

Analytical Chemistry in Microenvironments: Single Nerve Cells

ANDREW G. EWING,* TIMOTHY G. STREIN, AND YAU YI LAU

Department of Chemistry, 152 Davey Laboratory, Pennsylvania State University,
University Park, Pennsylvania 16802

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Introduction

The ultimate neuronal microenvironment is the single nerve cell. The heterogeneity between individual cells in the brain demands that attempts be made to determine the development, function, and regulation of individual neurons. When developing techniques to carry out analysis in neurochemical microenvironments, it is important to realize the incredible variability not only in chemistry from cell to cell but also in physiological response time and compartments within single cells. Analyses of cytoplasm, organelles, extracellular fluid, and single whole cells must be considered primary goals for eventual understanding of single cell function. Voltammetric microelectrodes^{1,2} are ideally suited for dynamic chemical changes resulting from discrete neurochemical events as they possess rapid response times. Furthermore, many neurochemicals are easily oxidized. In addition, microcolumn separation methods including open tubular liquid chromatography³ and capillary electrophoresis^{3,4} are capable of profiling the chemistry of single cells. Hence, voltammetry is useful for experiments where changes in chemical composition are to be monitored and microcolumn separation methods allow determination of chemical composition at a specific sampling time. This Account will describe experimental schemes and discuss results obtained using these methods to monitor neurochemistry at the level of a single cell.

Small voltammetric probes have been used to monitor easily oxidized substances in the cytoplasm of single nerve cells.⁵⁻⁸ Slightly larger carbon fiber electrodes, originally developed for voltammetry in the extracellular fluid of mammalian brains,^{9,10} have recently been used to monitor the secretion of catecholamines from single adrenal cells in culture.^{11,12} The amazing aspect of this latter experiment is the ability to

discriminate neurotransmitter secretion from single neuromessenger vesicles containing approximately 5×10^6 catecholamine molecules.

Microcolumn separation methods have been developed to the point where successful analyses have been carried out on single whole nerve cells, including large invertebrate cells^{1,13} and small mammalian adrenal cells.¹⁴ In addition, capillary electrophoresis has been used to inject, separate, and detect picoliter samples of cytoplasm from single large invertebrate neurons.¹⁵ In principle, these separation techniques should find great applicability in many areas. These might include profiling the amino acid content of single cells, investigating the possible coexistence of neurotransmitters in specific cells, determining interactions between pharmacological agents or neuroleptic drugs, and examining cytoplasmic levels of neurotransmitter.

Most chemical studies in the neurosciences have concentrated on chemical changes in specific regions of the nervous system with single cell work mainly approached by use of microchemical techniques including enzyme activity measurements, spectrophotometric techniques,¹⁶ microscale ion-selective electrodes,^{17,18} and fluorescence imaging techniques.¹⁹ The

* Author to whom correspondence should be addressed.

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Andrew G. Ewing received a B.S. degree with highest honors in chemistry from Saint Lawrence University in 1979. He received a Ph.D. degree from Indiana University in 1983 working with R. Mark Wightman on in vivo electrochemistry. He then spent 13 months as a postdoctoral research associate at the University of North Carolina working with Royce W. Murray on chemically modified microelectrodes. In 1984 he joined the faculty at Pennsylvania State University and was promoted to Professor of Chemistry in 1992. His current interests include electrochemistry at ultrasmall electrodes, capillary zone electrophoresis, TOF SIMS imaging techniques, and the application of these methods to single cell neurochemical analysis.

Timothy G. Strein received a B.S. degree in chemistry and textile chemistry from the honors program at North Carolina State University in 1988. He is currently supported by a fellowship sponsored by the Proctor and Gamble Company from the ACS Analytical Division and is completing his Ph.D. on development and application of submicron tip carbon electrodes.

Yau Yi Lau received a B.S. degree in chemistry from Hong Kong Baptist College in 1984 and an M.S. degree from Baylor University in 1989. She is currently completing her Ph.D. Thesis on voltammetric studies of neurotransmitter dynamics at single nerve cells.

latter two methods have been highly successful at monitoring the dynamics of inorganic ion concentrations at the single cell and subcellular levels. In the presentation below, the use of voltammetric microelectrodes to monitor the larger molecular neurotransmitters and the use of microcolumn separation techniques to profile and compartmentalize single nerve cells will be discussed. These methods are sensitive at the attomole level for detection of molecular species, thereby opening new avenues for understanding single cell neurochemistry. Furthermore, they should be generally applicable to the study of biological events in single cells.

Overview of Relevant Techniques for Analysis of Microenvironments

Small Electrodes Suitable for Biological Microenvironments. Achievement of chemical monitoring at the single cell level requires that significant attention be given to the miniaturization of chemical analysis technologies. Several techniques have been developed for dynamic chemical monitoring in small-volume environments. Among these, voltammetry at ultrasmall electrodes stands out as a rapid and sensitive means to monitor easily oxidized or reduced species. A key element in the use of electrochemistry in ultrasmall environments is the total structural tip dimension of the electrode employed. Electrode tip diameters in the micrometer or submicrometer range are necessary. A large repertoire of electrode designs and principles has been developed for applications in microenvironments. Ultrasmall noble-metal electrodes have been reported;^{20,21} however, ultrasmall carbon electrodes have been used for most work in this area.^{7,10,12} Several procedures have been developed for the construction of small carbon electrodes. The most widely employed small carbon electrodes use a carbon fiber as the electrochemical substrate.^{2,9} Carbon fiber electrodes have been constructed in both the disk-shaped² and cylindrical geometry.⁹ These electrodes are typically constructed using carbon fibers having 5–11 μm diameters, although 35 μm diameter electrodes are also used.²² Hence, disk-shaped carbon fiber electrodes typically have 15–20 μm tip diameters including insulation. These can be employed for applications outside single cells. In contrast, electrodes small enough to place through a single cell membrane for use in single cell cytoplasm should have total structural diameters in the 1–5 μm range.

One new electrode design that provides electrode tips with total structural diameters in the 1–5 μm range is the carbon ring electrode.²³ Carbon ring electrodes are routinely constructed by drawing silica tubes to small tip diameters in a methane–oxygen flame. After the tip has cooled, it is reheated while a flow of methane is maintained through the tube. Methane pyrolysis at approximately 1200 $^{\circ}\text{C}$ results in the deposition of a

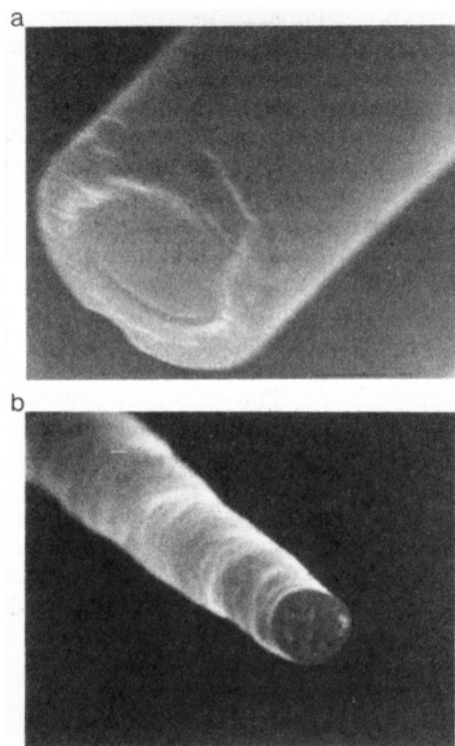


Figure 1. Scanning electron micrographs of two different carbon voltammetric electrodes: (a) carbon ring electrode (large scale bar represents 1 μm); (b) etched and insulated carbon fiber electrode (scale bar represents 100 nm). Figure 1a is reprinted with permission from Kim, Y. T.; Scarnulis, D. M.; Ewing, A. G. *Anal. Chem.* 1986, 58, 1782–1786. Copyright 1986 American Chemical Society.

conducting carbon film inside the small tip. The inside of the carbon is insulated with epoxy, and electrical contact is established inside the tube using conducting silver epoxy. To expose a ring of carbon, the tip of the structure is carefully cleaved with a scalpel while it is viewed under a microscope. A scanning electron micrograph of a carbon ring electrode is shown in Figure 1a. These electrodes have already proven useful for both intracellular and extracellular voltammetry (vide infra). However, carbon electrodes with even smaller total tip diameters are under development for use in still smaller biological microenvironments (i.e., single synapses).

Carbon fibers can be etched by electropolishing to provide submicrometer conical tips that produce extremely small electrodes.^{6,12} Indeed, carbon electrodes as small as carbon ring electrodes have been developed by these approaches. It has, however, been difficult to construct disk-shaped carbon fiber electrodes with submicrometer total tip diameters because most strategies to insulate the electrode tips require insulation thicknesses that are greater than 1 μm . We have recently developed a procedure to form insulating films with 100 nm thickness.²⁴ Electropolymerization of phenol and 2-allylphenol results in thin copolymer films that can be cross-linked by heating. This technology has been employed to construct carbon electrodes with total tip diameters as small as 400 nm (Figure 1b). Although these electrodes do not have electroactive diameters as small as those described by Penner et al.,²⁰ the total structural size of the etched carbon electrodes

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is considerably smaller. The tips of the electrodes described by Penner et al. are occluded by a large glass structure immediately behind the tip. This prevents electrode placement in microenvironments while viewing with a microscope. We expect the etched carbon electrodes to prove highly useful in future studies of the smallest microenvironments.

Microcolumn Separations for Neurochemical Analysis. *Open Tubular Liquid Chromatography (OTLC).* OTLC is carried out using capillary tubes with inner diameters of 2–50 μm and a typical length of 1–3 m. Separation is based on the differential retention of solutes as they interact with a stationary phase in the capillary. This stationary phase is adsorbed or bonded to the inner wall of the capillary instead of to particles that are packed into a column as in HPLC. When OTLC is operated at very small capillary diameters, the resolving power obtained is much greater than that obtained with packed-bed columns.²⁵ Theoretical predictions for OTLC, assuming reasonable operating conditions for time and pressure, suggest that optimum resolving power is obtained with columns having 2–5 μm inner diameters.²⁶ A key advantage to this technique is its ability, in fact necessity, to employ extremely small sample volumes.

Capillary Electrophoresis. Capillary electrophoresis^{4,27,28} provides a means to obtain the high-efficiency separations promised by microcolumns without the need for a coated or bonded stationary phase. The term capillary electrophoresis comprises several related techniques. Capillary zone electrophoresis generally implies an electrophoretic separation inside a solution-filled capillary. In this system, separation is based on the differential electrophoretic mobilities of ionic solutes as they migrate in the potential field. No gel support is used, and capillary dimensions are typically 2–100 μm inner diameter and 10–100 cm length. Other modes of capillary electrophoresis include micellar electrokinetic capillary chromatography,²⁹ capillary gel electrophoresis,^{30,31} capillary isotachopheresis,³² and capillary isoelectric focusing.³³ Virtually all of the work performed with single cells has been carried out in the capillary zone electrophoresis mode; hence, this technique will be emphasized here.

Capillary zone electrophoresis provides rapid, highly efficient separations of ionic species in extremely small volume samples. One unique advantage of narrow-bore capillary tubes is the enhanced heat dissipation via the capillary wall. This permits the use of very high potential fields and open solutions for fast, efficient separations. In addition to the enhanced heat dissipation, other advantages to the use of capillaries for electrophoresis exist. Electroosmosis is the flow of solvent in a capillary when a potential field is applied across the length of the capillary.³⁴ Electroosmotic flow

offers three key advantages for the separation of small biological samples. First, this flow, if not deliberately altered, is often strong enough to cause all solutes to elute at one end of the capillary. Thus, by placing a single detector at one end of the column, capillary electrophoresis is easily automated. Second, the ultrasmall volume flow rates in capillary electrophoresis permit sampling from microenvironments (i.e., single cells). Indeed, volumes as low as 270 fL have been injected using electroosmotic flow.¹⁵ Finally, electroosmotic flow provides a flat flow profile, since there is no stationary support between the origin of flow (capillary wall) and the bulk of solution. This leads to high-efficiency separations, and unless the capillary diameter is the same dimension as the solution double layer, electroosmotic flow is largely independent of diameter, pressure, and sample viscosity. This is, of course, a major advantage in the use of electroosmotic flow over externally applied pressure to acquire cellular or cytoplasmic samples.

Detection Methods for Microcolumn Separation. A great deal of work has been invested in the development of detectors for OTLC and capillary electrophoresis. Key developments in detector technology useful for neurochemical analysis have involved mass spectrometric, electrochemical, and fluorescence detectors. Although mass spectrometric detection has been used for neurochemicals³⁵ and shows great promise for the future, it is presently too insensitive to permit analysis of the extremely low levels of material found in single cells. Only amperometric^{3,4,13,15,36–39} and laser-based fluorescence detectors³ have been used with OTLC or capillary electrophoresis for single cell analysis.

Sensitive amperometric detection schemes have been developed for both OTLC⁴⁰ and capillary electrophoresis.^{4,38} In both of these systems, detection is accomplished using a small carbon fiber electrode placed inside the end of the capillary. To carry out amperometric detection in capillary electrophoresis, the detection electrode must be isolated from the high potential field employed for electrophoresis. This can be accomplished with a porous glass tube that is epoxied around a small crack in the electrophoresis capillary.^{4,38} Figure 2 shows one system employed for amperometric detection in capillary electrophoresis. Here, the separation potential is applied between the inlet of the capillary and the porous glass joint. Electroosmotic flow serves as a pump to push separated solutes past the porous glass coupler and to the detector.

Laser-based fluorescence has recently been employed with OTLC and capillary electrophoresis to detect amino acids in single nerve cells following homogenization of the cell and derivatization of the amino acids with naphthalene-2,3-dicarboxaldehyde.³ This work has allowed profiles of several amino acids to be obtained for single large invertebrate neurons by methods further elaborated on in the next section.

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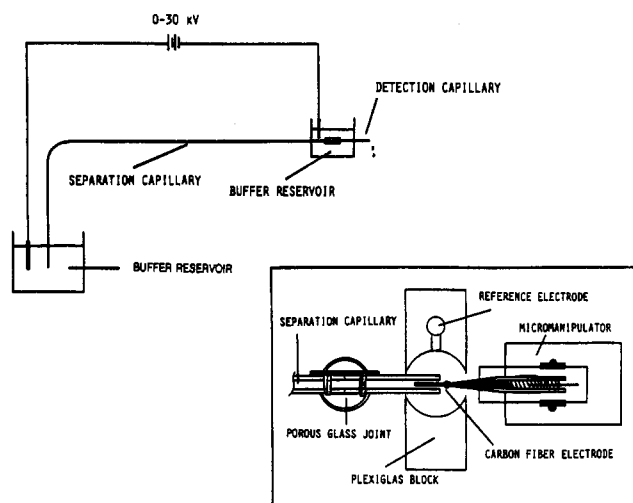


Figure 2. Schematic of CZE system with electrically conductive joint. Inset: Top view of amperometric detection system. Reprinted with permission from Ewing, A. G.; Wallingford, R. A.; Olefirowicz, T. M. *Anal. Chem.* 1989, 61, 292A-303A. Copyright 1989 American Chemical Society.

Other Techniques. Although this article will focus on voltammetric and separation-based probes of the single cell neuronal microenvironment, several other techniques for cell handling and chemical measurements have been developed for similar studies. These include methods involving enzyme activity, spectrophotometry, and immunoassay,^{16,41} microscale spectroscopic techniques,^{19,42} optical and electron microscopic techniques,^{43,44} and secondary ion mass spectrometry.⁴⁵

Experimental Results from the Neuronal Microenvironment

Voltammetric Analysis at Single Nerve Cells. Since the early use of carbon electrodes for in vivo neurochemistry,¹ several new electrodes, techniques, and applications have emerged. It is now possible to use voltammetric probes to follow real-time release of dopamine from nerve terminals in vivo following a single stimulus pulse.⁴⁶ A great deal of information has been obtained concerning neurotransmission, the interaction of drugs, and neurochemical dynamics in the extracellular fluid of the mammalian brain. More recently, these techniques have been scaled down to the level where neurotransmitter dynamics can be followed immediately outside and inside single hormone or nerve cells.

Extracellular Voltammetry at Single Nerve Cells. Carbon fiber electrodes have been used to monitor stimulated release from single bovine adrenal cells in

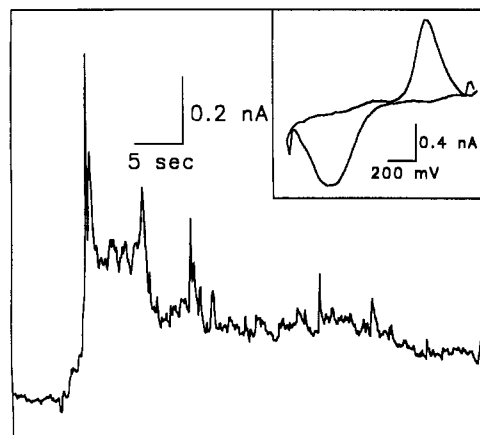


Figure 3. Current versus time profile of the response of a single adrenal modulatory chromatin cell to 100 μ M nicotine. Detection was performed with a carbon fiber microelectrode scanned at 300 V/s. Inset shows the background-subtracted cyclic voltammogram obtained at the maximal response. This voltammogram matches that for norepinephrine in vitro. Reprinted with permission from Ciolkowski, E. L.; et al. *J. Am. Chem. Soc.* 1992, 114, 2815-2821. Copyright 1992 American Chemical Society.

culture.^{11,12} The experiment is carried out by placing a micropipette and a microelectrode very close to a single adrenal cell. The micropipette is used to stimulate the cell with approximately 20 nL of a nicotine solution, and the neurotransmitter released is monitored voltammetrically at the carbon electrode (Figure 3). The exciting aspect of this experiment is the ability of this technique to discriminate release of single vesicles (termed granules for adrenal cells) of neurotransmitter from the single cell. Each of the large "spikes" observed on the current vs time plot represents neurotransmitter released from a single granule within the cell. This work has been extended to demonstrate the release of both norepinephrine and epinephrine, two neurotransmitters, from single cells,⁴⁷ and this result has been substantiated with microcolumn separation methods (see below). Analysis of the current observed for each granule release event provides an estimate of 5-8 amol of catecholamine in each granule.¹¹

Stimulated release of dopamine from a single neuronal cell body in the pond snail, *Planorbis corneus*, also results in single chemical "spikes" superimposed on the bulk release observed by voltammetry at carbon ring electrodes placed just outside the nerve cell (Figure 4). We have not yet confirmed that these spikes correspond to neurotransmitter release from single vesicles. However, the pattern displayed is very similar to that observed for release from adrenal cells, and difference voltammograms between the high and low responses shown in Figure 4b are similar to those for dopamine in vitro (Figure 4c). The average spike corresponds to approximately 0.1-0.5 fmol of dopamine molecules, which is consistent with the larger vesicles found in this system. Interestingly, vesicular release is not expected to occur at neuronal cell bodies in invertebrates. The voltammetric micromethods described collectively allow monitoring of microscopic phenomena never directly observed before (vesicular release) and chemical differentiation between vesicles

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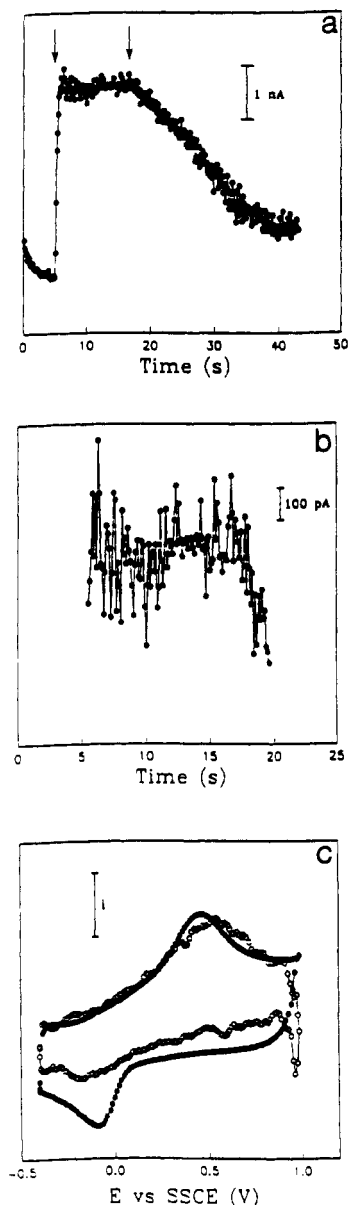


Figure 4. Oxidation current monitored at a $10\ \mu\text{m}$ carbon ring electrode placed immediately outside the cell body of the large dopamine neuron of *Planorbis corneus* following stimulation (at 5 s) with $15\ \mu\text{L}$ of 3 M KCl. Voltammograms were obtained every 110 ms at a scan rate of 300 V/s. (a) Current vs time obtained by averaging the current between 0.42 and 0.54 V on the positive scan of each voltammogram. (b) An expansion of the current vs time plot between the arrows shown in a. (c) Difference cyclic voltammograms: background-subtracted cyclic voltammograms obtained during an in vitro precalibration for a $10\ \mu\text{M}$ DA concentration increment. (●); cyclic voltammogram obtained by subtracting the average of 10 scans at the bottom of the observed peaks from 10 scans at the top of the observed peaks shown in b (○). Current scale: ●, $i = 1.3\ \text{nA}$; ○, $i = 0.1\ \text{nA}$.

in the same cell and may well lead to the discovery of new cellular phenomena.

Intracellular Voltammetry. Voltammetry has also been used to monitor drugs and neurotransmitters in the cytoplasm of single large nerve cells. Meulemans et al.⁶ have used electrochemically polished and glass-coated carbon fiber electrodes with tip diameters in the micrometer range for intracellular voltammetry. In these experiments, differential pulse voltammetry was used to monitor penetration of two electroactive drugs, metronidazol and antipyrine, into identified cholinergic

neurons of the marine snail, *Aplysia californica*. The results have been used to evaluate the kinetics of penetration and clearance of these drugs along with their maximum cytoplasmic concentration.

We have evaluated the use of carbon ring electrodes for voltammetric monitoring of neurotransmitters in single cell cytoplasm. Carbon ring electrodes have been placed in the large identified dopamine neuron of *Planorbis corneus*.^{7,8} Under normal cell resting conditions, the background level of dopamine in the cytoplasm of this cell is below the voltammetric detection limit for carbon ring electrodes (approximately $2 \times 10^{-6}\ \text{M}$). Voltammetric monitoring following cell membrane permeabilization with 50% ethanol allows a "snapshot" measurement of total dopamine in the cell. The ratio of total dopamine to the detection limit provides an estimate of the dopamine stores that are not in the cytoplasm and are presumably in vesicles. This ratio has been estimated at 98%, and it provides the first direct measurement of vesicular to cytoplasmic neurotransmitter stores in a single nerve cell.

The future of single cell voltammetric methods will include the development of electrodes capable of monitoring neurotransmitters and other substances that are not easily oxidized or reduced. Kuhr and co-workers are presently investigating the use of extremely small enzyme electrodes for rapid monitoring of the excitatory neurotransmitter glutamate at the single cell level.⁴⁸ We have recently developed a $2\ \mu\text{m}$ tip diameter enzyme electrode capable of monitoring glucose transients in single cell cytoplasm.⁴⁹ Figure 5 shows the amperometric response of one of these electrodes placed inside the large dopamine cell of *Planorbis corneus* following intracellular injection of picoliter volumes of glucose and control substances. Although oxygen levels can interfere with these measurements, the ability to qualitatively monitor glucose transients in single cell cytoplasm should allow the investigation of transient changes in single cell metabolic and respiratory processes during cell stimulation, neurotransmitter release, and neurotransmitter re-uptake.

Single Cell Analysis by Microcolumn Separation Techniques. A key aspect in the use of microcolumn separation techniques for analysis at the single cell level is the method of sample acquisition. Sample preparation and injection methods differ considerably between OTLC and capillary electrophoresis techniques as well as between analysis at the whole cell vs cytoplasmic levels.

Whole Cell Analysis. To profile compounds in single cells using OTLC, the general procedure is shown in Figure 6. This procedure^{36,37} involves isolation of a single cell by microdissection techniques and transfer of the cell via a micropipet to a 500-nL microvial. After addition of an internal standard, the cell is homogenized and centrifuged. A sample of supernatant is then injected by pressure from a micropipet into an OTLC column. A sample separation with electrochemical detection of cell components is shown in Figure 7. In this example, the electrode voltage has been scanned to provide a chromatovoltammogram to enhance se-

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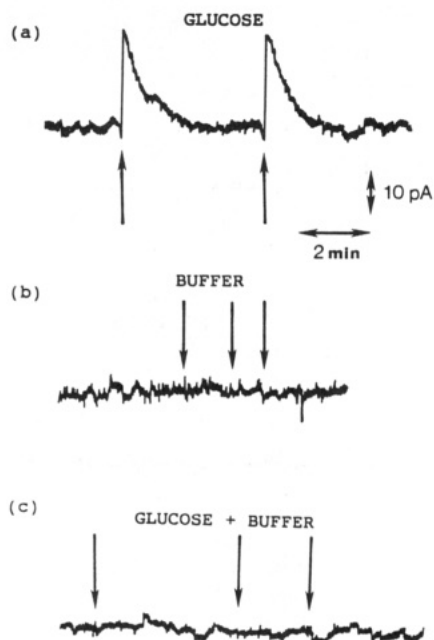


Figure 5. Current observed at a glucose oxidase electrode placed in the large dopamine neuron of *Planorbis corneus*: (a) response to a 2-pL 3 M intracellular glucose injection; (b) response to an 8-pL intracellular injection of pH 7.4 buffer only; (c) response of an electrode not coated with glucose oxidase to an 8-pL 3 M intracellular glucose injection. The electrode potential was 0.6 V vs SSCE in all cases. Reprinted with permission from Abe, T.; Lau, Y. Y.; Ewing, A. G. *Anal. Chem.* 1992, 64, 2160-2163. Copyright 1992 American Chemical Society.

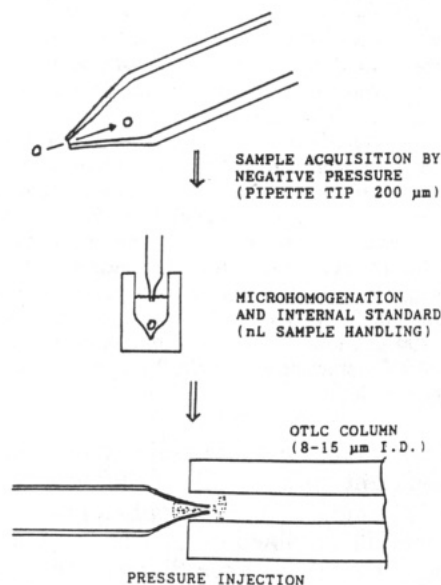


Figure 6. Procedure for whole cell analysis by OTLC.

lectivity. This method has been used to demonstrate the coexistence of dopamine and serotonin in a single neuron in *Helix aspersa*.^{3,37} Additionally, whole cells have been removed from *Helix aspersa* and the amino acids derivatized with naphthalene-2,3-dicarboxaldehyde followed by OTLC separation with amperometric detection.³⁹ The methods described here have also been used with this added derivatization step to apply both the OTLC and capillary electrophoresis techniques with laser fluorescence detection to whole cell profiling of amines.³ Finally, Jorgenson and co-workers have combined another microcolumn separation technique, microbore liquid chromatography, with similar proce-

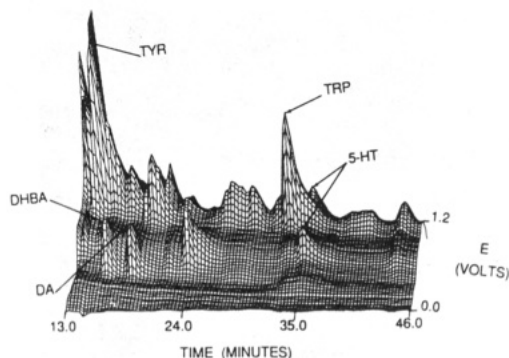


Figure 7. Chromatovoltammogram of cell F1 obtained using the method described in the text and shown in Figure 6. The column diameter was 19 μm i.d. The peak labels are as follows: DA, dopamine; DHBA, 3,4-dihydroxybenzylamine; 5-HT, serotonin; TYR, tyrosine; TRP, tryptophan. The voltammogram for serotonin has two peaks due to the two functionalities which are oxidizable at the electrode, namely, the phenol group and the indole nitrogen. Reprinted with permission from Kennedy, R. T.; Jorgenson, J. W. *Anal. Chem.* 1989, 61, 436-440. Copyright 1989 American Chemical Society.

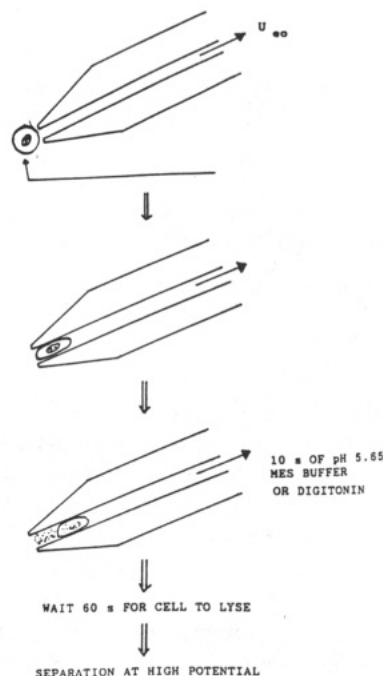


Figure 8. Procedure for whole cell injection and separation with CE.

dures to identify individual adrenal cells that contain norepinephrine, epinephrine, or the coexistence of both neuromodulators.¹⁴

Whole cell sampling can also be accomplished for capillary electrophoresis by directly drawing a cell into the tip of a capillary with electroosmotic flow and subsequently lysing the cell membranes inside the capillary.¹³ This holds the distinct advantage of providing 100% sampling of the cell components, leading to better detection limits for low-level species. However, direct sampling presently precludes sample derivatization and, hence, limits the detection modes available.

The procedure for direct sampling of whole cells by electroosmotic flow is shown in Figure 8. This experiment is performed with a capillary tip that has been chemically etched to a small diameter. With the aid of a microscope, the tip is placed in direct contact with

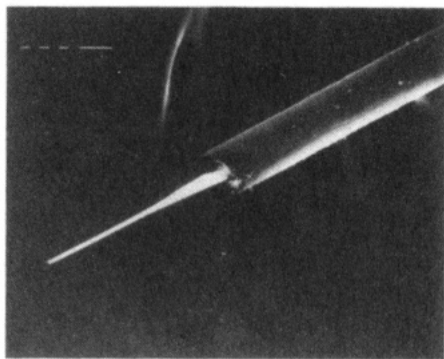


Figure 9. Scanning electron micrograph of an etched microinjector for acquiring and injecting cytoplasmic samples, constructed at the high-voltage end of a 5 μm i.d. electrophoresis capillary. Bar represents 100 μm . Reprinted with permission from Olefirowicz, T. M.; Ewing, A. G. *Anal. Chem.* **1990**, *62*, 1872–1876. Copyright 1990 American Chemical Society.

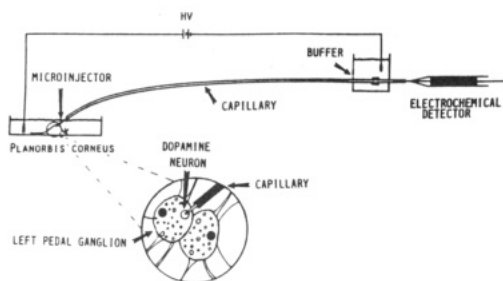


Figure 10. System used for removal, separation, and detection of cytoplasmic samples from single nerve cells with an expanded view of the left and right pedal ganglia of the brain of *Planorbis corneus*. Reprinted with permission from Olefirowicz, T. M.; Ewing, A. G. *Anal. Chem.* **1990**, *62*, 1872–1876. Copyright 1990 American Chemical Society.

the cell and an injection potential of 1–10 kV is used to transport the cell into the capillary. After the neuron is drawn into the capillary, a 10-s injection of non-physiological buffer is drawn into the capillary and the voltage is turned off for 60 s. This delay allows time for the cell to lyse. Following cell lysing, the cell components are separated and detected in the electrophoresis system. Separation of small, easily oxidized neurochemicals from single cells of *Planorbis corneus* has been carried out in this manner.¹³ However, we have also obtained capillary electropherograms for picoliter aliquots of single cell cytoplasm.

Sampling Cytoplasm. The extremely low volume capabilities of capillary electrophoresis allow manipulation of subcellular samples. Figure 9 shows an SEM of a capillary tip that has been chemically etched to provide an 8- μm microinjector for acquiring and injecting samples from single cell cytoplasm.^{7,50} A schematic diagram of the capillary electrophoresis system used for acquiring, separating, and detecting cytoplasmic samples directly obtained from single, intact neurons is shown in Figure 10. The etched electrophoresis capillary is inserted directly into a cell body of a single large dopamine (or serotonin) containing nerve cell. The neurons chosen for these studies have

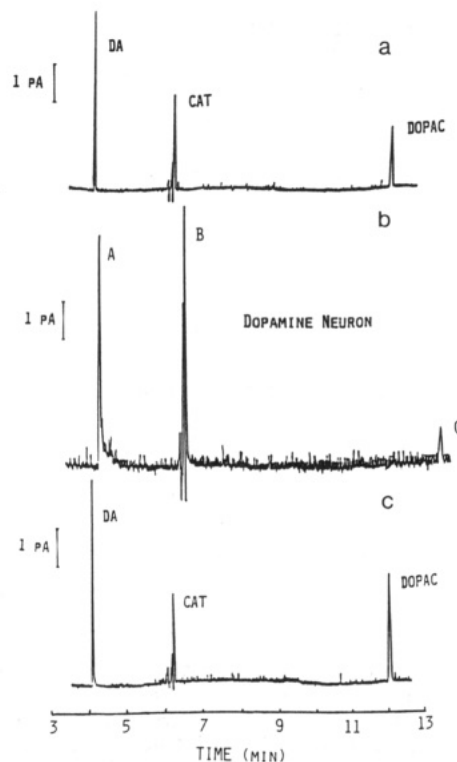


Figure 11. Capillary electrophoretic separations of standard solutions and a separation of a cytoplasmic sample. (a) Separation of authentic standards before cytoplasmic injection. DA = dopamine (105 amol), electrophoretic mobility is $2.40 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; CAT = catechol (60 amol) and DOPAC = dihydroxyphenylacetic acid (175 amol), electrophoretic mobility is $-2.22 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. The injection volume was 69 μL , based on electroosmotic flow. (b) Capillary electrophoretic separation of solutes in a sample of cytoplasm removed from inside the giant dopamine neuron of *Planorbis corneus*. The injection volume was 66 μL . Peaks A, B, and C have been identified as dopamine, a nonionic species, and dihydroxyphenylacetic acid on the basis of their calculated electrophoretic mobilities. The electrophoretic mobility of peak A is $2.40 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ and that of peak C is $-2.28 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. (c) Capillary electrophoretic separation of same authentic standards as in a obtained after the cellular experiment shown in b. Conditions for all separations: electrophoresis capillary length, 66 cm; buffer, 0.025 M MES, pH 5.65; injection, 5 s at 10 kV through a 700 μm long, 10 μm o.d. microinjector; separation potential, 25 kV; detection electrode, 0.7 V vs saturated calomel reference electrode.

been the relatively large and easily identified cells on the left and right pedal ganglia of *Planorbis corneus*.⁵¹

Figure 11 compares an electropherogram of a cytoplasmic sample obtained from the large dopamine neuron of *Planorbis corneus* (Figure 11b) to electropherograms of dopamine, catechol, and dihydroxyphenylacetic acid obtained from a standard solution (Figure 11a,c) before and after separation of the cytoplasmic sample, respectively. The peaks in the electropherogram of the cytoplasmic sample have been identified by calculating the electrophoretic mobilities of each peak and comparing them to those of authentic standards. The electrophoretic mobility of peak A from the cytoplasmic sample corresponds to that of the dopamine standard. The neutral peak (peak B) cannot be identified, even tentatively, by zone electrophoresis; however, it might contain a nonionic precursor of

(50) Olefirowicz, T. M.; Ewing, A. G. *J. Neurosci. Methods* **1990**, *34*, 11–15.

(51) (a) Marsden, C. A.; Kerkut, G. A. *Comp. Gen. Pharmacol.* **1970**, *1*, 101–116. (b) Penreath, V. W.; Berry, M. S.; Cottrell, G. A. *Cell Tissue Res.* **1974**, *151*, 369–384.

dopamine and possibly more than one component. The anionic peak (peak C) removed from the dopamine cell has an electrophoretic mobility similar to that of dihydroxyphenylacetic acid and appears to be this major metabolite, although it is not present in all of the dopamine cells examined.

Injection volumes for the cytoplasmic analysis, based on electroosmotic flow, ranged from 50 to 66 pL. However, since the injection of cations and anions involves both electrophoresis and electroosmotic flow, the apparent injection volume for these ions is different than the liquid injected. Thus, an apparent injection volume must be determined when using calibration standards that are based in total moles in order to calculate the concentration of the injected sample. This apparent injection volume, V , can be calculated by the equation

$$V = [\mu_e + \mu_{eo}]EA t_{inj}$$

where μ_e is the electrophoretic mobility of the substance, μ_{eo} is the coefficient of electroosmotic flow, E is the field strength, A is the cross-sectional area of the capillary bore, and t_{inj} is the injection time. In the experiment shown here, the apparent volume of injected cation was 103 pL. On the basis of the apparent injection volumes, results from several experiments indicate that the average cytoplasmic level of dopamine in the dopamine cell of *Planorbis corneus* is $3.0 \pm 0.79 \mu\text{M}$ ($n = 7$; SEM, standard error of the mean).

An additional point of interest in the data shown is that the peaks that appear to represent neurotransmitters removed from single cells (both dopamine and also for serotonin) are highly asymmetric. One explanation for this observation might be the sampling of vesicles from within single cell cytoplasm. This theory is currently under investigation.

Present experimental and long-range goals for microcolumn separation techniques include measuring and identifying neurotransmitters in single vesicles,⁵² measuring components of specific regions of neuronal membranes (i.e., growth cones, regions having a high

density of receptors or ion channels), and perhaps even measuring efflux of neurotransmitter from single synapses. The further miniaturization of microcolumn separations is bringing these goals closer to reality.

Concluding Remarks

Miniaturization of analytical techniques is providing new methods to examine neurochemical and neurophysiological events at the single cell level. In this article, we have presented an overview of recently developed techniques involving voltammetry at ultra-small electrodes and microcolumn separations. Voltammetry can be used to monitor neurotransmitter dynamics outside single nerve cells, in single cell cytoplasm, and perhaps at single synapses. One of the most exciting new revelations brought to light by these measurements is the apparent ability to observe the physiological event corresponding to the release of single neurotransmitter vesicles into the extracellular fluid. In addition, microcolumn separation methods provide a powerful approach to understanding single cell neurochemistry. Open tubular liquid chromatography and capillary electrophoresis have been scaled down to levels where picoliter and femtoliter volume samples can be separated. This provides the means to profile the chemistry of single whole nerve cells and to sample subcellular regions. The ability to quantitate and monitor chemical species in single cells and cellular compartments by these techniques should be widely applicable to other systems, including the determination of compartmentalization of catecholamines in adrenal cells. Clearly, the techniques in this area have led to many intriguing discoveries concerning single cell neurochemistry and with further development will lead to subcellular measurements of peptides, proteins, and intracellular second messengers, all en route to uncovering the way in which single nerve cells function.

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