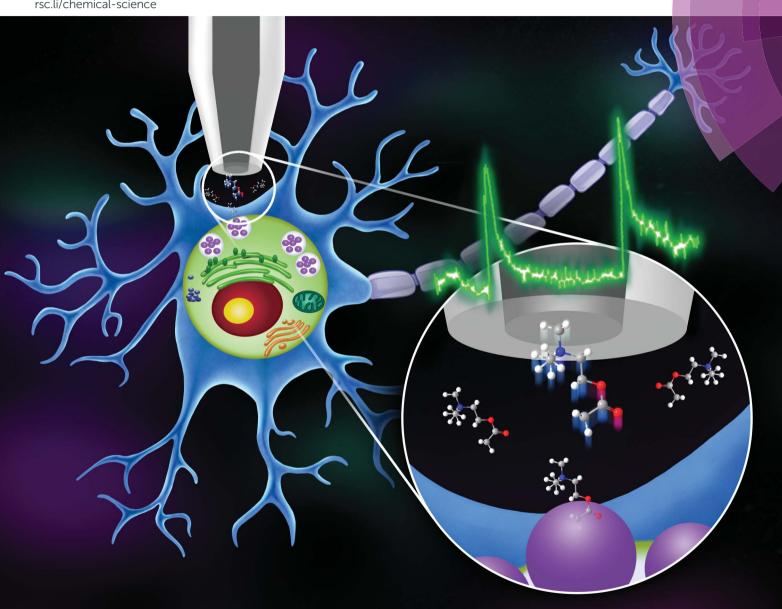
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## A high spatiotemporal study of somatic exocytosis with scanning electrochemical microscopy and nanoITIES electrodes†

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Extra-synaptic exocytosis is an essential component of cellular communication. A knowledge gap exists in the exocytosis of the non-redox active transmitter acetylcholine. Using the nano-interface between two immiscible electrolyte solutions and scanning electrochemical microscopy (SECM), a high resolution spatiotemporal study of acetylcholine exocytosis is shown from an individual neuronal soma. The nanoelectrode was positioned  $\sim$ 140 nm away from the release sites on the soma using an SECM. The quantitative study enables the obtaining of key information related to cellular communication, including extracellular concentration of the neurotransmitter, cellular permeability, Ca<sup>2+</sup> dependence on somatic release, vesicle density, number of molecules released and the release dynamics. Measurements were achieved with a high signal to noise ratio of 6–19. The released neurotransmitter with a concentration of 2.7 ( $\pm$ 1.0)  $\mu$ M was detected at the nanoelectrodes with radii of 750 nm to 860 nm.

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Extra-synaptic release of neurotransmitters, such as somatic exocytosis, is an essential part of neurotransmission and brain communication, playing a critical role in developmental processes in organisms, mediation of sensory systems, and human health. Measuring the neurotransmitter release concentration and dynamics simultaneously with high spatiotemporal resolution is important to understand cellular communication. Useful information has been generated *via* the study of extra-synaptic exocytosis of serotonin, dopamine, noradrenaline, octopamine, and neuropeptides. However, there is still much to be learned about acetylcholine (ACh) somatic exocytosis. While the possibility of ACh somatic release has been reported, 22,23 notably, there has been a lack of knowledge on simultaneous ACh concentration and release dynamics from a living neuronal soma. Besides, the mechanism of

neurotransmitter exocytosis from the soma is not fully understood.<sup>24</sup> Here, we present an nm spatial and ms temporal resolution study of ACh exocytosis from a living neuronal soma, unveiling its concentration and release dynamics simultaneously, which was achieved using nanometer-resolution scanning electrochemical microscopy (SECM)<sup>25</sup> and nanoelectrodes. Other pieces of information, *i.e.* vesicle density, cellular permeability, and number of transmitter molecules, were generated as well. This fundamental knowledge can offer insights into exocytosis and drug delivery, which may lead to a broader understanding of the chemical aspects of biology.<sup>11</sup>

The neuronal model in the current study is *Aplysia californica*, which has been demonstrated as an important model to understand cellular communication and fundamental mechanisms of learning and memory. <sup>26–31</sup> *Aplysia* contains a large number of neurons of varying sizes, facilitating its isolation and experimentation at the single cell level. <sup>29,31,32</sup> The release of ACh from the living pedal ganglion neurons of this organism in response to chemical stimulation has not been extensively studied; however, the presence of choline acetyl-transferase, the enzyme responsible for the synthesis of acetyl-choline, has been observed, <sup>33,34</sup> indicating cholinergic activity within these neurons. <sup>34–36</sup> The study of cholinergic release from *Aplysia californica* neurons contributes to a greater understanding of the role that acetylcholine plays in cellular communication.

SECM has been useful for biological study under actual physiological conditions, *e.g.* studying single cells and PC12 neuron cells, enabling biochemical measurements in

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† Electronic supplementary information (ESI) available: Experimental description; schematic representation of the experimental setup for neuronal stimulation experiments (and controls); in vitro ACh detection in ASW background solution with the calibration curve; cyclic voltammograms of components of ASW solution; COMSOL simulation of the pipet approach and experimental SECM approach curve to the neuronal soma surface; quantitative and kinetic parameters of release events; control experiments to confirm somatic release instead of physical disturbance that could occur during the stimulation process; results of control experiments demonstrating probe selectivity against stimulating solution; and distance dependence results on acetylcholine somatic release, before and after nano-positioning with an SECM. See DOI: 10.1039/c8sc01131a

‡ Equal contribution.

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real-time.<sup>37–46</sup> SECM does not require the use of labels/stains and is a non-invasive platform for measuring biochemical release from individual cells, especially while the cell is still alive.<sup>47–54</sup> Although SECM has been used in the study of various metabolic and secretory activities, including oxygen consumption or ROS/RNS release,<sup>46–48,50,53</sup> our work would be the first demonstration of using SECM in the study of another important signaling molecule, ACh.

The detection of ACh is based on the ion transfer across a polarized interface between two immiscible electrolyte solutions (ITIES), where the detection potential follows the Nernst equation (a detailed detection mechanism is included in the Experimental section of the ESI†). ACh detection has been studied using macro- or micro-ITIES pipet electrodes, as well as dual pipet electrodes.55-60 We recently developed nanometer scale ITIES pipet electrodes for the quantitative detection of ACh in vitro, 61,62 laying the foundation for studying ACh signaling with nanometer spatial resolution at the single living cell level as presented here. NanoITIES pipet electrodes with radii of 210 nm to 860 nm were used in the current study with the Cyclic Voltammograms (CV) corresponding to ACh detection shown in Fig. 1 (blue dashed curve). The first demonstration of ITIES electrodes in the study of extra-synaptic exocytosis from a living neuronal soma is presented, showing ITIES electrodes as powerful complementary analytical approaches to the conventionally used carbon electrodes in the study of extrasynaptic exocytosis.

The nm-resolution positioning capabilities of SECM enabled us to place the nanoITIES pipet electrodes at a nanometer distance from the release sites on the soma, where transmitter release was studied before its dilution. SECM also allows noncontact measurements, which prevent artifacts that could interfere with current recordings; this is difficult to achieve by optical microscopy alone. Neurotransmitters are diluted as they diffuse away following their release from the release sites, which poses challenges when studying their release temporal profile. Nanoelectrodes can be positioned at a much closer distance to

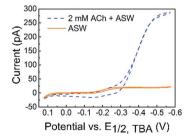


Fig. 1 Cyclic voltammograms (CVs) of acetylcholine (ACh) detection in vitro with the nanoITIES pipet electrode in the cellular medium of Aplysia, and artificial seawater (ASW). The electrochemical cell diagram is shown in cell 1. The CV of the ASW background solution is shown as an orange solid line, and the sigmoidal shape of the CV is due to the transfer of ASW ions facilitated by 1 mM DB18C6 inside the pipet (detailed current contributions of ASW ions are included in Fig. S3†). The CV for ACh detection is shown as a sigmoidal blue dashed line. The nanoelectrode radius is  $\sim\!370$  nm. Cell 1: Pt|1 mM DB18C6 + 5 mM TDDATFAB + 1,2-DCE||ASW|AqCl|Aq.

the neuron surface with an SECM than a macro- or microelectrode because the feedback distance is proportional to the electrode radius.<sup>25,63</sup>

Optical images before and after the SECM approach are shown in Fig. 2A and C, respectively. It is challenging to position the nanoelectrode to be at a nanometer distance from the soma surface by visual observation only. Using an SECM, we brought the nanoITIES electrode close (at a nm distance) to the soma surface while simultaneously recording the current measured on the nanoelectrode using a non-invasive electroactive mediator (ASW background ions, solid orange curve in Fig. 1, CVs of ASW and each component that makes up the ASW at nano-ITIES electrodes are shown in Fig. S4†), generating a current-distance curve, i.e. an SECM approach curve (solid purple line, Fig. 2B). The SECM approach curve can be simulated using COMSOL Multiphysics to determine accurately the distance down to the nm scale between the electrode and the cellular surface after the SECM approach; cellular permeability has also been derived from the simulation of the SECM approach curve. 50,64-66 Considering that we are approaching a living neuron, it is critical to consider the permeability in the SECM approach curve simulation. The simulated SECM approach curves with different cellular permeabilities to ASW ions are shown in Fig. 2B. As shown in Fig. 2B, without taking into account the cellular permeability  $(P_{\rm m}=0)$ , the simulated approach curve does not fit the experimental approach curve. Based on the fitting between the experimental approach curve and the simulated approach curve, it can be seen that the nanoITIES pipet electrode was

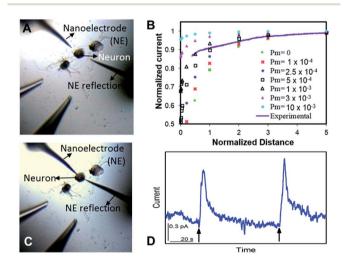


Fig. 2 High resolution spatiotemporal observation of acetylcholine (ACh) somatic exocytosis. The nanoITIES electrode for ACh detection was positioned with a nm-resolution scanning electrochemical microscope (SECM). Optical microscope images prior to SECM positioning (A) and after SECM positioning (C). Experimental SECM approach curve to the neuron soma ((B) solid purple curve) overlaid with simulated approach curves at varying permeability coefficient values ( $P_{\rm m}$ ). Amperometric recordings (D) of ACh release from a single Aplysia neuron in response to high concentration K<sup>+</sup> stimulation, indicated by the arrows. The nanoelectrode was positioned at a normalized distance of  $\sim$ 0.2, corresponding to an electrode–soma distance of  $\sim$ 140 nm (B). The nanoelectrode radius is 860 nm.

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positioned  $\sim$ 140 nm away from the soma surface, and the permeability of the *Aplysia californica* neuron membrane to ASW ions is between  $5 \times 10^{-4}$  and  $1 \times 10^{-3}$  m s<sup>-1</sup>.

Once positioned at a nm distance from the soma with the SECM, we employed amperometry to study the release of ACh in response to high concentration K<sup>+</sup> stimulation, to learn about its concentration and dynamics (Fig. 2D) with a time resolution on the order of ms. The applied potentials are the diffusion limiting potential for ACh detection, from approximately -0.5 Vto  $-0.6 \text{ V} \text{ vs. } E_{1/2.\text{TBA}}$ . At this potential under the condition of cell 1, the nanoITIES pipet electrodes are selective for ACh detection against other substances known to exist or to be released from the animal model in the current study, Aplysia californica, such as GABA, dopamine, glutamate, serotonin, amino acids, and pedal peptide, as well as high concentration K<sup>+</sup> in the stimulating solution, and H<sup>+</sup> variation accompanying exocytosis, as detailed in our recent study.<sup>67</sup> Arrows in Fig. 2D indicate the application of high concentration K<sup>+</sup> stimulation. As shown in Fig. 2D, ACh release from the soma was observed immediately in response to high concentration K<sup>+</sup> stimulation. Control experiments confirmed that the amperometric peaks in Fig. 2D are due to somatic release of the Aplysia cell, rather than mechanical perturbation of the electrode interface during the stimulation process (Fig. S5†), or from the ions present in the stimulating solution (Fig. S6†). The in situ release result in Fig. 2D was acquired right after the SECM approach with the optical image shown in Fig. 2C; in contrast, there is no significant observation of somatic release (Fig. S7†) before the SECM approach in the optical image shown in Fig. 2A, indicating the low concentration of the transmitter further away from the release sites.

The concentration of ACh released from the single cell measured in situ is 2.7  $\pm$  1.0  $\mu$ M, calculated from the maximum amperometric peak current (Table 1), using procedures described in the ESI† under the Data acquisition and analysis section. A lower concentration is expected at a location much further away from the cell soma due to dilution that happens when the transmitter diffuses away from the soma after release. The number of moles for each peak event is 80 ( $\pm 20$ )  $\times$  $10^{-18}$  mol, corresponding to 4.8 ( $\pm 1.3$ )  $\times$   $10^{7}$  molecules (Table 1). This amount of transmitter is much higher than the number of ACh molecules estimated by electrophoretic application of ACh in the rat diaphragm,68 iontophoretic application of ACh at the neuromuscular junction,69 and the amount measured with a 15 nm radius ITIES electrode around a single synapse of Aplysia. 67 Besides, the observed amperometric half peak width of 9.3 ( $\pm$ 3.5) s is significantly higher than that expected for

Table 1 Quantitative parameters of amperometric acetylcholine release peaks. Temporal resolution: ms

	Total $(n = 8)$
$t_{1/2}$ (s)	$9.3~(\pm 3.5)$
Concentration (µM)	$2.7\ (\pm 1.0)$
Moles released	$80~(\pm 20) \times 10^{-18}$
Number of	$4.8~(\pm 1.3) \times 10^{7}$
molecules	•

single vesicular events, which is in the order of hundreds of ms. <sup>70</sup> The above result suggests the measurements of multiple vesicular contents released from the single neuron soma *via* nanoITIES electrodes with radii of 750–860 nm during each peak event. Using the content per vesicle as suggested in our recent single synaptic study of acetylcholine release <sup>67</sup> from the same animal model, we calculated the releasable vesicle density to be  $25 \pm 2$  vesicles per  $\mu$ m² (Fig. 3, the calculation procedure is detailed in the ESI†). This number has a similar order of magnitude to the value of 90 vesicles per  $\mu$ m² obtained in a recent study using 3-D reconstruction of synaptic boutons from serial transmission electron microscopy sections. <sup>71</sup>

We further examined in situ the role of calcium in the somatic release of acetylcholine (Fig. 4). In these experiments, we replaced the standard ASW cell medium with calcium-free ASW solution, and measured the neurotransmitter release in response to high concentration-K<sup>+</sup>, Ca<sup>2+</sup>-free ASW stimulating solution; afterwards, Ca2+ was added back into the cellular medium and stimulating solution, followed by neurotransmitter release measurements. As shown in Fig. 4A, when calcium was not present in the cell medium and stimulating solution, no significant somatic release events were observed. In contrast, very strong release peaks (Fig. 4B) were observed after we added Ca2+ back into the cell medium and stimulating solution. These experiments demonstrated that acetylcholine exocytosis from the soma in Aplysia neurons is calciumdependent. Our results concur well with the calciumdependence of somatic release of catecholamines,5-7 and ACh somatic release from other animal models studied with radiochemical assay and excised patch probes<sup>22,23</sup> where simultaneous concentration and release dynamics of ACh somatic exocytosis are lacking.

In summary, a high resolution spatiotemporal quantitative study of acetylcholine exocytosis from a single living soma of *Aplysia* has been demonstrated. To the best of our knowledge, this is the first such report. A nanometer ITIES sensing probe was positioned  $\sim$ 140 nm vertically from the release sites using scanning electrochemical microcopy; exocytosis events were studied with ms temporal resolution. Due to the nanoscale structure of the electrodes as compared to the approximate 100–150  $\mu$ m diameter of the *Aplysia* soma, it is expected that we do not detect all of the transmitter molecules released. However, the scale of the nanoprobes is advantageous because it allows the determination of vesicle density, as well as the nm resolution and non-contact positioning of the sensing probe from the

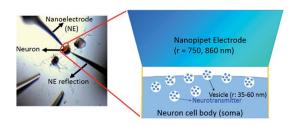


Fig. 3 Measurement of vesicle density. The measured vesicle density is  $\sim$ 25  $\pm$  2 vesicles per  $\mu$ m<sup>2</sup>. The vesicle radius (r) is reported in ref. 30.

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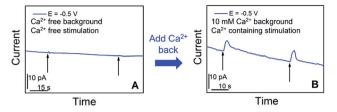


Fig. 4 Calcium-dependence of acetylcholine exocytosis from an *Aplysia californica* neuron in response to high concentration  $K^+$  stimulation. (A) Amperometric current response in response to chemical stimulation ( $Ca^{2+}$  free, elevated  $K^+$  solution, indicated by black arrows) measured at 50 min after replacing standard ASW with  $Ca^{2+}$ -free ASW. (B) Amperometric current response from the cell upon stimulation ( $Ca^{2+}$ -containing, elevated  $K^+$  solution, indicated by black arrows), 10 min after the addition of  $Ca^{2+}$  solution to a final concentration of 10 mM. The applied potential is the diffusion limited potential for acetylcholine detection shown in Fig. 1. The nanoelectrode radius is 780 nm.

release sites. Key pieces of information related to cellular communication were obtained, including extracellular concentration of acetylcholine during somatic exocytosis, number of molecules released, the dynamics of exocytosis, vesicle density, and Ca<sup>2+</sup> dependence on somatic exocytosis. The Ca<sup>2+</sup> dependence study contributes to the mechanistic understanding of somatic exocytosis, which is currently not fully understood. When Ca<sup>2+</sup> was not present, no exocytosis events were observed in response to high concentration K<sup>+</sup> stimulation; in contrast, acetylcholine exocytosis events recovered once Ca<sup>2+</sup> had been added back. Ca<sup>2+</sup> is known to be involved in the vesicular release of transmitters;<sup>11</sup> our findings suggest the vesicular release pathway of acetylcholine somatic exocytosis from *Aplysia*.

### Conflicts of interest

There are no conflicts to declare.

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