Electronic Supplementary Material (ESI) for Chemical Science.

Electronic Supplementary Information

Concentration of stimulant regulates initial exocytotic plasticity at single cells

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Chemicals and Solutions. Unless otherwise specified, all reagents were purchased from Sigma-Aldrich. All chemicals, of analytical grade, were obtained from Sigma-Aldrich and used as received. All solutions were prepared using 18 MΩ·cm water from Purelab Classic purification (ELGA, Sweden), adjusted to pH 7.4 and filtered prior for use. The isotonic saline solution included 150-mM NaCl, 5-mM KCl, 1.2-mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose and 10 mM HEPES, pH 7.4. The K^+ stimulation solution contained 10-, 30-, 50- or 100-mM KCl, (155-x, where x is the KCl conc) mM NaCl (i.e., 145-, 125-, 105-, or 55-mM, respectively), 1.2-mM $MgCl_2$, 2-mM $CaCl_2$, 5-mM glucose, and 10-mM HEPES, pH 7.4. 10× Locke's buffer included 1540-mM NaCl, 56-mM KCl, 36-mM $NaHCO_3$, 56-mM glucose, 50-mM HEPES, 1% (v/v) penicillin, pH 7.4. This stock solution was diluted 10 times with distilled water (1× Locke's buffer) the day before the experiment.

Isolation of Adrenal Chromaffin Cells. Bovine adrenal glands were obtained from a local slaughterhouse, and the chromaffin cells were isolated as previously described[1]. Briefly, the vein was perfused with Locke's buffer to clear away blood cells. The medulla was isolated after collagenase (0.2%, Roche, Sweden) treatment, and cells were isolated using a series of homogenization and centrifugation steps. For single cell experiments, ~700 000 cells were seeded on collagen (IV) coated plastic dishes (D=60 mm, Corning Biocoat, VWR, Sweden) and maintained in a humidified incubator at 37 °C, 5% CO_2 for a maximum of 3 days prior to experiments.

Fabrication of Disk Carbon Fiber Microelectrodes. The fabrication of disk microelectrodes was previously described[2]. Briefly, a 5 µm diameter carbon fiber was aspirated into a glass capillary (Sutter Instrument Co., Novato, CA). A micropipette puller (model PE-21, Narishige, Inc., Japan) was used to pull the glass capillary into two separate electrodes and epoxy (Epoxy Technology, Billerica, MA, U.S.A.) was used to seal the electrodes. The glued electrodes were then cured at 100°C overnight and subsequently beveled at 45° angle (EG-400, Narishige Inc., London, UK). Before the experiment, each electrode was tested with cyclic voltammetry (-0.2 to 0.8 V vs Ag/AgCl, 100 mV/s) in a solution of 100 µM dopamine in PBS (pH 7.4). Only electrodes showing good reaction kinetics and stable steady-state currents were used for experiments.

Fabrication of Nano-Tip Conical Carbon Fiber Microelectrodes. The fabrication of nano-tip conical carbon fiber microelectrode was previously described[3]. Briefly, a 5-µm carbon fiber was aspirated into a borosilicate glass capillary (1.2-mm o.d., 0.69-mm i.d., Sutter Instrument Co., Novato, CA). The glass capillary was subsequently pulled into two separate electrodes with a commercial micropipette puller (model PE-21, Narishige, Inc., Japan). The fiber extending from the glass was cut to 100-150 µm with a scalpel under a microscope. To flame etch the carbon fiber, the electrodes were held on the edge of the blue part of a butane flame (Multiflame AB, Hässleholm, Sweden) for less than 2 s. As the end of the tip became red, the electrode was rotated in order to ensure even etching. Fibers with a needle-sharp tip about 50- to 100-nm tip diameter and about 30- to 100-µm shaft length were sealed with epoxy (Epoxy Technology, Billerica, MA). Each electrode was then tested with cyclic voltammetry (-0.2 to 0.8

V vs Ag/AgCl, 100 mV/s) in a solution of 100-µM dopamine in PBS (pH 7.4). Only electrodes showing good reaction kinetics and stable steady-state currents were used for experiments.

Electrochemical measurements. Before single cell amperometry, the medium was removed and the cells were rinsed three times with isotonic saline solution. The cells were kept 37 °C in the isotonic solution during the whole experimental process. Electrochemical recordings from single chromaffin cells were performed on an inverted microscope (IX71, Olympus), in a Faraday cage. The working potential was +700 mV vs an Ag/AgCl reference electrode (Scanbur, Sweden) under the control of an Axopatch 200B potentiostat (Molecular Devices, Sunnyvale, CA). The output was filtered at 2.1 kHz and digitized at 5 kHz (Axoscope 10.4 software, Axon Instruments Inc., Sunnyvale, CA, USA). All the experiments were observed under an inverted microscope (IX81, Olympus) with 10x and 40x objectives. For single cell exocytosis, a disk carbon-fiber microelectrode was moved slowly by a Patch-Clamp Micromanipulator (PCS-5000, Burleigh Instruments, Inc., USA) to place it on the membrane of a chromaffin cell without causing any damage to the surface. Ten seconds after the start of recording, a 10-, 30-, 50-, or 100-mM K^+ stimulating solution in a glass micropipette was injected into the volume surrounding the chromaffin cell with an electrode placed on it with a single 30-s injection pulse.

Data Acquisition and Analysis. The amperometric traces were processed using an Igor Pro 6.22 routine originating from David Sulzer's group. The filter for the current was 10 kHz (binomial sm). The threshold for peak detection was three times the standard deviation of the noise. The traces were carefully inspected after peak detection and false positives were manually rejected. The number of molecules released by single cells was pooled, and the median of the data was calculated for each experimental condition. These parameters (Fig. S1), the rise time (t_{rise}), defined as the time separating 25% of the maximum from 75% of the maximum on the ascending part of the spike; the half peak width ($t_{1/2}$), defined as the width of the exocytotic at half of its magnitude; the fall time (t_{fall}), defined as the time separating 75% of the maximum from 25% of the maximum on the descending part of the spike. To compare between different conditions, the mean of median of molecules number calculated was used. The responding cells were also calculated from each experiment. Pairs of data sets were compared with a two-tailed Mann-Whitney rank-sum test, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Calcium Imaging Experiments. Chromaffin cells were cultured on collagen-coated dishes as described above. Cells were first washed with isotonic solution and then incubated with 0.4-µM Fura-2 (Invitrogen, Sweden) for 20 min at 37 °C. After loading of florescence, the loaded cells were washed with isotonic solution again and were bathed in isotonic saline for the further imaging experiment.

An inverted microscope (IX71, Olympus, Japan) was used to perform the fluorescent imaging. The microscope was equipped with an MT-20 illumination system and a Xenon are lamp with 150 W was used as the light source. Fura-2 in the cells was ratiometrically imaged at 380 nm and 340 nm for 30-s K^+ (e.g., 10, 30, 50, or 100 mM) stimuli, and the images were collected with an ORCA-ER camera (Hamamatsu, Japan) on the Cell-R software (Olympus). After a

10-s baseline, cells were stimulated with e.g., 10-, 30-, 50-, or 100-mM K^+ stimulation solution for 30 s, the same as for single-cell exocytosis. Cell-R software was also used to perform background subtraction and region of interest analysis. The calcium responses from the cells under different concentration K^+ stimuli were pooled and analyzed and further plotted in Origin.

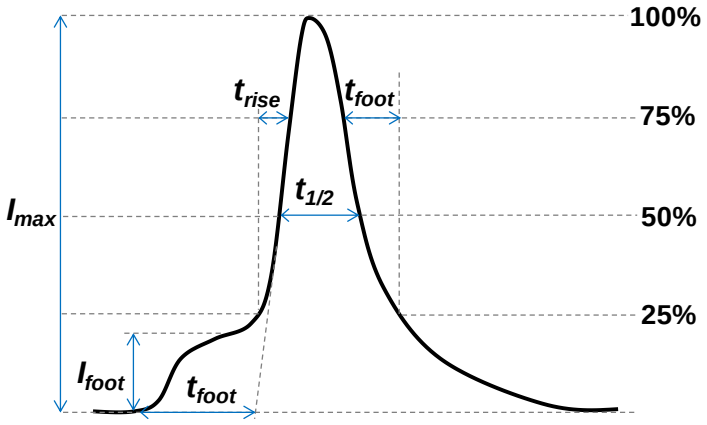

Fig. S1. Scheme of the different parameters used for the peak analysis for exocytosis. I_{max}=peak current, t_{rise}=rise time, $t_{1/2}$=half peak width, t_{fall}=fall time, I_{foot}=foot current, t_{foot}=foot duration.

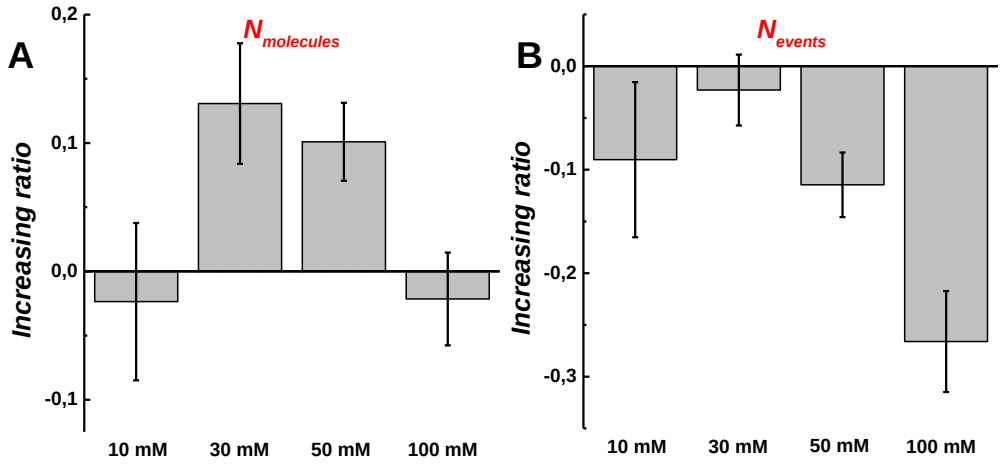

Fig. S2. Increased ratio of $N_{molecules}$ (A) and N_{events} (B) obtained from paired 1st and 2nd 15-s time of stimulation for single chromaffin cells following stimulation by different concentrations of K^+ (10, 30, 50, and 100 mM). Data represent means ± SEM. The number of cells was 20.

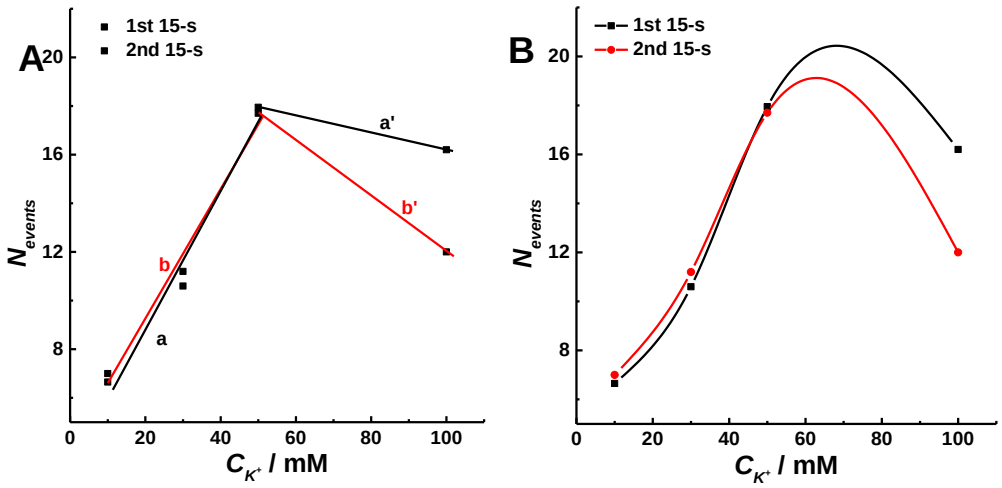

Fig. S3. Plots of N_{events} and the concentration of K^+ stimulation solution with linear lines (A) and curves (B). The values of N_{events} were obtained from the Fig. 2.

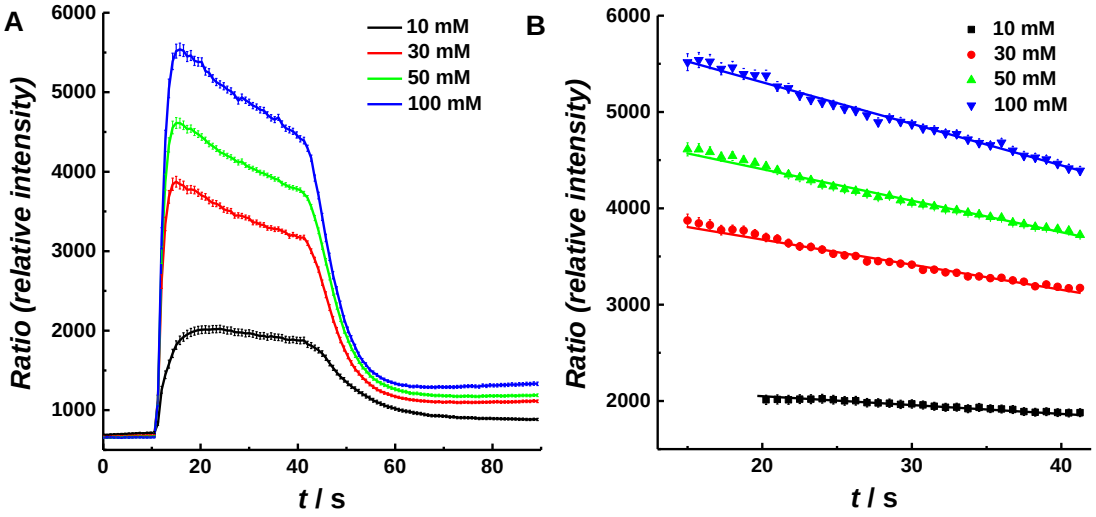

Fig. S4. A) Calcium imaging results for 10-, 30-, 50-, 100-mM K^+ stimuli. The number of the cells analyzed for all stimuli was 183 and the error bars represent SEM. B) Plot of calcium imaging results versus time. The black line (10 mM): y = (2195.66±8.69) - (7.87±0.27)x, R^2=0.97; red line (30 mM): y=(4198.02±20.44) - (25.96±0.64)x, R^2=0.98; green line (50 mM): y=(5072.39±18.49) - (33.04±0.59)x, R^2=0.99; and blue line (100 mM): y=(6168.98±23.95) - (43.06±0.77)x, R^2=0.99.

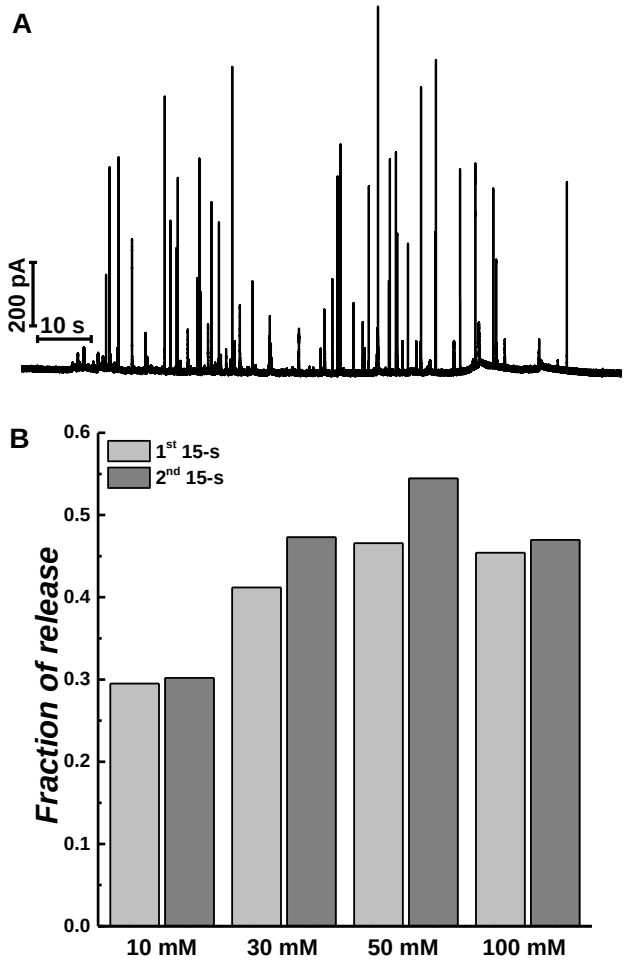

Fig. S5. A) A representative IVIEC amperometric trace from a single chromaffin cell without stimulation. B) Fraction of release calculated for consecutive 15-s time periods after K^+ stimulation with 10-, 30-, 50- and 100-mM K^+.

Table S1. P values to compare the number of events (N_{events}) obtained from SCA.

N_{events}	10 mM 1st 15-s	30 mM 1st 15-s	50 mM 1st 15-s	100 mM 1st 15-s	10 mM 2nd 15-s	30 mM 2nd 15-s	50 mM 2nd 15-s	100 mM 2nd 15-s
10 mM 1st 15-s	×	$<10^{-4}$ ***	$<10^{-4}$ ***	$<10^{-4}$ ***	0.668 ns	×	×	×
30 mM 1st 15-s	$<10^{-4}$ ***	×	$<10^{-4}$ ***	$<10^{-4}$ ***	×	0.436 ns	×	×
50 mM 1st 15-s	$<10^{-4}$ ***	$<10^{-4}$ ***	×	0.0988 ns	×	×	0.866 ns	×
100 mM 1st 15-s	$<10^{-4}$ ***	$<10^{-4}$ ***	0.206 ns	×	×	×	×	0.0002 ***
10 mM 2nd 15-s	0.668 ns	×	×	×	×	$<10^{-4}$ ***	$<10^{-4}$ ***	$<10^{-4}$ ***
30 mM 2nd 15-s	×	0.436 ns	×	×	$<10^{-4}$ ***	×	$<10^{-4}$ ***	0.437 ns
50 mM 2nd 15-s	×	×	0.866 ns	×	$<10^{-4}$ ***	$<10^{-4}$ ***	×	$<10^{-4}$ ***
100 mM 2nd 15-s	×	×	×	0.0002 ***	$<10^{-4}$ ***	0.437 ns	$<10^{-4}$ ***	×

Table S2. P values to compare number of molecules ($N_{molecules}$) released per exocytosis event obtained from SCA.

$N_{molecules}$	10 mM 1st 15-s	30 mM 1st 15-s	50 mM 1st 15-s	100 mM 1st 15-s	10 mM 2nd 15-s	30 mM 2nd 15-s	50 mM 2nd 15-s	100 mM 2nd 15-s
10 mM 1st 15-s	×	0.0002 ***	$<10^{-4}$ ***	$<10^{-4}$ ***	0.723 ns	×	×	×
30 mM 1st 15-s	0.0002 ***	×	0.0288 *	0.0643 ns	×	0.0199 *	×	×
50 mM 1st 15-s	$<10^{-4}$ ***	0.0288 *	×	0.606 ns	×	×	0.0004 ***	×
100 mM 1st 15-s	$<10^{-4}$ ***	0.0643 ns	0.606 ns	×	×	×	×	0.723 ns
10 mM 2nd 15-s	0.723 ns	×	×	×	×	$<10^{-4}$ ***	$<10^{-4}$ ***	$<10^{-4}$ ***
30 mM 2nd 15-s	×	0.0199 *	×	×	$<10^{-4}$ ***	×	0.0012 **	0.473 ns
50 mM 2nd 15-s	×	×	0.0004 ***	×	$<10^{-4}$ ***	0.0012 **	×	$<10^{-4}$ ***
100 mM 2nd 15-s	×	×	×	0.723 ns	$<10^{-4}$ ***	0.473 ns	$<10^{-4}$ ***	×

Table S3. P values to compare the peak parameter I_{max} obtained from SCA.

I_{max}	10 mM 1st 15-s	30 mM 1st 15-s	50 mM 1st 15-s	100 mM 1st 15-s	10 mM 2nd 15-s	30 mM 2nd 15-s	50 mM 2nd 15-s	100 mM 2nd 15-s
10 mM 1st 15-s	×	$<10^{-4}$ ***	$<10^{-4}$ ***	$<10^{-4}$ ***	0.547 ns	×	×	×
30 mM 1st 15-s	$<10^{-4}$ ***	×	$<10^{-4}$ ***	0.0089 **	×	0.107 ns	×	×
50 mM 1st 15-s	$<10^{-4}$ ***	$<10^{-4}$ ***	×	0.0025 **	×	×	0.0797 ns	×
100 mM 1st 15-s	$<10^{-4}$ ***	0.0089 **	0.0025 **	×	×	×	×	$<10^{-4}$ ***
10 mM 2nd 15-s	0.547 ns	×	×	×	×	$<10^{-4}$ ***	$<10^{-4}$ ***	$<10^{-4}$ ***
30 mM 2nd 15-s	×	0.107 ns	×	×	$<10^{-4}$ ***	×	$<10^{-4}$ ***	0.0532 ns
50 mM 2nd 15-s	×	×	0.0797 ns	×	$<10^{-4}$ ***	$<10^{-4}$ ***	×	$<10^{-4}$ ***
100 mM 2nd 15-s	×	×	×	$<10^{-4}$ ***	$<10^{-4}$ ***	0.0532 ns	$<10^{-4}$ ***	×

Table S4. P values to compare the peak parameter $t_{1/2}$ obtained from SCA.

$t_{1/2}$	10 mM 1st 15-s	30 mM 1st 15-s	50 mM 1st 15-s	100 mM 1st 15-s	10 mM 2nd 15-s	30 mM 2nd 15-s	50 mM 2nd 15-s	100 mM 2nd 15-s
10 mM 1st 15-s	×	$<10^{-4}$ ***	0.351 ns	0.899 ns	0.733 ns	×	×	×
30 mM 1st 15-s	$<10^{-4}$ ***	×	$<10^{-4}$ ***	$<10^{-4}$ ***	×	0.0195 *	×	×
50 mM 1st 15-s	0.351 ns	$<10^{-4}$ ***	×	0.0003 ***	×	×	0.0073 **	×
100 mM 1st 15-s	0.899 ns	$<10^{-4}$ ***	0.0003 ***	×	×	×	×	$<10^{-4}$ ***
10 mM 2nd 15-s	0.733 ns	×	×	×	×	0.0067 **	0.968 ns	0.108 ns
30 mM 2nd 15-s	×	0.0195 *	×	×	0.0067 **	×	$<10^{-4}$ ***	$<10^{-4}$ ***
50 mM 2nd 15-s	×	×	0.0073 **	×	0.968 ns	$<10^{-4}$ ***	×	0.0007 ***
100 mM 2nd 15-s	×	×	×	$<10^{-4}$ ***	0.108 ns	$<10^{-4}$ ***	0.0007 ***	×

Table S5. P values to compare the peak parameter t_{rise} obtained from SCA.

t_{rise}	10 mM 1st 15-s	30 mM 1st 15-s	50 mM 1st 15-s	100 mM 1st 15-s	10 mM 2nd 15-s	30 mM 2nd 15-s	50 mM 2nd 15-s	100 mM 2nd 15-s
10 mM 1st 15-s	×	$<10^{-4}$ ***	0.0002 ***	0.398 ns	0.183 ns	×	×	×
30 mM 1st 15-s	$<10^{-4}$ ***	×	$<10^{-4}$ ***	$<10^{-4}$ ***	×	0.414 ns	×	×
50 mM 1st 15-s	0.0002 ***	$<10^{-4}$ ***	×	$<10^{-4}$ ***	×	×	0.0022 **	×
100 mM 1st 15-s	0.398 ns	$<10^{-4}$ ***	$<10^{-4}$ ***	×	×	×	×	$<10^{-4}$ ***
10 mM 2nd 15-s	0.183 ns	×	×	×	×	$<10^{-4}$ ***	0.327 ns	0.0067 **
30 mM 2nd 15-s	×	0.414 ns	×	×	$<10^{-4}$ ***	×	$<10^{-4}$ ***	$<10^{-4}$ ***
50 mM 2nd 15-s	×	×	0.0022 **	×	0.327 ns	$<10^{-4}$ ***	×	$<10^{-4}$ ***
100 mM 2nd 15-s	×	×	×	$<10^{-4}$ ***	0.0067 **	$<10^{-4}$ ***	$<10^{-4}$ ***	×

Table S6. P values to compare the peak parameter t_{fall} obtained from SCA.

t_{fall}	10 mM 1st 15-s	30 mM 1st 15-s	50 mM 1st 15-s	100 mM 1st 15-s	10 mM 2nd 15-s	30 mM 2nd 15-s	50 mM 2nd 15-s	100 mM 2nd 15-s
10 mM 1st 15-s	×	$<10^{-4}$ ***	0.0002 ***	0.0491 *	0.429 ns	×	×	×
30 mM 1st 15-s	$<10^{-4}$ ***	×	0.0001 ***	$<10^{-4}$ ***	×	0.0035 **	×	×
50 mM 1st 15-s	0.0002 ***	0.0001 ***	×	0.0047 **	×	×	0.0024 **	×
100 mM 1st 15-s	0.0491 *	$<10^{-4}$ ***	0.0047 **	×	×	×	×	0.0004 ***
10 mM 2nd 15-s	0.429 ns	×	×	×	×	$<10^{-4}$ ***	0.0080 **	0.799 ns
30 mM 2nd 15-s	×	0.0035 **	×	×	$<10^{-4}$ ***	×	0.0039 **	$<10^{-4}$ ***
50 mM 2nd 15-s	×	×	0.0024 **	×	0.0080 **	0.0039 **	×	0.0002 ***
100 mM 2nd 15-s	×	×	×	0.0004 ***	0.799 ns	$<10^{-4}$ ***	0.0002 ***	×

Table S7. P values calculated to compare the ratio (relative intensity) of fluorescence at 25 and 40 s.

Ratio	10 mM at 25 s	30 mM at 25 s	50 mM at 25 s	100 mM at 25 s	10 mM at 40 s	30 mM at 40 s	50 mM at 40 s	100 mM at 40 s
10 mM at 25 s	×	$<10^{-4}$ ***	$<10^{-4}$ ***	$<10^{-4}$ ***	0.0603 ns	×	×	×
30 mM at 25 s	$<10^{-4}$ ***	×	$<10^{-4}$ ***	$<10^{-4}$ ***	×	$<10^{-4}$ ***	×	×
50 mM at 25 s	$<10^{-4}$ ***	$<10^{-4}$ ***	×	$<10^{-4}$ ***	×	×	$<10^{-4}$ ***	×
100 mM at 25 s	$<10^{-4}$ ***	$<10^{-4}$ ***	$<10^{-4}$ ***	×	×	×	×	$<10^{-4}$ ***
10 mM at 40 s	0.0603 ns	×	×	×	×	$<10^{-4}$ ***	$<10^{-4}$ ***	$<10^{-4}$ ***
30 mM at 40 s	×	$<10^{-4}$ ***	×	×	$<10^{-4}$ ***	×	10^{-4} ***	10^{-4} ***
50 mM at 40 s	×	×	$<10^{-4}$ ***	×	$<10^{-4}$ ***	$<10^{-4}$ ***	×	$<10^{-4}$ ***
100 mM at 40 s	×	×	×	$<10^{-4}$ ***	$<10^{-4}$ ***	$<10^{-4}$ ***	$<10^{-4}$ ***	×

Table S8. P values to compare the peak parameter I_{foot} obtained from SCA.

$\mathbf{I_{foot}}$	10 mM 1st 15-s	30 mM 1st 15-s	50 mM 1st 15-s	100 mM 1st 15-s	10 mM 2nd 15-s	30 mM 2nd 15-s	50 mM 2nd 15-s	100 mM 2nd 15-s
10 mM 1st 15-s	×	0.121 ns	0.0155 *	0.718 ns	0.841 ns	×	×	×
30 mM 1st 15-s	0.121 ns	×	0.0155 *	0.0051 **	×	0.0132 *	×	×
50 mM 1st 15-s	0.0155 *	0.0155 *	×	$<10^{-4}$ ***	×	×	0.904 ns	×
100 mM 1st 15-s	0.718 ns	0.0051 **	$<10^{-4}$ ***	×	×	×	×	0.841 ns
10 mM 2nd 15-s	0.841 ns	×	×	×	×	$<10^{-4}$ ***	0.0007 ***	0.232 ns
30 mM 2nd 15-s	×	0.0132 *	×	×	$<10^{-4}$ ***	×	0.398 ns	$<10^{-4}$ ***
50 mM 2nd 15-s	×	×	0.904 ns	×	0.0007 ***	0.398 ns	×	$<10^{-4}$ ***
100 mM 2nd 15-s	×	×	×	0.841 ns	0.232 ns	$<10^{-4}$ ***	$<10^{-4}$ ***	×

Table S9. P values to compare the peak parameter t_{foot} obtained from SCA.

t_{foot}	10 mM 1st 15-s	30 mM 1st 15-s	50 mM 1st 15-s	100 mM 1st 15-s	10 mM 2nd 15-s	30 mM 2nd 15-s	50 mM 2nd 15-s	100 mM 2nd 15-s
10 mM 1st 15-s	×	0.286 ns	$<10^{-4}$ ***	$<10^{-4}$ ***	0.779 ns	×	×	×
30 mM 1st 15-s	0.286 ns	×	$<10^{-4}$ ***	$<10^{-4}$ ***	×	$<10^{-4}$ ***	×	×
50 mM 1st 15-s	$<10^{-4}$ ***	$<10^{-4}$ ***	×	0.640 ns	×	×	0.0073 **	×
100 mM 1st 15-s	$<10^{-4}$ ***	$<10^{-4}$ ***	0.640 ns	×	×	×	×	0.0002 ***
10 mM 2nd 15-s	0.779 ns	×	×	×	×	$<10^{-4}$ ***	$<10^{-4}$ ***	0.0005 ***
30 mM 2nd 15-s	×	$<10^{-4}$ ***	×	×	$<10^{-4}$ ***	×	0.301 ns	0.0171 *
50 mM 2nd 15-s	×	×	0.0073 **	×	$<10^{-4}$ ***	0.301 ns	×	0.0350 *
100 mM 2nd 15-s	×	×	×	0.0002 ***	0.0005 ***	0.0171 *	0.0350 *	×

Table S10. P values to compare the peak parameter $N_{molecules}$ in foot obtained from SCA.

$N_{molecules}$ in foot	10 mM 1st 15-s	30 mM 1st 15-s	50 mM 1st 15-s	100 mM 1st 15-s	10 mM 2nd 15-s	30 mM 2nd 15-s	50 mM 2nd 15-s	100 mM 2nd 15-s
10 mM 1st 15-s	×	0.127 ns	0.0155 *	0.108 ns	0.883 ns	×	×	×
30 mM 1st 15-s	0.127 ns	×	0.0304 *	0.883 ns	×	0.0051 **	×	×
50 mM 1st 15-s	0.0155 *	0.0304 *	×	0.0122 *	×	×	0.183 ns	×
100 mM 1st 15-s	0.108 ns	0.883 ns	0.0122 *	×	×	×	×	0.0263 *
10 mM 2nd 15-s	0.883 ns	×	×	×	×	0.0003 ***	0.0155 *	0.925 ns
30 mM 2nd 15-s	×	0.0051 **	×	×	0.0003 ***	×	0.0596 ns	$<10^{-4}$ ***
50 mM 2nd 15-s	×	×	0.183 ns	×	0.0155 *	0.0596 ns	×	$<10^{-4}$ ***
100 mM 2nd 15-s	×	×	×	0.0263 *	0.925 ns	$<10^{-4}$ ***	$<10^{-4}$ ***	×

Table S11. P values to compare the percent of foot in all events (N_{foot}/N_{events}) obtained from SCA.

N_{foot}/N_{events}	10 mM 1st 15-s	30 mM 1st 15-s	50 mM 1st 15-s	100 mM 1st 15-s	10 mM 2nd 15-s	30 mM 2nd 15-s	50 mM 2nd 15-s	100 mM 2nd 15-s
10 mM 1st 15-s	×	10^{-4} ***	$<10^{-4}$ ***	10^{-4} ***	0.0139 *	×	×	×
30 mM 1st 15-s	10^{-4} ***	×	0.742 ns	0.425 ns	×	0.0111 *	×	×
50 mM 1st 15-s	$<10^{-4}$ ***	0.742 ns	×	0.550 ns	×	×	0.122 ns	×
100 mM 1st 15-s	10^{-4} ***	0.425 ns	0.550 ns	×	×	×	×	0.0178 *
10 mM 2nd 15-s	0.0139 *	×	×	×	×	0.0003 ***	0.432 ns	0.930 ns
30 mM 2nd 15-s	×	0.0111 *	×	×	0.0003 ***	×	10^{-4} ***	10^{-4} ***
50 mM 2nd 15-s	×	×	0.122 ns	×	0.432 ns	10^{-4} ***	×	0.105 ns
100 mM 2nd 15-s	×	×	×	0.0178 *	0.930 ns	10^{-4} ***	0.105 ns	×

References

1. D. T. O'Connor, S. K. Mahata, M. Mahata, Q. Jiang, V. Y. Hook, and L. Taupenot, *Nat. Protoc.* 2007, **2**, 1248–1253.
2. K. L. Adams, J. Engelbrektsson, M. Voinova, B. Zhang, D. J. Eves, R. Karlsson, M. L. Heien, A. S. Cans, and A. G. Ewing, *Anal. Chem.* 2010, **82**, 1020-1026.
3. a) T. G. Strein, and A. G. Ewing, *Anal. Chem.* 1992, **64**, 1368-1373; b) K. T. Kawagoe, J. A. Jankowski, and R. M. Wightman, *Anal. Chem.* 1991, **63**, 1589-1594; c) A. M. Strand, and B. J. Venton, *Anal. Chem.* 2008, **80**, 3708-3725.