Supporting Information for: High Spatiotemporal Study of Somatic Exocytosis with Scanning Electrochemical Microscopy and NanoITIES Electrodes

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Section S1. Experimental Description

Figure S1. Graphic of experimental setup for neuronal stimulation experiments (and controls).

Figure S2. In vitro Acetylcholine (ACh) detection experiments using amperometry (A) and cyclic voltammetry (B) in ASW using the same type of nanopores and under the same experimental conditions as living neuron experiments. Calibration curve for ACh detection based on amperometric $i$-$t$ recordings (C).

Figure S3. Cyclic voltammograms of components of ASW solution.

Figure S4. COMSOL simulation of pipet approach and experimental SECM approach curve to neuronal soma surface.

Table S1. Quantitative and kinetic parameters of release events.

Figure S5. Control experiments to confirm somatic release instead of physical disturbance that could occur during stimulating process.

Figure S6. Control experiments with configuration shown in Figure S1B, to confirm that the signal detected in Figure 2D was due to cellular release rather than from the ions present in the stimulating solution.
Figure S7. Distance dependence results on acetylcholine somatic release, with cellular exocytosis results before nm resolution SECM positioning shown in (A) and after SECM positioning shown in (B).
EXPERIMENTAL SECTION

Reagents. Potassium tetrakis(pentfluorophenyl)borate (TFAB) was obtained from Boulder Scientific Company (Mead, CO). Tetradodecylammonium (TDDA) chloride, dibenzo-18-crown-6 (DB18C6), 1, 2-dichloroethane (1, 2-DCE), Fast Green FCF Dye, chlorotrimethylsilane, calcium chloride (CaCl2), and tetrabutylammonium chloride (TBACl) were purchased from Sigma Aldrich (St. Louis, MO). Sodium chloride (NaCl) was obtained from EMD Chemicals (Gibbstown, NJ), potassium chloride (KCl) was obtained from VWR (Radnor, PA). Magnesium chloride (MgCl2) was from Amresco (Solon, OH). Magnesium sulfate (MgSO4) and HEPES were from Fischer Scientific (Pittsburgh, PA). Artificial seawater contained the following: 460 mM NaCl, 10 mM KCl, 10 mM CaCl2, 22 mM MgCl2, 26 mM MgSO4, 10 mM HEPES (pH 7.8). Calcium-free artificial seawater contained the following: 470 mM NaCl, 10 mM KCl, 22 mM MgCl2, 26 mM MgSO4, 10 mM HEPES. NaCl concentration was adjusted to maintain the same osmolarity. All reagents were used as received. Solutions were prepared using 18.3 MΩ cm deionized water (ELGA, Woodridge, IL).

Animal dissection and tissue preparation. Sea slugs Aplysia californica (20-100 g) are supplied by National Resource for Aplysia in Miami, FL. Animals are kept in circulated, aerated seawater at 14 °C and anesthetized via injection into the body cavity with isotonic magnesium chloride solution (roughly half the body weight) prior to dissection. Pedal ganglia were extracted from the Aplysia and incubated in artificial seawater (ASW) supplemented with proteases (1% type XIV, Sigma-Aldrich) at 34 ºC for 15-60 min, after which the ganglia were removed from the protease solution and washed in ASW for 60-180 min at 21-26 ºC. Approximately 30 animals were used to optimize the protocols, with two cultures prepared per animal. The data shown in Figures 2, S5-S6 are from three different culture preparations.

Cell isolation and culturing. Silicon wafers were coated with poly-L lysine for 15-20 min to facilitate cell adhesion, and then submerged in ASW supplemented with antibiotics (100 units/mL penicillin G,
100 mg/L streptomycin, 100 mg/L gentamicin, pH 7.7). Individual Aplysia cells were mechanically extracted from ganglia and isolated in ASW, then transferred onto the silicon wafer. These cells sat on the wafers submerged in ASW with antibiotic overnight at room temperature to allow for adhesion and growth of neurites.

**Pipet fabrication and characterization.** Recording nanoelectrodes were fabricated via laser pulling of quartz capillary tubes (Sutter Instrument Co., Novato CA; outer diameter = 1.0 mm, inner diameter = 0.7 mm, length = 7.5 cm) using a P-2000 capillary puller (Sutter Instrument Co., Novato CA). The approximate radii of these nanopipets were included in Figure captions, ranging from 210 nm to 860 nm. Detailed nanoITIES pipet electrode fabrication and characterization were reported in our recent work.\(^1,2,3,4\) To ensure that a stable interface formed at the opening of the pipet, the glass surface was treated with chlorotrimethylsilane using a plastic desiccator, where a vacuum was created and the pipets were exposed to silane vapors. Nanopipets were backfilled with a solution of 5 mM TDDATFAB + 1 mM DB18C6 in 1, 2-DCE using a 10 µL Hamilton syringe. The solution was pushed to the nano-tip using gentle vibrations. A Pt wire of 50 µm diameter was etched at the tip and inserted into the pipet and secured with wax. Our recent study show that current measured on the nanoITIES pipet electrode corresponding to ACh detection increases linearly with the concentration of ACh.\(^3\) Probe characterization experiments (Figure S2) show that these nanoelectrodes have a limit of detection of approximately 26 µM for probes with radii approximating 210 nm. The current is increasing linearly with increasing concentration of ACh ranging from 0.25 to 2 mM for these nanoelectrodes as shown in the calibration curve (Figure S2C).

Stimulating pipets were fabricated via laser pulling borosilicate capillaries (Sutter Instrument Co., Novato CA; outer diameter = 1.0 mm, inner diameter = 0.58 mm, length = 10 cm) with the P-2000 capillary puller. The radii of these pipets are approximately 5-10 µm. The pipet was connected via plastic tubing to a 5 mL syringe filled with a solution of elevated-K\(^+\) ASW (50 mM KCl, 420 mM NaCl,
10 mM CaCl₂, 22 mM MgCl₂, 26 mM MgSO₄, 10 mM HEPES) and Fast Green FCF Dye. For calcium-free experiments, the syringe was filled with elevated-K⁺, calcium-free ASW (50 mM KCl, 430 mM NaCl, 22 mM MgCl₂, 26 mM MgSO₄, 10 mM HEPES) and Fast Green FCF Dye.

**Electrochemical experiments.** The transfer of neurotransmitters across the 1, 2-DCE/water interface was studied by cyclic voltammetry and amperometry. All electrochemical recordings were taken using CHI920D Scanning Electrochemical Microscope (SECM) (potentiostat rise time: < 1 µs, 0.8 µs typical) (CH Instruments, Austin, TX). The nanopipet filled with 5 mM TDDATFAB + 1 mM DB18C6 in 1, 2-DCE was immersed in aqueous solution (ASW) for the detection of neurotransmitters. The aqueous solution used was ASW since it is the biological media for the neuronal model used, *Aplysia californica*. A Pt wire was inserted inside the pipet and secured with wax. An Ag wire (diameter = 250 µm) coated with AgCl was immersed in ASW outside the pipet, acting as the external reference electrode. In these experiments, the voltage was applied between the platinum wire and the external Ag/AgCl reference wire. Potential was also calibrated with respect to the transfer of TBA for the probe characterization experiments.

**Data acquisition and analysis.** Silicon wafers with plated cells submerged in ASW were placed on the SECM stage. A labbuilt LED light source and a DS-20S Sony CCD USB 2.0 digital microscope camera from Caltex Scientific (Irvine, CA) were used to locate cells of interest and position the stimulating pipet roughly within a few hundred microns of the cell body. The external reference electrode was submerged in the ASW and the recording nanoelectrode was positioned directly over the cell of interest roughly using lab-built side view optical microscope and stepper motors of the SECM, followed by fine positioning using SECM via piezo nanopositioner.

During electrochemical experiments, cyclic voltammetry was used to check the stability of the nanoelectrode and measure the steady state transfer potential of the ASW assisted by 1 mM DB18C6.
The transfer of ASW at this potential was used during SECM approach curves to bring the probe close to the cell surface. A SECM probe approach curve measured the current at the nanoelectrode while incrementally moving the nanoelectrode downward using a Piezo motor. After the SECM approach curve, we performed neuron stimulation experiments, where we measured neurotransmitter release concentration and dynamics in response to high concentration K\(^+\) stimulation. Amperometric \(i-t\) curves measured the current at the recording nanoelectrode over time, while 1-s puffs of high concentration-K\(^+\) ASW solution were applied at specific intervals. Figure S1 features a graphic depiction of the experimental setup for these stimulation experiments both conducted (A) over the Aplysia cell body; and (B) over the bare silicon wafer, as a control experiment to confirm the somatic release of transmitter.

We conducted several stimulation experiments, and consistently recorded response peaks corresponding to the release of transmitter in response to chemical stimulations. We further analyzed data from those with well-defined, consistent peaks and there was minimal baseline drift. Before calculating quantitative and kinetic parameters, the data to be analyzed were smoothed through the CHI recording software using the least squares technique with a least square point value of 49 assigned in order to minimize noise while preserving the underlying patterns in the data.

The concentration of the neurotransmitter released from the cell soma was calculated based on the maximum peak current using well established expression for diffusion limiting current,\(^5\)  
\[
\hat{i} = 4nFDca
\]
In this equation, \(\hat{i}\) is the diffusion limiting current, we used peak current in the amperometry for calculation, \(x\) is a function of the quantity \(RG = rg/a\) (\(rg\) being the outer tip radius and \(a\) being the inner tip radius) which here equals 1.23, \(n\) is the number of transferred charges in the tip reaction (which is equal to 1 for acetylcholine), \(F\) is Faraday’s constant, \(D\) is the diffusion coefficient of the neurotransmitter measured (\(D = 7.5 \times 10^{-10} \text{ m}^2/\text{s}\) for acetylcholine),\(^3\) \(c\) is the concentration and \(a\) is the radius of the nanopipet electrode. The number of molecules under each amperometric peak can be estimated based on the integrated current \((Q)\), expressed as \(Q = nFN\), where \(n\) is the number of moles of
neurotransmitter, \( F \) is Faraday’s constant, and \( N \) is number of charges transferred (here \( N = 1 \) for acetylcholine).

**Ca\(^{2+}\) dependent experiment.** Ca\(^{2+}\) acts as a common integrator of environmental cues that influence neurite growth. We first tried cell cultures in ASW without Ca\(^{2+}\), where not much growth was observed. From then on, the cells were first cultured in Ca\(^{2+}\) containing ASW, then the ASW background was replaced with a Ca\(^{2+}\)-free ASW one hour before the exocytosis experiments. The calcium was diluted out of the background solution by adding Ca\(^{2+}\)-free ASW to double the original volume and then removing half of the overall ASW volume (effectively halving the calcium concentration). This step was repeated 20 times and then followed by addition of 0.1 mM EDTA to the solution. After running cyclic voltammograms, and SECM approach curves to position the nanopipet electrode to be hundreds of nm over the cell body, we ran amperometric \( i-t \) curves and applied 1-s puffs of elevated-K\(^{+}\), Ca\(^{2+}\)-free ASW solution at specific intervals. After amperometric recordings in a Ca\(^{2+}\)-free environment, we proceeded by adding CaCl\(_2\) back to be 10 mM in the cell medium and studied somatic release using calcium-containing stimulating solution.

Silicon wafers with plated cells submerged in ASW were placed on the SECM stage. The same LED light source and digital microscope were used to locate cells of interest and position the stimulating pipet within a few hundred microns of the cell body. The external reference electrode was submerged in the ASW and the recording electrode was positioned over the cell of interest using SECM.

**Detection mechanism of the nanoITIES pipet electrode.** ACh detection on the nanopipet electrode is based on ion transfer across a nanopipet supported interface between two immiscible electrolyte solutions (ITIES).\(^1,2,3,4\) Chemical detection with ITIES pipet electrodes have been well established.\(^2,6-7\) The detection with ITIES pipet electrodes is based on potential driven ion transfer that is related to the structure of each analyte, and the detection potential follows a Nernstian equation.\(^8\) When a potential is
applied (the ITIES is polarized), ion transfer is induced and the movement of ionic neurotransmitter is
detected as a current response on the ITIES pipet electrode. The nanopipet electrode used in the present
study was able to detect its release less than 1 s following chemical stimulation of the neurons.

**COMSOL Simulation.** Using COMSOL Multiphysics version 4.4, simulated SECM approach curves
with different permeability coefficient values were obtained. A 2D model of the experimental SECM
setup was generated, with symmetry plane through the center of the nanoelectrode and neuron cell
(Figure S5A). The solution domain follows Fick’s second law of diffusion

\[
\frac{\partial c_s(r,z)}{\partial t} = D \left( \frac{\partial^2 c_s(r,z)}{\partial r^2} + \frac{1}{r} \frac{\partial c_s(r,z)}{\partial r} + \frac{\partial^2 c_s(r,z)}{\partial z^2} \right)
\]

The variables \( r \) and \( z \) are the cylindrical coordinates used in the 2D axially symmetric modeling. The
initial bulk concentration of artificial sea water is set to \( c_s = 470 \) mol/m\(^3\) to match the bulk solution
concentration used in the actual experiment, the flux of ASW ions across the electrode follows the
Butler-Volmer equation.

\[
i = i_0 \left[ \frac{C_0(0,t)}{C_0^*} e^{\alpha n_f \eta} - \frac{C_R(0,t)}{C_R^*} e^{(1 - \alpha) n_f \eta} \right]
\]

\[
k = k_0 (e^{\alpha n_f \eta} - e^{(1 - \alpha) n_f \eta}) = k_f(\eta) \Delta k_b(\eta)
\]

The cell membrane is defined as a flux boundary, the flux of ASW ions across the cell membrane
boundary is dependent on the permeability coefficient value (Pm).

\[
flux = Pm \times c_s
\]

The SECM cell was set as no flux boundary to simulate its insulating characteristics. The COMSOL
model geometry was then meshed using a free triangular mesh. The size of the mesh was customized to
have a maximum element size of $1.06 \times 10^{-5}$ and a minimum of $6 \times 10^{-8}$. A growth rate of 1.3 and curvature factor of 0.3 (Figure S4B)

A time dependent study was conducted, while the normalized distance $(d/a)$, the height between the electrode and the cell $(d)$ divided by the radius of the electrode $(a)$, was decreased to simulate a SECM approach to the cell. A simulated SECM approach curve was obtained by plotting the normalized current (the probe current divided by the current when the probe is far away) versus the normalized distance. Since the permeability value was parameterized, simulated SECM approach curves were obtained with varying permeability coefficient values. The normalized experimental approach curve was overlaid with the simulated approach curves at varying permeabilities. The permeability of *Aplysia californica* neuron to ASW ions was determined based on the best fitting between the experimental and simulated approach curves (Figure S4D). Based on the fitting of the experimental approach curve, the normalized distance $(d/a)$ was determined, then the distance between the nanoelectrode and the neuron surface $(d)$ can be calculated by plugging in the radius of the electrode $(a)$.

**Vesicle Density Calculation.** The number of releasable vesicles under each amperometric peak in the current work can be calculated based on the division of the number of molecules by the single vesicular contents suggested in our recent work on single synaptic study of the cholinergic transmitter release\textsuperscript{82}. The calculated number of vesicles can be further divided by the electrode area to generate the releasable vesicle density information.
Figure S1. Schematic representation of experimental setup when the recording nanoelectrode is positioned over the single cell soma to detect the release of neurotransmitter from cell body (A); and in control experiments when the nanoelectrode is moved laterally away from the cell surface over bare silicon wafer, while maintained at the same depth (B). Control experiments were conducted to confirm that the signals observed were due to the release of neurotransmitter from the cell body.
**Figure S2.** *In vitro* Acetylcholine (ACh) detection experiments using amperometry (A) and cyclic voltammetry (B) in ASW using the same type of nanoprobes and under the same experimental conditions as living neuron experiments. Calibration curve for ACh detection based on amperometric $i-t$ recordings (C). The applied potential in amperometry is the diffusion limiting potential for ACh detection approximately -0.465 V vs. $E_{1/2,TBA}$ (C). Probe radius was approximately 210 nm (A-C).
Figure S3. Cyclic voltammograms of ASW and each component that makes up ASW at nanoITIES electrodes. Each CV was taken using a different nanoITIES electrode, so current is normalized to electrode radius.
Figure S4. (A) 2D axially symmetric COMSOL model geometry of SECM setup. (B) The resulting mesh, which is finely focused at the Pt tip. (C) Surface concentration map of the artificial sea water domain following computation. (D) Normalized experimental approach curve to the neuron soma (solid purple curve) overlaid with simulated approach curves at varying permeability coefficient values (Pm).
Table S1. Quantitative and kinetic parameters of amperometric peaks detected during exocytosis of pedal ganglia of *Aplysia californica* neuronal cells with nanopipet electrode.

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1 ($n = 2$)</th>
<th>Experiment 2 ($n = 6$)</th>
<th>Total ($n = 8$)</th>
</tr>
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<tbody>
<tr>
<td>$I_{max}$ (pA)</td>
<td>1.09 ± 0.11</td>
<td>0.46 ± 0.09</td>
<td>0.62 ± 0.30</td>
</tr>
<tr>
<td>$Q$ (pC)</td>
<td>10.3 ± 0.2</td>
<td>6.8 ± 1.6</td>
<td>7.7 ± 2.1</td>
</tr>
<tr>
<td>$t_{1/2}$ (s)</td>
<td>4.77 ± 0.52</td>
<td>10.82 ± 2.54</td>
<td>9.31 ± 3.53</td>
</tr>
<tr>
<td>$t_{rise}$ (s)</td>
<td>1.46 ± 0.98</td>
<td>2.88 ± 1.69</td>
<td>2.52 ± 1.61</td>
</tr>
<tr>
<td>$t_{fall}$ (s)</td>
<td>8.54 ± 0.14</td>
<td>12.52 ± 5.10</td>
<td>11.52 ± 4.69</td>
</tr>
<tr>
<td>Concentration (µM)</td>
<td>4.3 ± 0.4</td>
<td>1.9 ± 0.5</td>
<td>2.7 ± 1.0</td>
</tr>
<tr>
<td>Attomoles released</td>
<td>108 ± 2</td>
<td>70 ± 16</td>
<td>80 ± 20</td>
</tr>
<tr>
<td>Number of molecules</td>
<td>6.50 ± 0.12 x 10⁷</td>
<td>4.23 ± 0.99 x 10⁷</td>
<td>4.79 ± 1.33 x 10⁷</td>
</tr>
</tbody>
</table>

Here we present data from two stimulation experiments done on two distinct *Aplysia* neurons, where $n$ represents the number of peaks observed from the cell in each experiment. Data presented as mean ± SD.
Figure S5. (A) Amperometric $i-t$ recording of acetylcholine release from *Aplysia* single neuron cell upon application of high concentration $K^+$ stimulating solution, indicated by arrows. (B) Control experiment to confirm that results observed in (A) are due to somatic release instead of physical disturbance that could occur during stimulating process. Radius of nanoelectrode: 750 nm (A, B).

Applied potential values ($E$) of approximately -0.43 V (A) and -0.13 V (B) vs. $E_{1/2, TBA}$. In the control experiment (B), all experimental conditions remained the same (position of recording nanoelectrode, application of stimulating solution, etc.), except the applied amperometric potential ($E$) was adjusted to -0.13 V vs. $E_{1/2, TBA}$, a potential that would not induce detection of ACh at the interface. This control result in Fig. S5B show that no current signal was observed following chemical stimulation at control potential of -0.13 V vs. $E_{1/2, TBA}$. This confirms that the amperometric peaks observed in Fig. S5A are due to somatic release of the *Aplysia* cell, rather than mechanical perturbation of the electrode interface during the stimulation process.
Figure S6. Control experiments with configuration shown in Figure S1B, to confirm that the signal detected in Figure 2D was due to cellular release rather than from the ions present in the stimulating solution. The amperometric $i-t$ recording was taken above bare silicon wafer in response to high-concentration $K^+$ stimulation (indicated by arrows). The absence of peaks confirms that the peaks in Fig. 2D are due to cellular release. The applied potential is the diffusion limiting potential of ACh, approximately $-0.53$ V vs. $E_{1/2,TBA}$. Radius of nanoelectrode: 340 nm.
Figure S7. Distance dependence results on acetylcholine somatic release, with cellular exocytosis results before nm resolution SECM positioning shown in (A) and after SECM positioning shown in (B). Amperometric $i-t$ curve (A) shows no significant response detected upon stimulation prior to SECM approach curve where the electrode was far away. In contrast, well-defined peak response (B) was recorded upon stimulation after performing SECM approach curve, where the nanoelectrode was moved ~7.2 µm closer to the cell surface. Application of chemical stimulation is represented by arrows. Applied potential values ($E$) of approximately -0.53 V (A, B) vs. $E_{1/2}$, TBA. Insets are optical microscopic pictures of the nanoelectrodes and neurons.

References.

4 N. T. Iwai; M. Kramaric; D. Crabbe; Y. Wei; R. Chen; M. Shen. *Anal. Chem.*, 2018, **90**, 3067-3072.