Bioanalysis

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Coupling Amperometry and Total Internal Reflection Fluorescence Microscopy at ITO Surfaces for Monitoring Exocytosis of Single Vesicles**

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Water-soluble hormones and neurotransmitters are packaged in secretory vesicles and secreted into the extracellular medium by exocytosis, a process involving the fusion of the vesicle membrane with the cell membrane.[1] Transport of the secretory vesicles to the cell's periphery, the maturation stages they undergo there to acquire fusion competence, and the factors controlling the fusion process itself (including the dynamics of the fusion pore) are important biological questions that are not fully understood.

To elucidate secretory mechanisms at the single-vesicle level, currently only a few analytical methods exist, which can be grouped into electrical or optical recordings.[2] The great advantage of electrical recordings (patch–clamp membrane capacitance and electrochemical amperometry) is their excellent time resolution (ca. tens of microseconds), which allows studies of the dynamics of the fusion pore itself. However, a major disadvantage is the fact that signals appear only after fusion has commenced; that is, the dynamics of the secretory vesicle itself or any labeled regulatory protein prior to the fusion event cannot be detected. In contrast, optical recordings allow secretory vesicles or regulatory proteins to be visualized and tracked prior to their fusion, yet generally they lack the time resolution required to follow the dynamics of the fusion pore (typical time resolution is ca. 100 ms). In addition, depending on the technique, secretion may be probed from different areas of a cell (top or bottom), which makes comparison of the results obtained by different approaches difficult.

Because of their complementary nature, it would be a great advance if electrical and optical measurements could be made simultaneously from the same side of a cell at the single-vesicle level. This will enable a comprehensive and precise analysis of the whole exocytotic event, from predocking through fusion steps up to the dynamics of vesicular release. Herein, we report a device based on transparent indium tin oxide (ITO) electrodes, which allows simultaneous total internal reflection fluorescence microscopy (TIRFM) and amperometric measurements (Figure 1). As a proof of concept, the ability of our device in the coupled optical and electrochemical detections of exocytotic events is demonstrated using enterochromaffin BON cells.

Amperometry is based on detection at a microelectrode surface positioned near the emitting cell of electroactive vesicular contents that are released into the extracellular medium.[3,4] With very high temporal resolution and sensitivity, the flux of the vesicular content (released through an initial fusion pore that is only a few nanometers wide) corresponding to an exocytotic event appears as a current spike, which features (frequency, time length, area, magnitude) the dynamics of release from single vesicles. Generally, amperometry involves placing a large collecting electrode near the investigated cell. The whole cell active surface area is covered so the spatial localization of a particular exocytotic event cannot be achieved. Nevertheless, a few studies involving smaller microelectrodes[5] or microelectrode arrays[6,7] allowed amperometric signals from different releasing sites to be identified, but with a random positioning for the small microelectrode and a spatial resolution necessarily limited by the array dimensions, respectively. Coupling of amperometric and optical recordings would allow precise localization of exocytosis events in space and time.

The most widely used optical approach to study exocytosis, TIRFM, is based on the total internal reflection of a laser beam at the glass/water interface, which creates an evanescent field in the aqueous medium whose characteristic decay length (ca. 100 nm) provides a high signal-to-noise ratio and an axial resolution of about 10 nm.[8,9] When a vesicle fuses with the plasma membrane, its labeled contents are released toward the glass/water interface where the excitation

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Supporting information for this article (details of ITO device fabrication, experimental information on the amperometric and optical detections, and the preparation and stimulation of BON cells) is available on the WWW under http://dx.doi.org/10.1002/anie.201101148.

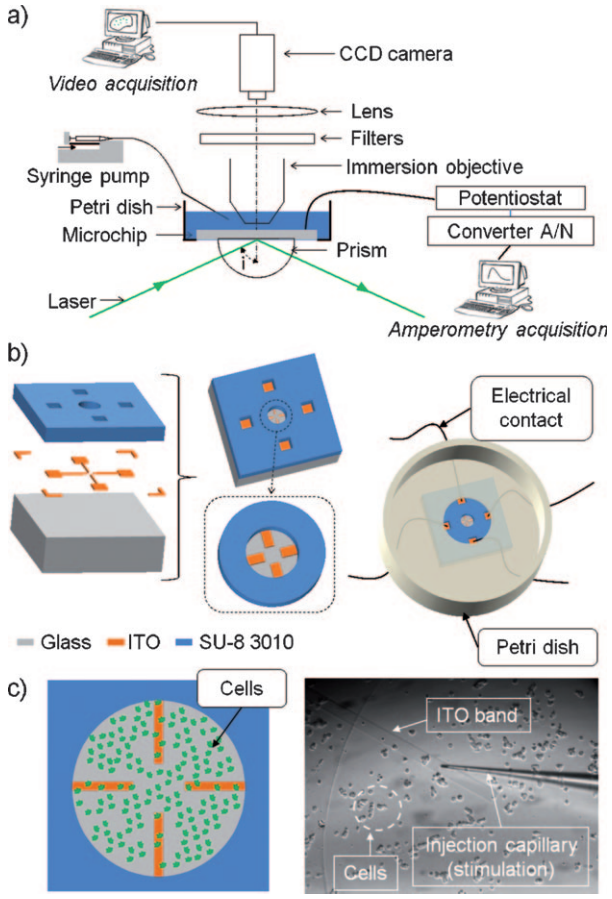

Figure 1. a) Setup required for TIRFM–amperometry coupling. b) Schematic representation of the device with four ITO bands used for the combined detections. c) Left: scheme of cells adhered on the device. Right: inverted microscopy image of BC21 BON cells adhering to the well containing the ITO microelectrodes. Only a portion of the well (one band) is shown for clarity.

field is stronger. Because the fluorescent probe is typically pH dependent, the signal transiently brightens during release and then disappears as the fluorophores diffuse away, that is, single exocytotic events are visualized as "puffs" of light which accompany the disappearance of fluorescent spots.[10–12]

Combining TIRFM and amperometric detection is technically challenging because of the different, and even conflicting, requirements for the two techniques.[13] TIRFM detects secretion from the bottom side of a cell adhering onto a good-optical-quality coverslip. The area of the coverslip should be large enough so that several different single cells can be recorded successively in the same run. Amperometry is traditionally carried out using a carbon-fiber microelectrode positioned near the top of a cell. To detect fusion events from the bottom side of a cell, surface microelectrodes of an optically transparent material with an excellent electrical conductivity are required. A few years ago, ITO-coated glass slides were shown to globally match these conditions[14,15] and allow the electrochemical detection of exocytotic events (catecholamine release) from adrenal chromaffin cells, though without any adaptation to TIRFM.

Here, we found that the thickness of the ITO layer had to be reduced to 150 nm to provide suitable resolution for TIRFM images without severely increasing the electrical resistance. For amperometry, the electrode surface must be limited to ensure the minimization of nonfaradaic information (electrical noise, capacitive currents) while allowing a high signal-to-noise ratio of detection. To restrict the active electrochemical surface without compromising the probability of finding observable cells on the electrode, we designed devices consisting of four ITO bands (width 200 μm/length ranging from 2 mm to 750 μm), the active surfaces of which were delimited by a well in the thin, insulating, SU8 photoresist layer (10 μm in thickness; see Figure 1 b,c and the Supporting Information).

Basically, a very thin film of ITO (150 nm in thickness) was sputtered onto a glass slide and subsequently chemically etched into four independent band-shaped microelectrodes. Photolithography techniques were used to create a circular well (2.6 mm in diameter) in an SU8 photoresist insulating layer to limit the ITO exposed surfaces and minimize the capacitive noise source. It must be emphasized that our microsystem finally presents three different surfaces (SU8/ITO/glass) exposed to the solution. A surface treatment was thus required to uniformly promote cell adhesion. This was firstly achieved through an oxygen plasma treatment, which provided hydrophilicity to the SU8 photoresist, and a subsequent collagen modification, for better cell behavior and adherence on the substratum. On such ITO surfaces and geometries, a good root-mean-square (rms) noise (less than 0.2 pA) for amperometric detection of individual exocytotic events can be reached after adhesion of a controlled number of living cells under the experimental conditions described below.

Independent amperometric and optical detections of exocytosis have already been evidenced in enterochromaffin BON cells, a cell line derived from a carcinoid tumor.[8,9,16,17] To test the suitability of our device to concomitantly detect optical and electrochemical signals of individual exocytotic events, we used BC21 cells, a stable clone of BON cells that release the electrochemically active neurotransmitter serotonin and stably expresses green fluorescent protein-tagged neuropeptide Y (NPY-GFP), a soluble luminal marker of secretory granules. All secretory vesicles thus optically appear as green dots (Figure 2). BC21 cells (10^6 cells mL^{-1}) were plated into the well delimiting the four ITO bands (Figure 1 c) and analyzed 24 h later. A few cells adhered onto each ITO band. An isolated cell could then be stimulated during 60 s by local perfusion of a saline solution supplemented with 5 μM of the calcium ionophore ionomycin.

During the stimulation, the ensuing secretory process was followed by amperometry (oxidation potential of +650 mV vs. Ag/AgCl) and optically (excitation and emission maxima at 488 and 509 nm, respectively). The low frequency of exocytosis (0.1 Hz) observed at these cells ($n = 6$), as well as the controlled and spatially orientated stimulation of a single isolated cell, allowed us to relate unambiguously amperometric spikes and individual optical events as displayed in Figure 2. Among all the exocytotic events recorded, around 30% corresponded to clearly observable simultaneous

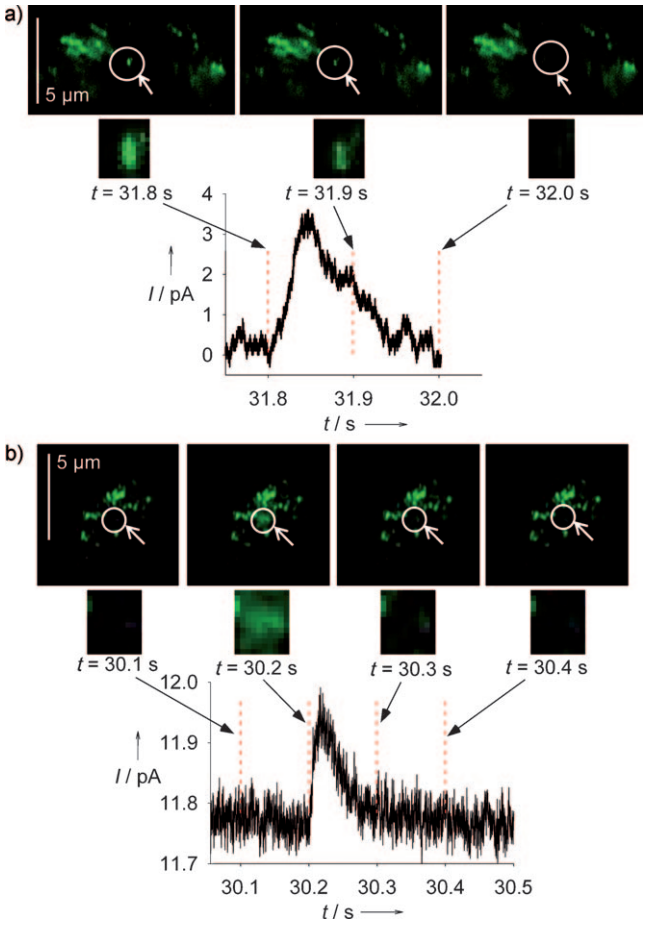

Figure 2. a,b) Two representative examples of combined analyses of an exocytotic event (top: TIRFM; bottom: amperometry) obtained with the device described. The initial time of the two amperometric and optical recordings is the same and refers to the onset of secretagogue injection. For TIRFM, the whole investigated cell is displayed and the secretory vesicles appear as green dots illuminated by the evanescent field. The single event is shown with an arrow/circle or zoomed (500%). From an optical point of view, exocytotic events are seen as rapid disappearance of the fluorescence corresponding to NPY-GFP diffusing out of the vesicles (see (a)), often preceded by a "flash" (transient increase in fluorescence and appearance of a cloud of fluorescent molecules surrounding the vesicle) as in (b). The temporal resolution of the two techniques (40 kHz, i.e., one acquisition per 25 µs for amperometry and 10 Hz, i.e., one acquisition per 100 ms for TIRFM) is suitable for the coupled detection of these individual exocytotic events (at least 100 ms duration) even if the resolution of the TIRFM camera may be enhanced in future investigations.

TIRFM/spike signals. This fraction of correlated signals stems from the resolution of the camera used here and the low vesicular serotonin concentration.[18] The isolated amperometric spikes detected ($n = 25$) ranged from 0.2 to 3.5 pA in magnitude and were globally consistent with those obtained independently at carbon microelectrodes on cells from the same origin stimulated with the same secretagogue ($Q = (112 \pm 15)$ fC; $I_{max} = (2.5 \pm 0.3)$ pA; $t_{1/2} = (34 \pm 3)$ ms; six cells, 110 analyzed spikes), considering the established differences between apex/bottom releasing sites.[19]

In summary, we have presented a microdevice that allows coupling of two complementary analytical techniques for the direct and real-time analysis of exocytotic phenomena. The simultaneous optical and electrochemical detection of single exocytotic events was successfully demonstrated on a transparent ITO electroactive material, by using enterochromaffin human BON cells. The present methodology could be transposed in the future to other neuroendocrine cell types (e.g., PC12 cells, chromaffin cells, pancreatic β cells) or any immobilized secretory cells (e.g., neurons) that emit electroactive compounds through vesicular release and that may be tagged with fluorescent probes. This work thus sets the basis of a new methodology for quantitative, temporally and spatially resolved monitoring of single-vesicle motions and exocytosis. Notably, it will allow a correlation of the fusion-pore behavior (opening/closure dynamics, stability, measured by amperometry) with the motion of a vesicle in three dimensions (tethering, docking, fusion, retrieval, detected by TIRFM), with the recruitment of fluorescently tagged molecules onto secretory vesicles (using dual-color TIRFM), or with actin or calcium dynamics. Imaging of exocytotic events will also help to identify small amperometric signals as transient fusion pores and thus to fully benefit from the high time resolution of amperometry to study the dynamics of the fusion pore. In addition, the technique will permit comparison of the release kinetics of different-sized molecules (small transmitters measured by amperometry and large fluorescent proteins by TIRFM), as an index of fusion-pore enlargement, to simultaneously measure content mixing and lipid mixing (e.g., to detect hemifusion) or to reveal whether vesicular content/size (measured by amperometry) depends on the secretion area of the plasma membrane (by TIRFM).[20] We thus think that this combined method will help to answer several important questions related to exocytosis (regulation of the fusion-pore dynamics, relationship between "kiss and run" and "full fusion" mechanisms, impact of the cell mechanics on fusion-pore dynamics, existence of specific spatial zones or hotspots, temporally limited or not, and devoted to particular sizes of vesicles). To the best of our knowledge, currently there are no other methods capable of such measurements.

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