Supporting Information for

Quaternary Ammonium-modulated SERS Effect: Discovery, Mechanism and Application for Highly-sensitive *in-vitro* Sensing Acetylcholine

Lang Li, Shenfei Zong, Yang Lu, Yizhi Zhang, Ziting Qian, Kai Zhu, Zhile Wang, Kuo Yang, Zhuyuan Wang*, Yiping Cui

Advanced Photonics Center, Southeast University, Nanjing 210096, Jiangsu, China

wangzy@seu.edu.cn

Table of content

Experiments……2
Figure S1. Characterization of the Ag nanospheres and SERS substrate……8
Figure S2. Real-time evolution for the SERS spectra of MB……9
Figure S3. The fluorescence spectra of MB solution……10
Figure S4. The chemical structure of ACh, DA, Gly and L-dopa……10
Figure S5. SERS spectra of MB influenced by ACa, Ch, Bet and TMAOH……11
Figure S6. Variations of SERS spectra of MB after involving of TMAB, TEAB, TPAB and TBAB……12
Figure S7. SERS spectra obtained when using Au NPs instead of Ag NPs……13
Figure S8. SERS based quantification of ACh with MB (1 μM) in the presence of other neurotransmitters……14
Figure S9. The feasibility of ACh sensing in the presence of biomacromolecules……15
Figure S10. SERS based quantification of ACh at different R6G concentrations……16
Figure S11. SERS based quantification of ACh with R6G (100 μM) in the presence of other neurotransmitters……17
Figure S12. Bright image of PC12 cell in a petri dish……18
Figure S13. Time-dependent evolution of the secretion rate of ACh from PC12 cells in PBS……18
Figure S14. The response of the SERS intensity of MB to different concentrations of ACh in serum environments……19
Table S1. Raman peak assignments of MB……19
Table S2. Comparison of different ACh sensing methods……20
Table S3. Calculated average ACh secretion of PC12 cells in petri dish (unit: fmol /106 cells)……20
Table S4. Calculated average ACh secretion of PC12 cells in 10 min under different treatment……21
Reference……21

Methods

Reagents and Instruments

Poly dimethyl diallyl ammonium chloride (PDDA), acetylcholine (ACh), choline (Ch), rhodamine 6g (R6G), 4-mercaptobenzoic acid (4MBA), 5-hydroxytryptamine (5-HT), levo dopamine (L-DOPA) and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich. Silver nitrate ($AgNO_3$) and dopamine (DA) were purchased from Alfa-Aesar. Sodium citrate, methylene blue (MB), ethanol (EtOH), tetramethylammonium hydroxide (TMAOH), magnesium chloride ($MgCl_2$) and calcium chloride ($CaCl_2$) were purchased from Sinopharm Chemical Reagent Co., Ltd. Phosphate buffered saline (PBS, 10 mM, PH=7.4) was purchased from Wuhan Boster Biological Technology., Ltd. Hydrochloric acid (HCl) was purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd. Sodium hydroxide (NaOH) was purchased from Xilong Chemical Co., Ltd. Glycine (Gly), acetamide (ACa) and collagenase type 1 was purchased from Aladdin Reagent (shanghai) co., Ltd. Betaine (Bet) was purchased from Wokai Reagent co., Ltd. Glucose was purchased from Biosharp. Tetramethylammonium bromide (TMAB), Tetraethylammonium bromide (TEAB), Tetrapropylammonium bromide (TPAB) and Tetrabutylammonium bromide (TBAB) were purchased from Adamas Reagent Ltd. Neostigmine was purchased from Maya Reagent co., Ltd. Quinacrine hydrochloride (QH) was purchased from Shanghai Yuanye Reagent co., Ltd. 4-bromophenacyl bromide (BPB) was purchased from Macklin Reagent co., Ltd. All solvents and reagents were of analytical grade and used without further purification. PC12 cells, fetal bovine serum and RPMI1640 were purchased from

Jiangsu Keygen biotechnology co. LTD. Glass slides were purchased from Haimen Xinxing experiment company. PDMS microfluidic chips were purchased from Suzhou Wenhao chip technology Co., Ltd.

SERS spectra was measured with a confocal laser scanning microscope (FV1000, Olympus, Japan) and spectrograph (Sharmrock, Andor, UK). TEM images were obtained by a transmission electron microscope (FEI Tecnai G2T20). SEM images were obtained by a scanning electron microscope (Zeiss Ultra Plus). UV−Vis spectra were recorded by UV-Vis spectrometer (UV-3600, Shimazu, Japan). Injection pump (LSP04-1A) was purchased from Longer Precision Pump Co., Ltd. AFM images were performed by a atomic force microscope (Bioscope, Bruker).

Synthesis of Ag nanospheres

Ag nanospheres were prepared according to the procedure developed by Lee.[1] Briefly, 44 mg of $AgNO_3$ was dissolved in 245 mL of deionized water in a erlenmeyer flask, then 50 mg of sodium citrate dissolved in deionized water was added into the erlenmeyer flask. The mixed solution was heated to boiling under stirring conditions for 45 min and then naturally cooled. Ag nanospheres were ready for use after centrifugation (8000 rpm, 15 min).

Fabrication of SERS substrate in a microfluidic chip

The PDMS chips with microfluidic channels (width:500 μm, height:100 μm) on the surfaces were first sonicated with EtOH for 30 min. Then, the microfluidic chips were prepared by bonding PDMS chips with the glass slides that previously sonicated with

piranha solution. The SERS substrate inside the microfluidic chip was prepared by electrostatic interaction between negatively charged Ag nanospheres and positive charged PDDA using the following steps. First, 30 μL of 1% w/t PDDA solution was injected (0.5 μL/min) into the microfluidic chip, followed by 3-min of water washing with a flow rate of 2 μL/min. PDDA molecules were adsorbed on the glass slides because of the presence of hydroxy on glass surfaces. Second, the solution of Ag nanospheres were injected (0.5 μL/min) into the microfluidic chip to be deposited on the PDDA layer due to the electrostatic force, followed by a wash of 3-min of deionized water at a flow rate of 2 μL/min. Then, a layer of Ag nanospheres was successfully assembled on the bottom of the microfluidic chips for further SERS measurements.

Mechanism analysis

1. Reaction sequence

Experiment 1 (ACh first). 10 μL of ACh aqueous solution (1 mM) was injected (0.5 μL/min) into the microfluidic chip to react with the SERS substrate and then washed with deionized water. Then 10 μL of MB aqueous solution (1 μM) was injected (0.5 μL/min) into the microfluidic chip and washed with deionized water. Finally, SERS spectra were measured with a Raman spectroscopy.

Experiment 2 (MB first). 10 μL of MB aqueous solution (1 μM) was injected (0.5 μL/min) into the microfluidic chip to react with the SERS substrate and washed with deionized water. Then 10 μL of ACh aqueous solution (1 mM) was injected (0.5 μL/min) into the

microfluidic chip and washed with deionized water. Finally, SERS spectra were measured with a Raman spectroscopy.

Experiment 3 (mixed). 10 μL of the mixed solution of MB (1 μM) and Ach (1 mM) was injected (0.5 μL/min) into the microfluidic chip to react with the SERS substrate and washed with deionized water. Then, SERS spectra were measured with a Raman spectroscopy.

In the experiments of using other neurotransmitters instead of ACh, DA (1 mM), L-dopa (1 mM) or Gly (1 mM) was used to replace ACh in the experiment 1 and experiment 2.

In the experiments of using other QA molecules instead of ACh, QA molecules were used to replace ACh in the experiment 1 and experiment 2.

2. Fluorescence analysis

Experiment 1. The glass slides were decorated with Ag nanospheres to form the SERS-active substrates, followed by an immersion of ACh solution at different concentrations for 1 h. Afterwards, the glass slides were rinsed with deionized water and subsequently immersed into MB solution for a fixed time span. Finally, the glass slides were removed from MB solution and the fluorescence spectra of MB in the remained solution were measured.

Experiment 2. The glass slides were decorated with Ag nanospheres to form the SERS-active substrates, followed by an immersion of MB solution for 1 h. Afterwards, the glass slides were rinsed with deionized water and subsequently immersed into ACh solution for

a fixed time span. Finally, the glass slides were removed from ACh solution and the fluorescence spectra of MB in the remained ACh solution were measured.

3. SERS analysis

To characterize the amount of residual MB after reacting with the SERS substrate in the microchannels, the solution of MB or the mixed solution of MB and ACh was injected into the microchannels, the outflow was collected and then mixed with Ag colloids respectively. Eventually, a droplet of the mixed solutions was dropped onto the glass slides for SERS measurements.

To verify if MB molecules could cause the desorption of ACh on Ag NPs, the solution of ACh was inject into microchannels to react with the SERS substrate, then the first SERS spectra of ACh was obtained. Afterwards, the solution of MB was injected into the same microchannels for a certain time, and the second SERS spectra of ACh was obtained after washing.

4. The chemical structures of molecules were drawn using the software of Chemoffice.

SERS quantification of ACh

The detection of ACh using SERS spectra were easily realized as follows. First, ACh aqueous solution was mixed with MB aqueous solution. Then, 15 μL of the mixed solution was injected (0.5 μL/min) into the SERS-active microfluidic chip, which was then washed with deionized water (2 μL/min, 3 min). After that, the SERS spectra of MB were obtained by a Raman spectroscopy. In the case of using MB solution at a concentration of 0.01 μM, in order to increase the SERS intensity, the incubation time for the adsorption of PDDA

and Ag nanospheres was increased. Then 30 μL of the mixed solution was injected (0.5 μL/min) to react with the SERS substrate. The remained other procedures were the same.

To investigate the feasibility of the quantification of ACh in the presence of other neurotransmitters, the solution of dopamine, levodopa or glycine at a final concentration of 1 nM was added to the mixed aqueous solution of ACh and MB (1 μM), respectively. Then 10 μL of the mixed solution was injected (0.5 μL/min) into the SERS-active microfluidic chip, which was then washed with deionized water (2 μL/min, 3 min). After that, the SERS spectra from the microfluidic chip were obtained by a laser scanning confocal microscope.

To confirm the feasibility for the detection of ACh in the environment with serum, ACh solution at different concentrations were added to serum. Then MB solution was added with a final concentration of 10 μM. The mixture was injected into the microfluidic chip to react with the SERS substrate. Finally, the SERS spectra of MB in the microfluidic chip were measured after the chip was rinsed with PBS.

Culture of PC12 cells and monitoring of the secretion of acetylcholine

PC12 cells were cultured in fresh RPMI1640 containing 5% fetal bovine serum. Prior to the extraction, cell culture medium was replaced with medium A (10 mM PBS, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose and 500 nM neostigmine, pH 7.4) and cultured for a required time. After incubation, the medium A was extracted and centrifuged (3000 rpm, 20 min) as the sample solution. In a stimulated or inhibited ACh release experiment, K^+ or drug at certain concentrations was added into medium A to culture PC12 cells.

Then, the quantitative analysis of ACh secreted from PC12 cells was performed as mentioned above by mixing MB solution with the sample solution. The mixture was then injected into the microfluidic chip to react with the SERS substrate.

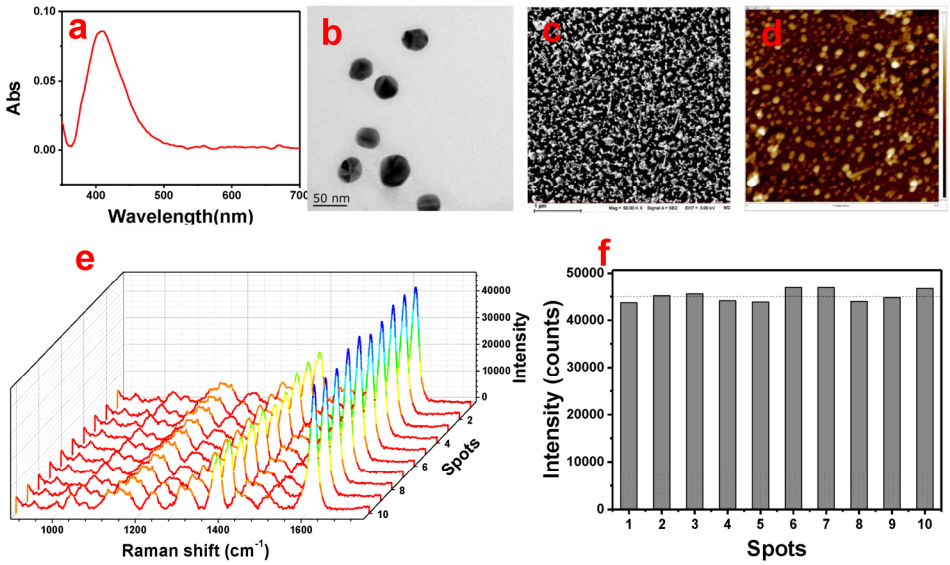

Figure S1. Characterization of the Ag nanospheres and SERS substrate. (a) Extinction spectrum of Ag nanospheres with a surface plasmon resonance (SPR) peak at 405 nm; (b) TEM image of Ag nanospheres; (c) SEM image of the SERS substrate self-assembled with Ag nanospheres, showing the uniform distribution of the Ag nanospheres; (d) AFM image of the SERS substrate self-assembled with Ag nanospheres, showing the mono-layer distribution formation of the Ag nanospheres; (e) uniformity of the SERS intensity using MB (1 μM) as the Raman reporter and (f) peak intensity at 1617 cm^{-1} in (e), the relative standard deviation (RSD) was as low as 6.92 % which indicating that the prepared SERS substrate has a good reliability uniformity of SERS enhancement and can be used for the quantitative SERS detection.

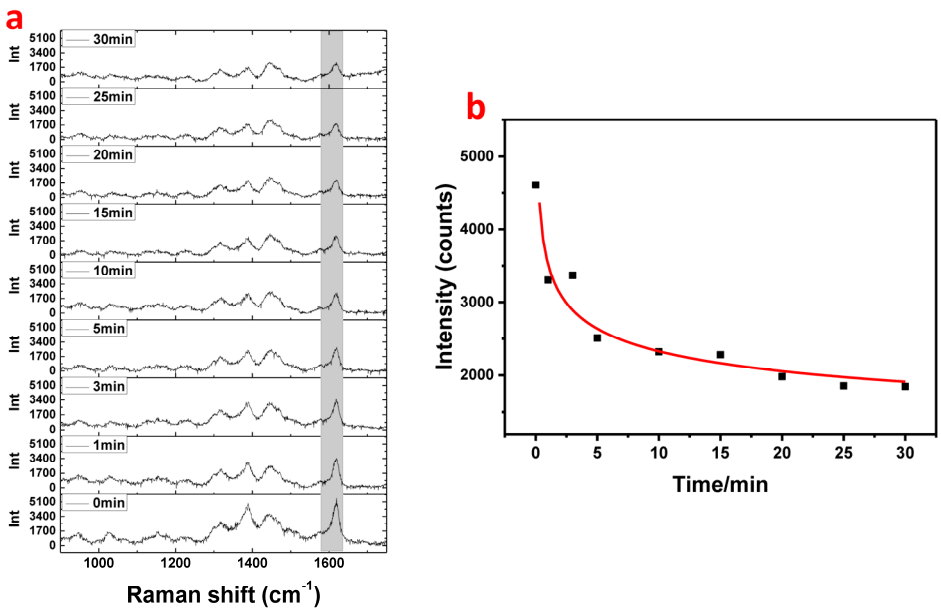

Figure S2. (a) Real-time evolution for the SERS spectra and (b) SERS intensity of MB at 1617 cm^{-1} peak during the injection of ACh solution into the microchannel adsorbed with MB molecules. The SERS intensity at 1617 cm^{-1} peak dramatically decreased and then gradually saturated.

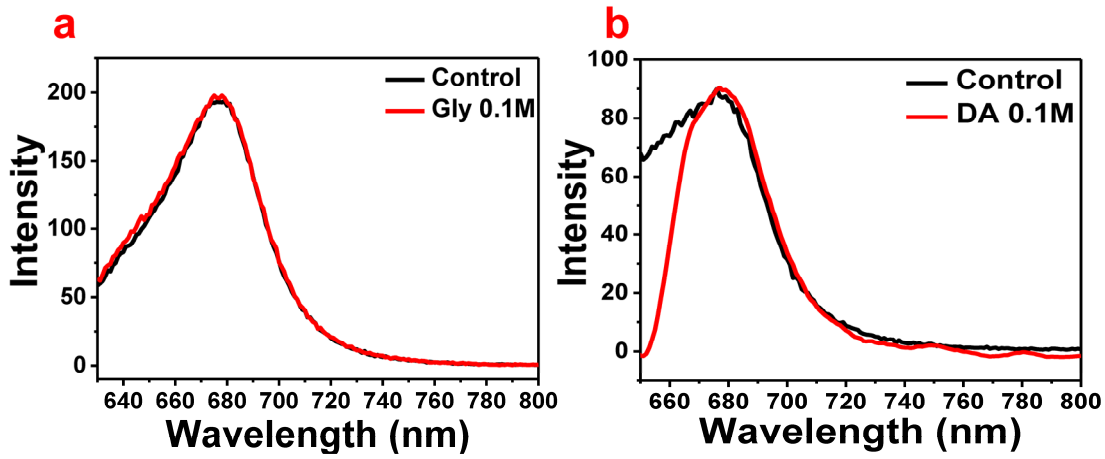

Figure S3. The fluorescence spectra of MB solution after being immersed with glass slides-based SERS substrate which has been previously immersed with the solution of (a) Gly and (b) DA;

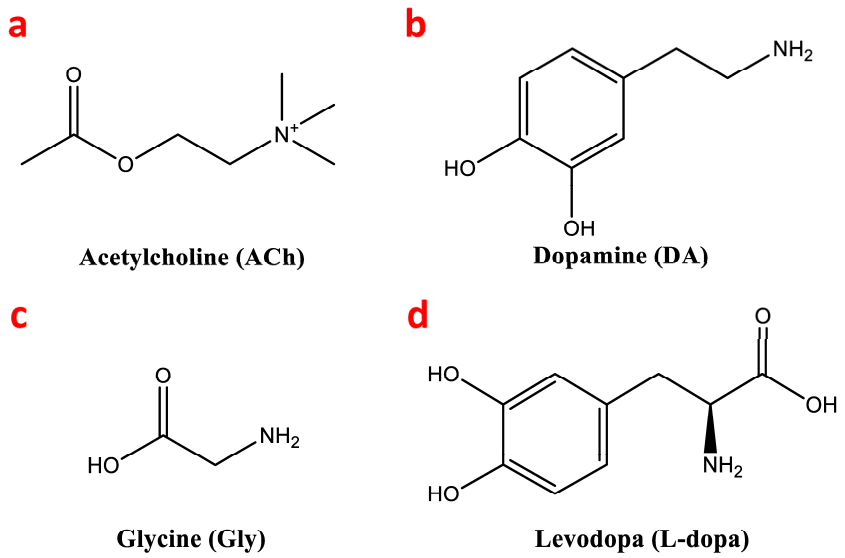

Figure S4. The chemical structure of (a) ACh, (b) DA, (c) Gly and (d) L-dopa.

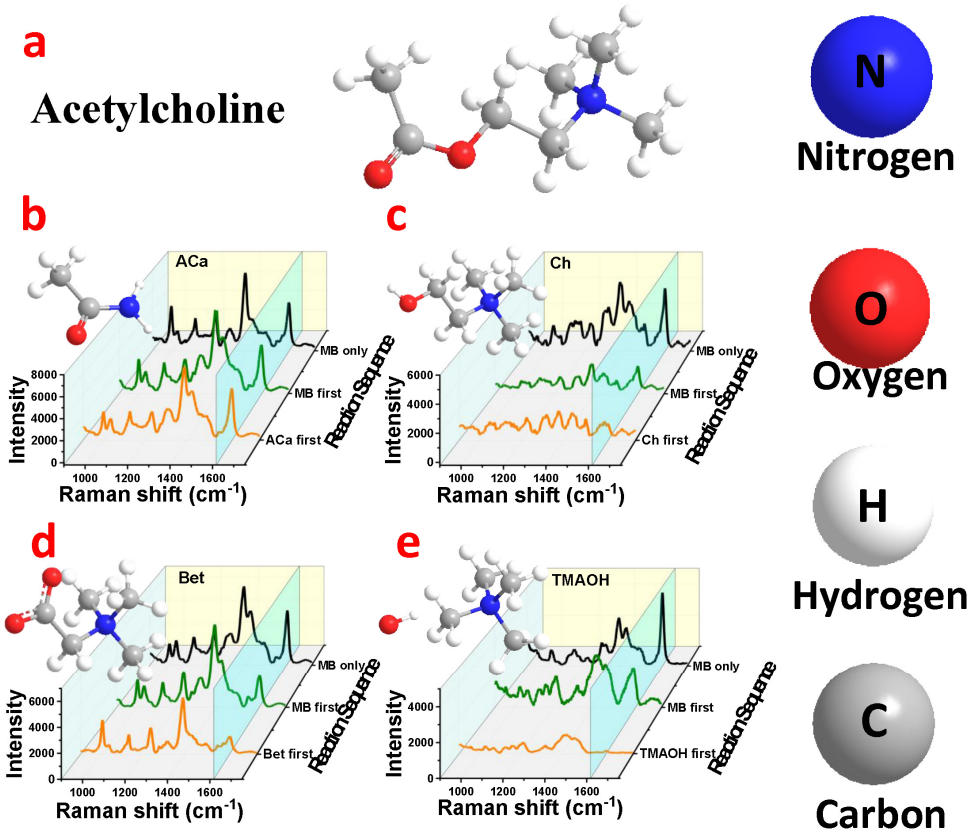

Figure S5. (a) The molecular structure of ACh. (b-e) SERS spectra of MB influenced by ACa, Ch, Bet and TMAOH with different reaction orders.

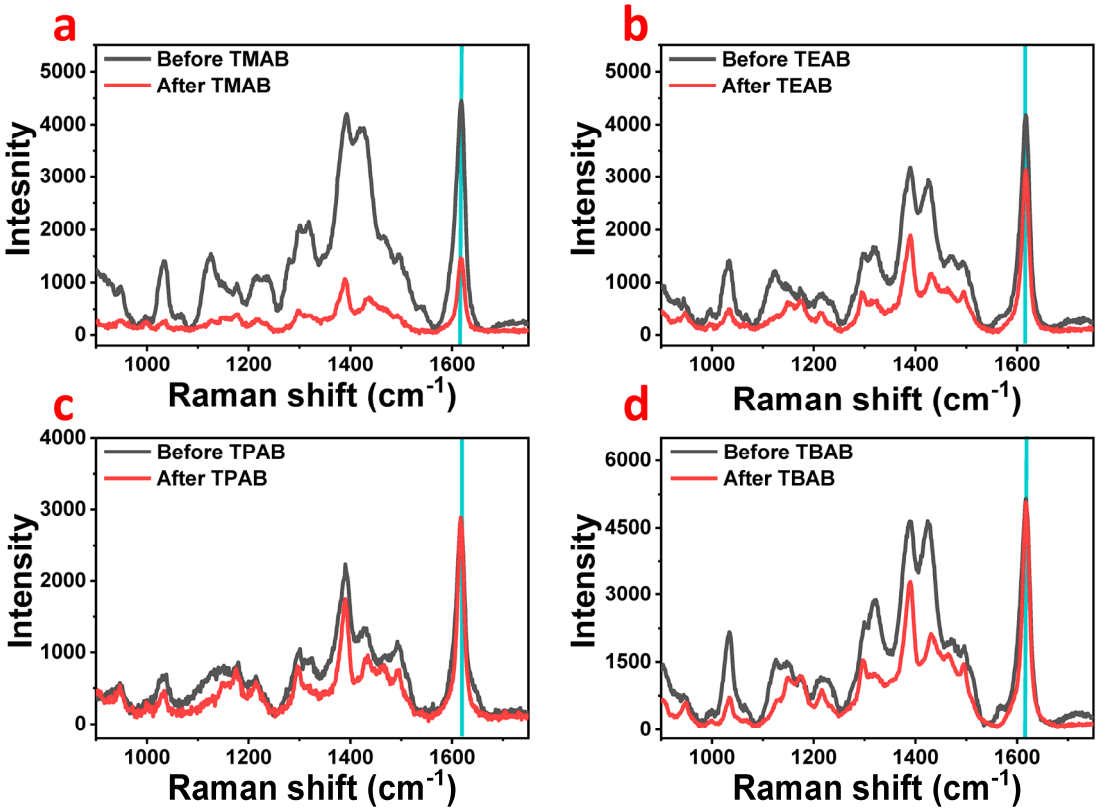

Figure S6. Variations of SERS spectra of MB after involving of (a) TMAB, (b) TEAB, (c) TPAB and (d) TBAB.

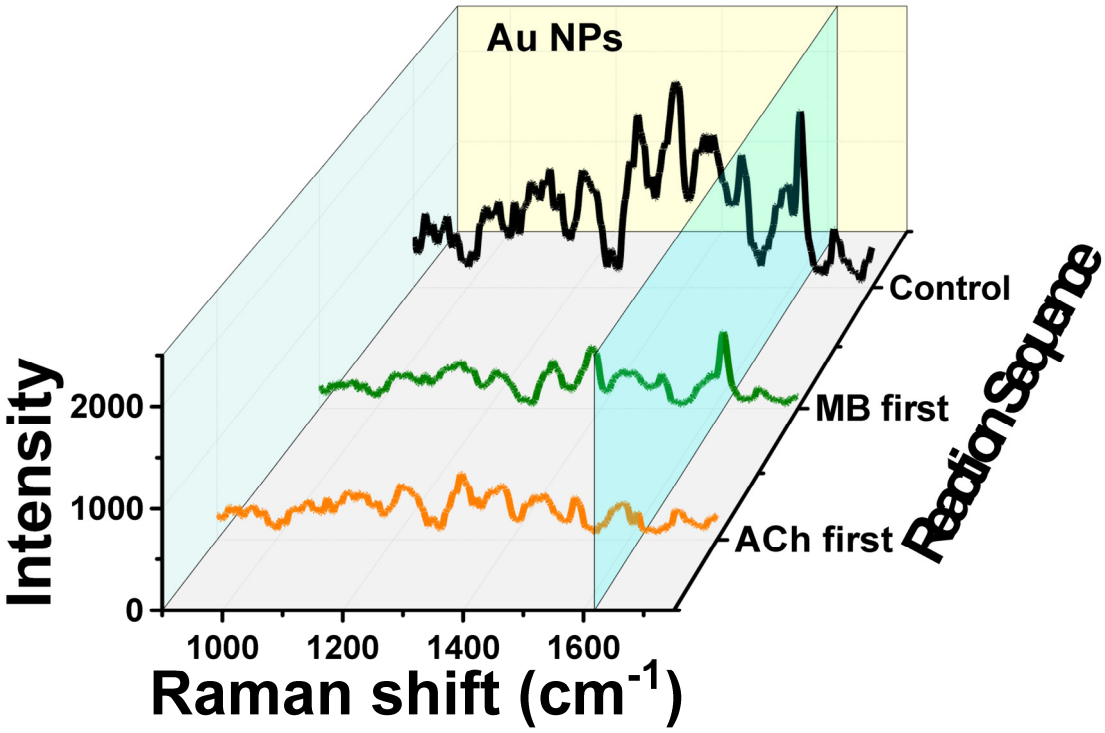

Figure S7. SERS spectra obtained when using Au NPs instead of Ag NPs.

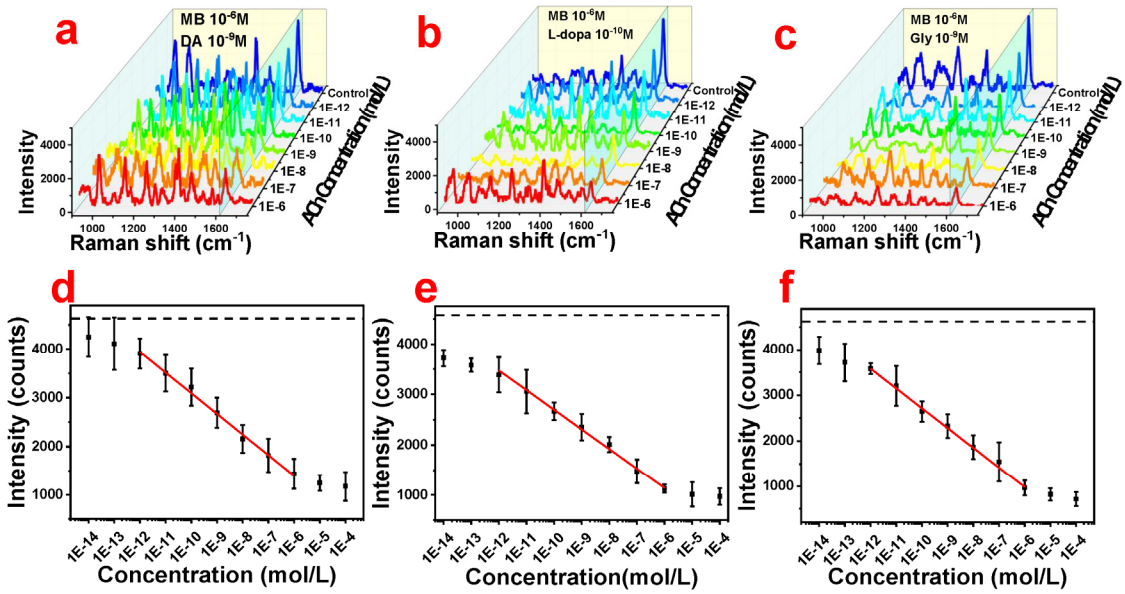

Figure S8. SERS based quantification of ACh with MB (1 μM) in the presence of other neurotransmitters. SERS spectra(a) and calibration curve(d) between the concentration of ACh and the SERS intensity of MB with respect to 1617 cm^{-1} in the presence of DA (1 nM); SERS spectra(b) and calibration curve(e) between the concentration of ACh and the SERS intensity of MB with respect to 1617 cm^{-1} in the presence of L-dopa(1 nM); SERS spectra(c) and calibration curve(f) between the concentration of ACh and the SERS intensity of MB with respect to 1617 cm^{-1} in the presence of Gly (1 nM). The results show the possibility of detecting ACh in much more complicated environments.

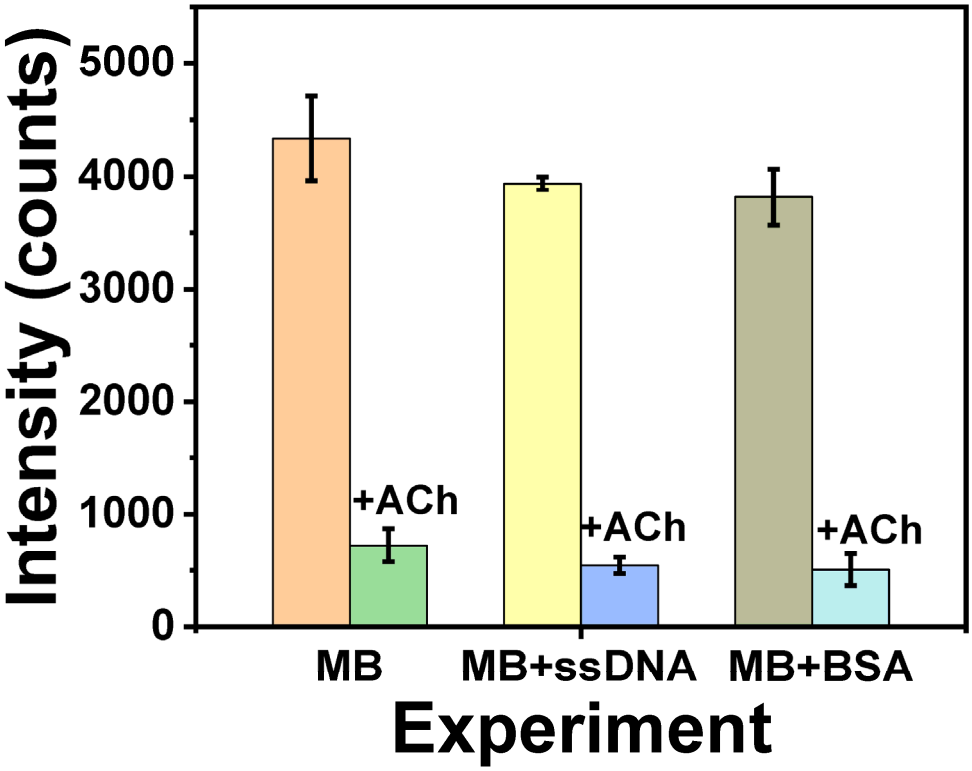

Figure S9. The feasibility of ACh sensing in the presence of 0.1 mg/mL bovine serum albumin (BSA) and 0.1 mg/mL salmon sperm DNA (ssDNA). The concentration of MB and ACh is 1 M and 1 mM.

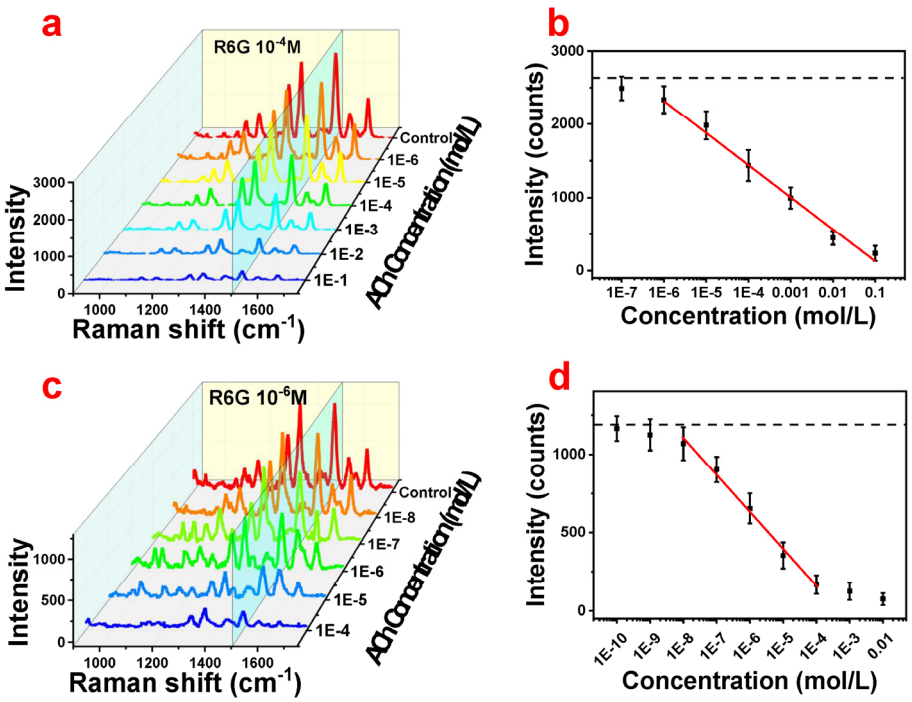

Figure S10. SERS based quantification of ACh at different R6G concentrations. (a) SERS spectra and (b) calibration curve between the concentration of ACh and the SERS intensity of R6G(100 μM) with respect to 1507 cm^{-1} peak; (c) SERS spectra and (d) calibration curve between the concentration of ACh and the SERS intensity of R6G(1 μM) with respect to 1505 cm^{-1} peak. The results show that R6G can also be used as the Raman reporter for the detection of ACh, which indicates of the optional diversity for the selection of Raman reporters.

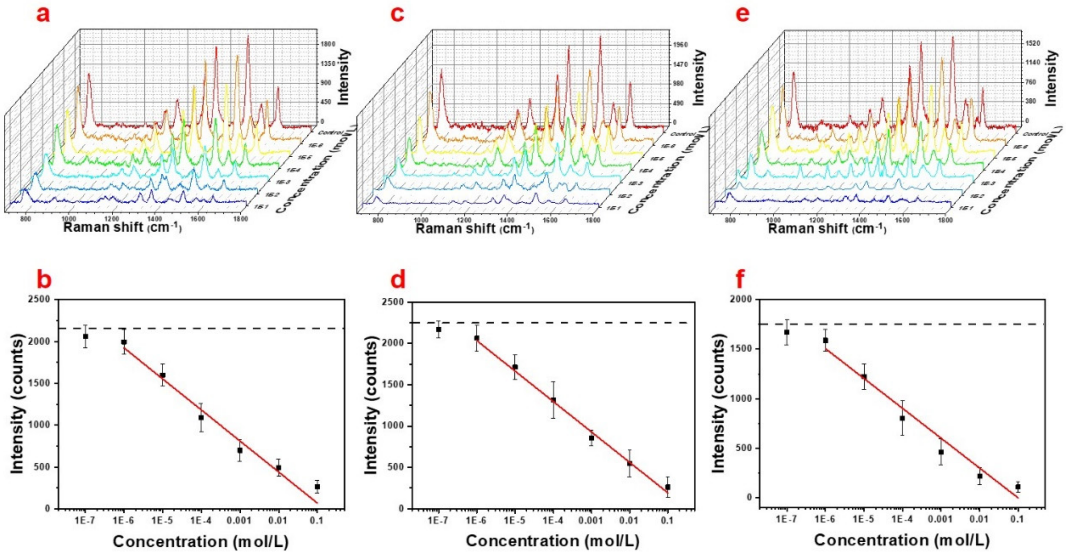

Figure S11. SERS based quantification of ACh with R6G (100 µM) in the presence of other neurotransmitters. (a) SERS spectra and (b) calibration curve between the concentration of ACh and the SERS intensity of R6G with respect to 1507 cm^{-1} in the presence of DA(100 µM); (c) SERS spectra and (d) calibration curve between the concentration of ACh and the SERS intensity of R6G with respect to 1507 cm^{-1} in the presence of L-dopa (100 µM). (e) SERS spectra and (f) calibration curve between the concentration of ACh and the SERS intensity of R6G with respect to 1507 cm^{-1} in the presence of Gly(100 µM).

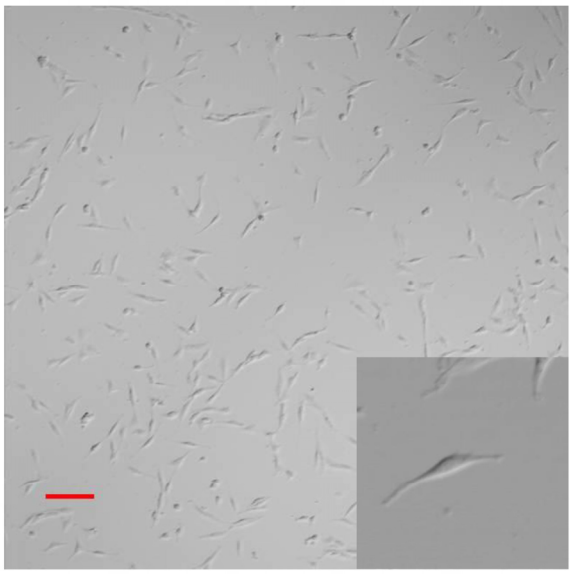

Figure S12. Bright image of PC12 cell in a petri dish. The inset shows an enlarged one. Scale bar = 100 μm.

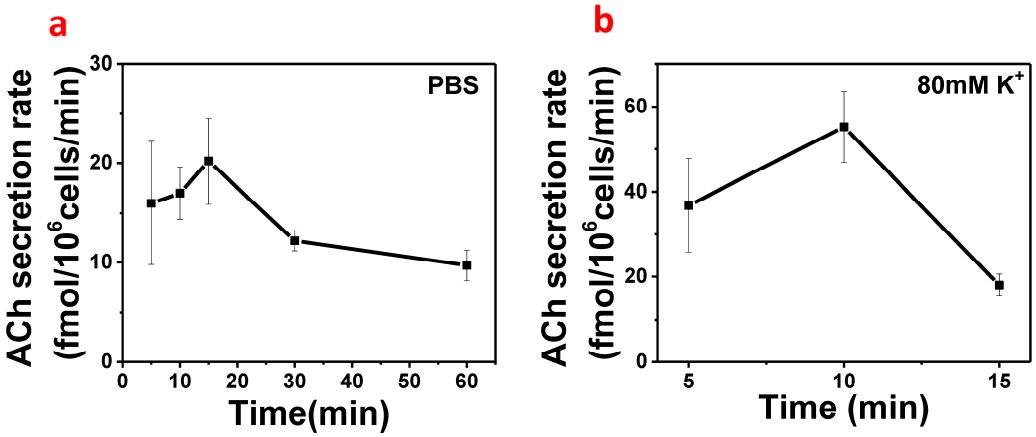

Figure S13. (a) Time-dependent evolution of the secretion rate of ACh from PC12 cells in PBS. (b) Time-dependent evolution of the secretion rate of ACh from PC12 cells under the stimulation of 80 mM K^+.

$SR_{t_{x+1}} = \frac{S_{t_{x+1}} - S_{t_x}}{t_{x+1} - t_x}$, SR: secretion rate, S: secretion, t: time.

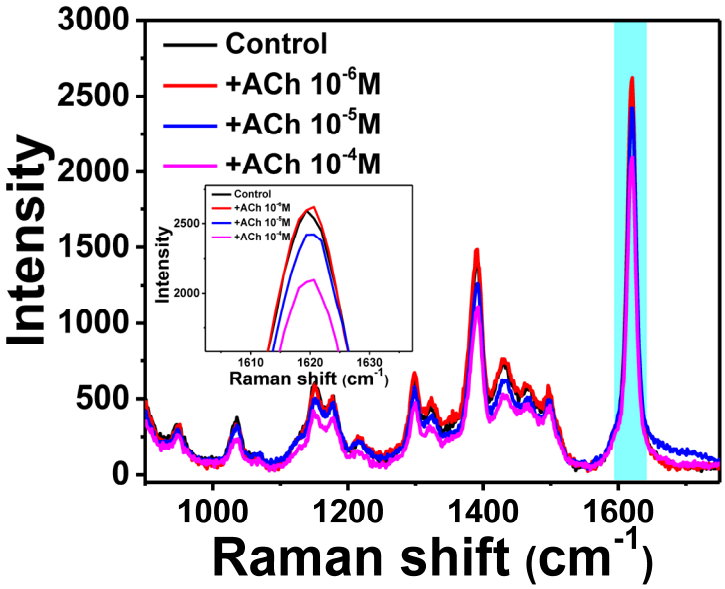

Figure S14. The response of the SERS intensity of MB to different concentrations of ACh in serum environments.

Table S1 Raman peak assignments of MB.[2-4]

Peaks(cm^{-1})	Band assignments
1617(s)	ν (C-C)$_{RING}$
1445(w)	α (N-C-H)$_{AMG}$
1396(m)	β (C-H)； ν (C-N)$_{RING}$
1322(w)	ν (C-N)$_{RING}$
1232(w)	β (C-H)
1040(w)	β (C-H)
769(m)	ν (C-N ring)$_{AMG}$； α (C-N-C)$_{RING}$

ν :stretching; α , β : in-plane bending; AMG: attached to methyl group; s: strong; w: weak; m:medium

Table S2 Comparison of different ACh sensing methods. *(**HPLC**: high-performance liquid chromatography; **TMS**: tandem mass spectrometry; **GACh**: genetically encoded G-protein-coupled receptor activation-based sensors for ACh; **SRS**: stimulated Raman scattering.)*

Methods	Linear range	LOD	Ref
Electrochemical	4 - 800 nM	4 nM	5
	100 pM - 10 mM	100 pM	6
Enzyme degradation & Fluorescence	1 – 100 nM	0.21 nM	7
	0.1 - 20 nM	5 pM	8
HPLC & TMS	*	28 pM	9
	*	100 pM	10
HPLC & Electrochemical	*	700 pM	11
Genetically encoded (GACh)	0.01 – 100 µM	10 nM	12
SERS	*	4 µM	13
SRS	*	20 mM	14
QAM-SERS	**10 fM - 100 µM**	**10 fM**	

Table S3*. Calculated average ACh secretion of PC12 cells in petri dish (unit: fmol /10^6 cells).*

Time	***5 min***	***10 min***	***15 min***	***30 min***	***60 min***
Secretion (in PBS)	*80±31*	*165±25*	*266±57*	*450±38*	*741±119*
Average secretion rate/min (in PBS)	*16±6.2*	*17.8±2.6*	*20.2±4.3*	*12.2±1.03*	*9.7±1.55*
Secretion (80 mM K^+)	*184±55*	*460±70*	*570±83*	---	---
Average secretion rate/min (80 mM K^+)	*36.8±11*	*55.2±8.4*	*18±2.6*	---	---

$SR_{t_{x+1}} = \frac{S_{t_{x+1}} - S_{t_x}}{t_{x+1} - t_x}$ *, SR: secretion rate, S: secretion, t: time.*

***Table S4**. Calculated average ACh secretion of PC12 cells in 10 min under different treatment.*

	ACh Secretion (fmol/10^6 cells/10min)
PBS	*165±25*
80 mM K^+	*460±70*
80 mM K^+ + 20 μM QH	*285±26*
80 mM K^+ + 100 μM QH	*205±65*
80 mM K^+ + 5 μM BPB	*290±47*
80 mM K^+ + 30 μM BPB	*185±25*

Reference

1. Lee, P. C.; Meisel, D. ADSORPTION AND SURFACE-ENHANCED RAMAN OF DYES ON SILVER AND GOLD SOLS. *J. Phys. Chem.* **1982,** *86* (17), 3391-3395.
2. Naujok, R. R.; Duevel, R. V.; Corn, R. M. FLUORESCENCE AND FOURIER-TRANSFORM SURFACE-ENHANCED RAMAN-SCATTERING MEASUREMENTS OF METHYLENE-BLUE ADSORBED ONTO A SULFUR-MODIFIED GOLD ELECTRODE. *Langmuir* **1993,** *9* (7), 1771-1774.
3. Xiao, G.-N.; Man, S.-Q. Surface-enhanced Raman scattering of methylene blue adsorbed on cap-shaped Silver nanoparticles. *Chem. Phys. Lett.* **2007,** *447* (4-6), 305-309.
4. Li, C.; Huang, Y.; Lai, K.; Rasco, B. A.; Fan, Y. Analysis of trace methylene blue in fish muscles using ultra-sensitive surface-enhanced Raman spectroscopy. *Food Control* **2016,** *65*, 99-105.
5. Chauhan, N.; Chawla, S.; Pundir, C. S.; Jain, U. An electrochemical sensor for detection of neurotransmitter-acetylcholine using metal nanoparticles, 2D material and conducting polymer modified electrode. *Biosensors & Bioelectronics* **2017,** *89*, 377-383.
6. Xue, W.; Cui, T. A High-Resolution Amperometric Acetylcholine Sensor Based on Nano-Assembled Carbon Nanotube and Acetylcholinesterase Thin Films. *Journal of Nano Research* **2008,** *1*, 1-9.
7. Wang, C.-I.; Chen, W.-T.; Chang, H.-T. Enzyme Mimics of Au/Ag Nanoparticles for Fluorescent Detection of Acetylcholine. *Anal. Chem.* **2012,** *84* (22), 9706-9712.
8. Li, H.; Guo, Y.; Xiao, L.; Chen, B. A fluorometric biosensor based on H2O2-sensitive nanoclusters for the detection of acetylcholine. *Biosensors & Bioelectronics* **2014,** *59*, 289-292.
9. Zhu, Y. X.; Wong, P. S. H.; Cregor, M.; Gitzen, J. F.; Coury, L. A.; Kissinger, P. T. In vivo microdialysis and reverse phase ion pair liquid chromatography/tandem mass spectrometry for the determination and identification of acetylcholine and related compounds in rat brain. *Rapid Commun. Mass Spectrom.* **2000,** *14* (18), 1695-1700.
10. Hows, M. E. P.; Organ, A. J.; Murray, S.; Dawson, L. A.; Foxton, R.; Heidbreder, C.; Hughes, Z. A.; Lacroix, L.; Shah, A. J. High-performance liquid chromatography/tandem mass spectrometry assay for the rapid high sensitivity measurement of basal acetylcholine from microdialysates. *J. Neurosci. Methods*

2002, *121* (1), 33-39.

11. Kato, T.; Liu, J. K.; Yamamoto, K.; Osborne, P. G.; Niwa, O. Detection of basal acetylcholine release in the microdialysis of rat frontal cortex by high-performance liquid chromatography using a horseradish peroxidase-osmium redox polymer electrode with pre-enzyme reactor. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **1996,** *682* (1), 162-166.

12. Jing, M.; Zhang, P.; Wang, G.; Feng, J.; Mesik, L.; Zeng, J.; Jiang, H.; Wang, S.; Looby, J. C.; Guagliardo, N. A.; Langma, L. W.; Lu, J.; Zuo, Y.; Talmage, D. A.; Role, L. W.; Barrett, P. Q.; Zhang, L. I.; Luo, M.; Song, Y.; Zhu, J. J.; Li, Y. A genetically encoded fluorescent acetylcholine indicator for in vitro and in vivo studies. *Nat. Biotechnol.* **2018,** *36* (8), 726-+.

13. Siek, M.; Kaminska, A.; Kelm, A.; Rolinski, T.; Holyst, R.; Opallo, M.; Niedzioka-Jonsson, J. Electrodeposition for preparation of efficient surface-enhanced Raman scattering-active silver nanoparticle substrates for neurotransmitter detection. *Electrochimica Acta* **2013,** *89*, 284-291.

14. Fu, D.; Yang, W.; Xie, X. S. Label-free Imaging of Neurotransmitter Acetylcholine at Neuromuscular Junctions with Stimulated Raman Scattering. *Journal of the American Chemical Society* **2017,** *139* (2), 583-586.