, epinephrine and serotonin) can absorb and fluoresce in the UV/Vis range. Due to this characteristic, with proper equipment, those neurotransmitters can be detected by their native fluorescence. A lot of work has been reported on this method.

As shown in Figure 1-11, Maiti and coworkers developed a ratiometric fluorescent method to image the native fluorescence of serotonin.⁴⁶ Upon three-photon excitation of serotonin, the wavelength of serotonin emission at high concentrations is red shifted by forming transient oligomers. This method works well for the concentration of serotonin in synaptic vesicles.

Figure 1-11. Work flow for detection native fluorescence of serotonin

1.3.3. Biosensors

Compared to neurotransmitter chemosensors, biosensors are powerful methods to detect neurotransmitters with molecular specificity and relatively high temporal resolution. These genetically encoded fluorescent sensors are targeted to the cell membrane and use a neurotransmitter specific binding site, a fluorescent protein and a linker to connect them. Upon binding of neurotransmitters, the conformation of the neurotransmitter binding site changes, which leads to a conformation change of the fluorescent protein, consequently increasing fluorescence. Looger's lab has engineered a genetically encoded sensor (iGluSnFR) for imaging glutamate with good signal-noise

ratio and good resolution (Figure 1-12).⁴⁷ It was able to differentiate glutamate from other neurotransmitters except aspartic acid, both of which have similar structures. The reporter is able to monitor glutamate release in glutamatergic neurons and astrocytes. It has also been tested in mouse retina, worms, zebrafish and living mice to validate the applicability, and is the best genetically encoded fluorescent reporter for glutamate currently available.

Figure 1-12. iGluSnFR reporter for sensing glutamate

Another example here is the FRET sensor (GluSnFR) for imaging glutamate developed by Tsien and coworkers (Figure 1-13). They engineered a glutamate specific binding protein with two fluorescent proteins (GFP and YFP) on the hippocampal neurons.⁴⁸ Upon glutamate binding, the conformation change of glutamate binding led to a distance change between GYP and YFP, which led to FRET. This sensor was tested in hippocampal neurons, showing some promising results. However, due to the low efficiency of FRET, it should be improved for real application.

Figure 1-13. Schematic of surface-displaced GluSnFR in a ligand-free state

1.3.4. Supramolecular Chemistry Methods

Supramolecular chemistry has been widely used for encapsulation and detection of small molecule analytes. Among them, several macrocyclic structures, such as cyclodextrins, calixarene and cucurbiturils, are very popular encapsulation tool to detect neurotransmitters.⁴⁹⁻⁵¹

One example presented here is using cucurbit[8]uril (CB[8]) complexation with a 2,7-dimethyldiazapyrenium dication ($\text{Me}_2\text{DAP}^{2+}$) (Figure 1-14).⁵² This stable complex was confirmed by NMR and mass spectrometry. When the dye $\text{Me}_2\text{DAP}^{2+}$ was encapsulated into CB[8], the excitation spectrum showed there was a decreased

absorbance at 245 nm. To the contrary, the fluorescence intensity had an enhanced signal when encapsulated into CB[8]. This complex can hold dopamine inside the hydrophobic cavity, due to the hydrophobic effect of CB[8] and the charge-charge interaction between the electron poor dye Me₂DAP²⁺ and electron rich dopamine. The fluorescence was quenched when dopamine interacted with the dye, and a broad absorbance at 475 nm was observed due to charge transfer interaction of dopamine and Me₂DAP²⁺.

Figure 1-14. Mechanism of encapsulation of dopamine into cucurbi[8]uril complex

Thomas Schrader and coworker have developed a color sensor for catecholamines, by incorporating a xylylene bisphosphonate moiety with a phenyl boronic acid via an aliphatic linker (Figure 1-15).⁵³ Upon addition of catecholamines, the amino group of catecholamines could enable a charge-charge interaction with bisphosphate moiety as well as the formation of a boronate ester by phenyl boronic acid and the catechol group. NMR titration indicated that a number of proton signals of both host and guest appeared. Also, UV/Vis spectroscopy showed that absorption blue shifted from 530 nm (unbound sensor) to 460 nm (bound form of the complex), which demonstrated the interaction of

catecholamines and the color sensor. The binding constant is 280 M^{-1} for dopamine, 350 M^{-1} for norepinephrine and 310 M^{-1} for epinephrine.

Figure 1-15. Structure of the color sensor for detection catecholamines

1.3.5. Fluorescent False Neurotransmitters

We have discussed several methods to detect neurotransmitters above, and they are useful methods to some extent. Unfortunately, some of them are invasive and indirect, others are not applicable for living cell, brain slice, or *in vivo* imaging. Sames and Sulzer have developed a series of fluorescent probes called fluorescent false neurotransmitters (Figure 1-16).⁵⁴⁻⁵⁹ Those sensors are designed to mimic structures of neurotransmitters and help to monitor the function of neurotransmitters when neurons do signal transduction. This series of FFNs are taken up at neuronal sites by vesicular monoamine transporter 2 (VMAT2) and packed into synaptic vesicles, which could be visualized as punctate staining at living cells under confocal microscopy.

Figure 1-16. Structures of FFNs

FFN511 was the first available fluorescent false neurotransmitter, which was delivered from cytoplasm to synaptic vesicles by neuronal vesicular monoamine transporter 2 (VMAT2). It was able to label synaptic vesicles in adrenal chromaffin cells, and dopamine terminals in living cortical-striatal brain slice. Upon the electrical stimulation, the live brain slices undergo destaining, which demonstrates that FFN511 can actually mimic the structure of dopamine and as a fluorescent tracer for this neurotransmitter (Figure 1-17).⁵⁴

Figure 1-17. Mechanism of FFNs taken up into synaptic vesicles

After FFN511 was validated to be an effective fluorescent tracer for neurotransmitters, a pH sensitive version of fluorescent tracer FFN102 was developed.⁵⁶ Synaptic vesicles are at pH 5.5, but the extracellular space (synapse cleft) is pH 7.4. According to this difference, they appended a pH sensitive group on a FFN511 analogue, making the pK_a of this molecule at 6.2. While FFN102 was taken up into synaptic vesicles, the acidic environment quenched the fluorescence of FFN102, upon the stimulation, the dopaminergic synaptic terminal started to release neurotransmitters with FFN102 into synaptic cleft, which turned the fluorescence on by deprotonating the hydroxyl group of FFN102 and delocalizing the electrons. This molecule was also validated in cells and tissue.

Thereafter, several fluorescent tracers (FFN270, FFN246, FFN200)⁵⁷⁻⁵⁹ were developed based on FFN511 structure. FFN 270 could label the norepinephrine in

cultured neurons and live brain slices. Upon the electric stimulation, FFN 270 was used to monitor the release of norepinephrine in cells and tissue. The highlight of this work is the tracer also worked in live animals to visualize the neuron functions. FFN246 is another fluorescent tracer for imaging serotonin. Currently, there is no available small molecule imaging agents to image serotonin, though serotonin is a very important neurotransmitter. They tested this molecule FFN246 in cells and tissue, showing some promising data towards imaging serotonin, which opens a door to solve this problem.

1.3.6. FM Dyes

FM dyes were developed by Bewick and colleagues to monitor exocytosis and endocytosis of firing neurons.⁶⁰⁻⁶² These water-soluble FM dyes are composed of a hydrophobic moiety and a hydrophilic moiety (Figure 1-18). These dyes are completely nonfluorescent in extracellular environment (synaptic cleft), but they give intense fluorescence upon the hydrophobic moiety of the dye interacting with the hydrophobic environment of lipid membrane. Upon stimulation, the firing stimulated by action potential start to take up the nonfluorescent FM dyes dispersed in the extracellular via endocytosis while release neurotransmitters via exocytosis. The hydrophobic moiety of the uptaken FM dyes in the synaptic vesicles insert into the lipid membrane of synaptic vesicles, which becomes very fluorescent (Figure 1-19). Therefore, those FM dyes could stain single vesicles brightly and well stain the nerve terminals macroscopically. Another stimulation is able to fire the neurons again to release both neurotransmitters and FM dyes into extracellular environment via exocytosis and recycle neurotransmitters. The

bright single vesicles and nerve terminals become dim. Currently, there are two versions of FM dyes, which are more convenient to use especially for colocalization studies.

Figure 1-18. Structures of FM dyes

Figure 1-19. Mechanism of FM dyes to monitor exocytosis and endocytosis

1.4. Fluorescent Sensors for Direct Detection Neurotransmitters

We have already talked about a number of methods to detect neurotransmitters in this dissertation, some of which are invasive and indirect, others are not applicable for living cells and tissue imaging. Genetically encoded fluorescent sensors are a very popular method to detect neurotransmitters sensitively and noninvasively. However, to append this sensor on the membrane, the DNA sequence is edited and manipulated,

which must occur in transgenic mice. Therefore, it is necessary to design and synthesize fluorescent sensors to visualize neurotransmitters.

1.4.1. Fluorescent Sensor for Dopamine

Most chemically synthesized sensors are based on supramolecular interactions, such as hydrogen bonding, the hydrophobic effect, and charge-charge interactions, which for many reasons, are not applicable to living cells and tissue imaging. Beside these, there are quite a few fluorescent sensors for directly imaging neurotransmitters. The Yoon lab has developed an anthracene based fluorescent sensors for imaging catecholamines, which incorporates an aldehyde group and phenyl boronic acid (Figure 1-20).⁶³ Due to photoinduced electron transfer, the electron-rich catechol of catecholamines strongly quenched the fluorescence of the fluorophore. Therefore, only little fluorescence was detected and it is not capable of being used in biological systems.

Figure 1-20. Dopamine binding to the sensor

1.4.2. Fluorescent Sensor for Amino Acids

Our group started to work on development of fluorescent sensors for neurotransmitters with a fluorescent sensor for amino acids. In 2003, the group made a

fluorescent sensor to detect amino acids, which gave a turn-on response when amino acids bind to it by forming an iminium ion from the aldehyde group of the sensor and the amino group of amino acids in aqueous buffer (Figure 1-21).⁶⁴ Upon binding, the absorbance red shifted from 440 nm to 495nm, when the bound complex was excited at 495 nm, the emission wavelength was located at 520 nm. It should be noticed that this sensor only had an aldehyde group, which could react with all the analytes bearing a primary amine. It has no selectivity towards amino acids and neurotransmitters.

Figure 1-21. Reversible interaction of the sensor with amino acids

1.4.3. Fluorescent Sensor for Catecholamines

After we successfully made the amino acid sensor, we started to think about appending another recognition element on the dye with the aldehyde group, making it a selective fluorescent sensor for catecholamine neurotransmitters. It was reported that boronic acid can bind to catechol, diol and glucose to form a boronate ester with moderate affinity. Therefore, our group reported a selective fluorescent sensor **4** for catecholamine with primary amino group (dopamine and norepinephrine) with an aldehyde and a boronic acid group on it (Figure 1-22).⁶⁵

Figure 1-22. Dopamine binding to the sensor

This sensor bound to catecholamines (dopamine and norepinephrine) by forming both an iminium ion and a boronate ester. The UV/Vis spectra showed the absorbance red shifted from 440 nm to 490 nm upon dopamine binding, which indicated the new complex formed. Upon excitation at 484 nm, the fluorescence of the sensor was strongly quenched by more than a half. For epinephrine, there was no red shift in absorbance, indicating that no iminium ion formation in this titration. However, when the sensor bound to other analytes (tyramine, lysine and etc.), the binding affinity towards them is pretty low with very large fluorescent enhancements. The binding affinity for those catecholamines is very strong ($3,400\text{ M}^{-1}$ for dopamine and $6,500\text{ M}^{-1}$ for norepinephrine). But this sensor is not applicable in living cell imaging due to the large loss of fluorescence.

Our group focuses on the development of selective fluorescent sensors for imaging neurotransmitters in real applications. In this process we met two difficulties in designing and synthesizing sensors. One is that high binding affinity and selectivity towards neurotransmitters are hard to obtain, due to only a few binding sites on applicable of analytes. The other is that the fluorescence of the sensor is strongly quenched by the electron rich quencher (catecholamines and serotonin). Since we

obtained high binding affinity and good selectivity from this work, manipulating the fluorophore to change the electronic properties was the next priority.

1.4.4. NeuroSensor 521

We spent a couple of years making and testing sensors, finally making some progress to reach this goal. In 2013, my group member developed a selective fluorescent sensor for visualizing catecholamines and tested this sensor in fixed and live cells. In this process, they tested a number of moieties to manipulate the electronic properties of this sensor. The sensor (NS521) bound to all the analytes with a primary amine group by forming an iminium ion (Figure 1-23).⁶⁶ The UV/Vis spectroscopy produced red shift from 440 nm to 480 nm upon norepinephrine binding. Upon excitation at 488 nm, which is an Argon laser line, the fluorescence spectroscopy showed a maximum emission at 521 nm. However, the selectivity for catecholamines over other analytes with a primary amine group is not great. The values of the binding constant are 112 M^{-1} for dopamine, 78 M^{-1} for norepinephrine, 10 M^{-1} (glutamate), 11 M^{-1} for lysine and no binding for epinephrine. From these data, the iminium ion formation was not able to provide a strong binding affinity towards all the analytes, considering the norepinephrine concentration in vesicles is about 0.5 M, this should not be a problem to image norepinephrine in synaptic vesicles. Epinephrine without a primary amine group does not react with NS521 to produce a fluorescence response. However, except these catecholamines, other analytes showed very large fluorescence enhancements with NS521, which may cause background fluorescence. Sensor NS521 was tested in live chromaffin cells and was able to differentiate norepinephrine from epinephrine chromaffin cells.⁶⁶ Immunostaining

experiments were done to confirm the fluorescence produced in norepinephrine enriched cells came from the interaction of NS521 and norepinephrine.

Although the sensor NS521 did not provide a good selectivity for catecholamines over other analytes, this did move this project forward. We had sensors in hand with either high selectivity or with turn-on response for catecholamines. In the future, we hoped to make fluorescent sensors with both high selectivity and turn-on response and apply them in live cell imaging.

Figure 1-23. Norepinephrine binding to NS521

Since my research focused on the development of fluorescent sensors to monitor exocytosis of neurotransmitters in live cells and glutamate fluorescent sensors, I would like to talk about other work we have done previously.

1.4.5. ExoSensor 517

I have discussed a pH sensitive fluorescent false transmitter developed by Sames and Sulzer, which is indirect way to image neurotransmitters.⁵⁶ Herein our group has developed a fluorescent sensor ExoSensor 517 to monitor neurotransmitter exocytosis (Figure 1-24).⁶⁷ When neurotransmitters bound to ES517 in synaptic vesicle, where the

environment is acidic (pH = 5.5), the complex was slightly fluorescent, because the lone pair electrons of nitrogen were unable to delocalize in the conjugated π system. While when neurotransmitters bound to ES517 at synaptic cleft (pH = 7.4), the nitrogen of sulfonamide group was deprotonated, and the lone pair electrons could delocalize in the π system, which would produce stronger fluorescence compared to that at pH 5.5 (Figure 1-25). Although ES517 successfully showed different fluorescence strength at different pH in the cuvettes, it was not applicable for monitoring neurotransmitter exocytosis due to low binding affinity towards neurotransmitters and low fluorescence ratio at pH 5.5 and pH 7.4. Future work may focus on pH sensitive fluorescent sensor for imaging neurotransmitters with high affinity.

Figure 1-24. Neurotransmitter binding and deprotonation of ES517

Figure 1-25. Graphic mechanism for ES517 with neurotransmitters

1.4.6. NeuroSensor 715

Serotonin is one of the most important neurotransmitters, involved in the neurotransmission that is related to several neurodegenerative diseases.^{68,69} Unfortunately, there are no currently available fluorescent sensors for the direct imaging of serotonin, most of which target to the precursor of serotonin in biochemically synthetic pathway. Recently, we have developed a fluorescent sensor for visualizing serotonin (Figure 1-26).⁷⁰ Upon addition of serotonin, NS715 bound to serotonin to give the fluorescence at 715 nm with a large Stokes shift. Considering the serotonin is electron-rich, it quenches most fluorophores. This sensor gave a turn-on response when serotonin bound. We titrated the sensor NS715 with several important neurotransmitters (serotonin, dopamine, norepinephrine, glutamate and epinephrine). The sensor NS715 gave small selectivity for serotonin over other neurotransmitters (409 M^{-1} vs 145 M^{-1}) and largest turn-on

fluorescent enhancements (8 fold vs 4 fold). Therefore, NS715 is a successful sensor to detect serotonin. Although we made some progress, the binding affinity with serotonin was relatively low. Efforts should be paid to improve the binding affinity towards serotonin.

Figure 1-26. NS715 binding to serotonin to give red fluorescence

1.4.7. Fluorescent Sensor for Zn^{2+} and Glutamate

Glutamate is one of main neurotransmitters involved in neurotransmission and related to several neurodegenerative diseases.^{71,72} Though glutamate is very important to the health of human beings, there are not very effective methods to visualize glutamate. I discussed the genetically encoded fluorescent reporter iGluSnFR, which engineered a glutamate binding protein and green fluorescent protein via a linker.⁴⁷ However, this reporter can be utilized in transgenic mice, and this limitation hampered its widespread use. We are striving to develop the fluorescent sensor for glutamate. We finally made a fluorescent sensor for glutamate and zinc based on two-input logic gate strategy (Figure 1-27).⁷³ The sensor we developed showed slightly quenched fluorescence upon addition of glutamate and the binding constant for glutamate is very low as well as other analytes.

In addition, the sensor also gave quenched fluorescent response towards only zinc. But if both glutamate and zinc were present, the sensor gave very large fluorescent enhancements (Figure 1-27). It was reported that zinc is important and tightly involved in neurotransmission, moreover, zinc and glutamate can be both found in some glutamatergic neurons at certain age of mice. This sensor can help image glutamate in the presence of zinc.

Figure 1-27. Graphic scheme for the sensor binding to both zinc and glutamate

However, this method showed very promising way to image glutamate, but similarly this sensor did not work well in live cells and tissue, because it is difficult to find both zinc ion and glutamate together in cells and tissue. So we also need to develop a fluorescent sensor for glutamate to fill this blank.

We have talked about some knowledge about fluorescence and quenching mechanisms (ICT, PET and FRET) and summarized several methods to detect neurotransmitters (invasive methods and noninvasive methods). They all suffered from

some drawbacks, such as water solubility, low binding affinity, utilization limitations, therefore we should aim to develop some fluorescent sensors for selectively imaging neurotransmitters and focus on their application in live cells and tissue.

Chapter Two

Selective Fluorescent Sensor for Imaging Norepinephrine and Application

2.1 Overview

2.1.1. Introduction

Understanding the function of the brain is a grand challenge of modern science.⁷⁴ As part of this work, measurements of the uptake, storage and release of neurotransmitters are of central importance. Norepinephrine, epinephrine, dopamine, and glutamate, are the major neurotransmitters in the sympathetic nervous system,⁷⁵⁻⁷⁷ and are involved with many functions, including memory, attention, learning, emotion, sleep and movement.^{78,79} Aberrant levels of neurotransmitters are implicated in various disease states, thus measuring these simple organic compounds has become an important area of research. Fluorescence imaging of neurotransmitters has distinct advantages over methods such as capillary electrophoresis and microelectrochemistry because it is less invasive and has better spatial resolution.⁸⁰⁻⁸³ For this reason, Sames and Sulzer have developed a series of fluorescent false neurotransmitters, which are taken up into synaptic vesicles because they mimic the structure of genuine neurotransmitter, and can therefore be used to image vesicle accumulation at neuronal release sites and the loss of fluorescence that accompanies transmitter release.⁵⁴⁻⁵⁹ However, tools for direct labeling of neurotransmitters are still lacking.

Dopamine is synthesized via three steps from L-Phenylalanine with two enzymes (AAAH, AADC). Norepinephrine was converted from dopamine with the key enzyme

differentiate norepinephrine from epinephrine via immunostaining assays (Figure 2-1).⁸⁴

Figure 2-1. Biochemical pathway for catecholamine neurotransmitters

2.1.2. Previous Work

Figure 2-2. Binding of Sensor 4 to dopamine

Figure 2-3. Binding of NS521 to norepinephrine

Our group is developing fluorescent sensors that label transmitter via direct binding. Sensor **4** (Figure 2-2) was prepared some time ago as a selective catecholamine sensor with a boronic acid moiety to bind the catechol group and an aldehyde that forms an iminium ion upon binding to primary amines.⁶⁵ While the sensor binds catecholamines with excellent affinity and appreciable selectivity, the fluorescence is quenched by the electron-rich catechol. More recently, we developed NS521 (Figure 2-3), which lacks the boronic acid group and thus has lower selectivity for catecholamine compared to sensor **4**.⁶⁶ However, NS521 has the advantage of having a several-fold turn-on fluorescence response to catecholamines in part because of electronic effect of the methoxyphenyl substituent on the fluorophore as well as the fact that the neutral catechol is not as strong of a quencher as the boronate formed when sensor **4** reacts with catecholamine.

2.2 NeuroSensor 16

2.2.1 Design and Synthesis

Some years ago, our group has developed a quinolone dimer based fluorescent sensor for imaging diamine, which showed larger fluorescence enhancements and

quantum yield.⁸⁵ So we started to think about design and synthesized a quinolone based fluorescent sensor for neurotransmitters to confirm if this fluorophore is actually more electron-rich than coumarin.

Scheme 2-1. Synthesis of NS16

NS16 was prepared in 2 steps from the known compound **6** (Scheme 2-1).⁸⁵ The Vilsmeier-Haack reaction both chlorinated and formylated the quinolone to give compound **7**. Suzuki coupling to compound **8** furnished sensor NS16 in good overall yield.

2.2.2 Spectroscopic Data

NS16 was screened with various biogenic amines by UV/visible spectroscopy and fluorescence spectroscopy. NS16 bound to all the primary amines tested by formation of an iminium ion, producing a red shift in absorption from 436 nm to 488 nm. Upon addition of the analyte norepinephrine, a 12-fold increase in fluorescence of NS16 was measured upon excitation at 488 nm (Figure

2-4). Table 2-1 summarizes the binding and spectroscopic data of the interaction of NS16 with six relevant amines.

Figure 2-4. UV/vis (a) and fluorescence (b) titration of NS16 (20 μ M) with norepinephrine in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, pH 7.4, 0.8% DMSO). $\lambda_{\text{ex}} = 488$ nm. Inset is the fit to a binding isotherm using $\lambda_{\text{em}} = 521$ nm ($K_a = 170 \text{ M}^{-1}$).

As with the other amine sensors in this class, NS16 did not interact with the secondary amine epinephrine. NS16 binds to the analytes with a primary amine with relatively low binding affinity and high fluorescence enhancements. The binding constant between NS16 and norepinephrine was 4 fold larger than that between NS16 and dopamine and 20 fold higher binding constants than alkyl amines such as glutamate. However, the fluorescence intensity of NS16 bound to norepinephrine was smaller than that of NS16 bound to alkyl analytes. It should be noted that the concentration of norepinephrine within secretory vesicles is on the order of 500 mM, therefore a K_a value of $\sim 170 \text{ M}^{-1}$ should be sufficient to ensure labeling norepinephrine vesicles within neurons and neuroendocrine cells.

Table 2-1. Association constants and spectroscopic parameters for titration of NS16 with various analytes

amine guest	$K_a(\text{M}^{-1})^a$	I_{sat}/I_0^b ($\lambda_{\text{ex}}=488 \text{ nm}$)
epinephrine	nd	nd
norepinephrine	170	11.2
dopamine	38	8.5
glutamate	1.9	50.8
lysine	2.5	77.7
glycine	2.9	46.6

^a K_a measured by fluorescence spectroscopy of NS16 (20 μM) with analytes in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, pH 7.4, 0.8% DMSO). $\lambda_{\text{ex}} = 488 \text{ nm}$. Error in K_a values are $\pm 10\%$ based on triplicate titrations. ^b I_{sat} = fluorescence intensity at saturation taken from the theoretical fit to the binding isotherm.

It was important for us to compare the responses of NS16 to NS521, since the latter compound had already been validated in chromaffin cells.⁶⁶ Interestingly, NS16 gave larger fluorescence enhancements and better binding of catecholamines compared to NS521. In particular, the quantum yield of sensor NS16 was greatly improved over that of NS521. NS521 has a rather low quantum yield ($\phi=0.0053$) which drops even further when norepinephrine binds ($\phi=0.0033$). NS521 produces a turn-on response only because the iminium ion can be selectively excited due to the red-shift in absorbance. The sensor-analyte complex of NS521 is actually less fluorescent than the parent sensor. In contrast, NS16 starts with a higher quantum yield due to the quinolone core ($\phi=0.022$). More importantly, the quantum yield of the sensor increases upon binding norepinephrine ($\phi=0.054$) producing good fluorescence turn-on and overall high fluorescence in the bound state. Based on these results, we pursued biological imaging with this sensor.

2.2.3 Cell Studies

Figure 2-5. (a) Confocal and (b) TIRF images of norepinephrine-enriched cells incubated with NS16 (5 μM) ($\lambda_{\text{ex}} = 488 \text{ nm}$).

We utilized adrenal chromaffin cells as a model cell to test the fluorescent catecholamine sensor NS16. Chromaffin cells store norepinephrine and epinephrine in acidic vesicles and release them via exocytosis much like neurons. Subpopulations of chromaffin cells enriched in norepinephrine versus epinephrine can be isolated by centrifugation on a Percoll gradient.^{86,87} Live norepinephrine-enriched chromaffin cells were stained and imaged with an Olympus Optical FluoView FV1000 confocal laser scanning biological microscope. Cells were treated with 5 μ M NS16 for 45 min at 37 °C and rinsed twice. The cells were excited with a 488 nm laser.

Norepinephrine-enriched chromaffin cells were brightly stained by NS16 (Figure 2-5). Punctate staining of the chromaffin cells suggests that the sensor is labeling the secretory vesicles found throughout the cell where the norepinephrine is present in high concentrations. Next, we imaged labeled chromaffin cells using TIRF microscopy (Figure 2-5). TIRF imaging selectively illuminates the bottom surface of the cell with the evanescent field that penetrates only ~100 nm past the glass/cell interface. It therefore can better resolve the catecholamine-containing vesicles adsorbed to the bottom surface of the cell without contamination of fluorescence from other regions of the cell. Through-objective TIRF images were obtained using an Olympus IX-80 inverted microscope fitted with an Olympus UAPO 150X oil N.A 1.45 TIRF objective. Images were acquired using a cooled CCD camera (Hamamatsu EM-CCD Digital camera C9100, Japan) controlled by Slidebook software. Distinct fluorescent puncta were observed in norepinephrine-enriched chromaffin cells loaded with 5 μ M NS16 that are consistent with labeling of granules (Figure 2-5). Upon stimulation with a high-K⁺ solution, we recorded

amperometric spikes using underlying electrodes (Figure 2-6). These data indicate that staining with NS16 does not interfere with the ability of the cells to release catecholamines via quantal exocytosis. However, loss of punctae with stimulation indicative of exocytosis was not observed.

Figure 2-6. A sample trace of quantal catecholamine release from a single bovine adrenal chromaffin cell on an Au electrode demonstrated that the cells can still undergo exocytosis after being loaded with NS16. The inset is an expanded view of amperometric spikes occurring during the interval marked with the dashed box.

2.3 NeuroSensor 510

2.3.1. Design and Synthesis

We have designed and synthesized the fluorescent sensor NS16 for imaging norepinephrine and labeled the norepinephrine vesicles, visualized by confocal and total internal fluorescence microscope. But initial goal to make this sensor is to verify that

quinolone is a more electron rich and brighter fluorophore than coumarin. Since we have the titration data of NS16 and NS521 that was made by my colleague, we should be able to compare the data and draw conclusions.

Table 2-2. Comparison data between NS16 and NS521

	NS16		NS521	
amine guest	$K_a(M^{-1})^a$	I_{sat}/I_0 ($\lambda_{ex}=488nm$)	$K_a(M^{-1})^a$	I_{sat}/I_0 ($\lambda_{ex}=510nm$)
norepinephrine	170	11	78	5.4
dopamine	38	9	112	3.0
glutamate	2	51	10	7.8

^a K_a measured by fluorescence spectroscopy. Error in K_a values are $\pm 10\%$ based on triplicate titrations. ^b I_{sat} = fluorescence intensity at saturation taken from the theoretical fit to the binding isotherm.

We picked the data of three major neurotransmitters for comparison (Table 2-2), NS16 shows larger fluorescent response towards all three neurotransmitters. And we discussed the quantum yield of NS16 was much larger than NS521. So we can conclude

that the quinolone is a more electron-rich fluorophore than coumarin, we started to think about building a phenyl boronic acid and an aldehyde group on the quinolone core to make it a selective sensor for imaging norepinephrine with high binding affinity.

Here we report the development of a next-generation sensor NeuroSensor 510 (NS510, Figure 2-7) with stronger affinity and reduced quenching of fluorescence upon catecholamine binding. We utilize a quinolone fluorophore since it is much more electron rich than coumarin,⁸⁸ and therefore is quenched less upon binding the electron-rich catechol.⁸⁹ NS510 possesses the base quinolone fluorophore with a boronic acid appended to an aromatic substituent to confer significantly enhanced affinity and therefore selectivity for catecholamine binding, similar to sensor **4**.

Figure 2-7. Structures of sensor **4** and NS510

NS510 was prepared in 4 steps (Scheme 2-2). A hydroxymethylphenyl group was coupled to the tosylate of compound **6** to furnish compound **10** via Suzuki coupling. It was most efficient to chlorinate compound **10** prior to formylation to give intermediate **12**. Alkylation of **12** produced the target sensor NS510 in moderate yield.

Scheme 2-2. Synthesis of NS510

2.3.2. Spectroscopic Data

Table 2-3 summarizes the binding and spectroscopic data of the interaction of NS510 with relevant amines. NS510 showed a red shift in absorption from 428 nm to 473 nm upon addition of norepinephrine (Figure 2-8). The fluorescence at 510 nm roughly doubles upon binding and the binding constant was substantially higher compared to NS521 (Figure 2-8), which indicates that the boronic acid is participating in binding. Dopamine binds equally well to NS510, though the fluorescence is slightly quenched upon binding since dopamine is a more quenching analyte.⁸⁹ Thus NS510 is selective for norepinephrine over dopamine based on fluorescence enhancement. To our best knowledge, this is the first sensor that can differentiate norepinephrine from dopamine.

Figure 2-8. (a) UV/vis titration and (b) fluorescence titration of the sensor NS510 (10 μ M on 25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, pH = 7.4) with norepinephrine (10 mM in buffer, λ_{ex} = 488 nm). Inset in (b) is a plot of emission intensity at 510 nm versus concentration with a fit to a one-site binding isotherm.

Epinephrine does not produce a shift in absorption because it does not form an iminium ion with its secondary amine and binds only through the catechol group. Interestingly, epinephrine binds better than either norepinephrine or dopamine (Table 2-3), suggesting that norepinephrine and dopamine suffer from a small amount of strain in the bound state which lowers the overall affinity slightly compared to just the catechol binding. As with dopamine, epinephrine binding produces a fluorescence decrease because it does not form the iminium ion. Most importantly, the binding affinity of NS510 for norepinephrine is much larger than that for other amines such as glutamate (over 400 fold). In particular, amino-sugars such as glucosamine do not bind well to NS510, indicating that the binding pocket of the sensor does not match the more compact sugar structure.

Table 2-3. Binding and spectroscopic properties of NS510 with various amines

amine guest	$K_a(\text{M}^{-1})^a$	I_{sat}/I_0^b
35,900 ^c	0.80
epinephrine
13,500	1.9
norepinephrine
17,900 ^c	0.88
dopamine
32	26
glutamate
24	24
lysine
83	28
glucosamine		

^a K_a measured by fluorescence spectroscopy (see Figure 2-8 for conditions). Error in K_a values are $\pm 10\%$ based on triplicate titrations. ^b I_{sat} = fluorescence intensity at saturation taken from the theoretical fit to the binding isotherm. ^c K_a measured by UV/vis spectroscopy.

Compared to NS521, the quantum yield of sensor NS510 was greatly improved. NS521 has a rather low quantum yield ($\phi=0.0053$), which drops even further when norepinephrine binds ($\phi=0.0033$). NS521 produces a turn-on response only because the iminium ion can be selectively excited due to the red-shift in absorbance. The sensor-analyte complex of NS521 is actually less fluorescent than the parent sensor. In contrast, NS510 starts with a higher quantum yield due to the quinolone core ($\phi=0.025$). More importantly, the quantum yield of the sensor increases upon binding norepinephrine ($\phi=0.031$) producing good fluorescence turn-on and overall high fluorescence in the bound state. Thus, the quinolone-based sensor is brighter than NS521.

2.3.3. Cell Studies

We utilized bovine adrenal chromaffin cells as a model cell to test the fluorescent catecholamine sensor NS510. Chromaffin cells store norepinephrine and epinephrine in acidic vesicles and release them via exocytosis much like neurons. Subpopulations of chromaffin cells enriched in norepinephrine versus epinephrine can be isolated by centrifugation on a Percoll gradient.^{86,87} Live norepinephrine-enriched chromaffin cells were stained and imaged with an Olympus FluoView FV1000 confocal laser scanning inverted microscope. Cells were treated with 0.5 μ M NS510 for 45 min at 37 °C and rinsed twice. The cells were excited with a 488 nm laser. Norepinephrine-enriched chromaffin cells were brightly stained by NS510 (Figure 2-9). Punctate staining of the chromaffin cells suggests that the sensor is labeling the secretory vesicles found throughout the cell where the norepinephrine is present in high concentrations (~ 0.5 M).

Figure 2-9. Confocal image of norepinephrine-enriched cells incubated with NS510 (0.5 μM) ($\lambda_{\text{ex}} = 488 \text{ nm}$)

Then we incubated NS510 with both norepinephrine and epinephrine enriched chromaffin cells, to determine if this sensor can differentiate those two different cells by a fluorescence intensity difference (Figure 2-10). From the figure below, although the norepinephrine enriched chromaffin cells stained by NS510 is brighter than epinephrine enriched cells, the difference between the two was not large.

Figure 2-10. Confocal images of norepinephrine enriched chromaffin cells (a) and epinephrine enriched chromaffin cells incubated with NS510 (0.5 μM)

To confirm this conclusion is correct, we labeled cells with different concentrations (0.1, 0.5, 1.0 and 1.5 μM) of NS510, finally we found that with 0.5 μM concentration of NS510, we could see the largest fluorescent intensity difference between the two kinds of cells. We repeated these experiments with the same concentration of the sensor. We statistically summarized and compare the data in Figure 2-11.

Figure 2-11. Average ratio of fluorescence intensity at 510 nm

We could draw the conclusion that the average fluorescence intensity of norepinephrine enriched chromaffin cells labeled by NS510 was about two times of that of epinephrine enriched chromaffin cells. After we carefully reviewed all the data, we found this data actually matched the data we got for the titration in cuvettes. About 10% percent of the fluorescence of the sensor was quenched by epinephrine, but for norepinephrine, it had about 1 fold fluorescent enhancement (Table 2-3).

To further validate the fluorescence, we visualized in confocal images came from the binding of the sensor NS510 with norepinephrine. We used histochemical staining to identify norepinephrine and epinephrine containing cells (Figure 2-12).⁹⁰ An antibody specific for binding to phenylethanolamine *N*-methyltransferase (PNMT) was used here

to label fixed chromaffin cells. This enzyme can convert norepinephrine to epinephrine by appending a methyl group. Then a secondary antibody conjugated with a cyanine 3 (a red fluorescent dye) can bind to the primary antibody to visualize the enzyme-antibody interaction. There should be plenty of PNMTs in epinephrine-enriched chromaffin cells, we expected to see the red fluorescence in those cells. To the contrary, low red fluorescence should appear in norepinephrine enriched cells. For these histochemical staining experiments, we could visualize the strong green fluorescence in norepinephrine enriched cells that was attributed to the binding of NS510 with norepinephrine, compared with very low green fluorescence in epinephrine enriched cells. For the antibody assays, we did visualize little red fluorescence in norepinephrine enriched cell populations and relatively large red fluorescence in those epinephrine enriched cells. These results did match the expectations we gave above. We also used antibody staining in mixed cells, and found the cells with strong green fluorescence did not fluoresce red well, which were norepinephrine enriched cell populations, while the cells labeled with clear red fluorescence did not show bright green fluorescence, which are epinephrine enriched chromaffin cells. This method is able to validate the sensor NS510 could label norepinephrine containing vesicles better than epinephrine containing vesicles to some extent.

Figure 2-12. Immunostaining assays for NS510 in norepinephrine and epinephrine enriched chromaffin cells at green channel, red channel and bright field

2.3.4. Monitoring Exocytosis via Correlation of TIRF and Amperometry

We have discussed several methods for monitoring exocytosis of neurotransmitters in Chapter one, such as FM dyes, pH sensitive fluorescent false neurotransmitters and synaptophysins (Figure 2-13).^{54,61,91} FM dyes are amphiphilic molecules, which include both a hydrophobic moiety and a hydrophilic part. When these dyes are dissolved in aqueous buffer solution, the fluorescence is strongly quenched. Neuron cells take up FM dyes via vesicle cycling while the neurotransmitters are

released into synaptic cleft via exocytosis. The hydrophilic tails insert into the outer layer of lipid membrane and give strong fluorescence. This method is used to monitor exocytosis of neurotransmitters. A pH sensitive group is appended on the fluorescent false neurotransmitters. These molecules are taken up into synaptic vesicles that will quench the fluorescence of pH sensitive FFNs at pH 5.0. Upon neurotransmitters released into synaptic cleft, the pH sensitive group is deprotonated and shows turn-on fluorescence response, which is one of the main methods for exocytosis monitoring. The third method is synaptophysins, in which a pH sensitive protein is genetically engineered on vesicular membrane protein. Neurotransmitters are included in synaptic vesicles, protonation of the pH sensitive residue causes low fluorescence. When exocytosis occurs, deprotonation of the pH sensitive residue gives a turn-on fluorescence towards the neurotransmitter releasing. I presented three major and popular methods here. They are validated to monitor exocytosis of neurotransmitters, however these methods are indirect visualization of neurotransmitters release.

Figure 2-13. Three methods for visualizing neurotransmitter endocytosis and exocytosis

Herein, NS510 binds to norepinephrine with turn-on fluorescent response and very high affinity. We developed a method to monitor norepinephrine exocytosis by combining total internal reflective fluorescence (TIRF) microscopy and amperometry. First, we imaged labeled chromaffin cells using TIRF microscopy. TIRF imaging selectively illuminates the bottom surface of the cell with the evanescent field that penetrates only ~100 nm past the glass/cell interface. It therefore can better resolve the catecholamine-containing vesicles adsorbed to the bottom surface of the cell without contamination of fluorescence from other regions of the cell. Through-objective TIRF images were obtained using an Olympus IX-80 inverted microscope fitted with an Olympus UAPO 150X oil N.A 1.45 TIRF objective. Images were acquired using a cooled

CCD camera (Hamamatsu EM-CCD Digital camera C9100, Japan) controlled by Slidebook software. TIRF imaging revealed distinct fluorescent puncta in norepinephrine-enriched chromaffin cells that are consistent with labeling of granules. Abrupt, punctate loss of fluorescence were observed upon stimulation with high- K^+ solution that are likely to reflect exocytosis of a vesicle containing norepinephrine labeled with 10 μ M NS510. In order to validate that punctate drops in NS510 correspond to release of catecholamine from individual vesicles, experiments were performed using an amperometric ring microelectrode patterned underneath the cell being imaged.⁹² Amperometric microelectrodes immediately adjacent to release sites on the cell surface measure pA-magnitude spikes of current as the catecholamine released from an individual vesicle is oxidized on the surface of the electrode.⁹²⁻⁹⁴ Figure 2-14 presents three examples of amperometric spikes recorded synchronously in time with abrupt, punctate drops in NS510 fluorescence indicated by the regions of interest. In example A, note that two release events were recorded, each with a decrease in fluorescence in the region of interest, which is presumably due to two nearby vesicles sequentially undergoing exocytosis. Note in examples B and C that an increase in fluorescence is observed for one frame before a drop, which may be due to dye brightening as it enters the stronger evanescent field on the surface of the coverslip before it diffuses away, as previously observed.⁹⁵ These data demonstrate that destaining events correspond to exocytotic events, thus NS510 can be used to monitor exocytosis in live cells.

Figure 2-14. Combination of amperometry with TIRF imaging demonstrates that NS510 can resolve catecholamine release from an individual vesicle. Three single-vesicle release events from three different bovine adrenal chromaffin cells on an Au ring electrode demonstrate punctate drops in NS510 fluorescence synchronous with amperometric spikes resulting from oxidation of catecholamine release from a single vesicle. Circles depict the regions of interest where drops in fluorescence were observed as quantified in the horizontal lines below the amperometric traces (red).

2.4. Conclusions

In conclusion, a fluorescent sensor was designed and synthesized for high affinity for catecholamines and visualize norepinephrine in live cells. NS510 demonstrated that the quinolone was brighter than the corresponding coumarin and gave punctate staining of secretory vesicles using confocal microscopy. More importantly the boronic acid group of NS510 gave it excellent affinity and selectivity for catecholamines over other amines. Although the fluorescence of the sensor was somewhat quenched by the catechol group, it could be used to image quantal norepinephrine release in live cells. These data indicate that NS510 is the most selective and highest affinity catecholamine sensor for use in live cells.

Chapter Three

Near-IR High Affinity Fluorescent Sensor for Imaging Serotonin

3.1 Overview

3.1.1. Introduction

Serotonin (5-Hydroxytryptamine) is one of the most important neurotransmitters involved in functions of animals and human beings. It is biochemically synthesized from the amino acid tryptophan through the hydroxylation of indole ring and decarboxylation (Figure 3-1).⁹⁶ Serotonin is mainly produced in epithelial enteroendocrine (EE) cells, which is able to regulate critical functions of the gastrointestinal (GI) tract.⁹⁷⁻⁹⁹ It also can be found in the central nervous system (serotonergic neurons) which mainly regulates emotions such as happiness and helps regulate learning, memory and cognition and other functions with other neurotransmitters.^{45,100} In addition, it has been reported that serotonin is involved in agitation and vasoconstriction via restoration and release from blood platelets.¹⁰¹ Like other neurotransmitters such as dopamine and glutamate, serotonin is also taken up by vesicular monoamine transporters (VMAT2) into secretory vesicles with high concentrations (50-270 mM), the axon terminal releases serotonin into synaptic cleft upon neurons firing.¹⁰²⁻¹⁰⁵

Figure 3-1. Synthetic pathway of serotonin from L-tryptophan

Currently available methods to detect serotonin are chromatographic and electrochemical methods.^{106,107} As we discussed these methods in the introduction chapter, chromatographic methods can identify serotonin with accurate concentration from other analytes in the mixture. However, this technique requires cell lysis, which is an invasive method. The disposition of serotonin within live cells may be different from that obtained after utilization of chromatographic methods. Electrochemical methods are a noninvasive approach compared to the chromatographic method, quantitative detection and temporal resolution of serotonin can be successfully achieved with this method, but spatial resolution is lacking.

Imaging techniques became popular for neurotransmitter detection with high temporal and spatial resolution. Positron emission tomography (PET) is a powerful tool to image serotonin, the mechanism of which is development of ^{11}C and ^{18}F labeled radiotracers specific binding to serotonin receptors and transporters to study the biodistribution of receptors and transporters.^{108,109} Even though this method is pretty effective to image serotonin receptors and transporters, it is still not a direct method to visualize serotonin with low spatial resolution.

Recently, fluorescence imaging has started to draw attention for serotonin labeling. I have discussed about a multi-photon microscopy method to quantitative and direct detection of serotonin in the first chapter, because serotonin has an aromatic structure and can autofluoresce.^{46,105} However, this method suffers from strong background fluorescence at certain wavelengths and requires deep UV. Fluorescence-based immunostaining is widely used for labeling serotonin, unfortunately this method is not able to image serotonin in live cells and tissue.

I have discussed most of the popular methods widely used for selectively imaging serotonin. These approaches are all not applicable in live biological samples for different reasons, therefore a small-molecule fluorescent sensor for serotonin is in much need. However, recalling the fluorescence of NS510 was strongly quenched by catecholamines, only about 1 fold fluorescence increase for norepinephrine and 10% fluorescence quenched for dopamine, 5-hydroxyindole ethylamine is much more electron-rich than catecholamines (dopamine and norepinephrine). The difficulty to develop a fluorescent sensor for serotonin is most of the fluorescence would be quenched by electron-rich

serotonin (Figure 3-2), which is definitely inapplicable for selectively imaging and detection of serotonin *in vitro* and *in vivo*.

Figure 3-2. Fluorescence quenching trend of catecholamines and serotonin

3.1.2. Previous Work

After several years struggling with searching for suitable fluorophores, finally our group has developed the first fluorescent sensor (NeuroSensor 715) for selective imaging of serotonin (Figure 3-3).⁷⁰ Upon serotonin binding to NS715, the formation of iminium ion produced a 46 nm shift in UV/Vis spectroscopy. The complex was excited at 559 nm and it fluoresced at the wavelength of 715 nm in the NIR region (Figure 3-4). Sensor NS715 was screened with several main neurotransmitters and it turned out to bind to serotonin at the affinity of 409 M^{-1} with 8 fold fluorescence enhancements. NS715 bound to catecholamines with a small fluorescence increase and low binding affinity (Table 3-1). In conclusion, the sensor is not only a NIR fluorescent sensor, but also gives a turn-on fluorescent response with relatively low binding affinity.

Figure 3-3. Binding of NS715 to serotonin

Figure 3-4. UV/Vis spectra (a) and fluorescence spectra (b) of NS715 binding to serotonin

Table 3-1. Titration of NS715 with different analytes

neurotransmitter	$K_a(M^{-1})^a$	$\Delta\lambda_{abs} (nm)$	I_{sat}/I_0^b
serotonin	409	46	8.0
dopamine	145	37	4.0
norepinephrine	129	37	3.4
glutamate	22	30	3.0
epinephrine ^c	nd	nd	nd

^a K_a measured by fluorescence spectroscopy Error in K_a values are $\pm 10\%$ based on triplicate titrations. ^b I_{sat} = fluorescence intensity at saturation taken from the theoretical fit to the binding isotherm. ^c nd = not detectable.

Figure 3-5. Confocal images of norepinephrine and epinephrine enriched chromaffin cells incubated with NS715

3.2 NeuroSensor 659

3.2.1. Design and Synthesis

After we developed the NIR fluorescent sensor NS715 for selective imaging serotonin, we have made great progress towards serotonin labeling. However, the binding affinity towards serotonin and catecholamines are too low to image neurotransmitters at lower concentration. Inspired by my colleagues' and my work (NS715 and NS510), I proposed a selective fluorescent sensor **14** to image serotonin in the near IR region.

A boronic acid group is known to bind to diols, especially catechol with moderate binding affinity. Based on the previous work, we believe that the 5-hydroxyl group of serotonin should be able to bind to a boronic acid with appreciable affinity, due to the activity of hydroxyl group on a very electron-rich aromatic ring. Actually we tested NS510 with serotonin, and the fluorescence was too strongly quenched by serotonin. We can borrow the part of NS715 and combine it with the boronic acid part of NS510 to make a more electron-rich sensor with large binding affinity.

Figure 3-6. Design of selective fluorescent sensor for serotonin

Therefore, we combined the moiety labeled with red color of NS715 and the part labeled with blue color of NS510 to make a structure for NIR selective imaging serotonin (Figure 3-6). We expected this sensor can improve the binding affinity towards serotonin.

A few of synthetic pathways were proposed in order to achieve this boronic acid based coumarin aldehyde sensor. The first trial to synthesize this sensor was following the procedure for the synthesis of NS715 (Scheme 3-1).

Scheme 3-1. Proposed synthesis of sensor 14

The strategy was to reduce **15** to **16** via hydrogenation, followed by reaction of **16** with glyoxal to furnish compound **17**. Then compound **17** underwent reductive ethylation to make compound **18**. A failure of Friedel Crafts reaction for appending compound **19** on compound **18** would not be able for us to furnish compound **20**. The reason we

proposed was the chloromethyl group may involve in reaction with AlCl_3 , which probably disturbed the Friedel Crafts reaction.

Scheme 3-2. Proposed synthesis of sensor **14**

The second strategy we proposed was to use compound **25** to react with the very reactive intermediate **24** generated in situ after demethylation reaction to form compound **26**, after several steps were taken to make compound **14** bases on literature procedures (Scheme 3-2).¹¹⁰ However, this reaction did not work. We propose that the reason was the very reactive intermediate generated in situ was oxidized quickly, due to its very electron-rich character.

After we were unable to use these two synthetic pathways to make the proposed sensor **14**, the other structure NS659 was proposed with a quinolone core (combination of

the moiety of NS715 labeled with red color and moiety of NS510 labeled with blue color) (Figure 3-7). We expect this sensor could show even better turn-on fluorescence response towards serotonin because the quinolone core was demonstrated much more electron-rich than the coumarin fluorophore in Chapter Two.

Figure 3-7. Design of NS659

My first attempt to make NS659 was to take commercially available starting material compound **27** for reductive ethylation reaction to produce compound **28**. Then we wanted to couple a benzyl amine on compound **28** to furnish compound **30** via Buchwald-Hartwig cross coupling, however, the coupling reaction did not go successfully and formed one major unwanted byproduct (debromination of starting material, compound **31**) (Scheme 3-3). The reason why this reaction did not work is

probably Buchwald-Hartwig cross coupling reaction did not favor the very electron-rich aromatic system. Also, we have tried optimized Ulmann coupling at mild conditions, and we did not give the desired product.

Scheme 3-3. Proposed synthesis of NS659

After we tried a couple of synthetic strategies, finally we found an important paper published by Hans Wynberg and colleagues.¹¹¹ They described a new method for aromatic amination reactions by substituting dimethoxy aromatic system with lithium amide (Scheme 3-4).

Scheme 3-4. Synthetic route for aromatic amination reaction

The successful synthetic route for making NS659 was described in Scheme 3-5. First, a condensation of compound **37** with benzaldehyde furnished compound **38**, which reacted with compound **25** to form quinolone core **39** via Pechmann condensation. Suzuki cross coupling reaction of compound **9** and tosylated compound occurred to form compound **41**. We ran the key step following the procedures we discussed above to make compound **43**.¹¹¹ The benzyl alcohol was brominated to yield compound **44**. We furnished compound **45** via Vilsmeier reaction. Alkylation of Compound **45** yielded the final product NS659.

Scheme 3-5. Synthesis of NS659

We also wanted to synthesize a pH sensitive version of NS659 with this method to monitor neurotransmitter release. However, the reaction could only give the starting material with the same reaction conditions (Scheme 3-6). We believe that because sulfonamide is not a good nucleophile as secondary amine.

Scheme 3-6. Proposed synthesis of pH sensitive version of NS659

3.2.2. Spectroscopic Data

NeuroSensor 659 (NS659) was screened with several important neurotransmitters by UV/visible and fluorescence spectroscopy. NS659 actually is the red version of NS510, and it bound to catecholamines by forming an iminium ion group as well as a boronate ester with very high affinity ($17,428 \text{ M}^{-1}$ for dopamine, $20,186 \text{ M}^{-1}$ for norepinephrine and $16,906 \text{ M}^{-1}$ for epinephrine), however, the fluorescence of the sensor NS659 was all quenched to some extent. But for serotonin, the binding constant of this sensor was $2,619 \text{ M}^{-1}$, which is more than four folds higher than that for binding NS715 to serotonin. We believe that NS659 bound to serotonin through not only iminium ion formation, but also the 5-hydroxyl group of indole ring forming a reversible covalent bond with boronic acid (Figure 3-8). Usually, the binding between phenol and boronic acid should provide high binding affinity, but NS659 didn't bind serotonin so well, it was probably because iminium ion formation actually disturbed the binding of 5-hydroxyl

group of indole to phenyl boronic acid. However, this is the first fluorescent sensor for imaging serotonin with high binding affinity compared to our previous work NS715. Upon binding, it gave the emission in the near IR region with a large Stokes shift (more than 150 nm) (Figure 3-9), though the fluorescence was still somewhat quenched, this would not be a problem since it was a near IR sensor, and there is little background fluorescence at this region. Moreover, the concentration of serotonin in vesicles are very high, the fluorescence can still be accumulated in vesicles. In addition, sensor NS659 bound to glutamate with very small binding affinity, which provides appreciable selectivity (Table 3-2).

Figure 3-8. Binding of NS659 to serotonin

Figure 3-9. (a) UV/Vis and (b) fluorescence spectroscopy of NS659 (20 μ M) with serotonin in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, 2% DMSO, pH 7.4), $\lambda_{\text{ex}} = 532$ nm, $\lambda_{\text{em}} = 659$ nm. Inset is the fit to a one-site binding isotherm.

Figure 3-10. (a) UV/Vis and (b) fluorescence spectroscopy of NS659 (20 μ M) with norepinephrine in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, 2% DMSO, pH 7.4), $\lambda_{\text{ex}} = 532$ nm, $\lambda_{\text{em}} = 665$ nm. Inset is the fit to a one-site binding isotherm.

Table 3-2. Association constants (K_a) and spectroscopic parameters for binding of NS659 to several important neurotransmitters

NS659		
amine guest	$K_a(\text{M}^{-1})^a$	I_{sat}/I_0^b
epinephrine	16,906	0.58
norepinephrine	20,186	0.15
dopamine	17,428	0.23
Serotonin	2,619	0.23
glutamate	109	0.31

^a K_a measured by fluorescence spectroscopy. Error in K_a values are $\pm 10\%$ based on triplicate titrations. ^b I_{sat} = fluorescence intensity at saturation taken from the theoretical fit to the binding isotherm.

3.2.3. Cell Studies

After we completed the synthesis of NS659 and the titration data in cuvettes, and it seemed promising for practical use in biological system. We incubated the norepinephrine enriched chromaffin cells with the sensor NS659. We could see NS659 labeled the secretory vesicles with red fluorescence (Figure 3-11). We sent NS659 to our collaborator to image serotonin in dissociated organoids and enterochromaffin cells.

Figure 3-11. Confocal image of norepinephrine enriched chromaffin cells incubated with NS659 ($\lambda_{\text{ex}} = 515 \text{ nm}$)

3.3 Conclusions

In summary, we developed a near IR fluorescent sensor with very high binding affinity and appreciable selectivity. Upon binding to serotonin, it gave a quenched fluorescence in near IR region (659 nm) with a large Stokes shift. NS659 serves as a useful tool to investigate the function of serotonin in nervous system and gastrointestinal tract. Preliminary data we got in chromaffin cells are very promising for potential use in

live biological system and we expect to further investigate the eventual utility of this sensor in live cells and tissue.

Chapter Four

Fluorescent sensor for imaging glutamate in cells and tissue

4.1. Overview

4.1.1. Introduction

Glutamate is the most critical excitatory neurotransmitter in central nervous system. Glutamate is involved in most major excitatory functions of the brain and plays a dominant role in neural circuit communication.¹¹² Similar to other neurotransmitters, glutamate is stored by vesicular glutamate transporters (VGLU) in smaller secretory vesicles (40-50 nm in diameter), compared to the size of catecholamine secretory vesicles (200-400 nm in diameter).¹¹³⁻¹¹⁵ The concentration of glutamate stored in those secretory vesicles is much lower than that for catecholamines.¹¹⁶ With glutamic acid decarboxylase, glutamate is converted to gamma-aminobutyric acid (GABA), and it has been reported that glutamate and GABA are co-released upon neurons firing in hippocampus.^{117,118} For these reasons, development of a noninvasive selective and sensitive method to detect glutamate is significant and challenging.

Figure 4-1. Biochemical pathway of glutamate

Glutamine is the metabolic precursor of glutamate, converting to glutamate in the presence of glutamate dehydrogenase and glutaminase. Consumption of glutamate can convert to GABA with glutamic acid decarboxylase and glutamine with glutamine synthetase. With these processes, the concentration of glutamate is able to keep at a constant level, ~100 mM in secretory vesicles, ~2 mM in astrocyte and ~10 mM in postsynaptic neurons.¹¹⁹ Abnormal levels and dysfunction of glutamate are associated with many nervous system diseases and disorders, such as amyotrophic lateral sclerosis (ALS).^{120,121} Thus glutamate detection is gaining more attention in recent years.

Figure 4-2. Glutamate distribution in nervous system

Magnetic resonance imaging (MRI) was applied to detect glutamate, amine group of glutamate underwent chemical exchange saturation transfer (GluCEST) with bulk water.¹²² With this approach, a change of glutamate concentration was measured in human samples. This method is radiolabel-free, noninvasive and of high temporal resolution, however, it was not able to discern the functions of glutamate in neurotransmission.

Another popular method involving in glutamate detection is to use genetically encoded glutamate fluorescent reporter. Recently genetically encoded fluorescent

reporter (iGluSnFR) for imaging glutamate developed by Looger and coworkers is of high selectivity, high temporal and spatial resolution and enable to measure the dynamics of glutamate.⁴⁷ Unfortunately, it suffered from the limited utilization, only use in transgenic mice, extracellular glutamate level measurement with limited fluorescence enhancements and easy photobleaching. Therefore, we hope to develop a chemosensor to image glutamate without those limitations of biosensors.

4.1.2. Previous Work

Our group has developed a fluorescent sensor for zinc and glutamate, upon binding to glutamate, the fluorescence was quenched, but upon addition of glutamate and zinc, it gave a turn-on response. However, this sensor can only be used for neurons which copackage both glutamate and Zn^{2+} .⁷³ Our group also has designed and synthesized a fluorescent sensor (structure in Figure 4-3) for glucosamine with high selectivity. It bound to glucosamine with high affinity ($4,100 \text{ M}^{-1}$) compared to weak binding to other analytes (Table 4-1).¹²³

Figure 4-3. Structure of Sensor **49**

After we carefully reviewed the titration data for sensor **49**, we found it did bind to glutamate with moderate affinity (105 M^{-1}) (in Table 4-1) as well as other α -amino acids (glycine, aspartic acid) (Figure 4-4). The sensor was selective against catecholamines due to strong quenching upon addition of catecholamines. This sensor binds to glucosamine with large affinity, but it does not appear in glutamatergic neurons. We believe sensor **49** should be able to image glutamate in cells and tissue, however, this sensor has never been tested in cultured glutamatergic neurons due to the synthetic (a) limitations.

Figure 4-4. (a) UV/Vis and (b) fluorescence titration of sensor **49** upon addition of glutamate, inset is the fit to a one site binding isotherm. $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 572 \text{ nm}$

Table 4-1. Titration data of sensor **49** with different analytes

Guest	λ_{ab} (nm)	λ_{em} (nm)	K_a^a (M ⁻¹)	I_{sat}/I_0^b
D-Glucosamine	485	568	4100	32
Norepinephrine	487	NA	25	0
L-Glutamic acid	492	572	105	41
L-Aspartic acid	492	575	107	41
Glycine	484	565	93	22
N-Butylamine	455	550	73	13

^a K_a measured by fluorescence spectroscopy. Error in K_a values are $\pm 10\%$ based on triplicate titrations. ^b I_{sat} = fluorescence intensity at saturation taken from the theoretical fit to the binding isotherm.

4.2. NeuroSensor 560

4.2.1. Design and Synthesis

After a number of trials to make glutamate sensor, we developed a quinolone-based fluorescent sensor for imaging glutamate (NS560) (Structure in Figure 4-5). Compared to the complicated synthesis of Sensor **49**, it is much easier to synthesize.

Figure 4-5. Structure of NS560

The synthesis of NS560 was straightforward. The starting material **6** was readily tosylated to form compound **50**, which underwent Suzuki cross coupling with compound **51** to furnish compound **52**. NS560 was produced by formylation of compound **52** via Vilsmeier reaction (Scheme 4-1).

Scheme 4-1. Synthesis of NS560

The mechanism for NS560 binding to glutamate is that pinacol dissociate from boron in aqueous solution to form boronic acid version of this sensor. Then, the amino group binds to the aldehyde group to form an iminium ion and the α -carboxylic acid group binds to the boronic acid group by forming a boronate ester (Figure 4-6).

Figure 4-6. Binding of NS560 to glutamate

4.2.2. Spectroscopic Data

NS560 was screened with several important amine analytes. Upon addition of glutamate, the formation of iminium ion produced a red shift from 425 nm to 480 nm in UV/Vis spectroscopy. When excited at 488 nm, fluorescence spectroscopy showed a turn-on response at 560 nm. It is noteworthy that there was little background fluorescence for NS560 itself at 560 nm, and it gave more than 100 fold fluorescence enhancement upon addition of 10 mM glutamate, compared to the genetically encoded fluorescent

reporter iGluSnFR, which gave about 5 fold fluorescence enhancement towards addition of 10 mM glutamate. When the titration reached saturation, it had a 740 fold fluorescence enhancement.

Figure 4-7. (a) UV/Vis and (b) fluorescence spectroscopy of NS560 (10 μ M) with glutamate in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, 1% DMSO, pH 7.4), $\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 560$ nm.

All the titration data is summarized in Table 4-2, NS560 can bind to α -amino acids with moderate binding affinity and give very large fluorescence enhancements. Given the fact that there is only small concentration of other amino acids (about 10 μ M) except glutamate, and binding of NS560 to glutamate gave the largest fluorescent response, 3 fold for lysine and 5 fold for glycine, this sensor could be utilized to label glutamate in neurons and tissue. We will validate the utility of this sensor with immunostaining later. For catecholamine neurotransmitters, NS560 bound to them and gave no fluorescence response at 520 nm, which can help differentiate glutamate from catecholamines, though they are distributed in different areas in brain.

Glutamate is the main excitatory neurotransmitters in the nervous system, while GABA is the most abundant inhibitory neurotransmitters in nervous system. It is reported that there are GABAergic neurons, glutamatergic neurons and the mixed GABAergic and glutamatergic neurons in the cortex and hippocampus. Due to lack of valid chemical tools, it is really a hurdle to differentiate glutamate and GABA in neurons. From the data we got in Table 4-2, NS560 bound to glutamate with similar affinity to GABA. Moreover, the binding of the sensor to glutamate produced a 740 fold fluorescence enhancements at 560 nm, while the binding of the sensor NS560 produced only 33 fold fluorescence increase at 520 nm upon binding GABA. Based on this data, we can conclude that the sensor should be able to differentiate glutamate from GABA by the difference of both the emission wavelength and brightness, which is the most important advantage of this sensor. We would like to validate the utility of this fluorescent sensor NS560 to

successfully stain glutamate in cultured neurons and the capability of NS560 to differentiate glutamatergic and GABAergic neurons in cultured hippocampus neuron.

Table 4-2. Titration data of NS560 binding to different analytes

amine guest	$K_a(\text{M}^{-1})^a$	NS560 I_{sat}/I_0	Abs (nm)	Emission (nm)
glutamate	47.2	740	467	560
norepinephrine	-	-	466	518
dopamine	-	-	463	520
lysine	257	256	467	560
glycine	242	150	461	560
GABA	59.4	33	460	520

^a K_a measured by UV/Vis spectroscopy. Error in K_a values are $\pm 10\%$ based on triplicate titrations.

4.2.3. Cell and Tissue Studies

As part of a collaboration with Wallace Thoreson, glutamate dye was loaded into rods and cones by incubating isolated salamander retina for 10 min. with 1 μ M dye in the presence of 20 mM KCl to stimulate vesicle cycling. Retinas were washed three times with amphibian saline and then incubated in 30 u/ml papain for 35 min. After enzymatic treatment, cells were washed with amphibian saline plus 1% BSA and DNAase (1 mg/ml), followed by two more rinses in amphibian saline without BSA. Cells were plated on a coverslip coated with Cell-Tak and allowed to settle for 20 min.

Figure 4-8. Epifluorescence image of photoreceptor cells incubated with NS560

Epifluorescence microscopy revealed that the outer segment was sensitive to light, so autofluorescence was observed. Synaptic terminals were brighter than the soma, consistent with preferential loading of synaptic vesicles. This could build a self-control system that NS560 labeled glutamate at terminal (green fluorescent area) compared to soma (dim area) (Figure 4-8).

Figure 4-9. Brightfield image of another rod (A). Regions of the membrane that adhere to the coverslip can be visualized by TIRFM (B). Maximum intensity image from a series (200 frames) of images shows the region of membrane contact and a few bright spots in the terminal. These bright spots are vesicles that advanced to the membrane during the trial and could therefore be seen by TIRFM (B). C) A single fluorescent synaptic vesicle from this terminal is shown advancing to the membrane (40 ms/frame) and then abruptly disappearing, consistent with fusion and release of glutamate.

Since glutamate is predominant neurotransmitter in retina cells, it is easy to get clear results with low background. We initiated our biological test of NS560 with TIRF microscopy. The data we got showed NS560 successfully labeled glutamate stored in vesicles of small size (bright spots). Upon stimulation with high- K^+ solution, the disappearance of bright spots was observed. We were able to monitor glutamate release with this method. The diameter of vesicles for glutamate is about 50 μm , while it is about

200-300 μm for catecholamine vesicles. Therefore it is difficult to monitor glutamate release via TIRF microscopy compared to monitoring catecholamine release via TIRF microscopy (Figure 4-9).

Figure 4-10. (A) The likelihood of putative fusion events (rapidly disappearing vesicles, N=66 vesicles) increased during a 2 s puff of high KCl (50 mM). (B) Average time course of 29 individual brightly fluorescent vesicles showing that they disappeared rapidly with a time constant of 43 ms (N=29).

We could observe just moderate number of events before and significantly after stimulation with high-K⁺ solution, however, within 2 s after KCl solution, significantly more events were observed (Figure 4-10). In conclusion, NS560 is able to stain glutamate at high concentration and monitor glutamate release with high-K⁺ solution in photoreceptor cells.

Since NS560 worked pretty well in photoreceptor cells, which are reported to have high concentration of glutamate, we moved to test if NS560 can label glutamate at low concentration in astrocytes (about 1 mM) and cultured glutamatergic neurons (about 10 mM) in cytosol.

Figure 4-11. (a) Confocal image of cultured glutamatergic neurons incubated with NS560 (0.5 μ M) (b) epifluorescence image of cultured astrocytes incubated with NS560 (5 μ M)

Cultured glutamatergic neurons were brightly stained by NS560 (Figure 4-11), punctate staining of cultured glutamatergic neurons suggests that the sensor is labeling the secretory vesicles found throughout the whole cell in which glutamate is found. A number of brightly green fluorescent spots were observed close to the membrane, which suggested that NS560 labeled the secretory vesicle clusters of glutamate (Figure 4-11). This data actually matched the reported conclusion that there are vesicular compartments for exocytosis of glutamate in astrocytes to establish cell-cell communication.¹²⁴ In summary, NS560 also could stain glutamate with low concentration and label secretory vesicles of glutamate in cultured glutamatergic neurons and astrocytes.

NS560 was validated to label glutamate in several different cells, therefore we tested NS560 in tissue. We incubated the fresh and live brain slice with 10 μ M NS560 for 45 minutes at 37 °C and moved it to two-photon fluorescence microscope (Figure 4-12).

Figure 4-12. Two-photon image of brain slice incubated with NS560 (10 μ M)

NS560 could penetrate the deep tissue very well and label cell body (bright triangle) and axons (bright line) of glutamatergic neurons. This project is in progress and we need to validate that NS560 actually labels glutamatergic neurons with immunostaining. Moreover, we expect to observe the destaining of glutamatergic neurons which indicates the neurons are communicating with each other via releasing glutamate upon electrical stimulation to complete this project.

Chapter Five

Conclusions and Future Work

5.1. Conclusions

Neurotransmitters are playing very important role in neuroscience, so both invasive and noninvasive methods were developed, as summarized in the first chapter. Since we began to work on the development of fluorescent sensors for direct detection of neurotransmitters, we have already developed a series of sensors. We started with the first fluorescent sensor **4** (Figure 5-1) for catecholamines, upon catecholamines binding, the fluorescent of which was quenched. After that, we tried to develop a proof of concept fluorescent sensor NS521 with turn-on response. It did give several fold turn-on response upon binding norepinephrine and dopamine, however, the binding affinity for catecholamines was not large. The sensor was able to label norepinephrine vesicles in chromaffin cells with low binding affinity, due to the large concentration of norepinephrine in secretory vesicles, but we didn't observe destaining undergoing exocytosis with TIRF imaging. At the same time, a pH sensitive fluorescent sensor for catecholamines was developed by my colleague, which gave several fold turn-on response when the pH jumped from 5 to 7.4, however, the binding constant was not high enough to test in cells. A NIR fluorescent sensor NS715 was developed in our group for imaging serotonin, this sensor actually shares the same disadvantage with NS521. Upon serotonin binding, the emission moved to NIR region with turn-on response. With only an aldehyde group, the binding affinity was not ideal. In the process to develop these fluorescent sensors for catecholamines and serotonin, we also pursued a fluorescent

sensor for glutamate, this sensor was able to give turn-on response in the presence of both glutamate and zinc, but quenched fluorescence upon binding to either glutamate or zinc.

Figure 5-1. Structures of neurotransmitters fluorescent sensors developed in our group

Before I started to work on this project, we already had a toolkit of fluorescent sensors for neurotransmitters, they either lacked of high binding affinity or turn-on fluorescence. We aimed to have a fluorescent sensor for catecholamines with both turn-on response and high binding affinity. After a couple of trials, I did make the sensor NS510, which gave approximately 2 fold turn-on response upon norepinephrine binding, due to incorporation of boronic acid, we improved the binding affinity. We tested this sensor NS510 in norepinephrine enriched chromaffin cells with confocal imaging, TIRF imaging and also correlation of both TIRF imaging and amperometry. After the work was

done, we can claim that the sensor could be able to monitor norepinephrine exocytosis, besides the methods such as FFN, FM dyes I talked about in Chapter One.

Figure 5-2. Structures of fluorescent sensors I designed and synthesized

After my first sensor NS510 was successfully developed, we moved to develop a NIR fluorescent sensor for imaging serotonin. I took the diamine group from NS715 and attached it to the main part of NS510 to make NS659. After about 2 years efforts, we finally made NS659. This sensor gave quenched fluorescence with very large Stokes shift upon binding to catecholamines and serotonin. We tested this sensor in norepinephrine enriched chromaffin cells and observed punctate staining in cells. The reason that we could observe the quenched fluorescence is firstly the concentration of neurotransmitters are pretty high (0.5 M for norepinephrine, 0.25 M for serotonin), allowing the sensor to accumulate in secretory vesicles. Secondly, since the sensor gave emission at NIR region with very large Stokes shift, there was not much background fluorescence in the NIR region. Since there are no serotonin sensors available, we pursued to serotonin imaging with this sensor. One of our collaborators is working on imaging serotonin in both enterochromaffin cells and dissociated organoids with this sensor.

The final project in my dissertation is the development of a fluorescent sensor for imaging glutamate. Glutamate is the most important inhibitory neurotransmitter in the brain. Due to its structure, it is really difficult to incorporate recognition moieties to bind to it. Currently, the most popular glutamate reporter is iGluSnFR, which is genetically encoded sensor. The limitation of the sensors of this kind is the utility in transgenic animals, easily photobleached, limited turn-on response. After we synthesized NS560 with a straightforward synthetic route, tested in photoreceptor cells, the sensor was preferentially loaded into the nerve terminal and labeled glutamate vesicles, and we observed glutamate release via TIRF microscopy. We also use this sensor to label glutamate in hippocampus neurons in both somas and axons and expect to differential glutamate from GABA. Finally we observed that the sensor was able to label both cell bodies and axons of glutamatergic neurons in cortex brain slice with a two-photon microscope.

5.2. Future Work

As I mentioned in this chapter, we have developed a fluorescent sensor ES517 to monitor neurotransmitter release from vesicles to synaptic cleft. Due to the limited fluorescence change and lack of high binding affinity, we are unable to test it in neurons. Therefore, a pH sensitive fluorescent sensor is in need. The design strategy is to attach a pH sensitive group to the main part of NS510, which would give better binding towards catecholamines. I did the initial trial to synthesize this sensor, however, it was not successful. Efforts may be made to try different synthetic route to make this sensor.

Scheme 5-1. Proposed synthetic route for pH sensitive sensor

For glutamate sensor NS560, it has appreciable selectivity for glutamate over GABA and catecholamines with moderate binding affinity, however, it binds to all the α -amino acids with similar affinity. Considering the concentration of glutamate is much higher than that of other amino acids in glutamatergic neurons and the sensor binding to

glutamate gives the largest turn-on response, the sensor is definitely able to image glutamate in both cultured neurons and tissue. For future work, we could increase the binding affinity for this sensor by appending several electron-withdrawing groups on the phenyl ring where the pinacol boron ester was attached.

Figure 5-3. Proposed structures of glutamate sensor

Scheme 5-2. Proposed synthetic route for glutamate sensor

Also I did an initial trial to make these sensors for imaging glutamate with improved binding affinity. Due to the limited resources of the starting material, the synthesis was not successful. But if another synthetic route was found, we can utilize this sensor in biological systems.

APPENDIX

Experimental Procedures and Characterization Data

Part I. General Information

All reagents and solvents were purchased from commercial sources (Sigma Aldrich, Acros Organic, Fisher Scientific, Alfa Aesar, TCI America, or Combi-Blocks) and used without further purification unless stated otherwise. Anhydrous dichloromethane, acetonitrile, and trimethylamine were obtained by distillation from CaH_2 . Anhydrous THF and toluene were obtained by distillation from sodium metal and benzophenone. All reactions were conducted using oven or flame dried glassware and under N_2 atmosphere unless stated otherwise. Flash column chromatography was carried by using 32-63 μm silica gel.

NMR spectra were obtained by a Bruker ARX-250 MHz, DRX-300 MHz, or DRX-500 MHz in CDCl_3 or DMSO-d_6 using tetramethylsilane (TMS) as a reference. IR spectra were obtained from Nicolet FT-IR spectrometers. High resolution mass spectra (HRMS) were obtained from a Bruker Apex-Qe FTMS at Old Dominion University in Norfolk, VA.

Part II. Spectroscopic Data

UV-Visible spectra were obtained by a Varian Cary-1E UV-Visible spectrometer. Fluorescence spectra were obtained by a Shimadzu RF-5301 PC spectrofluorometer. Titrations were performed by using bis-tris propane or HEPES buffer with NaCl or

Na₂S₂O₃ added unless otherwise stated. Absorbance and fluorescence spectra were processed using SigmaPlotTM or Origin and best-fit lines using GraphPad Prism 6.01.

1 mg/ml stock solution of NeuroSensor 16 (NS16) in DMSO for UV/vis spectra and fluorescence spectra was prepared and diluted to 1 ml with buffer (2.0×10⁻⁵ M, 25 mM HEPES, 50 mM Na₂S₂O₃, pH= 7.4, 0.8% DMSO). The analytes (norepinephrine, dopamine, epinephrine, glutamate, lysine and glycine) were prepared by dissolving the analytes in buffer sensor solution (the concentration of NS16 was the same as described above) to ensure the concentration of NS16 was constant. Sodium thiosulfate was used to protect aromatic analytes from oxidation in solution. UV/vis spectra were recorded on a Cary 1E spectrophotometer at 25 °C. Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorometer at 25 °C.

1.0 mM stock solution of NeuroSensor 510 (NS510) in MeOH for UV/vis spectra and fluorescence spectra was prepared and diluted to 1 ml with buffer (1.0×10⁻⁵ M, 25 mM HEPES, 50 mM Na₂S₂O₃, pH= 7.4, 1% MeOH). The analytes (norepinephrine, dopamine, epinephrine, glutamate, lysine, and glucosamine) were prepared by dissolving the analytes in buffer sensor solution (the concentration of NS510 was the same as described above) to make sure the concentration of NS510 keeps constant. Sodium thiosulfate was used to protect aromatic analytes from oxidation in solution. UV/vis spectra were recorded on a Cary 1E spectrophotometer at 25 °C. Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorometer at 25 °C.

1.0 mM stock solution of NeuroSensor 659 (NS659) in DMSO for UV/vis spectra and fluorescence spectra was prepared and diluted to 1 ml with buffer (2.0×10⁻⁵ M, 25

mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, pH= 7.4, 2% DMSO). The analytes (norepinephrine, dopamine, epinephrine, glutamate, serotonin) were prepared by dissolving the analytes in buffer sensor solution (the concentration of NS659 was the same as described above) to make sure the concentration of NS659 keeps constant. Sodium thiosulfate was used to protect aromatic analytes from oxidation in solution. UV/vis spectra were recorded on a Cary 1E spectrophotometer at 25 °C. Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorometer at 25 °C.

Figure A1. (a) UV/vis and (b) fluorescence titration of NS16 (20 μM) of 2 M glutamate in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, pH 7.4, 0.8% DMSO). $\lambda_{\text{ex}} = 488 \text{ nm}$. Inset is the fit to a binding isotherm. $\lambda_{\text{em}} = 520 \text{ nm}$

Figure A2. (a) UV/vis and (b) fluorescence titration of NS16 (20 μM) of 2 M glycine in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, pH 7.4, 0.8% DMSO). $\lambda_{\text{ex}} = 488$ nm. Inset is the fit to a binding isotherm. $\lambda_{\text{em}} = 521$ nm

Figure A3. (a) UV/vis and (b) fluorescence titration of NS16 (20 μM) of 2 M lycine in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, pH 7.4, 0.8% DMSO). $\lambda_{\text{ex}} = 488$ nm. Inset is the fit to a binding isotherm. $\lambda_{\text{em}} = 520$ nm

Figure A4. (a) UV/vis and (b) fluorescence titration of NS16 (20 μM) of 1 M dopamine buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, pH 7.4, 0.8% DMSO). $\lambda_{\text{ex}} = 488$ nm. Inset is the fit to a binding isotherm. $\lambda_{\text{em}} = 517$ nm

Figure A5. (a) UV/vis and (b) fluorescence titration of NS510 (10 μM) of 10 mM dopamine in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, pH 7.4, 1% MeOH). $\lambda_{\text{ex}} = 488$ nm. Inset is the fit of the absorption of 426 nm to a binding isotherm. $\lambda_{\text{em}} = 510$ nm

Figure A6. (a) UV/vis and (b) fluorescence titration of NS510 (10 μ M) of 1 M glucosamine in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, pH 7.4, 1% MeOH). $\lambda_{\text{ex}} = 488$ nm. Inset is the fit of the fluorescence at 510 nm to a binding isotherm. $\lambda_{\text{em}} = 510$ nm

Figure A7. (a) UV/vis and (b) fluorescence titration of NS510 (10 μ M) of 1 M glutamate in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, pH 7.4, 1% MeOH). $\lambda_{\text{ex}} = 488$ nm. Inset is the fit of the fluorescence at 510 nm to a binding isotherm. $\lambda_{\text{em}} = 510$ nm

Figure A8. (a) UV/vis and (b) fluorescence titration of NS510 (10 μ M) of 1 M lysine in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, pH 7.4, 1% MeOH). $\lambda_{\text{ex}} = 488$ nm. Inset is the fit of the fluorescence at 510 nm to a binding isotherm. $\lambda_{\text{em}} = 510$ nm

Figure A9. (a) UV/vis and (b) fluorescence titration of NS510 (10 μ M) of 10 mM epinephrine in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, pH 7.4, 1% MeOH). $\lambda_{\text{ex}} = 488$ nm. Inset is the fit of the absorption of 426 nm to a binding isotherm. $\lambda_{\text{em}} = 510$ nm

Figure A10. UV/vis and fluorescence titration of red sensor NS659 (20 μM) of 10 mM dopamine in buffer (25 mM HEPES, 50 mM Na₂S₂O₃, pH 7.4, 2% DMSO). $\lambda_{\text{ex}} = 532$ nm. Inset is the fit of the fluorescence to a binding isotherm.

Figure A11. Fluorescence titration of red sensor NS659 (20 μM) of 10 mM epinephrine in buffer (25 mM HEPES, 50 mM Na₂S₂O₃, pH 7.4, 2% DMSO). $\lambda_{\text{ex}} = 532$ nm.

Figure A12. Fluorescence titration of red sensor NS659 (20 μ M) of 1 M glutamate in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, pH 7.4, 2% DMSO). $\lambda_{\text{ex}} = 532$ nm.

Figure A13. (a) UV/Vis and (b) fluorescence spectroscopy of NS560 (10 μM) with 1 M GABA in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, 1% DMSO, pH 7.4), $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$.

Figure A14. (a) UV/Vis and (b) fluorescence spectroscopy of NS560 (10 μ M) with 1 M dopamine in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, 1% DMSO, pH 7.4), $\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 520$ nm.

Figure A15. (a) UV/Vis and (b) fluorescence spectroscopy of NS560 (10 μ M) with 500 mM glycine in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, 1% DMSO, pH 7.4), λ_{ex} = 488 nm, λ_{em} = 560 nm.

Figure A16. (a) UV/Vis and (b) fluorescence spectroscopy of NS560 (10 μ M) with 500 mM lysine in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, 1% DMSO, pH 7.4), $\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 560$ nm.

Figure A17. (a) UV/Vis and (b) fluorescence spectroscopy of NS560 (10 μ M) with 1 M norepinephrine in buffer (25 mM HEPES, 50 mM $\text{Na}_2\text{S}_2\text{O}_3$, 1% DMSO, pH 7.4), $\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 520$ nm.

Relative Quantum Yield Determination To determine the relative quantum yield of NS16, NS16-norepinephrine complex, NS510 and NS510-norepinephrine complex, a stock solution of NS16 in buffer (25 mM HEPES, 50 mM Na₂S₂O₃, pH 7.4, 0.8% DMSO), NS510 in buffer (25 mM HEPES, 50 mM Na₂S₂O₃, pH 7.4, 1% DMSO) and a stock solution of norepinephrine in buffer (25 mM HEPES, 50 mM Na₂S₂O₃, pH 7.4) was prepared. A fluorescein solution was prepared by dissolving fluorescein in 0.1M NaOH (pH=13). The absorption of the fluorescein solution, sensor solution and sensor-analyte solution were recorded on a Cary 1E spectrophotometer at 25 °C ($A_{\text{NS16}} = 0.077$, $A_{\text{NS16-NE}} = 0.077$, $A_{\text{NS510}} = 0.065$, $A_{\text{NS510-NE}} = 0.076$, $A_{\text{standard}} = 0.06$). To ensure the accuracy, the absorptions of fluorescein solution, sensor solution and sensor-analyte solution must be smaller than 0.01. The reported quantum yield of fluorescein is 0.85 excited at 494 nm.⁸⁹ All the three solutions were excited at 494 nm, and the data were recorded on a Shimadzu RF-5301 PC spectrofluorometer at 25 °C. The area under the curve for the three solutions were integrated with excel starting from 504 nm ($FA_{\text{NS16}} = 339.47$, $FA_{\text{NS16-NE}} = 838.20$, $FA_{\text{NS510}} = 328.819$, $FA_{\text{NS510-NE}} = 476.432$, $FA_{\text{Standard}} = 10182.96$). Given the equation below,¹²⁵ the relative quantum yields ($\phi_{\text{NS16}} = 0.022$, $\phi_{\text{NS16-NE}} = 0.055$, $\phi_{\text{NS510}} = 0.025$, $\phi_{\text{NS510-NE}} = 0.031$) were obtained.

$$\text{Equation } QY_u = \frac{(QY_s)(FA_u)(A_s)}{(FA_s)(A_u)}$$

Part III. Protocol for Cell Studies

Chromaffin Cell Preparation. Chromaffin cells were isolated from bovine adrenal glands as previously described.⁸⁶ Following centrifugation at 18 °C at 13,500 rpm for 30 min in the Percoll (Sigma Life Science, P4937, St. Louis, MO) gradient, chromaffin cells were separated into fractions enriched with either epinephrine-enriched cells (EP, the bottom fraction) and norepinephrine-enriched cells (NE, the top fraction).⁴ Over 90% of the catecholamine content found in the cells in the bottom fraction is epinephrine, whereas approximately 67 % of the catecholamine content in the top fraction is norepinephrine.⁸⁷ The two cell fractions were collected from the Percoll gradient with careful pipetting and separately cultured in chromaffin cell regular medium (Dulbecco's Modified Eagle's Medium (DMEM), 11965-092 gibco[®] by life technologies[™] corporation, Grand Island, NU) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Sigma Life Science, F6178, St. Louis, MO) and 1% Penicillin/Streptomycin (GIBCO Invitrogen cell culture, 15140, Carlsbad, CA)) for 20 min. Then we used an alternative culture method to make it easier to detach the cells from the flasks and to reduce cell clumping. Chromaffin cells were cultured in Hibernate A media with calcium (BrainBits LLC, Springfield, IL) in a refrigerator (4 °C) and used 1–6 days after preparation.⁵

Solutions. The standard cell bath solution for amperometry measurements, confocal imaging and total internal reflection fluorescence (TIRF) imaging as well consisted of 150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM HEPES, and 11 mM glucose titrated to pH 7.3 with 5 M NaOH. The osmolality is ~310. A “high-

K⁺ solution was used to trigger exocytosis that consisted of 55 mM NaCl, 100 mM KCl, 5 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, and 10 mM glucose titrated to pH 7.3 with 1M NaOH. Chromaffin cells regular medium is DMEM supplemented with 10% (v/v) Fetal Bovine Serum and 1% Penicillin/Streptomycin.

Confocal imaging and TIRF imaging. Approximately 4 ml of culture media containing suspended norepinephrine-enriched cells or epinephrine-enriched cells were centrifuged at 1000 rpm for 5 min. The pellet was resuspended in 1 ml DMEM only without FBS and antibiotics to help cells intake the dye. Next, the cells were incubated on a 35 mm Petri dish with 0.5 μ M NS510 in a humidified incubator at 37 °C with 5% CO₂ for 45 min. Then two types of cells were transferred into 15 ml conical tubes, respectively, spun, washed twice by prewarmed 1XDPBS (Dulbecco's Phosphate Buffered Saline, 14190-144, gibco[®] by life technologies[™] corporation, Grand Island, NU) and resuspended with prewarmed standard cell bath solution. Finally, the coverslip that coated 0.0025% poly-L-lysine solution (Sigma Life Science, P4707, St. Louis, MO) was mounted onto the stage of Olympus Optical FluoView FV-1000 confocal laser scanning biological microscope, and 500 μ l norepinephrine or epinephrine cell in appropriate density was loaded into the coverslip. The confocal images were acquired using a laser with a wavelength of 488 nm, or TIRF images, evanescent illumination (EVA mode) after waited for ~20 min to let cells settle down.

Patterning of working electrodes. The Au/Ti (30 nm/5 nm) film on 3"x3" No. 2 cover glass was patterned into working electrodes using photolithography and wet etching as described previously.¹²⁶ A circular $\varnothing$ 10 μ m gap was etched in the electrodes to

allow imaging such that the working electrode consisted of a ring. The thick photoresist SU-8 2025 (MicroChem Corp, Newton, MA) was used both to fabricate Ø20 µm microwells and to insulate nonactive areas of the conductive film. Pattered electrode devices on 3"x3" cover glass were diced into nine biochips and assembled into nine devices, and Cyclic Voltammetry with the test analyte ferricyanide was used to validate the sensitivity of electrodes before cell studies.

Combination of amperometric recordings and TIRF recordings. Each device was further cleaned by air plasma and coated with 0.0025% poly-L-lysine at room temperature for 30 min, then washed with dd H₂O and dried. 100 µl of the cell suspension solution was loaded onto the device for combination of amperometric recordings and TIRF recordings. After waiting for ~20 min for the suspended cells in the solution to fall into each individual microwell and adhere to the device surface, 50 µl of the high-K⁺ stimulation solution was added to the reservoir cassette. Amperometric recordings were performed using a self-designed 16-channel potentiostat and a potential of 600 mV was applied relative to a Ag/AgCl wire inserted in the standard cell bath solution that served as the counter/reference electrode. Meanwhile, a series of TIRF images (1000 frames) were acquired with Hamamatsu EM-CCD camera (C9100-12, Hamamatsu Photonics, Japan) controlled by slidebook software and the TTL signal from slidebook was recorded for synchronization of image and amperometric recording. Amperometric signals were low-pass filtered with cutoff frequency of 1000 Hz and sampled at a rate of 10 ksamples/s.

Figure A18. Consecutive TIRF images of NS510 labeled vesicles (10 frames /s) (a, b and c present three different bovine chromaffin cells): Regions of interest depict abrupt, punctate loss of fluorescence that is assumed to exocytosis of a vesicle containing NE labeled with NS510 after stimulation with a high- K^+ solution.

NMR titration of NS510 with epinephrine

Figure A19. (a) NMR of NS510 aromatic region of NS510 (1.4 mM, 80/20 MeOD/D₂O). (b) Aromatic region upon addition of 2.1 equivalents of epinephrine. Large epinephrine peaks are evident between 6.7 and 6.9 ppm.

NS510 was dissolved in a 80/20 mixture of deuterio-methanol/D₂O. Small impurities in the NMR are due to small amounts of hemiacetal which forms with these types of aldehydes in methanol. Upon addition of epinephrine, changes in the aromatic region are observed. Notably, the aldehyde peak integrates for one proton after addition of epinephrine, indicating that no amine adduct forms.

Part IV. Synthetic Procedures and Characterization Data

Compound **7**. POCl₃ (5.2 ml, 56.1 mmol) was added to DMF (10.8 ml, 139.5 mmol) at 0 °C in a flame dried round bottom flask. The reaction mixture was stirred at ambient temperature for 2 hours. The Vilsmeier reagent (12 ml) was added to a solution of compound **6**⁸⁵ (586 mg, 2 mmol) in DMF (20 ml). The solution was stirred at ambient temperature for 16 hours. The resulting red solution was poured onto ice water, extracted with CH₂Cl₂. The combined organic layers were dried over Na₂SO₄ and the solvent was removed in *vacuo*. The residue was purified by chromatography (28:1 CH₂Cl₂/MeOH) to yield compound **7** (467.3 mg, 1.37 mmol, 69%) as a yellow solid (m.p. 234 °C): ¹H NMR (500 MHz, CDCl₃) δ 10.55 (s, 1H), 8.01 (d, 1H, *J* = 9.5 Hz), 7.23-7.33 (m, 6H), 6.65 (dd, 1H, *J* = 9.5, 2.5 Hz), 6.21 (d, 1H, *J* = 2 Hz), 5.49 (s, 2H), 3.00 (s, 6H); ¹³C NMR (125 MHz, CDCl₃) δ 189.4, 162.3, 153.8, 148.4, 142.3, 136.0, 129.8, 128.8, 127.4, 126.7, 116.0, 109.6, 95.3, 46.2, 40.1; IR (neat, cm⁻¹) 2914, 2846, 1704, 1678, 1651, 1591, 1553, 1534, 1501, 1395; HRMS calculated for C₁₉H₁₇ClN₂O₂Na (M+Na⁺): 363.0870, Found 363.0870.

NS16. A mixture of compound **7** (120 mg, 0.35 mmol), 2-thienyl boronic acid (54 mg, 0.42 mmol), Pd₂dba₃ (16.1 mg, 0.05 mmol), Sphos (21.5 mg, 0.15 mmol), K₃PO₄ (149.7 mg, 2 mmol) degassed for 20 minutes, dry, degassed THF (4 ml) was added and the solution was stirred at 50 °C for 24 h, the solution was filtered and extracted with CH₂Cl₂ (3× 20 ml) and removed in *vacuo*. The remaining residue was purified by flash chromatography (20:1 CH₂Cl₂/MeOH) to yield NS16 as a brown oil (55.6 mg, 0.14 mmol, 41%). ¹H NMR (500 MHz, CDCl₃) δ 10.21 (s, 1H), 7.53 (dd, 1H, *J* = 5.5, 1 Hz), 7.23-7.36 (m, 6H), 7.17 (dd, 1H, *J* = 4.5, 3.5 Hz), 7.06 (dd, 1H, *J* = 3.5, 1 Hz), 6.51 (dd, 1H, *J* = 9, 2 Hz), 6.28 (d, 1H, *J* = 2 Hz), 5.56 (s, 1H), 2.97 (s, 6H); ¹³C NMR (125 MHz, CDCl₃) δ 190.1, 161.5, 153.5, 149.4, 142.7, 136.5, 134.5, 131.2, 128.7, 128.7, 127.3, 127.2, 127.0, 126.9, 118.8, 111.7, 108.8, 95.5, 46.1, 40.0; IR (neat, cm⁻¹) 2922, 2852, 1694, 1600, 1506, 1486, 1401, 1179, 973; HRMS calculated for C₂₃H₂₀N₂O₂SNa (M+Na⁺): 411.1137, Found 411.1137.

Compound **10** TsCl (572 mg, 3 mmol) was added to a mixture of compound **6**⁸⁵ (800mg, 2.72 mmol) and Na₂CO₃ (862 mg, 8.13 mmol) in the solvent mixture THF (24 ml) and H₂O (1.2 ml), and the mixture was heated to 50 °C for 2 hours. Then the reaction mixture was cooled down to room temperature. 3-hydroxymethyl phenylboronic acid (497 mg, 3.27 mmol), Pd₂dba₃ (124.6 mg, 0.14 mmol) and Sphos (168.4 mg, 0.41 mmol) were added into the mixture, degassed for 15 minutes. The solution was heated at 50 °C overnight. The solution was filtered and extracted with CH₂Cl₂ (3×30 ml) and removed *in vacuo*. The remaining residue was purified by flash chromatography (40:1 CH₂Cl₂/MeOH) to furnish compound **10** as a light yellow amorphous solid (679 mg, 65%). ¹H NMR (500 MHz, CDCl₃) δ 7.42-7.47 (m, 3H), 7.28-7.33 (m, 6H), 7.21-7.24 (m, 1H), 6.48 (dd, 1H, *J* = 9, 2 Hz), 6.4 (s, 1H), 6.38 (d, 1H, *J* = 2.5 Hz), 5.56 (s, 2H), 4.77 (s, 2H), 2.90 (s, 6H), 2.65 (s, 1H) ; ¹³C NMR (125 MHz, CDCl₃) δ 163.1, 151.5, 141.4, 141.2, 137.9, 136.9, 128.7, 128.5, 128.5, 127.9, 127.3, 127.1, 126.9, 126.8, 115.1, 111.1, 107.9, 96.9, 64.9, 46.1, 40.0; IR (neat, cm⁻¹) 3387, 3048, 2987, 2986, 2921, 2365, 2325, 1646, 1601, 1552, 1532, 1397, 1254; HRMS calculated for C₂₅H₂₄N₂O₂Na (M+Na⁺): 407.1729, Found 407.1730.

Compound **11** To the solution of compound **10** (200 mg, 0.52 mmol) in dry dichloromethane (5 ml), POCl₃ (0.78 ml, 8.4 mmol) was added. The reaction was stirred at room temperature for 8 hours and quenched with H₂O. The mixture was extracted with DCM (3×10ml) and the solvent was removed *in vacuo*. The remaining residue was purified by flash chromatography (80:1 CH₂Cl₂/MeOH) to yield compound **11** as a yellow oil (142.6 mg, 68%). ¹H NMR (500 MHz, CDCl₃) δ 7.46-7.47 (m, 3H), 7.39-7.41 (m 1H), 7.29-7.33 (m, 5H), 7.21-7.23 (m, 1H), 6.51 (dd, 1H, *J* = 9, 2 Hz), 6.47 (s, 1H), 6.39 (d, 1H, *J* = 2 Hz), 5.58 (s, 2H), 4.64 (s, 2H), 2.90 (s, 6H); ¹³C NMR (125 MHz, CDCl₃) δ 162.8, 151.5, 150.7, 141.3, 138.3, 137.6, 136.9, 128.9, 128.8, 128.7, 128.6, 128.4, 128.3, 127.0, 126.7, 115.3, 110.7, 107.7, 96.8, 46.0, 45.8, 40.0; IR (neat, cm⁻¹) 2921.2, 2847.7, 1654.5, 1609.5, 1527.8, 1491.0, 1450.2, 1393.0, 1352.1, 1303.1, 1266.3, 1164.1; HRMS calculated for C₂₅H₂₃ClN₂ONa (M+Na⁺): 425.1391, Found 425.1391.

Compound **12** POCl₃ (0.87 ml, 9.3 mmol) was added to DMF (1.8 ml, 23.2 mmol) at 0 °C in a flame dried round bottom flask. The reaction mixture was stirred at ambient temperature for 1 hour. The Vilsmeier reagent (0.77 ml) was added to a solution of compound **11** (120 mg, 0.3 mmol) in DMF (4 ml). The solution was stirred at ambient temperature for 16 hours. The resulting red solution was poured onto ice water, extracted with CH₂Cl₂ (3×10ml). The combined organic layers were dried over Na₂SO₄ and the solvent was removed *in vacuo*. The residue was purified by chromatography (70:1 CH₂Cl₂/MeOH) to yield compound **12** (76 mg, 59%) as a yellow solid (m.p. 194 °C). ¹H NMR (500 MHz, CDCl₃) δ 10.24 (s, 1H), 7.20-7.49 (m, 9H), 7.01 (d, 1H, *J* = 9.5 Hz), 6.47 (dd, 1H, *J* = 9, 2 Hz), 6.30 (d, 1H, *J* = 2 Hz), 5.59 (s, 2H), 4.66 (d, 1H, *J* = 12 Hz), 4.64 (d, 1H, *J* = 12 Hz), 2.97 (s, 6H); ¹³C NMR (125 MHz, CDCl₃) δ 190.5, 162.4, 155.4, 153.4, 143.1, 137.5, 136.4, 135.9, 131.6, 129.0, 128.8, 128.5, 128.5, 128.3, 127.3, 126.9, 117.2, 111.2, 108.8, 95.7, 46.1, 45.9, 40.0; IR (neat, cm⁻¹) 3052.0, 2986.6, 2925.3, 2855.9, 2308.3, 1691.3, 1642.2, 1601.4, 1519.6, 1487.0, 1409.3, 1270.4, 1184.6; HRMS calculated for C₂₆H₂₃ClN₂ONa (M+Na⁺): 453.1340, Found 453.1337.

NS510 Compound **12** (76 mg, 0.18 mmol), (2-((methylamino)methyl)phenyl)boronic acid (43.6 mg, 0.26 mmol) and K_2CO_3 (121.6 mg, 0.88 mmol) were placed in a sealed tube with THF (1.5 ml). The mixture was stirred at 50 °C for 16h. The mixture was cooled, poured onto H_2O (10 mL), extracted with CH_2Cl_2 (3 x 10 mL), dried on $MgSO_4$, and the solvent was removed *in vacuo*. The resulting residue was purified by prep reverse phase TLC plates (85% MeOH/15% H_2O) to give NS510 as a yellow amorphous solid (41.4 mg, 42%). 1H NMR (600 MHz, $CDCl_3$) δ 10.20 (s, 1H), 8.15 (s, 1H), 7.91 (dd, 1H, $J = 6.6, 1.2$ Hz), 7.91 (t, 1H, $J = 1.2$ Hz), 7.33-7.49 (m, 8H), 7.21 (d, 1H, $J = 1.8$ Hz), 7.18 (m, 2H), 6.98 (d, 1H, $J = 9$ Hz) 6.44 (dd, 1H, $J = 9.6, 2.4$ Hz), 6.30 (d, 1H, $J = 2.4$ Hz), 5.58 (s, 2H), 3.64-3.75 (m, 4H), 2.96 (m, 6H), 2.19 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 190.8, 162.5, 156.2, 153.6, 143.3, 141.6, 136.8, 136.5, 136.3, 135.7, 131.8, 130.8, 130.3, 130.1, 129.6, 129.0, 128.6, 128.1, 127.7, 127.5, 127.2, 117.6, 111.5, 108.9, 95.9, 64.3, 60.9, 46.4, 40.6, 40.3; IR (neat, cm^{-1}) 2933.5, 2851.7, 1687.2, 1597.3, 1511.5, 1478.8, 1450.2, 1393.0, 1360.3, 1172.3; HRMS NS510 was dissolved in MeOH, generating the boronic acid dimethyl ester. Calculated for $C_{36}H_{38}BN_3O_4Na$ ($M+Na^+$): 610.2847, Found 610.2846.

Compound **40** Compound **39** (3.11 g, 9.98 mmol) was mixed with THF (50 ml) and H₂O (2.5 ml) at ice/water bath, then Na₂CO₃ (1.27 g, 12.0 mmol) was added into the cloudy mixture. The mixture was stirred at 0 °C for 15 min. *p*-Toluenesulfonyl chloride (2.29 g, 12.1 mmol) was added into the cloudy mixture and stirred it at 0 °C for 3 hrs. The mixture was warmed to room temperature and quenched with H₂O, and extracted with CH₂Cl₂ (3 x 30 mL), dried on MgSO₄, and the solvent was removed *in vacuo*. The resulting residue was purified by chromatography (2:1 Hex/EtOAc) to give compound **40** as a light yellow powder (4.56 g, 98%, M.P. 144-148 °C). ¹H NMR (500 MHz, CDCl₃) δ 7.89 (d, 2H, *J* = 8.5 Hz) 7.38 (d, 2H, *J* = 8 Hz), 7.31 (t, 2H, *J* = 1 Hz), 7.24-7.30 (m, 1H), 7.20 (d, 2H, *J* = 7 Hz), 6.68 (s, 1H), 6.39 (s, 1H), 5.49 (s, 2H), 3.85 (s, 3H), 3.73 (s, 3H), 2.46 (s, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 162.5, 154.4, 153.0, 146.3, 145.4, 136.1, 135.4, 132.4, 130.2, 128.9, 128.4, 127.5, 126.6, 109.6, 108.3, 104.1, 98.4, 56.1, 56.0, 46.5, 21.8; IR (neat, cm⁻¹) 3429.2, 3052.2, 2993.3, 2938.8, 2944.9, 2305.6, 2305.6, 1649.4, 1600.0, 1564.0, 1528.1, 1429.2, 1271.9, 1195.5, 1060.7, 1024.7, 741.6; HRMS calculated for C₂₅H₂₃NO₆SNa (M+Na⁺): 488.1138, found 488.1135.

Compound **41** Compound **40** (1.2 g, 3 mmol), 3-hydroxymethyl phenylboronic acid (497 mg, 3.27 mmol), Pd₂dba₃ (137.4 mg, 0.15 mmol), Sphos (184.7 mg, 0.45 mmol) and K₃PO₄ (1.27 g, 6 mmol) were placed in a sealed tube with THF (15 ml) and H₂O (0.75 ml). After the mixture was degassed for 20 min, it was heated to 110 °C for 36 hrs. The solution was filtered and extracted with CH₂Cl₂ (3×50ml) and removed *in vacuo*. The remaining residue was purified by flash chromatography (80:1 CH₂Cl₂/MeOH, 40:1 CH₂Cl₂/MeOH) to furnish compound **41** as a light yellow amorphous solid (1.15 g, 96%).
¹H NMR (500 MHz, CDCl₃) δ 7.50 (s, 1H), 7.44-7.46 (m, 2H), 7.28-7.31 (m, 3H), 7.23-7.28 (m, 3H), 6.92 (s, 1H), 6.75 (s, 1H), 6.55 (s, 1H), 5.58 (s, 2H), 4.78 (d, 2H, *J* = 3 Hz), 3.74 (s, 3H), 3.63 (s, 3H), 3.55 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 162.2, 151.7, 151.0, 144.7, 142.1, 137.3, 136.4, 135.1, 128.8, 128.5, 127.5, 127.3, 127.1, 127.1, 126.6, 118.1, 113.7, 108.4, 98.5, 64.5, 55.9, 55.8, 46.3; IR (neat, cm⁻¹) 3406.7, 3051.6, 3002.2, 2970.7, 2934.8, 2665.1 2512.3, 2359.5, 2341.5, 2076.4, 1959.5, 1644.9, 1577.5, 1555.0, 1519.1, 1267.4, 1074.1, 1029.2; HRMS calculated for C₂₅H₂₃NO₄Na (M+Na⁺): 424.1519, found 424.1517.

Compound **43** Compound **41** (803 mg, 2 mmol) was dissolved in dry toluene in a flame-dried round bottom flask, then the reaction mixture was cooled to 0 °C in ice/water bath. To this mixture, *n*-Buli (0.1 ml in hexanes, 4.2 mmol) was added. After stirring the mixture at 0 °C for 30 min, it was warmed to RT for 1 hr. *N, N'*-Diethylethylenediamine (0.3 ml, 2.1 mmol) was added into the reaction mixture, which was heated to 75 °C overnight. The reaction was quenched with H₂O, followed by extraction with CH₂Cl₂ (3×20ml) and removed *in vacuo*. The remaining residue was purified by flash chromatography (80:1 CH₂Cl₂/MeOH, 40:1 CH₂Cl₂/MeOH) to furnish compound **43** as a light yellow oil (390 g, 43%). ¹H NMR (600 MHz, CDCl₃) δ 7.47 (s, 1H), 7.41-7.45 (m, 2H), 7.34 (dt, 2H, *J* = 5.4, 1.8 Hz), 7.29 (d, 4H, *J* = 5.4 Hz), 7.18-7.23 (m, 1H), 6.53 (s, 1H), 6.38 (s, 1H), 6.28 (s, 1H), 5.56 (s, 2H), 4.75 (d, 2H, *J* = 3 Hz), 3.37 (t, 2H, *J* = 4.8 Hz), 3.19 (q, 2H, *J* = 7.2 Hz), 3.16 (t, 2H, *J* = 4.2 Hz), 3.06 (q, 2H, *J* = 7.2 Hz), 2.71 (s, 1H), 1.01 (t, 3H, *J* = 7.2 Hz), 0.85 (t, 3H, *J* = 7.2 Hz); ¹³C NMR (150 MHz, CDCl₃) δ 162.3, 150.6, 141.3, 138.7, 138.5, 137.3, 134.4, 130.7, 128.7, 128.4, 127.9, 127.3, 126.9, 126.7, 126.5, 114.8, 111.2, 107.0, 95.9, 64.9, 47.2, 46.2, 45.5, 45.3, 45.2, 9.8, 9.7; IR (neat, cm⁻¹) 3429.2, 3060.6, 2984.2, 2687.6, 2521.3, 2305.6, 1644.9, 1613.4, 1559.5, 1537.0, 1420.2, 1384.2, 1186.5, 1092.1; HRMS calculated for C₂₉H₃₁N₃O₂Na (M+Na⁺): 476.2308, found 476.2307.

Compound **44** To the solution of compound **43** (310 mg, 0.68 mmol) in dry dichloromethane (5 ml), PBr_3 (0.08 ml, 0.82 mmol) was added. The reaction was stirred at room temperature for 3 hours and quenched with H_2O . The mixture was extracted with DCM ($3 \times 10\text{ml}$) and the solvent was removed *in vacuo*. The remaining residue was purified by flash chromatography (80:1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$) to yield compound **44** as an impure foaming solid (189.6 mg, 54%). Because the product was not stable, we used it for the next step right away after we furnished it.

Compound **45** POCl₃ (0.65 ml, 6.97 mmol) was added to DMF (1.35 ml, 17.4 mmol) at 0 °C in a flame dried round bottom flask. The mixture was stirred at ambient temperature for 1 hours. The Vilsmeier reagent (0.38 ml) was added to a solution of compound **44** (80 mg, 0.147 mmol) in DMF (1.4 ml). The solution was stirred at ambient temperature for 2.5 hours. The resulting red solution was poured onto ice water, extracted with CH₂Cl₂ (3×10ml). The combined organic layers were dried over Na₂SO₄ and the solvent was removed *in vacuo*. The residue was purified by chromatography (70:1 CH₂Cl₂/MeOH) to yield compound **45** (26 mg, 33%) as an impure red oil. Because the product was not stable enough, we set the reaction for the final product with this product right away after we furnished it.

NS659 Compound 45 (45 mg, 0.083 mmol), (2-((methylamino)methyl)phenyl)boronic acid (21.5 mg, 0.13 mmol) and K_2CO_3 (57.4 mg, 0.42 mmol) were placed in a sealed tube with THF (1.5 ml). The mixture was stirred at 50 °C for 16h. The mixture was cooled, poured onto H_2O (10 mL), extracted with CH_2Cl_2 (3 x 10 mL), dried on $MgSO_4$, and the solvent was removed *in vacuo*. The resulting residue was purified by prep reverse phase TLC plates (95% MeOH/5% H_2O) to give NS659 as a red amorphous solid (29.2 mg, 56%). 1H NMR (600 MHz, MeOD) δ 10.1 (s, 1H), 7.56-7.63 (m, 4H), 7.43 (s, 1H), 7.19-7.38 (m, 9H), 7.13 (d, 1H, $J = 7.2$ Hz), 6.29 (s, 1H), 6.04 (s, 1H), 5.66 (s, 2H), 4.14 (s, 2H), 4.06 (s, 2H), 3.48 (t, 2H, $J = 4.8$ Hz), 3.32 (t, 2H, $J = 7.2$ Hz), 3.13 (q, 2H, $J = 4.8$ Hz), 2.94 (q, 2H, $J = 7.2$ Hz), 2.41 (s, 3H), 0.85 (q, 6H, $J = 6.6$ Hz), ^{13}C NMR (150 MHz, MeOD) δ 190.3, 162.3, 154.4, 143.3, 140.0, 138.0, 137.0, 132.7, 131.9, 131.0, 130.4, 128.8, 128.7, 128.6, 128.3, 127.0, 126.1, 115.0, 111.7, 107.0, 94.4, 61.7, 58.5, 45.5, 45.4, 44.6, 44.4, 38.9, 31.3, 29.3, 22.3, 13.0, 9.0, 8.7, 8.4; IR (neat, cm^{-1}): 3445.3, 2993.1, 2906.9, 2328.3, 1986.7, 1666.6, 1518.8, 1438.8, 1411.1, 1340.3, 1309.6, 1260.3, 1146.4, 1051.0, 949.5; HRMS NS659 was dissolved in MeOH, generating the boronic acid dimethyl ester. Calculated for $C_{40}H_{45}BN_4O_4Na$ ($M+Na^+$): 679.3426, Found 679.3429.

Compound **50** Compound **6**⁸⁵ (2.94 g, 10 mmol) was mixed with THF (50 ml) and H₂O (2.5 ml) at ice/water bath, then Na₂CO₃ (1.27 g, 12.0 mmol) was added into the cloudy mixture. The mixture was stirred at 0 °C for 15 min. *p*-Toluenesulfonyl chloride (2.29 g, 12.1 mmol) was added into the cloudy mixture and stirred it at ambient temperature for 5 hrs. The mixture was warmed to room temperature and quenched with H₂O, and extracted with CH₂Cl₂ (3 x 20 mL), dried on MgSO₄, and the solvent was removed *in vacuo*. The resulting residue was purified by chromatography (2:1 Hex/EtOAc) to give compound **50** (4.3 g, 96%) as a yellow powder (M.P. 151-154 °C). ¹H NMR (600 MHz, CDCl₃) δ 7.86 (d, 2H, *J* = 8.4 Hz), 7.55 (d, 1H, *J* = 9 Hz), 7.32 (d, 2H, *J* = 8.4 Hz), 7.26 (t, 2H, *J* = 5.4 Hz), 7.18-7.25 (m, 3H), 6.50 (dd, 1H, *J* = 9, 2.4 Hz), 6.22 (d, 2H, *J* = 2.4 Hz), 5.41 (s, 2H), 2.87 (s, 6H), 2.41 (s, 3H); ¹³C NMR (150 MHz, CDCl₃) δ 163.4, 155.2, 152.5, 146.1, 141.4, 136.6, 132.5, 130.2, 128.8, 128.4, 127.3, 126.8, 124.6, 108.3, 106.2, 104.4, 96.2, 46.1, 40.1, 21.8; IR (neat, cm⁻¹) 3060.6, 2993.2, 2683.1, 2305.6, 1649.4, 1622.4, 1595.5, 1528.0, 1402.2, 1271.9, 1191.0, 1164.0, 1065.1, 1029.2, 975.2; HRMS calculated for C₂₅H₂₄N₂O₄SNa (M+Na⁺): 471.1348, found 471.1349.

Compound **52** Compound **50** (1.2 g, 3 mmol), 3-hydroxymethyl phenylboronic acid (497 mg, 3.27 mmol), Pd₂dba₃ (137.4 mg, 0.15 mmol), Sphos (184.7 mg, 0.45 mmol) and K₃PO₄ (1.27 g, 6 mmol) were placed in a sealed tube with THF (15 ml) and H₂O (0.75 ml). After the mixture was degassed for 20 min, it was heated to 110 °C for 36 hrs. The solution was filtered and extracted with CH₂Cl₂ (3×50ml) and removed *in vacuo*. The remaining residue was purified by flash chromatography (80:1 CH₂Cl₂/MeOH, 40:1 CH₂Cl₂/MeOH) to furnish compound **52** as a light yellow amorphous solid (663 mg, 46%). ¹H NMR (500 MHz, CDCl₃) δ 7.83 (dd, 1H, *J* = 7, 0.5 Hz), 7.52 (td, 1H, *J* = 7.5, 1 Hz), 7.43 (td, 1H, *J* = 7.5, 1 Hz), 7.26-7.35 (m, 5H), 7.22 (t, 1H, *J* = 7 Hz), 7.03 (d, 1H, *J* = 9 Hz), 6.45 (s, 1H), 6.40 (dd, 1H, *J* = 9, 2.5 Hz), 6.36 (d, 1H, *J* = 2.5 Hz), 5.93 (s, 1H), 5.29 (s, 1H), 2.87 (s, 6H), 1.09 (s, 1H), 0.89 (s, 6H); ¹³C NMR (125 MHz, CDCl₃) δ 163.2, 153.1, 151.6, 143.4, 140.8, 137.4, 134.7, 130.7, 128.8, 128.7, 127.5, 127.1, 126.9, 115.7, 113.5, 107.8, 97.1, 83.6, 45.8, 40.3, 24.6, 24.4; IR (neat, cm⁻¹) 3433.7, 3056.1, 2984.2, 2692.1, 2305.6, 1658.4, 1613.4, 1541.4, 1429.2, 1406.7, 1352.8, 1276.4, 1146.0, 894.3; HRMS calculated for C₃₀H₃₃BN₂O₃Na (M+Na⁺): 503.2476, found 503.2477.

NS560 POCl₃ (0.87 ml, 9.3 mmol) was added to DMF (1.8 ml, 23.2 mmol) at 0 °C in a flame dried round bottom flask. The reaction mixture was stirred at ambient temperature for 1 hours. The Vilsmeier reagent (0.78 ml) was added to a solution of compound **52** (144.1 mg, 0.3 mmol) in DMF (3 ml). The solution was stirred at ambient temperature for 16 hours. The resulting red solution was poured onto ice water, extracted with CH₂Cl₂ (3×10ml). The combined organic layers were dried over Na₂SO₄ and the solvent was removed *in vacuo*. The residue was purified by chromatography (80:1 CH₂Cl₂/MeOH) to yield compound NS560 (56.4 mg, 37%) as dark a yellow foaming solid. ¹H NMR (500 MHz, CDCl₃) δ 10.18 (s, 1H), 7.93 (dd, 1H, *J* = 7, 0.5 Hz), 7.54 (td, 1H, *J* = 7.5, 1.5 Hz), 7.45 (td, 1H, *J* = 7.5, 1 Hz), 7.36 (d, 2H, *J* = 7 Hz), 7.32 (t, 2H, *J* = 2 Hz), 7.25 (d, 1H, *J* = 8.5 Hz), 7.19 (d, 1H, *J* = 7 Hz), 6.85 (d, 1H, *J* = 9 Hz), 6.38 (dd, 1H, *J* = 9.5, 2.5 Hz), 6.27 (d, 1H, *J* = 2.5 Hz), 5.97 (s, 1H), 5.29 (s, 1H), 2.93 (s, 6H), 1.06 (s, 6H), 0.89 (s, 6H); ¹³C NMR (125 MHz, CDCl₃) δ 190.8, 162.4, 158.4, 153.2, 142.7, 141.5, 137.0, 135.3, 131.5, 130.5, 128.8, 128.5, 127.4, 127.3, 127.0, 117.9, 112.8, 108.4, 95.7, 83.5, 45.8, 40.1, 24.7, 24.4; IR (neat, cm⁻¹) 3438.2, 3056.1, 2984.2, 2310.1, 1703.3, 1604.4, 1528.0, 1483.1, 1424.7, 1352.8, 1267.4, 1164.0, 1146.0; HRMS calculated for C₃₁H₃₃BN₂O₄Na (M+Na⁺): 531.242559, found 531.242176.

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VITA

Le Zhang was born in Inner Mongolia, China and grew up there. He decided to pursue studies in Chemistry at Xidian University in China. After receiving his bachelor's degree in 2009, he went to University of Leuven, Belgium for his master's degree, where he became interested in bioorganic chemistry. In 2013, He began graduate studies at the University of Missouri-Columbia and worked with Dr. Timothy Glass on development of selective and high affinity fluorescent sensors for imaging neurotransmitters. After graduating with PhD degree, he decided to pursue postdoc studies and work in the area of chemical biology and later start his independent career as a principle investigator in academia.