Supporting Information

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SI Text

Materials. Unless otherwise stated, all chemicals were purchased at standard grades and used as received. FC3283 (3M) was used as the carrier fluid with surfactant 1,1,2,2-tetrahydroperfluorooctanol (PFO; Alfa Aesar) or RfOEG (triethyleneglycol mono[1*H*,1*H*-perfluorooctyl]ether), which was prepared according to published procedures (1). Human insulin and albumin from bovine serum (BSA) were purchased from Sigma–Aldrich. Monoclonal antibody to human insulin was purchased from Meridian Life Science. Tween 20 and 2,5-dihydroxybenzoic acid were purchased from Acros Organics. Alexa Fluor 488 5-TFP, cell-impermeant fluo-4 pentapotassium salt, cell-permeant fluo-4 a.m. ester, dextran Alexa Fluor 594, and 8-methoxy-pyrene-1,3,6 trisulfonic acid (MPTS) were purchased from Bio-Rad. PTFE tubing was purchased from Zeus Industrial Products.

Fabrication and Use of the Chemistrode. To make the chemistrode, we first fabricated an enclosed V-shaped channel ($300 \times 300 \mu$ m) by rapid prototyping in poly(dimethylsiloxane) (PDMS) with geometry shown in Fig. S1*A*. Microchannels were rendered hydrophobic and fluorophilic by flowing tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane vapor into the device for 1 h (1). Second, the device was carefully cut by a blade along the red dashed lines shown in Fig. S1*A*. Subsequently, Teflon tubing was inserted into the channels as shown in Fig. S1*B*. The gap between the Teflon tubing and surrounding PDMS was filled with half-cured PDMS glue (Dow–Corning Sylgard 184 A and B at a ratio of 10:1, cured at 110 °C for 110 s), and then the device was baked at 65 °C for the PDMS glue to fully cure. The tip of the chemistrode could be cut smaller, typically with a bottom area of $\approx 1 \times 0.6$ mm, to reduce the outer dimension.

To use the chemistrode, the inlet tubing was connected to a plug-generating device or a premade cartridge (see *Device and Methods for Fig. 1 and Fig. 2*, below). The chemistrode was held by a clamp of an x-y-z micromanipulator, and brought into contact with the surface supporting the substrate. A slight pressure toward the interface was applied to prevent leakage.

Solution Properties and Calculations of Weber Number and f_{ca} . Properties of all aqueous solutions, including PBS buffer (1×, pH 7.4) and potassium phosphate buffer (32 mM, pH 8.2), were estimated as the values of water at room temperature. Viscosity and density of all aqueous solutions were estimated as 10^{-3} kg/(m·s) and 10^3 kg/m³, respectively. Viscosity and density of FC3283 at room temperature were 1.4×10^{-3} kg/(m·s) and 1.82×10^{3} kg/m³, respectively. Surface tension of potassium phosphate buffer and FC3283 containing 0.5 mg/mL RfOEG was ≈ 10 mN/m. Weber number, We, is calculated as $We = \frac{\rho U^2 d}{\gamma}$, where ρ (kg·m⁻³) is the density of the aqueous solution, d (m) is the tubing diameter, and γ (N·m⁻¹) is surface tension between the aqueous solution and the carrier fluid. U (m·s⁻¹) is the average flow velocity, which is calculated by $U = \frac{4Q}{\pi d^2}$, where Q (m³·s⁻¹) is the volumetric flow rate. The capillary number, Ca, limits the frequency at which plugs can flow over a surface for f_{0} can be

frequency at which plugs can flow over a surface, f_{Ca} . f_{Ca} can be calculated by using the following experimental parameters.

$$f_{Ca} = \frac{Ca \cdot \gamma}{\mu \cdot 6d} = \frac{0.1 \times 10 \times 10^{-3} \text{N/m}}{10^{-3} \text{kg/(m \cdot s)} \times 6 \times 200 \times 10^{-6} \text{m}} \approx 800 \text{s}^{-1}.$$

Device and Methods for Fig. 1 and Fig. 2. Device and methods for Fig. 1C. Stimulus plugs were generated in a 3-inlet PDMS device with microchannels of square cross-section (100 μ m) (Fig. S1C). The carrier fluid was 0.5 mg/mL RfOEG dissolved in FC3283. Plugs were formed by using an aqueous stream of buffer (32 mM potassium phosphate, pH 8.2) and an aqueous stream of 140 μ M fluorescein dissolved in the same buffer. To generate repeated arrays of a single fluorescent plug followed by ≈ 20 nonfluorescent plugs, the carrier fluid stream was flowed continuously at a flow rate of 4 μ L/min while a LabVIEW program switched between 2 aqueous streams in an alternating fashion. At any time, only 1 aqueous stream was flowing at a flow rate of 4 μ L/min. The total flow rate was 8 μ L/min. The resulting plugs were delivered to the chemistrode through PTFE tubing (200 μ m i.d., 250 μ m o.d.). Images were taken by using a high-speed Phantom 7.1 camera (Vision Research) at 1,000 fps (Movie S1). Four images from Movie S1 are shown in Fig. 1C.

Fig. 2A and Movie S2. Plugs were generated by using the same device, solutions, and program as above. Time-lapse fluorescent images were taken by using an inverted fluorescence microscope (IRE2; Leica) equipped with the high-speed Phantom camera at 500 fps (Movie S2).

Fig. 2B. Arrays of stimulus plugs were generated robotically (Fig. S3A) in 40-cm-long Teflon tubing (240 μ m i.d.) and delivered to a hydrophilic glass surface through a chemistrode at various flow velocities. The volume of individual aqueous plugs and the carrier fluid between 2 plugs were both 30 nL. Calibration plugs containing different concentrations of fluorescein (sequence as recorded in Fig. S2B) were included at the beginning of each array, so that the fluorescence intensity could be quantitatively converted back into fluorescein concentration. The sequence of the calibration array was 10 plugs of 0 μ M fluorescein in PBS buffer, 10 plugs of 25 μ M fluorescein in PBS buffer, 10 plugs of 50 μ M fluorescein in PBS buffer, and 10 plugs of 100 μ M fluorescein in PBS buffer. After the calibration array, 5 cycles of stimulus plugs were repeated, where each cycle consisted of 5 fluorescent plugs (100 μ M fluorescein in PBS buffer), followed by 20 nonfluorescent plugs (PBS buffer). The change in fluorescence intensity in the center of the wetting layer during the transition from fluorescent to nonfluorescent plugs was used to characterize the recording efficiency (Fig. S2A).

The fluorescence intensity on the surface of the substrate was monitored by using an inverted fluorescence microscope (IRE2; Leica), and images were acquired by using the Phantom camera. The images (Fig. S2A) were analyzed by using MetaMorph 6.0. Data were taken as the average intensity of a 200- μ m-diameter circle at the center of the substrate as shown in Fig. S2A, because we assumed that the region of interest corresponded to stimuli located in the center of the area under the tip of the chemistrode. Each calibration data point was the average of 10 plugs. Each data point in Fig. 2B represents the average concentration of fluorescein in plugs from 5 cycles of stimulus plugs. The data obtained with the chemistrode at flow rate of 7.4 mm/s is shown in Fig. S2B, including average intensities of the time-lapse images and a calibration curve for converting intensity into concentration of fluorescein.

Fig. 2C. To deliver a sequence of multiple molecular signals, an array of plugs as shown in Fig. S3B was generated in PTFE tubing (240 μ m i.d.) robotically as shown in Fig. S3A. Light-green, dark-green, light-red, and dark-red plugs contained 10 μ M fluorescein, 20 μ M fluorescein, 10 μ M sulforhodamine 101, and 20 μ M sulforhodamine 101, respectively. Gray plugs contained

only buffer. The volume of individual aqueous plugs and the carrier fluid between 2 plugs were both 30 nL. The carrier fluid was FC3283 containing 0.5 mg/mL RfOEG. Buffer for all aqueous solutions was potassium phosphate buffer (32 mM, pH 8.2). One array of plugs contained multiple repeating periods of the sequence. The chemistrode was brought into contact with a hydrophilic glass slide. Plugs in the preformed array were flowed into the chemistrode at a flow rate of 80 μ L/min (flow velocity = 29 mm/s).

A Leica SP5 tandem scanner spectral 2-photon confocal microscope was used to obtain fluorescence data with the following settings: xt scan mode; pinhole (Airy) 1.3; zoom 4.9; HCX PL APO CS 10.0×0.40 DRY UV objective; laser lines of 488-nm Ar and 561-nm diode; emission bandwidths of 500.0-545.0 nm and 600.2-720.1 nm; 8-bit PMT output. A Leica LAS AF Lite (1.7.0 build 1240) was used to control the microscope and analyze the data. Focus was adjusted on the surface of the glass slide, determined by reflected light. The position of the line scan is shown in Fig. S3C. The scan rate was 8,000 lines per second. Every 8 lines were averaged for 1 recording time point. Two beams of laser (488 and 561 nm) were switched by an acoustic optical tunable filter (AOTF). Emission light of 500.0-545.0 nm and 600.2–720.1 nm were detected by 2 photomultiplier tube (PMT) fluorescence detectors. Fluorescence intensities of both fluorescein and sulforhodamine 101 were averaged on the center 50% along the lengths of line scans (Fig. S3D), which corresponded to a physical length of $\approx 150 \ \mu m$.

Fig. 2D. To simulate the release of molecular signals from a surface, fluorescein solution was pulsed out of a microcapillary to a glass surface. A 5-cm-long fused silica capillary with square cross-section (50 μ m i.d.; Polymicro Technologies) was placed in a PDMS channel. The gap between the capillary and surrounding PDMS was filled with PDMS glue as described above. The other end of the capillary was connected to a 20-cm-long Teflon tubing (300 μ m i.d.) filled with fluorescein (500 μ M), and this tubing was connected to a microinjector (IM300; Narishige). The chemistrode was brought into contact with the wetting layer above the opening of the capillary.

Stimulus plugs were formed by injecting both a carrier fluid stream (0.5 mg/mL RfOEG in FC3283) and an aqueous stream $(1 \times PBS \text{ buffer, pH 7.4})$ into a T-junction (Fig. S4A). The volumetric flow rate of both streams was 16 μ L/min, and the total flow rate was 32 μ L/min. The microinjector delivered multiple pulses of fluorescein solution into the silica surface with well-defined injection time and intervals. A LabVIEW program was used to control the microinjector for automatic operation. The duration for each injection was 40 ± 2 ms, as measured by the recorded movie (Movie S3). The time interval between consecutive pulses was 1 s. Pulses of fluorescein collected by response plugs in the chemistrode were detected at 2 positions: the tip of the chemistrode (site 1) and 10 cm downstream (site 2) (Fig. S4A). In a separate experiment, pulses of fluorescein were also collected by single-phase laminar flow at a flow rate of 32 μ L/min in the same device geometry. The detection results from the chemistrode were compared with those from the single-phase laminar flow at the same sites. The fluorescence microscopic images (Fig. S4 B and C) and movie (Movie S3 and Movie S4) were acquired by using a Leica IRE2 fluorescence microscope with the Phantom camera.

Device and Methods for Fig. 3. The PDMS microfluidic device for achieving high spatial resolution (Fig. S5*A*) was fabricated by using rapid-prototyping multilayer soft lithography (2). Two molds with mirrored channel designs (Fig. S5 *B* and *C*) were made. The tips of the chemistrode and the end of pulsing device were connected in these molds. The channels had uniform thickness of 25 μ m and widths of 15 or 25 μ m. Dow–Corning Sylgard 184 A and B components were mixed at a mass ratio of

5:1, poured onto the mold for the top layer to a thickness of 5 mm, and incubated at 65 °C for 30 min. A 20:1 mixture of A and B was spin-coated onto the mold for the second layer pattern at 3,600 rpm for 30 s and then cured at 65 °C for 25 min. This spin-coating resulted in a thin PDMS layer of \approx 35 µm thick covering the channel patterns (membrane layer). This membrane layer was aligned to the top layer with a MJB3 contact Mask Aligner (Karl Suss) and cured at 65 °C for 15 min. The bonded layers were peeled from the mold, punched with access holes, sealed to a 1-mm-thick 5:1 (A/B) flat PDMS layer (preincubated the same as the top layer), and baked at 65 °C overnight. Finally, the chemistrode and the pulsing device were cut apart under a microscope by using a sharp blade. The spatial resolution was defined as the distance between the 2 closest corners of the 2 pulsing channels (Fig. S5F, \approx 15 µm). By controlling the thickness of the membrane layer and the alignment of the 2 chemistrodes, we can get different spatial resolution ranging from $\approx 10 \ \mu m$ to hundreds of micrometers.

Before use, the pulsing device was made hydrophilic by injecting 5 mg/mL BSA solution in 1× PBS buffer (pH 7.4) into the pulsing channels at a flow rate of 0.1 μ L/min for 30 min. Then, the pulsing channels were rinsed with PBS buffer to flush away residual BSA. This also made the surface (Fig. S5F) of the pulsing device, which was brought into contact with the chemistrode, hydrophilic. The channels of the chemistrode were rendered fluorophilic by flowing FC3283/PFO (5:1 vol/vol) into the chemistrode at a flow rate of 0.10 μ L/min for 20 min to saturate the PDMS surface with fluorinated carrier fluid-phase surfactant.

For spatial resolution experiments, a clamp was used to bring the chemistrode and the pulsing device into close proximity (Fig. S5 D and E). Syringes filled with solution and carrier fluid were connected to the pulsing channels with Teflon tubing (300 μ m i.d.) and driven by 4 syringe pumps (PHD2000; Harvard Apparatus) controlled by a LabVIEW program. Each pump simultaneously drove 2 syringes that were connected to the 2 different layers, ensuring simultaneous flow control in both layers of chemistrodes and pulsing devices (Fig. S5 E and G). An adjustable vacuum (adjusted to 50 ± 25 mmHg) was connected to the outlet channels of the chemistrode to balance the pressure drop at the tip of the chemistrode. Stimulus plugs were formed by flowing a carrier fluid stream (20% vol/vol PFO in FC3283) and an aqueous stream ($1 \times PBS$ buffer, pH 7.4) into the T-junction at flow rates of 0.075 μ L/min and 0.075 μ L/min, respectively (Fig. S5B). For fluorescence measurements, a Leica DMIRE2 microscope with a digital camera (ORCA-ER; Hamamatsu) was used. GFP and DAPI filter cubes were used to observe the fluorescence of fluorescein and MPTS, respectively.

Device and Methods for Fig. 4. *Preparation of solutions.* Hepes buffer (25 mM) was made by diluting 0.1 M Hepes buffer (pH 7.35) with Millipore filtered water. This Hepes buffer was stirred with Chelex 100 resin for 1 h to reduce the background Ca²⁺ ions. A solution of 0.1% Tween 20 in 25 mM Hepes buffer was treated by Chelex 100 resin with the same protocol. The sample solution contained 250 μ M CaCl₂, 500 nM insulin, 10 μ M MPTS, 50 mM glucose, and 0.1% Tween 20 in 25 mM Hepes buffer. All solutions were filtered with 0.45- μ m medium PTFE syringe filters (Fisher Scientific) before use. The concentration of insulin stock solution was quantified by using light absorption at 277 nm. All PDMS devices were rinsed with 50 μ M EDTA and then Millipore filtered water before experiments.

Fig. 4A recording of pulses of multiple molecules. Stimulus plugs were formed by flowing a carrier fluid stream (0.5 mg/mL RfOEG in FC3283) and an aqueous stream (25 mM Hepes buffer, pH 7.35) into a T-junction at flow rates of 2.5 μ L/min and 2.0 μ L/min, respectively. The resulting plugs were transported through the chemsitrode device at a flow rate of 4.5 μ L/min. Pulses of

chemicals were delivered to a hydrophilic surface through a 15 \times $25-\mu m$ orifice from a single layer PDMS device shown in Fig. S5C. Alternating pulses of buffer (0.1% Tween 20 in 25 mM Hepes buffer, pH 7.35) or sample solution (250 µM CaCl₂, 500 nM human insulin, 10 μ M MPTS, 50 mM glucose, and 0.1% Tween 20 in 25 mM Hepes, pH 7.35) were delivered to the surface at a flow rate of 0.5 μ L/min and duration of 8 s. The chemistrode was aligned and brought into contact with the outlet of the pulsing device, and the pulses were captured in response plugs. Under these experimental conditions, the volume of response plugs had a 1:1 ratio to the volume of carrier fluid in the resulting array. After recording, the response plugs were split (3) into 4 identical daughter arrays in Teflon tubing (100 μ m i.d.) for further analysis as discussed below. On the basis of flow rates and the starting concentrations of the sample, the concentrations in the response plugs (assuming 100% sample recovery) should be: 50 µM CaCl₂, 100 nM human insulin, and 2.0 µM MPTS. The average determined concentrations in response plugs were: 44 μ M CaCl₂, 102 nM human insulin, and 2.0 μ M MPTS.

Fig. 4B detection of Ca²⁺. The analyzing agent for detecting Ca²⁺ contained 400 µM cell-impermeable fluo-4 pentapotassium salt, 400 nM dextran Alexa Fluor 594, and 0.1% Tween 20 in 25 mM Hepes buffer (pH 7.35). This solution of reagent was injected into each plug of 1 daughter array with the average injection ratio of 0.5. The injection ratio is the ratio between the volume of injected reagent and the volume of the plug before injection. The device used for injection was the same as described in previous publications (4). Briefly, the device consisted of a PDMS T-junction with a hydrophilic glass capillary inserted in the vertical arm of the "T." Reagent solutions were injected into plugs through the hydrophilic glass capillary. The intensities of fluo-4-Ca²⁺ complex, dextran Alexa Fluor 594, and MPTS in each plug were measured by using a Leica DMI6000 fluorescence microscope with GFP, Texas red, and DAPI filter cubes, respectively. MetaMorph 6.0 was used to control the microscope and analyze the data.

To obtain a calibration curve for the concentration of Ca^{2+} in the response plugs, cartridges of calibration plugs containing solutions made as shown in Fig. S6*A* were generated. Each of the calibration plugs was injected with analyzing reagent used in the analysis of response plugs. Then, the intensities of fluo-4-Ca²⁺ complex and dextran Alexa Fluor 594 were measured.

The concentration of Ca^{2+} in the response plugs was calibrated and corrected by following the procedure below:

(*i*) Calculating the fraction of the analyzing reagent injected:

InjectionFraction =
$$\frac{I_{\text{Alexa594,postinjection}}}{I_{\text{Alex594,preinjection}}}$$
,

where $I_{Alexa594, preinjection}$ is the intensity of Alexa 594 in the analyzing reagent before injection, and $I_{Alexa594, postinjection}$ is the intensity of Alexa 594 in the plugs after injection.

(*ii*) Correcting the intensity of fluo-4- Ca^{2+} by subtracting the background intensity of the analyzing reagent:

$$I_{Ca^{2+}, \text{corrected}} = I_{\text{original}, Ca^{2+}} - I_{\text{background}, \text{analyzingagent}}$$

× InjectionFraction.

where $I_{Ca2+,corrected}$ is the corrected intensity of fluo-4-Ca²⁺, $I_{original,Ca2+}$ is the original intensity of fluo-4-Ca²⁺ measured in the plugs after injection, and $I_{background,analyzingagent}$ is the intensity of fluo-4-Ca²⁺ in the analyzing reagent before injection.

(*iii*) Calculating the final concentration of Ca^{2+} in the calibration plugs after injection:

$$c_{Ca^{2+}, \text{calibration}, \text{postinjection}} = c_{Ca^{2+}, \text{calibration}} \times (1)$$

- InjectionFraction),

where $c_{Ca2+,calibration,postinjection}$ is the final concentration of Ca²⁺ in calibration plugs after injection, and $c_{Ca2+,calibration}$ is the concentration of Ca²⁺ in calibration plugs before injection, equal to those in Fig. S6A.

(*iv*) By plotting $c_{Ca2+,calibration,postinjection}$ versus the corresponding fluorescence intensity of fluo-4-Ca^{2+,} a calibration curve of Ca²⁺ was obtained (Fig. S6B).

(v) By using the calibration curve and the intensity of fluo-4-Ca²⁺ in the response plugs, the concentration of Ca²⁺ in each response plug after injection ($c_{Ca2+,sample,postinjection}$ was obtained.

(*vi*) Finally, concentration of Ca^{2+} in the response plugs before injection ($c_{Ca2+,sample}$) was obtained.

$$c_{Ca^{2+},\text{sample}} = \frac{c_{Ca^{2+},\text{sample},\text{postinjection}}}{1 - \text{InjectionFraction}}.$$

The ntensity of MPTS in each could be corrected as well:

$$I_{\rm MPTS, corrected} = \frac{I_{\rm original, MPTS}}{1 - \rm InjectionFraction}$$

where $I_{\text{MPTS, corrected}}$ is the corrected intensity of MPTS, and $I_{\text{original, MPTS}}$ is the original measured intensity of MPTS.

Fig. 4B competitive immunoassay for insulin analysis. Labeled human insulin (insulin*) was prepared by reacting human insulin and Alexa Fluor 488 5-TFP according to the manufacturer's instructions. The resulting product was purified by HPLC to obtain a single pure monolabeled isomer (insulin*). The lypholized powder of insulin^{*} was dissolved in $1 \times PBS$ buffer (pH 7.4) to a concentration of 40 μ M and stored at -78 °C as 1- μ L aliquots. To perform the immunoassay, a reagent solution containing 72 nM monoclonal anti-insulin antibody (mAb), 1.2 nM insulin*, 0.3% BSA, and 0.3% Tween 20 in 25 mM Hepes (pH 7.35) was injected into each plug of 1 daughter array. The procedure for injecting reagents into plugs was the same as that described above for Ca²⁺ detection. The flow rate was 0.60 μ L/min for the array of response plugs and 0.15 μ L/min for the reagent solution. The volumetric ratio of plugs to injected reagent was $\approx 2:1$. The insulin and insulin* compete for binding to the mAb, thus changing the fraction of free insulin* in the solution. Insulin concentration was inferred by determining the fraction of free insulin* by using fluorescence correlation spectroscopy (FCS).

The plugs containing reagents and sample were analyzed by FCS performed by using a commercial instrument. ConfoCor 3 (Carl Zeiss). A 488-nm argon laser was used as the excitation light. BP 505–540 IR* was used as the emission filter. For FCS measurements on a plug, the curved carrier fluid-aqueous interface and the thin layer of carrier fluid surrounding the plugs could introduce artifacts. To avoid these potential problems, a coverglass-PDMS device was constructed to house the plug during FCS measurements (Fig. S7A). The device was fabricated by sealing a piece of PDMS with imprinted channels on the bottom surface to a no. 1 cover glass on both its top and bottom surfaces. The bottom cover glass formed an enclosed channel with the PDMS. The 2 ends of the PDMS channel were connected to Teflon tubing (100 µm i.d., 150 µm o.d.) to transport plugs in and out of the device. The center of the channel formed a chamber with dimensions of 50 μ m (height) \times 150 μ m (width) × 350 μ m (length) (Fig. S7A). This geometry of the chamber was chosen so that plugs become "flat," with minimal curvature of the aqueous-carrier fluid interface and minimal thickness of carrier fluid at the center of the bottom surface of the plug. Sealing both the top and bottom of the PDMS piece to the cover glass was also important to prevent the cover glasses from bending after sealing to the PDMS. FCS measurements were performed by focusing the light at the center of the plug and 25 μ m above the cover glass-liquid interface.

Control experiments indicated that performing FCS in this geometry did not introduce artifacts due to the aqueous–carrier fluid interface.

Next, the characteristic diffusion time for free insulin* and insulin*-mAb were determined under these experimental conditions. First, FCS measurements were obtained for a solution of insulin* in the absence of mAb. A single-component 3D free-diffusion model was used to fit the autocorrelation curve and obtain the characteristic diffusion time of free insulin*. Next, FCS measurements were obtained for a series of solutions containing insulin* and increasing concentrations of mAb. The autocorrelation curves were fit by using a 2-component 3D free-diffusion model with the diffusion time of free insulin* fixed to give the characteristic diffusion time of the insulin*-mAb complex. In these experiments, the characteristic diffusion time was 60 μ s and 230 μ s for free insulin* and insulin*-mAb, respectively.

To analyze an array of response plugs, the plugs were carefully moved into the PDMS-cover glass chamber by using a manual syringe pump. After 1 plug arrived in the chamber, the flow was stopped, and FCS measurements were performed on the plug in the chamber. After the measurement, the plug was moved out of the chamber, and the next plug was moved into the chamber for measurement. The autocorrelation curves were fit with a 2-component (free insulin* and insulin*–mAb complex), 3D freediffusion model to give 2 parameters: the average number of fluorescent insulin* molecules in focal volume and the fraction of insulin* unbound to mAb (free insulin*%). To determine the concentration of insulin, 4 calibration curves of free insulin*% at different concentrations of insulin were constructed, with the average number of insulin* molecules in the focal volume being 0.65, 0.75, 0.95, and 1.2, respectively (Fig. S7B).

Fig. 4B detection of glucose. The analyzing reagent for detecting glucose contained 0.1 M Girard's reagent T, 2% acetic acid, and 20 mM arabinose. This analyzing reagent was injected into each plug of 1 daughter array of response plugs with a volumetric ratio of plugs to injected reagent of 2:1. The procedure for injecting reagents into plugs was the same as that described above for Ca^{2+} detection. Glucose reacted with the Girard's reagent T to form a hydrazone (as illustrated below), resulting in increased detection sensitivity in MALDI-MS (5).

control; acquisition mass range 100–350 Da; focus mass 213 Da; total shots per spectrum 3,000; fixed laser intensity 4,000 V; default calibration type. The peak heights in MALD-MS were measured with Data Explorer version 4.8 (Applied Biosystems). The level of glucose in each response plug was presented as the ratio of the peak height of hydrazone of glucose (m/z 294) to the peak height of Girard's reagent T (m/z 132). Representative MALDI-MS spectra are shown in Fig. S8.

Fig. 4 C-E experiments monitoring insulin secretion of single islets by the chemistrode. Islets were isolated from the pancreas of C57BL/6J wild-type mice (The Jackson Laboratory), 8–12 weeks of age, by using collagenase digestion and Ficoll gradients by following procedures described in previously published literature (6). Isolated islets were transferred to glass-bottom culture dishes (Mattek Corporation) and cultured in RPMI medium 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μ g/mL streptomycin. Islets were maintained in a humidified incubator at 37 °C under an atmosphere of 95% air and 5% CO₂ and were used within 3 days of isolation.

Experiments to test compatibility of chemistrode with mouse islets are described below. Islets were loaded with fluo-4 (a calcium indicator) by incubating in Krebs-Ringer buffer (KRB) (119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.5 mM KH₂PO₄, 25 mM Hepes, pH 7.35) containing 5 µM cell permeable fluo-4 a.m., and 2 mM glucose for 40 min. The MatTek plate containing loaded islets was placed on a DMI6000 Leica fluorescence microscope that was kept at 37 °C. The staining medium was then replaced with KRB containing 2 mM glucose. A chemistrode was pressed down on the cover glass of MatTek plate by using a micromanipulator to trap 1 islet under the tip of the chemistrode. The PDMS tip of the chemistrode formed a conformal contact with the cover glass to isolate the space in the tip of the chemistrode from the bulk solution. Droplets of KRB containing either 2 mM glucose or 30 mM KCl plus 2 mM glucose plus 400 nM dextran Alexa Fluor 594 were formed and transported through the chemistrode by using the setup shown in Fig. 4C. All aqueous solutions and carrier fluid were oxygenated. The flow rates of the carrier fluid (0.5 mg/mL RfOEG in FC3283) and the aqueous stream were both 0.5 μ L/min, resulting in a plug being delivered to the islet every 2 s. Time-lapse images of the islet under stimulation were captured



After incubation at room temperature for 60 h, each response plug was deposited onto a MALDI plate (plate type: ABI 01–192-6-AB, 192-well; Applied Biosystems) and allowed to evaporate. A matrix solution containing 10 mg/mL 2,5dihydroxybenzoic acid in 1:1 acetonitrile/ethanol was deposited over each sample, which was dried and analyzed by MALDI-MS. MALDI spectra were acquired by using an ABI 4700 MALDI TOF/TOF MS instrument (Applied Biosystems).

All spectra were obtained with the same instrument settings: MS Reflector Positive operation mode; automatic acquisition with a DMI6000 Leica microscope every 3 s by using GFP and Texas red filter cubes. The incident excitation light was attenuated with an optical density 2.0 neutral density filter to reduce photo damage to the islet. Images were analyzed by using MetaMorph 6.0. Whereas data in the GFP channel recorded the $[Ca^{2+}]_i$ response of the islet under stimulation, intensity in the Texas red channel marked the aqueous solution being applied to the islet. Only the high glucose solution contained 400 nM dextran Alexa Fluor 594.

Concentration of insulin in the recording plugs were analyzed

as described above, with the following exceptions. The monoclonal antibody to insulin (CBL71) was purchased from Millipore. The standard sample of mouse insulin was extracted from mouse islets by soaking purified mouse islets in 1% Triton surfactant, followed by repeated freezing and thawing, and centrifugation to remove the debris. The concentration of insulin in the extracted sample was determined using an ELISA kit (ALPCO). Calibration curves were constructed by using appropriate concentrations of Alexa Fluor 488-labeled human insulin, antibody, and standard mouse insulin sample. When analyzing the concentration of insulin in the recording plugs, fluorescence of Alexa Fluor 594 was also detected. Only recording plugs of

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stimulant solution contained Alexa Fluor 594. Temporal profiles of $[Ca^{2+}]_i$ and insulin secretion were aligned by using Alexa Fluor 594 as the marker.

We confirmed the compatibility of the chemistrode with living cells for experiment with longer time scale. A continuous stimulation by plugs of KRB containing 14 mM glucose plus 400 nM dextran Alexa Fluor 594 was applied to the islets for 1 h through the chemistrode. The $[Ca^{2+}]_i$ response of the islet was imaged as described above. The islets displayed the expected $[Ca^{2+}]_i$ response—a slight decrease in $[Ca^{2+}]_i$, followed by a sharp increase and then a gradual decrease, followed by regular oscillations (Fig. S9).

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Fig. S1. Frabication and assembly of the chemistrode. (A) Design of the PDMS device of chemistrode. (B) An assembled chemistrode. (C) Schematic of the experimental setup for Figs. 1C and 2A.



Fig. 52. Experimental setup and supporting data for Fig. 2*B.* (*A*) Schematic drawing of the experimental setup (*Upper*). Time-lapse fluorescence images of the removal of fluorescein from the wetting layer by subsequent buffer plugs at flow velocity of 7.4 mm/s are shown (*Lower*). These images were taken from below the substrate and were focused on the center of the wetting layer. The dashed circle in the first microscopic image indicates the region from which the data in Fig. 2*B* were extracted. (*B*) Fluorescence intensity measured from below the substrate as a preformed array of plugs containing fluorescein or buffer flowed over the substrate. Flow velocity: 7.4 mm/s. See *Device and Methods for Fig. 1 and Fig. 2* for more details. The *Inset* on the left shows the calibration curve for determining fluorescein concentration in the wetting layer. The *Inset* on the right shows a zoomed-in view of the fluorescence-intensity decrease during the transition from fluorescent to nonfluorescent plugs.

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Fig. S3. Experimental design for Fig. 2*C*. (*A*) Schematic showing a complex array of stimulus plugs being generated with a laboratory-built robot by aspirating from a 96-well plate filled with various solutions and carrier fluid. (*B*) Schematic drawing showing the sequence of stimulus plugs in the array used for Fig. 2*C* (See section *Fig. 2C*, above, for details). (*C*) Schematic drawing showing the position of the confocal line scan in the wetting layer above substrate for Fig. 2*C*. (*D*) A time series of line scans showing fluorescence intensity of fluorescein and sulforhodamine 101 delivered to the surface by the chemistrode. Line scans of bright-field and fluorescence are overlaid. Data plotted in Fig. 2*C* are the intensities averaged between the 2 black dashed lines.



Fig. S4. Experimental setup and data acquisition for Fig. 2*D*. (*A*) Schematic drawing of the setup for generating stimulus plugs, pulsing fluorescein to the PDMS surface by using a microinjector, collecting the fluorescein in response plugs, and measuring fluorescence in the response plugs at sites 1 and 2. (*B*) Microscopic images of chemistrode recording 40-ms pulses of fluorescein. (*C*) Microscopic images of single-phase flow recording 40-ms pulses of fluorescein. Arrows indicate the direction of flow.

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Fig. 55. Device design and experimental procedures for Fig. 3. (*A*) Schematic of the experimental design for multilayer chemistrode with 15- μ m spatial resolution (see *Device and Methods for Fig. 3* for details). Gray shade represents PDMS membrane between 2 layers. (*B*) Designs for the 2 layers of the chemistrode. The channel features were designed to be 25 μ m wide and 25 μ m thick. (*C*) Designs for the 2 layers of the pulsing device. Channel features are 25 μ m wide and 25 μ m (blue line) or 15 μ m (red line) thick. The top layer and the membrane layer were aligned to make the tips of pulsing devices ~15 μ m apart. (*D*) A bright-field image of the chemistrode brought into contact with the pulsing device. (*E*) Fluorescence image of the chemistrode while recording. Two separate microscopic images obtained with DAPI filter (for MPTS, top layer) and GFP filter (for fluorescein, membrane layer) were overlapped. Fluorescent dyes were pulsed to the surface and recorded by chemistrode with high spatial resolution without cross-contamination. Arrows indicate the direction of flow. (*F*) A LabVIEW program for the chemistrode and pulsing device. The flow rates for carrier fluid and aqueous buffer (1× PBS buffer, pH 7.4) were both 0.075 μ L/min. Species 1 (0.4 mM fluorescein or MPTS) and species 2 (1× PBS buffer, pH 7.4) were pulsed with the cycling program as listed.

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Fraction of sample solution	0	0.08	0.16	0.24	0.32	sity (A.	2000 —					B		
Fraction of 25 mM HEPES buffer with 0.1% Tween 20	0.2	0.12	0.04	0	0	nt inten	1500 —			Å				
Fraction of 25 mM HEPES buffer	0.8	0.8	0.8	0.76	0.68	oresce	1000 —		ø					
Concentration of Ca2+ (μM)	0	20	40	60	80	Flu	500 —	<u>~</u>	0					
								Т				I		-
								0	10 20 30 40 50 Concentration of Ca ²⁺ (μM)					

Fig. S6. Method for generating a calibration curve for detecting Ca²⁺. (A) Solutions made to obtain a calibration curve for detecting Ca²⁺. (B) Calibration curve for detecting Ca²⁺.



Fig. 57. Device and methods for detecting insulin in response plugs. (A) Device for FCS measurements in an array of nanoliter plugs. (B) Calibration curves for detecting insulin by FCS. The horizontal axis is the concentration of insulin in the response plug before analyzing reagent was injected. Insulin concentrations below 20 nM or above 120 nM could not be accurately measured due to the limited dynamic range of the immunoassay. The error bars are the difference between 2 parallel measurements.



Fig. S8. Representative MALDI-MS spectra. (A) A representative MALDI spectrum for a response plug that captured a pulse of sample solution containing glucose. (B) A representative MALDI spectrum for a response plug that captured a pulse of buffer containing no glucose.



Fig. S9. Characteristic profile and oscillation of intracellular [Ca²⁺]_i (green) were observed upon stimulation with the chemistrode (red line), indicating normal response of islets.

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Movie S1. Bright-field images taken at 1,000 frames per second show plugs coalescing with the wetting layer on the substrate and reforming in a chemistrode. The 5.7-s movie corresponds to 0.9 s of real-time imaging data.

Movie S1 (MOV)

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Movie 52. Fluorescence images show the mixing and exchange between the wetting layer on the surface and the plugs. Fluorescence images were taken at 500 frames per second. The 19.0-s movie corresponds to 1.2 s of real-time imaging data.

Movie S2 (MOV)



Movie S3. Recording 40-ms fluorescein pulses in substrate with plugs in chemistrode. Fluorescence images were taken at 200 frames per second. The 9.1-s movie corresponds to 1.4 s of real-time imaging data.

Movie S3 (MOV)



Movie 54. Recording of 40-ms fluorescein pulses with single-phase laminar flow. Images were taken at 400 frames per second. The 10.0-s movie corresponds to 1.5 s real-time imaging data.

Movie S4 (MOV)