Release monitoring of single cells on a microfluidic device coupled with fluorescence microscopy and electrochemistry

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A method for monitoring the biological exocytotic phenomena on a microfluidic system was proposed. A microfluidic device coupled with functionalities of fluorescence imaging and amperometric detection has been developed to enable the real-time monitoring of the exocytotic events. Exocytotic release of single SH-SY5Y neuroblastoma cells was studied. By staining the cells located on integrated microelectrodes with naphthalene-2,3-dicarboxaldehyde, punctuate fluorescence consistent with localization of neurotransmitters stored in vesicles was obtained. The stimulated exocytotic release was successfully observed at the surface of SH-SY5Y cells without refitting the commercial inverted fluorescence microscope. Spatially and temporally resolved exocytotic events from single cells on a microfluidic device were visualized in real time using fluorescence microscopy and were amperometrically recorded by the electrochemical system simultaneously. This coupled technique is simple and is hoped to provide new insights into the mechanisms responsible for the kinetics of exocytosis. © *2010 American Institute of Physics.* [doi:10.1063/1.3491470]

I. INTRODUCTION

Exocytosis is an essential biological process for understanding the process of intercellular communication. It can be summarized as the docking of bioactive molecules (neurotransmitters, peptides, hormones, etc.) containing vesicles to the cell membrane and subsequent release of the contents by fusion of the vesicle and cell membranes. This process allows the conversion of an electrical signal to a chemical one, which is necessary for signal communication between cells.[1–3]

An alternative way to study the process of exocytotic release is by electrical measurements that mainly include electrophysiological (i.e., membrane capacitance measurements)[4,5] and electrochemical detection (e.g., constant-potential amperometry, high-speed chronoamperometry, fast-scan cyclic voltammetry).[6–10] Another possible approach to study exocytosis is through optical methods.[11–15] Among them, amperometry using microelectrodes and fluorescence microscopy especially in an evanescent wave field are considered to be two of the most powerful techniques.[16] By tracking the exocytotic process in real time through the simultaneous use of fluorescence microscopy and electrochemical monitoring, more detailed and intuitive information about the events can be collected.

The laboratory on chip provides a powerful platform for working with live cells due to the

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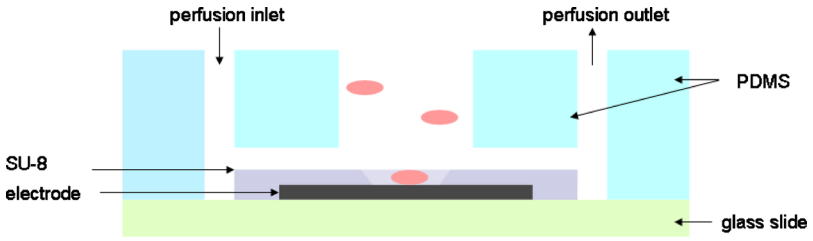

FIG. 1. Schematic diagram shown as the cross section of an analytical unit in the microfluidic device.

possibilities of miniaturization and integration of multifunctionalities in a single system. The development of multilayer soft lithography has greatly promoted the use of mechanically controlled microflows and the displacement of cells into microfluidic devices.[17,18] A variety of other functions such as cell culture,[19,20] cell manipulation,[21–23] cell analysis,[24–29] etc. has also been performed in these kinds of microfluidic devices. A transparent polydimethylsiloxane (PDMS) microfluidic chip comprising manipulations of single living cells and microelectrodes will benefit both the fluorescence imaging and electrochemical detection of exocytosis in a high throughput and automated way.

In this study, a technique combining both fluorescence microscopy and electrochemistry to monitor catecholamine release from single SH-SY5Y neuroblastoma cells is described based on a microfluidic device. The microfluidic device, which has integrated microelectrodes, can be fabricated to serve as an analytical platform. In the experiment, we used naphthalene-2,3-dicarboxaldehyde (NDA) to stain SHSY5Y neuroblastoma cells and successfully observed stimulated exocytotic release at the surface of SHSY5Y cells without refitting the commercial inverted fluorescence microscope. Using this microdevice, stimulated exocytosis was visualized in real time using fluorescence microscopy and was amperometrically recorded through the electrochemical detectors simultaneously.

II. EXPERIMENTAL

A. Chemicals and materials

NDA was obtained from Fluka Chemical Corp. (Switzerland). Stock solutions of NDA 50 mM were prepared in acetonitrile and stored in the dark. Catecholamine solutions were prepared as 10.0 mM solutions in 100 mM perchloric acid. A stock solution of 50 mM sodium cyanide (NaCN, BDH) was prepared in de-ionized (DI) water. All solutions were refrigerated at 4 °C when not in use. DI water obtained from a Milli-Q water purification system (Millipore, USA) was used throughout the experiment.

PDMS silicone elastomer (Sylgard 184, base and curing agent) was purchased from Dow Corning (Germany). Photoresist (SU-8 series) was purchased from MicroChem (Newton, MA) and the photoresist (AZ 5214) was from electronic materials K. K. (Japan). Poly(ethylene glycol) (PEG) was purchased from Sigma-Aldrich Chemical Corp. (St. Louis MO).

SH-SY5Y cells were obtained from the European Collection of Cell Cultures (ECACC) and cultured in Dulbecco's Modified Eagle Medium (DMEM) medium with 10% fetal bovine serum and 1% penicillin-streptomycin solution. Cells were kept in a 90% humidity atmosphere with 95% air/5% CO_2 at 37 °C. The cell medium was refreshed every 2 d or 3 d. HeLa cells were obtained from the American Type Culture Collection (ATCC) and prepared as described above.

B. Fabrication of microfluidic devices

The microfluidic device consists of PDMS molded slab reversibly sealed to a glass slide with patterned microelectrode structure (Fig. 1). The fabrication process of microelectrodes was comprised of photolithography, magnetron sputtering, and lift-off technologies. The protocol for fabrication of microelectrodes is as follows. First, a glass slide was cleaned with Piranha solution

($H_2SO_4:H_2O_2, 7:3$) then rinsed with DI water and dried with N_2 atmosphere. The clean slide was spincoated with AZ 5214 photoresist at 3000 rpm for 30 s, baked for 50 s at 110 °C to remove the solvent. The electrode pattern was transferred onto the photoresist layer by UV exposure at an intensity of 14 mW/cm^2 for 15 s through a mask followed by development. Metals (titanium/platinum) were then deposited by magnetron sputtering to produce a multilayer electrode structure. Following metal deposition, the unwanted metal coat was removed and the new microelectrode pattern was realized by lift-off procedure with acetone. The same process as above was fabricated to deposit silver electrode, silver/silver chloride microelectrode by electrochemical methods, or by immersing silver electrode in 6 wt % NaOCl solution (bleach) for 5–10 s. The fabricated microelectrodes were insulated using PEG modified SU-8 photoresist with UV contact photolithography.

The PDMS slab was fabricated by pouring PDMS prepolymer (Sylgard 184 base and curing agent, weight ratio 10:1) against a channel master on silicon with a thickness of 50 μm using SU-8 UV photolithography and curing the PDMS in an oven (80 °C, 3 h). After curing, the PDMS layer was peeled from the channel master and holes were punched for microreservoirs, perfusion inlets, and perfusion outlets. Finally, the PDMS slab was bonded to the above microelectrode layer.

C. Fluorescence imaging and analysis

An inverted microscope (IX71 inverted, Olympus, Japan) was used in the experiment. For fluorescence imaging, we used a 40× (NA 0.60, Olympus) objective lens and two Olympus filter set: BP 360/370 nm excitation filter, 400 nm dichroic filter, 420 nm emission filter and BP 400/440 nm excitation filter, 455 nm dichroic filter, 475 nm emission filter. Images were captured with a digital color charge-coupled device (CCD) camera (DP71, Olympus, Japan). Static images were taken at a resolution of 1360×1024 pixels (with a pixel width of 0.16 μm) and videos were recorded using 2×2 binning and 4×4 binning, respectively.

Videos (2×2 binning) were converted into a series of images with 688×512 pixels using VIRTUALDUB software. Image series were analyzed using MATLAB programs. It is assumed that the fluorescence values for each pixel would range between 0 and 255. The dimension of each pixel is 0.32×0.32 μm. For the video (4×4 binning) with frames of 352×250 pixels, the size of each pixel is 0.64×0.64 μm.

D. Electrochemical recording and analysis

Electrochemical experiments were conducted on an electrochemical workstation (CHI 660B, CH instruments, Shanghai, China) and Ag/AgCl electrodes were used as reference electrodes. In the amperometric mode, the applied potential was 0.5 V versus Ag/AgCl electrodes. Spike parameters were calculated and analyzed according to the methods described in the references.[16,30] Briefly spike amplitude, I_{max} (A), was measured between the current at T_{max} (time at spike maximum) and the baseline current under the spike maximum. Spike width, $t_{1/2}$ (s), was evaluated at 50% of I_{max}. The total electrical charge Q (A × s, C) was calculated using handwriting programs. I_{max}, $t_{1/2}$, and Q were presented as mean ± SD (standard deviation). All apparatus were grounded through a common ground and shielded by copper foil to minimize the electrical noise.

E. Single cell experiment

Before the experiment, SH-SY5Y cells and HeLa cells were incubated in DMEM medium supplemented with derivatization reagents containing NDA (1 mM), NaCN (1 mM), and borate buffer (1 mM, *p*H 9.1) for ~10 min before washing thoroughly and followed by resuspension in phosphate buffered saline (PBS) solution. Cells were placed in chamber within microfluidic devices and allowed to pass by an approximately cone-shaped aperture, which made single cells sediment on the microelectrode or position around microelectrodes. A syringe pump (TS2-60,

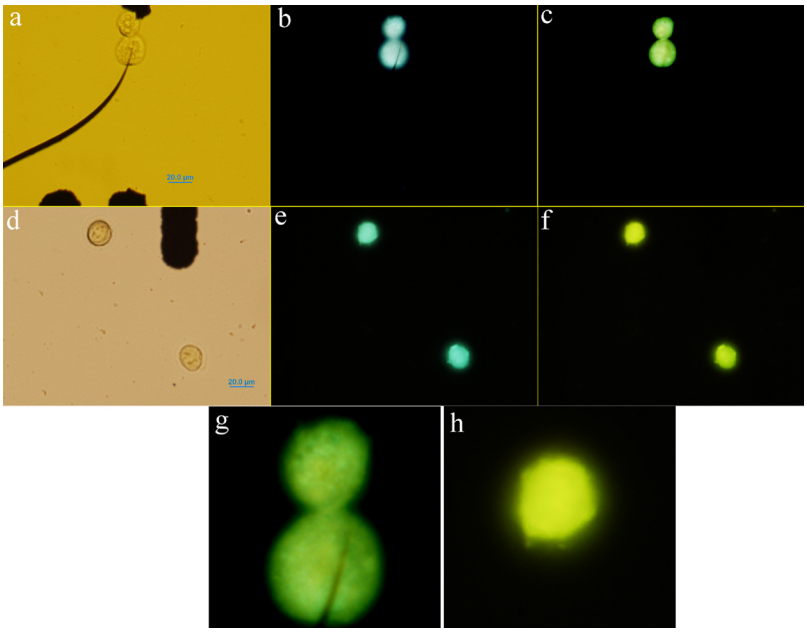

FIG. 2. Comparison of NDA labeled SH-SY5Y neuroblastoma cells [(a)–(c)] and NDA labeled HeLa cells [(d)–(f)]. Bright-field images [(a) and (d)], fluorescence images [(b) and (e)] with excitation at 360/370 nm, and fluorescence images [(c) and (f)] with excitation at 400/440 nm, respectively. Fluorescence images [(g) and (h)] are magnified [(c) and (f)] respectively. Scale bar, 20 μm.

Longer Precision Pump Co. Ltd., China) was used to drive high K^+ saline solution through the microfluidic channel to evoke exocytotic responses. Experiments were performed at room temperature (~25 °C).

III. RESULTS AND DISCUSSION

A. Fluorescence imaging evaluation

NDA, nonfluorescent in itself, can interact with primary amine in the presence of excess cyanide to yield 1-cyanobenz[f]isoindole products, which are both fluorescent and electroactive.[31] NDA has been used as catecholamines (e.g., dopamine, norepinephrine), labeled reagent in single cell analysis by capillary electrophoresis.[32] In this study we used NDA to stain SH-SY5Y neuroblastoma cells. As shown in Figs. 2(b), 2(c), and 2(g), although the surface of cells is fluorescent, punctuate fluorescence (bright fluorescent spots) can be observed on the surface of cells. To make comparison, the NDA labeling experiment was performed on nonsecreting cells (e.g., HeLa cells). However, in this case, the small bright spots were not observed on the surface of HeLa cells [Figs. 2(d)–2(f)] under the same experiment conditions. It is possible that the bright spots were due to high concentration of neurotransmitters at some specific zones on the surface of SH-SY5Y neuroblastoma cells, presumably corresponding to the docked vesicles because neurotransmitters are typically stored within vesicles which are close to the cells membrane.[10]

Dynamic exocytotic release of SH-SY5Y neuroblastoma cells was recorded after using high K^+ stimulation to trigger exocytosis. Figure 3 shows this exocytotic process clearly in a dot map of successive video frames. Related video record can be referred in supporting information. As expected, a sudden fluorescent flash around SH-SY5Y neuroblastoma cells was observed in the movie. The fluorescence imaging allows visualization of clouds of released material as high K^+ stimulation caused fluorescent spots to become brighter and spread then diffuse away from cells. Neurotransmitters released by exocytotic events elsewhere in the cell caused a general brightening of the plasma membrane. To enhance the contrast, we used the frame of the sequence obtained right before stimulation as background fluorescence; the background image was then subtracted

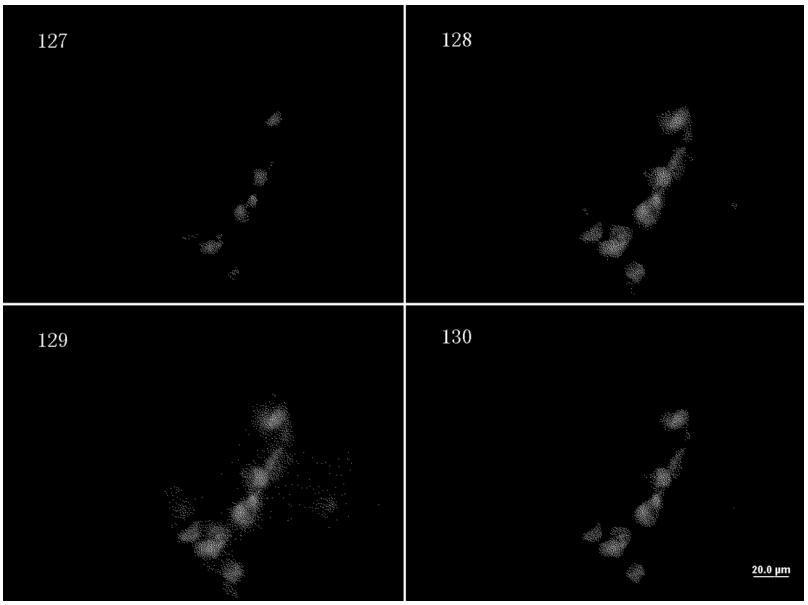

FIG. 3. A dot map of the NDA labeled SH-SY5Y neuroblastoma cells showing dynamic exocytotic process (frame 127–130). Fluorescence images were converted into bit mode using ADOBE PHOTOSHOP software (choose Image > mode > Grayscale; then choose Image > mode > bitmap). Time interval between pictures: 65 ms.

from other frames (Fig. 4). It can be noted that the size of these spots in diameter is close to the size of vesicles reported in the literature (ranging from 50 nm to 1 μm in diameter).[33]

To acquire temporal and spatially resolved information, fluorescence intensity for bright spots (five regions of interest selected) was plotted against time [Fig. 5(a)]. Although temporally resolved profiles in Fig. 4(a) show differences, the difference among them is rather insignificant. Signal response depends on the capture rate and therefore some hidden information is likely to be

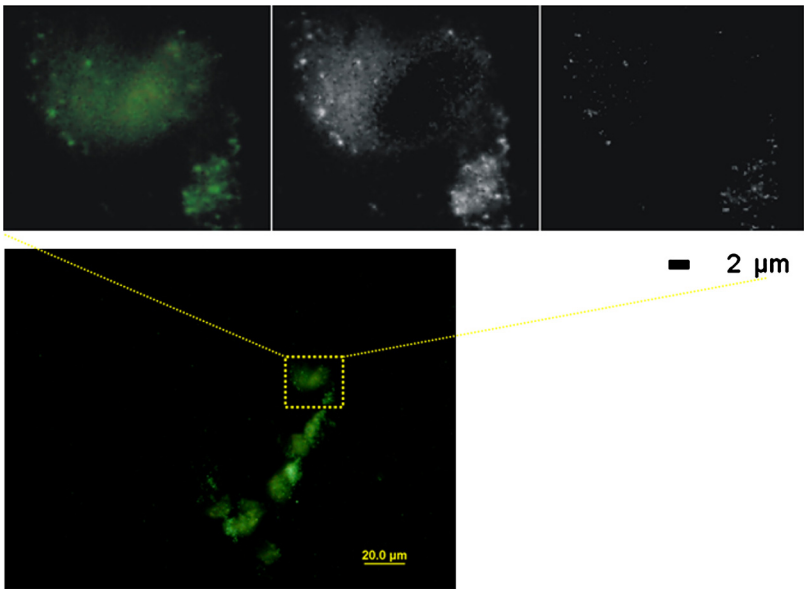

FIG. 4. Images before (left) and after (middle and right) subtracting background fluorescence using handwriting MATLAB programs. Middle, image after the first background subtraction; right, image after the second background subtraction. The selected cell is located on the top in NDA labeled SH-SY5Y neuroblastoma cells. Data were from the same video clips.

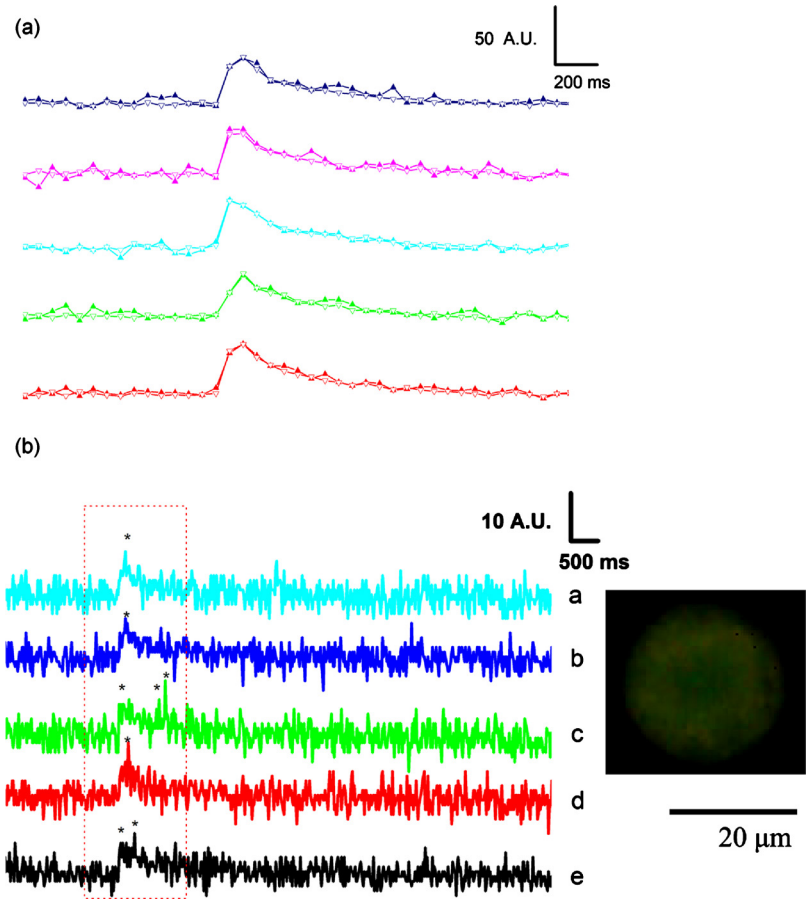

FIG. 5. (a) Fluorescence intensity for five regions of interest plotted against time in the same cell. Triangles (filled) indicate bright spots and triangles (empty) show the average of outer eight pixels around bright spots. The ordinate is given in arbitrary units of brightness. Successive frames captured using 2×2 binning; frames acquired at the rate of 65 ms/frame. (b) Fluorescence intensity for five regions of interest plotted against time in the same cell using 4×4 binning; frames were acquired at the rate of 16.7 ms/frame. Spikes with asterisks correspond to release of bright spots from different sites [(a)–(e)] when the cell was stimulated by elevated K^+. The inset image is a NDA-labeled cell image. The black dots represent the five regions of interest.

missing at a low capture rate. As an attempt to improve the above results, we increased the frame capture rate up to 16.7 ms/frame to capture the video before plotting the fluorescence intensity for the selected sites against time. Figure 4(b) shows typical fluorescence transients for the five regions of interest from the same cell. The images show relatively high noise levels because they were captured in high speed and resulted in damaged resolution (4×4 binning). Despite this, information about release from the selected site was obtained. It was found that more release events at the selected site (e.g., trace e) occurred at certain specific time (rectangular box) with the overlap of multiple exocytotic events in Fig. 5(b), which contributed to the fluorescent flash observed in the video during exocytosis. The occurrence of overlapped multiple exocytotic transients from traces in temporal scale corresponds exactly to the fluorescent flash in the video.

B. Amperometric monitoring of catecholamine release

High K^+ stimulation caused cell depolarization-evoked Ca^{2+} dependent release of neurotransmitters. This process can be monitored simultaneously using microelectrodes. Figure 6 (trace b) shows an amperometric recording of exocytotic events from SH-SY5Y cells. The current rises to form a broad envelope in which sharp spikes are superimposed. This indicates that sharp spikes

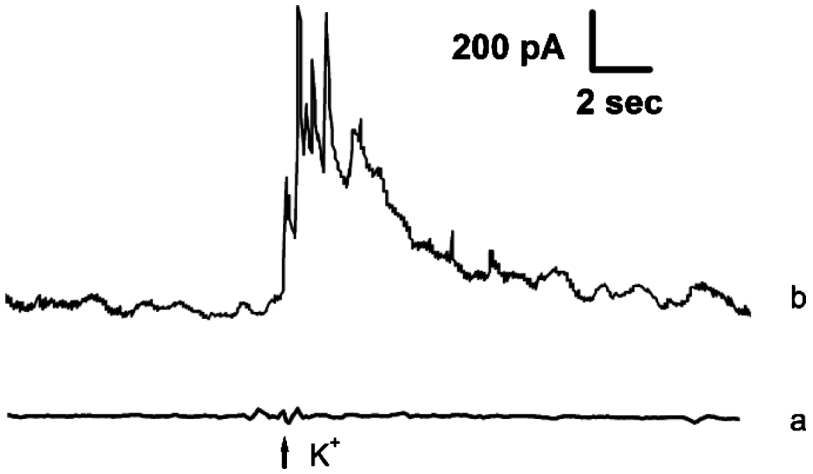

FIG. 6. Amperometric response to 50 mM potassium stimuli from NDA-labeled SH-SY5Y cells (b) and blank PBS (*p*H 7.2) solution (a) on the microelectrode. The shape of microelectrodes is shown in Fig. 2(a).

correspond to exocytosis of vesicles at the bottom of cells where the cell membrane directly contacts the working electrode.[3] Three cells touched the microelectrode in the above experiment so each cell contributed to the broad envelope in exocytotic events.

C. Simultaneous fluorescence and amperometric measurements

It should be noted that the above fluorescence imaging showed mostly the information about vesicles on the upper surface of cells, whereas the microelectrode monitored the response mainly from vesicles at the bottom of cells where the cell membrane directly contacted the working electrode. A more realistic result could be achieved if both the fluorescence microscopy and electrochemical methods were used simultaneously to monitor the region of interest. Thus, we conducted additional experiments in which another microelectrode was used to monitor the release from a single cell. By repositioning the cell and making the microelectrode contact only one side of the cell [Fig. 7(a)], the exocytotic events from the upper lateral vesicles of the cell could be electrochemically monitored at the same time during fluorescence imaging. As shown in Fig. 7(a), both fluorescent (trace a) and amperometric (trace b) responses of the selected region of interest were obtained when the cell was stimulated by the elevated K^+. We found that some peaks in fluorescence and current can be approximately correlated with each other [rectangular box in Fig. 7(a)] although the result showed some broad fluorescence signals in trace a. For simultaneous measurements, the experiments were conducted on 16 different cells using K^+ as the stimulant. Of the cells with detectable signals due to stimulation, $<10\%$ of events showed no correlation between the peaks in fluorescence and current. This leads to the belief that NDA can interact with primary amine/CN^- to produce fluorescent compounds, and other fluorescent signals besides signal molecules (e.g., catecholamines) may cause disturbance during exocytosis, although they are not released from the cell with signal molecules. On the other hand, the amperometric response was improved for the use of NDA probe. Amperometric response was obvious not only in a number of events but also in amplitude. It can be seen that some spikes resulting from release showed very high current response, as shown in both Fig. 6 and Fig. 7 (trace b) as the NDA probe could react with certain catecholamines (e.g., dopamine, norepinephrine) to form electroactive products which can remarkably increase peak currents. The kinetic parameters of amperometric response were calculated and the mean values for $t_{1/2}$, I_{max}, and Q were 73.7 ± 29.2 ms, 56.1 ± 30.4 pA, and 4.0 ± 2.3 pC (SD, n=62), respectively.

The working electrode, integrated in the microfluidic device in this work, is a planar electrode different from conventional carbon fiber electrode, which can be localized spatially on the selected site. The response from other sites in the cell may be acquired by the fabrication of multilayer or

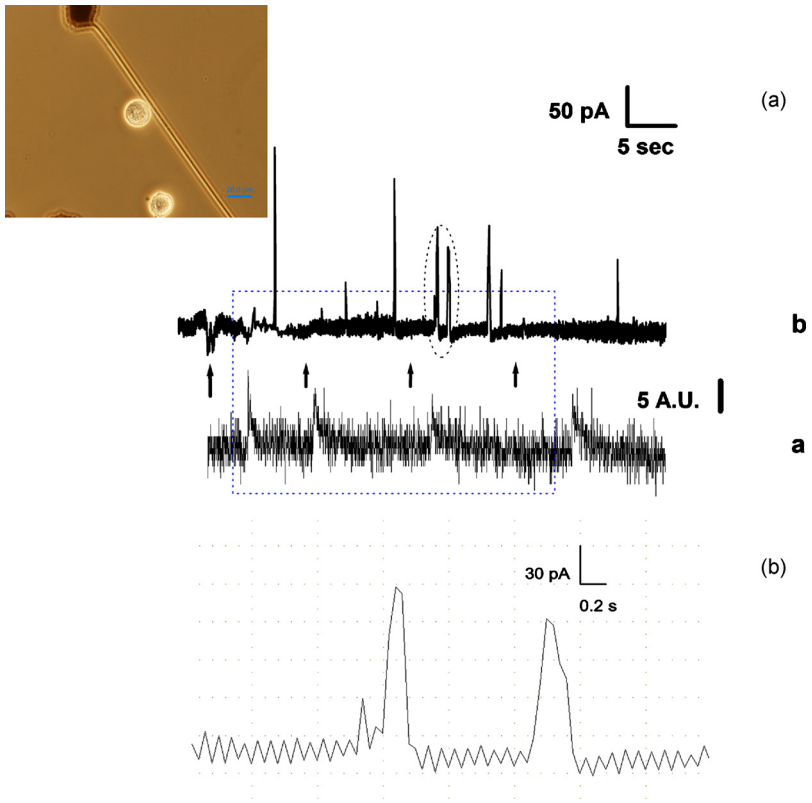

FIG. 7. (a) Simultaneous fluorescence and amperometric response from the upper lateral selected sites of the SH-SY5Y cell during exocytosis with 4× stimulation (50 mM K^+). The arrow indicates the beginning of the high K^+ stimulation. The inset image shows that the microelectrode is located at one side of the NDA labeled cell. Scale bar in the inset, 20 μm. (b) Expanded time scale view of the interval marked with the dashed ellipse in part a.

stereo microelectrode in view of cellular shape. Therefore the next step of this work should involve the fabrication of a microfluidic device with a multilayer and stereo-microelectrode structure and application of multichannel data collection,[9] which would be useful for obtaining more comprehensive data in the exocytotic events.

IV. CONCLUSION

The use of fluorescence microscopy could show excellent spatial information and dynamic pictures during exocytosis while electrochemical detection could simultaneously provide kinetic parameters of release events, which are complementary to each other. In this work we have demonstrated an analytical method to monitor real-time exocytotic events on a microfluidic device through a combination of fluorescence microscopy and electrochemical technique. Both fluorescence microscopy and electrochemical methods have been exploited to investigate stimulated exocytotic release of SH-SY5Y cells. The most important feature of this coupled technique is the ability to simultaneously record spatial and temporal information during exocytotic process. Such technique is envisaged to provide new insights into the mechanisms responsible for the kinetics of exocytosis.

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