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Single Neuron Analysis by Capillary Electrophoresis with Fluorescence Spectroscopy

Neurotechnique

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Summary

A technique to identify and quantitate simultaneously more than 30 compounds in individual neurons is described. The method uses nanoliter volume sampling, capillary electrophoresis separation, and wavelength-resolved native fluorescence detection. Limits of detection (LODs) range from the low attomole to the femtomole range, with 5-hydroxytryptamine (or serotonin [5-HT]) LODs being ~20 attomoles. Although the cellular sample matrix is chemically complex, the combination of electrophoretic migration time and fluorescence spectral information allows positive identification of aromatic monoamines, aromatic amino acids and peptides containing them, flavins, adenosine- and guanosine-nucleotide analogs, and other fluorescent compounds. Individual identified neurons from *Aplysia californica* and *Pleurobranchaea californica* are used to demonstrate the applicability and figures of merit of this technique.

Introduction

Simultaneous detection and quantitation of small signal molecules, metabolites, and cofactors at the level of individual cells provide background and fundamental information for cellular physiology. For identified neurons, such measurements aid in the identification of neurotransmitters and the mechanisms of their regulation. Among many modulators, 5-hydroxytryptamine (or serotonin [5-HT]) and related molecules are of particular interest because of widespread distribution in central and peripheral tissues, a broad spectrum of biological action, and critical roles in diverse pathologies (Cooper et al., 1996). Mapping of serotonergic neurons in invertebrate and vertebrate central nervous systems (CNS) reveals a number of specific cells (Steinbusch and Mulder, 1984; Walker, 1986), but only a few identified neurons have been characterized biochemically (e.g., Osborne, 1972; Goldman and Schwartz, 1974; Hanley et al., 1974); almost none of the cells have had multiple biologically active compounds simultaneously characterized. Indeed, absolute concentrations of 5-HT and the quantitative aspects of colocalization of 5-HT with other signaling molecules are not known for most cell types.

In general, past assays of 5-HT and other neuroactive compounds in single cells have generally been few, cell-system specific, and often dependent on highly molecule-specific detection methods. Over the years, various mass spectrometric, electrochemical, fluorescence, and enzymatic approaches have been devised to overcome some limitations of multicomponent single-cell analysis but often with limited applicability. Impressive research involving the detection of small molecules released from single cells has been reported in selective systems without chemical separation (Leszczyszyn et al., 1990; Wightman et al., 1991; Alvarez de Toledo et al., 1993; Bruns and Jahn, 1995; Chen et al., 1995). Mass spectrometry of single intact cells has also been shown to be useful in qualitatively determining multiple cellular compounds (Valaskovic et al., 1996). Researchers studying neuroactive compounds usually employ a separation method, such as high pressure liquid chromatography or capillary electrophoresis (CE), with a suitable detection scheme in order to avoid analytical interferences. CE has already proved to be useful for single-cell studies with various detection schemes (Kennedy et al., 1987; Jankowski et al., 1995; Lillard and Yeung, 1997; Swanek et al., 1997). Unfortunately, however, single-channel CE detection systems offer almost no qualitative information about a species being detected. Some detection schemes have been developed that address this limitation (Swanek et al., 1997), but chemical pretreatment and/or histological methods must complement most single-cell measurements in order to help identify analytes and are often performed on different cells than the one undergoing chemical analysis.

Combining CE with an information-rich spectroscopic detection scheme provides both qualitative and quantitative chemical information about the target species. We describe a technique using a CE separation coupled to a wavelength-resolved laser-induced native fluorescence detection system for the direct, single-neuron analysis of diverse aromatic monoamines and a variety of other compounds. This method uses ultraviolet laser excitation at 257 nm, which produces characteristic fluorescence emission spectra for many classes of biologically important compounds, thereby more completely identifying cellular constituents than does the separation alone. When the spectral information is combined with CE, the identification, purity, and amount of many natively fluorescent analytes can be simultaneously determined in a manner not possible with filter-based, single-channel fluorescence methods. We show that the method is direct, convenient, highly sensitive, and quantitative without the need for chemical derivatization. We successfully apply the technique to various identified neurons in feeding and locomotor neural networks of well-known neurobiological model systems, the gastropod mollusks *Aplysia californica* and *Pleurobranchaea californica*.

Results

General Description of the Method

The experimental equipment is schematically shown in Figure 1. A freshly isolated neuron is placed in a microvial, where it can be either stored at −60°C or −20°C

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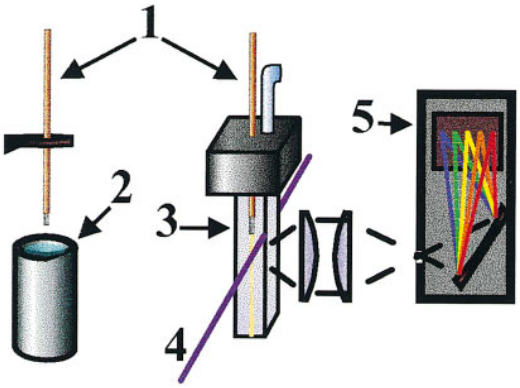

Figure 1. Schematic Drawing of Electrophoresis System

Drawing of the capillary electrophoresis and detection hardware. Cellular samples are electrokinetically drawn into the fused silica capillary (1) from a stainless steel microvial (2) after precise positioning with a motion controller system. The analytes separate in the capillary, elute into the square-cuvette sheath-flow cell (3), and are excited by 257 nm laser light (4). Light is collected orthogonally and directed into a CCD/spectrograph (5).

or mechanically and osmotically homogenized. Before analysis, the sample is diluted to 300 nl, and a small portion (typically, ~2 nl) of the sample is electroosmotically injected into a 50 μm i.d. capillary. Analytes separated by CE elute into a sheath flow detection cell and are excited by 257 nm laser light. The fluorescence emission from the analytes is collected by a CCD/spectrograph and analyzed as described below. The whole procedure starting from the sample isolation through data collection takes <40 min. Multiple analyses from the same individual neuron can be performed using modifications of the protocol to verify the identity of some of the peaks in the CE separation as well as to assay standards by spiking them into the same cellular sample. Many naturally occurring compounds are natively fluorescent and are detectable with this method, including the aromatic monoamines, aromatic amino acids, adenosine- and guanosine-nucleotide analogs, and others.

Analysis of Standards and Figures of Merit

A typical separation of selected standards is shown in Figure 2. The figure demonstrates the separation power and the spectral variability that can be recorded from a single analysis. Additionally, "wavelength-filtered" electropherograms (Figure 2B) are provided so that the reader can compare the richness of this method to standard filter-based methods. Many other detectable analytes of cellular importance were not included in Figure 2 to improve clarity in the figure. Some of these analytes include other indolamines (N-acetylserotonin, 5-hydroxytryptophan, 5-hydroxytryptophol, etc.), catecholamines (dopamine, norepinephrine, etc.), phenylalanine, guanosine-nucleotide analogs, adenosine-nucleotide analogs, coenzyme A, and others. Analytes studied and not detected under these conditions include NAD, NADP, uridine-nucleotide analogs, cytidine-nucleotide analogs, and pyridoxal-5-phosphate.

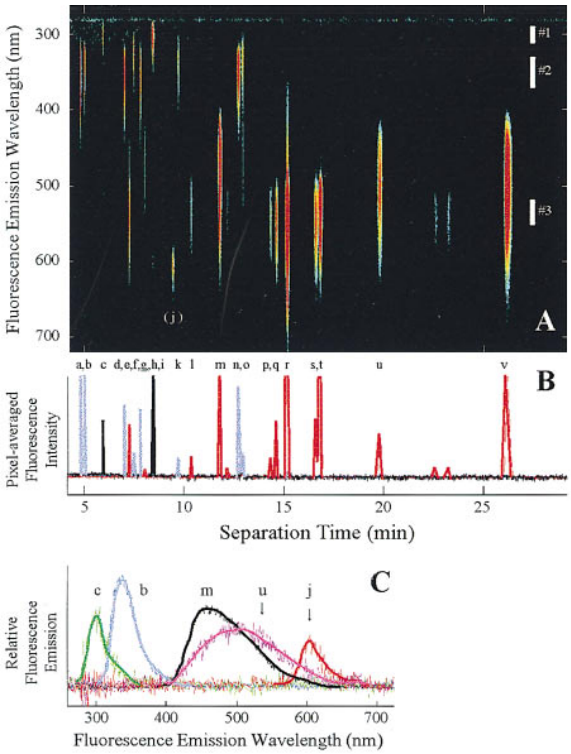

Figure 2. Analysis of Standards

(A) Single capillary electrophoresis separation showing complete fluorescence emission spectra (λ_{exc} = 257 nm) of several standards.

(B) Light intensity across three separate wavelength ranges (#1, 290–310 nm; #2, 330–365 nm; and #3, 520–550 nm) are averaged to generate "wavelength-filtered" electropherograms (black, blue, and red traces, respectively).

(C) Complete emission spectra of selected analytes from the same separation. Spectral intensities of 5-HT (b) and 6-biopterin (m) are divided by 2 and 4, respectively, to share the same intensity scale with the other analytes. Peaks were at 100–1000 times their limits of detection and were identified as follows: (a) tryptamine, 2.4 μM; (b) 5-HT, 2.4 μM; (c) octopamine, 12 μM; (d) melatonin, 2.3 μM; (e) THB, <2.5 μM; (f) epinephrine, 180 μM; (g) tryptophan, 3.6 μM; (h) THB impurity; (i) tyrosine, 59 μM; (j) sulforhodamine 101, 240 nM; (k) thiamine-5-phosphate, 1.2 mM; (l) 6-biopterin impurity; (m) 6-biopterin, <5.7 μM; (n) 5-HIAA, 23 μM; (o) cocarboxylase, 3.6 mM; (p) FMN impurity; (q) FAD impurity; (r) FMN, <29 μM; (s) FAD, <29 μM; (t) fluorescein, 2.4 μM; (u) β-NADH, 240 μM; and (v) β-NADPH, 1.2 mM. Identified impurities were present in the supplied reagent and/or were the result of rapid decomposition in solution. Unlabeled peaks are unknown impurities in the standards.

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); THB, 5,6,7,8-tetrahydrobiopterin; 5-HIAA, 5-hydroxyindole acetic acid; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; β-NADH, β-nicotinamide adenine dinucleotide, reduced form; β-NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; NAS, N-acetylserotonin (data not shown); 5-HTP, 5-hydroxytryptophan (data not shown).

Analytes in cellular samples are identified by correlating CE migration times and complete fluorescence emission spectra with standards. In some cases, a cellular analyte peak migrating at a time consistent with a given standard does not have the spectral characteristics of that same standard. In filter-based fluorescence systems, the detected signal might have been mistaken

Table 1. Figures of Merit for Model Analytes

Analyte	Limits of Detection (LOD) from Complete Spectra†			Emission Maximum† ($\lambda_{em,max}$) (nm)	Wavelength Range with Maximum Signal-to-Noise Ratio (nm)
	Best LOD (nM)	Mean LOD ± SEM‡ (nM)	n		
Tryptophan	5.0	9.1 ± 1.3	8	352 ± 2	350 – 375
Tryptamine	24	26 ± 3	2	349 ± 3	315 – 375
5-Hydroxytryptamine	3.0	38 ± 20	4	333 ± 2	330 – 355
Tyrosine	39	57 ± 6	6	300 ± 2	290 – 330
Phenylalanine	240	590 ± 140	4	280 ± 2	270 – 295
Dopamine	850	860 ± 10	2	322 ± 2	305 – 350

† 0.90 nm/binned CCD pixel, 500 wavelength elements (pixels), nonsmoothed data, 50 mM borate (pH 8.7).
‡ SEM, standard error of the mean, based on s_{n-1}.

for a known analyte and quantitated on the basis of migration times and fluorescence intensity alone. Our system was able to prevent the misidentification of many similarly migrating peaks by demonstrating minor spectral inconsistencies. The measurement of 5-HT and tryptamine is a good example of the need for wavelength-resolved fluorescence detection. These two analytes are easily confused, because they are usually found together as the first two detectable compounds to elute from most cellular samples with migration times within 10 s of each other. However, the fluorescence emission maxima and spectral shapes of the two compounds are sufficiently different to permit the compounds to be distinguished spectrally (Table 1). In some cases, compounds with similar fluorescence emission spectra with as little as 5–10 nm spectral shift were effectively identified as interferants.

All detected species are quantitatively determinable from calibration curves. Calibration curves are linear (typical $r^2 > 0.99$) from ~1 mM, or maximum soluble concentration, down to the limit of detection. Signal intensities of repetitively injected analytes are 5%–10% relative standard deviation. Table 1 presents the typical and the best limits of detection for selected model analytes.

Single-Cell Analyses

Data from a representative subset of cells are presented in Figures 3–5 and summarized in Table 2. These data demonstrate that chemical species can be accurately

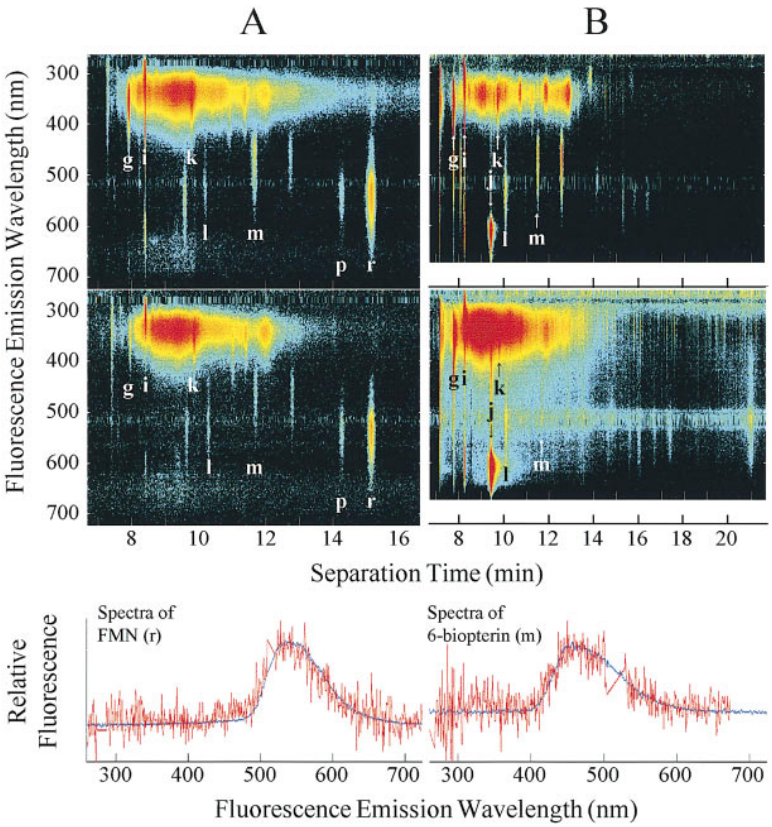

Figure 3. Comparison of Identified Neurons and Spectral Identification of Neuronal Components

(A) CE/native fluorescence analysis of two separate NADPH-diaphorase–reactive caudal cells (top, center) from the cerebropleural ganglion of *Pleurobranchaea*. Similarities in size (diameter, 400 μm), location, and NADPH-diaphorase staining suggest that these neurons are nearly identical, a result confirmed in this analysis. Flavin mononucleotide (FMN) is identified in these cells both electrophoretically and spectrally by comparison to standards. The FMN spectrum from the top electropherogram (red trace) is compared to the intensity-normalized standard spectrum (blue trace) studied similarly in Figure 2 (bottom). See Figure 2 for peak identification.

(B) Injections from the two giant pleural lobe cells (top, center) from the same ganglion. While the cells are similar in size (diameter, 350 μm) and have similar histochemistry to the caudal cells (above), results here suggest that the two cell-types contained differing amounts of many compounds at the time of dissection. 6-Biopterin is identified from the top electropherogram, compared to standard 6-biopterin, and displayed as described in Figure 3A (bottom). See Figure 2 for peak identification and Figure 4 for locations of the neurons.

Table 2. Summary of Analyte Amounts and Concentrations Calculated from *Pleurobranchaea* Single-Neuron Analysis

		Cell Type						
		Caudal NADPH-d Reactive Cell #1	Caudal NADPH-d Reactive Cell #2	Giant Pleural Lobe Cell #1	Giant Pleural Lobe Cell #2	Metacerebral Cell	Dorsal White Cell	Metacerebral Cell*
Cell diameter (±25 μm)		400	400	350	350	225	300	250
Cell volume (nL)		34 ± 7	34 ± 7	22 ± 5	22 ± 5	6.0 ± 2.4	14 ± 4	8.2 ± 2.9
Analyte†		Amount of Material in Cell (pmol)/Concentration in Cell (μM) ‡						
a	Tryptamine	**<0.0024 (<0.072)**	**<0.0027 (<0.079)**	**<0.0014 (<0.062)**	**<0.00098 (<0.045)**	**<0.0026 (<0.44)**	**<0.0019 (<0.14)**	0.0069 § (0.84)
b	5-HT	**<0.0025 (<0.072)**	**<0.0025 (<0.074)**	**<0.0012 (<0.057)**	**<0.00095 (<0.043)**	2.0 (340)	**<0.0018 (<0.13)**	0.75 (94)
w	NAS + 5-hydroxytryptophol	**<0.0025 (<0.072)**	**<0.0028 (<0.083)**	**<0.0020 (<0.091)**	**<0.0020 (<0.091)**	0.063 **(11)**	**<0.0025 (<0.18)**	0.0026 § (0.32)
n	5-HIAA	**<0.034 (<1.0)**	**<0.038 (<1.1)**	**<0.019 (<0.88)**	**<0.014 (<0.63)**	0.83 (140)	**<0.027 (<1.9)**	**<0.037 (<4.5)**
g	Tryptophan (+ 5-HTP)	0.66 †† (20)	0.13 †† (3.9)	0.97 †† (43)	2.0 †† (89)	2.1 (350)	2.8 †† (200)	1.8 (220)
i	Tyrosine	22 (660)	2.4 (72)	6.2 (280)	18 (800)	35 (5900)	17 (1200)	53 (6400)
m	6-Biopterin	0.029 (0.87)	0.027 (0.81)	0.0013 § (0.058)	0.016 (0.71)	0.014 (2.3)	0.10 (7.1)	0.029 (3.6)
l	6-Biopterin impurity	yes ‡‡	yes ‡‡	yes ‡‡	yes ‡‡	yes ‡‡	yes ‡‡	yes ‡‡
k	Thiamine-5-phosphate	170 (5100)	200 (5800)	14 ‡ (630)	6.6 § (290)	**<8.8 (<1500)**	**<7.0 (<500)**	**<9.3 (<1100)**
o	Cocarboxylase	**<2.1 (<60)**	**<2.5 (<75)**	**<1.2 §§ (<55)**	**<0.86 (<39)**	**<1.9 (<320)**	**<1.7 (<120)**	**<2.1 (<260)**
e	THB	**<0.0088 (<0.26)**	**<0.0078 (<0.23)**	**<0.0014 (<0.065)**	**<0.0032 (<0.15)**	**<0.0092 (<1.5)**	**<0.0017 (<0.12)**	**<0.0093 (<1.1)**
h	THB impurity	**n.d.**	**n.d.**	**n.d.**	**n.d.**	yes ‡‡	yes ‡‡	yes ‡‡
r	FMN	0.21 (6.3)	0.26 (8.3)	**<0.00065 (<0.030)**	**<0.0016 (<0.071)**	**<0.0044 (<0.73)**	0.0057 (0.39)	**<0.0051 §§ (<0.63)**
p	FMN impurity	yes ‡‡	yes ‡‡	**n.d.**	**n.d.**	**n.d.**	yes ‡‡	**n.d.**
s	FAD	**<0.062 (<1.8)**	**<0.061 (<1.8)**	**<0.0091 (<0.41)**	**<0.022 (<0.99)**	**<0.061 (<10)**	0.11 § (7.8)	**<0.072 (<8.7)**
q	FAD impurity	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	yes ‡‡	**n.d.**

The boldface entries indicate that the analyte was not detected (n.d.) and/or reliably identified; values therein represent detection limits (3σ) for the amount and concentration of the analyte contained within the cell. (Values in parentheses are concentration results.)

* Cell from *Aplysia*.

† See Figure 2 for abbreviations.

‡ Amounts of material found in cell are measured directly by fluorescence, while concentrations of analytes are calculated by estimated cell sizes and spherical volume calculations. Therefore, errors associated with cellular concentrations should be propagated from cell-sizing errors.

§ Integrated peak value (A) compared to noise (σ): $3\sigma < A < 10\sigma$. Compound is qualitatively identified but is not reliably quantitated.

†† This value accurately represents tryptophan quantities since no 5-HTP contribution is expected in cells where all other 5-HT metabolites are absent.

‡‡ Standard unknown/unavailable for quantitation purposes.

§§ Identified peak too small or has too many interferences for reliable quantitation/spectral identification.

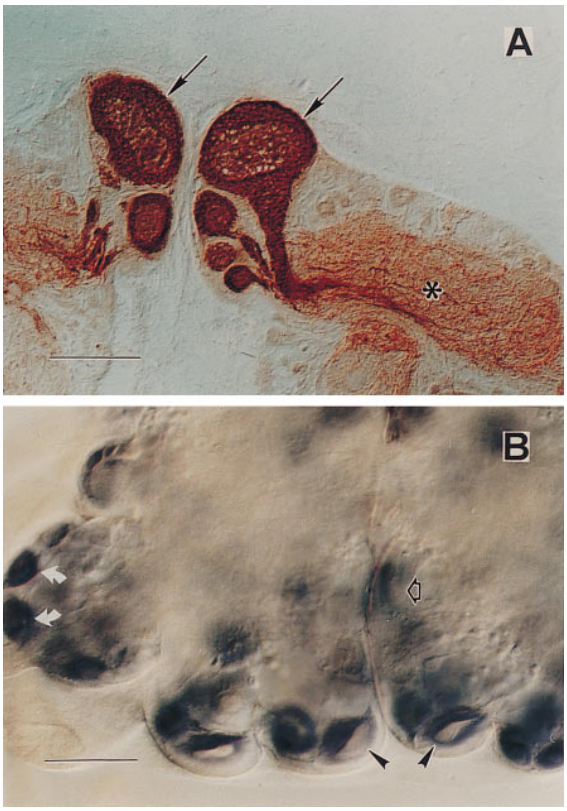

Figure 4. Comparison of Identified *Pleurobranchaea* Neurons Using Histochemistry

(A) A pair of 5-HT–immunoreactive metacerebral neurons (arrows) in the anterior area of the cerebropleural ganglion (cryostat section). The asterisk (*) indicates a 5-HT–immunoreactive neuropil. Scale bar, 200 μm.

(B) NADPH-diaphorase–reactive (NADPH-d–reactive) neurons in the posterolateral areas of the cerebropleural ganglion (whole-mount preparation, dorsal view). Black arrows show the location of the labeled caudal neurons; white arrows indicate the position of positively stained neurons in the pleural lobe of the ganglion; an open arrow points out the location of an NADPH-d–negative giant peptidergic neuron (the dorsal white cell); note that the weakly NADPH-d–reactive neurons (out of the photograph focus) are located on the ventral surface under the marked cells. Scale bar, 400 μm. (For staining protocol, see Moroz et al., 1997.)

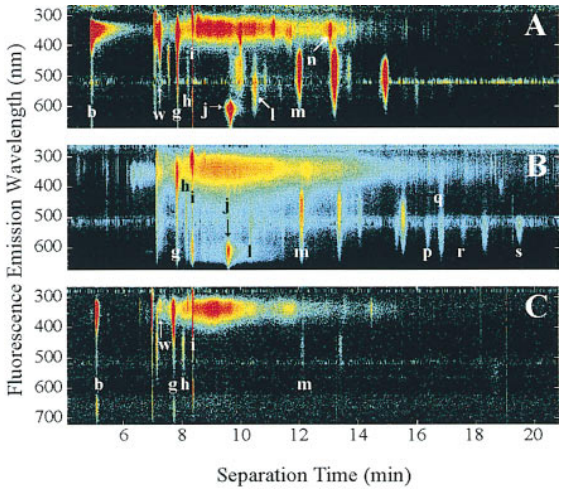

Figure 5. Comparison of Serotonergic Metacerebral Cells from *Pleurobranchaea* and *Aplysia* and a Third 5-HT–Negative, Peptide-Containing Dorsal White Cell from *Pleurobranchaea*

(A) The left metacerebral cell (MCC), known to be 5-HT–immunoreactive, has measurable quantities of several 5-HT pathway metabolites.

(B) A single peptide-containing dorsal white cell from the cerebropleural ganglion from the same *Pleurobranchaea* is obviously devoid of classical indolamines but has significant quantities of fluorescent amino acids and other housekeeping compounds.

(C) The *Aplysia* MCC predictably contains significant levels of 5-HT but has few strongly fluorescent components compared to its homologous *Pleurobranchaea* counterpart.

Peaks are labeled as in Figure 2. Sulforhodamine 101 served as an internal standard. Peak (w), corresponding to N-acetylserotonin and/or 5-hydroxytryptophol, is not shown in Figure 2 but is identified here both electrophoretically and spectrally with laboratory standards. Quantitative results for many of the compounds found in these cells are in Table 2.

identified with this technique and compare the contents of different identified neurons. For example, Figure 3A shows the analysis of two similar NADPH-diaphorase–reactive caudal cells from the cerebropleural ganglion of *Pleurobranchaea* (Moroz and Gillette, 1996). These cells appear histochemically similar (Figure 4B), and the electropherograms in Figure 3A document that these two cells are also similar in their natively fluorescent constituents. In contrast, histochemically equivalent neurons from the giant pleural lobe of the same ganglion (Figure 4B) appear very different in a single-cell assay (Figure 3B). Although many peaks appear to be present in both cell types, large differences in the concentrations of many analytes and even the presence of different species are apparent. The biological significance of this and other findings is under investigation, and the unknown peaks are currently being identified using additional standards.

An additional example of how this technique is useful is in the intercellular comparison between single homologous neurons from different species. Figure 5 rapidly highlights the similarities and differences between homologous 5-HT–containing feeding modulatory neurons: a single metacerebral cell (MCC) from *Pleurobranchaea* (Figure 4A) is compared to a 5-HT–negative, peptide-containing *Pleurobranchaea* dorsal white cell (Figure 4B) and also with an MCC from *Aplysia*. The homologous neurons from different species show a high degree of similarity in electropherogram patterns, verifying both characteristic cell-specific constituents and the reproducibility of the method. Careful analysis reveals that there are several identified and unidentified components present in measurable quantities in some but not all of the three cells (Table 2).

While the chemical differences between single cells is potentially interesting for comparative physiology studies, the similarities in cellular housekeeping components are also significant. The ubiquitous tyrosine, tryptophan, and 6-biopterin can be easily monitored and are at similar concentrations in most assayed cells. When maximum and minimum cellular concentration values are removed from consideration in Table 2, the steady-state concentrations of these housekeeping compounds can be estimated (mean $\pm$ s_{n-1}; n = 5): tyrosine, 810 $\pm$

Table 3. Single-Cell Statistics for Tryptamine and 5-Hydroxytryptamine

Metacerebral Cells		Tryptamine		5-HT	
		Amount (fmol)	Concentration (μM)	Amount (pmol)	Concentration (μM)
Left	Animal 1	21 $\pm$ 2	1.7 $\pm$ 0.5	2.2 $\pm$ 0.2	190 $\pm$ 60
	Animal 2	41 $\pm$ 4	3.4 $\pm$ 1.1	22 $\pm$ 2	1800 $\pm$ 600
	Animal 3	13 $\pm$ 1†	1.1 $\pm$ 0.3†	4.6 $\pm$ 0.5	390 $\pm$ 120
Right	Animal 1	45 $\pm$ 5	3.7 $\pm$ 1.2	3.6 $\pm$ 0.4	300 $\pm$ 100
	Animal 2	20 $\pm$ 2	1.7 $\pm$ 1.5	7.6 $\pm$ 0.8	630 $\pm$ 200
	Animal 3	10 $\pm$ 1†	0.83 $\pm$ 0.26†	1.5 $\pm$ 0.2	130 $\pm$ 40
Mean $\pm$ s_{n-1} (n = 4)‡		24 $\pm$ 14	2.0 $\pm$ 1.1	4.5 $\pm$ 2.6	380 $\pm$ 220

† Integrated peak value (A) compared to noise (σ): $3\sigma < A < 10\sigma$. Compound is qualitatively identified but not reliably quantitated.
‡ Minimum and maximum values removed from statistical analysis to minimize skewing.

410 μM; tryptophan, 78 $\pm$ 82 μM; and 6-biopterin, 1.1 $\pm$ 0.8 μM. Although the validity of reporting this data with parametric statistics is uncertain, these values are consistent with other data from our laboratory (not shown) and published elsewhere (Kennedy et al., 1987, 1989; Kennedy and Jorgenson, 1989; Oates et al., 1990).

Single-Cell Statistics

In addition to the comparative single-cell studies outlined above, six *Aplysia* MCCs were studied from three identically sized animals and quantitatively assayed for tryptamine and 5-HT. These MCCs were expected to have similar cellular contents and were chosen for statistical purposes. Each cell was observed to have a mean diameter of 285 $\pm$ 25 μm. Quantitation errors in any given measurement were estimated at 10%, while cellular concentration errors were estimated at $\sim$30% because of cell-sizing approximations. Individual values and statistics are reported in Table 3.

Discussion

The technique allows the simultaneous reliable identification and quantitation of cellular components with attomole to femtomole detection limits. Assays of both standard mixtures and multiple injections of the same types of identified neurons (e.g., MCC from *Aplysia*) show a high level of reproducibility, with absolute amounts and concentrations comparable to other literature reports (Brownstein et al., 1974; Ono and McCaman, 1984; Kennedy et al., 1987, 1989; Kennedy and Jorgenson, 1989; Oates et al., 1990). While a limitation of this method is its selectivity toward aromatic or π-conjugated analytes, the very same selectivity enables detected analytes to be quantitated directly, without concern for derivatization, enzymatic reactions, radiolabeling, or other chemistries. Along with many other useful electrochemical, enzymatic, immunological, and imaging techniques available, direct assays of cells by wavelength-resolved native fluorescence following a high efficiency separation promises to be very useful.

While laser-induced native fluorescence experiments with single cells have been documented in the literature (Chang and Yeung, 1995; Lillard et al., 1996; Tong and Yeung, 1997), prior work has been based on single-channel detection. As shown in the figures, much more reliable information on analyte identity is obtained when using spectral data. For example, CE of cells can have problems with nonreproducible migration times dues to proteins and lipids coating the walls of the fused silica capillary. We account for migration time and injection variability by spiking samples with the markers/internal standards fluorescein and/or sulforhodamine 101 at known concentrations, 10^{-8}–10^{-7} M. As these have distinguishing fluorescence properties different from all native cellular components, these internal standards do not interfere with the analysis and greatly increase the reliability and quantitative nature of our results. In addition, the cells studied contain measurable amounts of tyrosine and tryptophan, which serve as additional electrophoretic standards. Adding known amounts of biologically active compounds such as 5-HT to selected experimental samples allowed us to monitor decay and degradation of the analytes due to sample handling procedures. Overall, the technique is reproducible and robust, even when working with complex samples and sample matrices.

We can identify more than 30 components in single cells; in addition, we observe a number of unidentified peaks that we are currently attempting to identify. Many of the standard reagents contained a number of unidentified impurity peaks, presumably associated with the biological origin of some of the standards. Interestingly, many of the identified cells studied showed varying amounts of the same naturally occurring, unidentified "impurities" as determined by electrophoretic and spectral data. Thus, it is likely that compounds related to the metabolism of many of the standards were being observed in the cells.

There are limited data related to absolute intracellular concentrations of neurotransmitters, different metabolites, and cofactors in single cells. A comparison of the values described here with radioimmunoassay data for 5-HT in selected molluskan neurons shows the same order of calculated concentrations: 0.2–1 mM for MCC (Brownstein et al., 1974; Ono and McCaman, 1984) and 0.2–4.5 mM for some 5-HT–immunoreactive motoneurons (Ono and McCaman, 1984). Recent measurements of the 5-HT level in secretory granules of rat basophilic leukemia cells yield a value of $\sim$50 mM (Maiti et al., 1997). Thus, taking into account a very high cytoplasm:granule volume ratio, and assuming the presence of similar types of granules in the molluskan neurons, we predict high micromolar to low millimolar 5-HT concentrations

in many neuronal cell types, in accordance with our measurements. Our findings support the conclusion that high intracellular concentrations of 5-HT and related compounds are neuron-specific, as we observe a >10,000-fold difference in concentrations in nearly adjacent neurons. Additionally, we detect a number of 5-HT metabolites in serotonergic neurons (such as N-acetylserotonin and 5-hydroxyindole acetic acid) and their absence in 5-HT–immunonegative cells. Tryptophan and 5-hydroxytryptophan (5-HTP) concentrations were also higher in serotonergic neurons compared to other cell types. Interestingly, a wide range of concentrations were found for several cofactors such as 6-biopterin, which can vary by >100-fold among neurons.

Experiments have been done with cells from 50–500 μm in diameter that give clear, interpretable, multicomponent separations. For smaller cells, the effective sensitivity of the technique can be improved by minimizing sample dilution. In the current experiments, the cells are homogenized and diluted to ~300 nl before injection, ~10–30 times their internal volumes. Since only ~2 nl of the sample volume is injected for CE analysis, ~1% of the cellular material is actually being studied. The current injection system can easily be modified to a volume of 30 nl, allowing for an order-of-magnitude increase in signal; however, for the larger neurons assayed here, several compounds such as 5-HT might exceed the upper end of the dynamic range of the instrument if this change was made. In addition, a further 10-fold sensitivity enhancement is expected from our system if an entire cell was injected without dilution in a manner similar to other literature reports (Swanek et al., 1996; Cruz et al., 1997). Overall, the approach is expected to significantly optimize collection of chemical information from neuronal samples.

Experimental Procedures

Reagents

The running buffer was ~50 mM borate (3.0 g boric acid [H_3BO_4; EM Science, Gibbstown, NJ or Sigma, St. Louis, MO], 9.2 g sodium borate [$Na_2B_4O_7 \cdot 10\ H_2O$; EM Science or Sigma] in 1.00 l ultrapure water [Millipore, Bedford, MA] [pH 8.65 ± 0.04]). All standard solutions were prepared in borate buffer. All standards were obtained from Sigma and were reagent quality or better.

Animals

Specimens of *Pleurobranchaea californica* (Opistobranchia: Notaspidea) (300–1000 g) were collected from the Pacific coast of California or were obtained from Sea-Life Supply (Sand City, CA) and kept in artificial seawater at 15°C for up to a month before experiments. *Aplysia californica* were obtained from the Aplysia Research Facility (Miami, FL) and Marinus (Long Beach, CA) and were stored similarly. *Aplysia* weighed ~300 g for most experiments, except for the single-cell statistics experiments, which used 100 ± 10 g animals.

Cell Isolation and Sample Preparation

Individual ganglia from the CNS were dissected from *Aplysia* or *Pleurobranchaea* under cold anesthesia in molluskan physiological saline. After a 20 min incubation in proteolytic enzyme mixture (1% protease dispase and 0.2% protease, Type XIV, Sigma) at room temperature, ganglionic connective tissues were removed with tungsten needles, identifiable neuron somata were isolated under a stereomicroscope using a microsyringe or micropipettes, and cells were maintained in physiological saline for up to 2–3 min. Freshly isolated molluskan neurons were sized microscopically to ±25 μm and then transferred by micropipettes into 100 or 200 nl of borate buffer in a microvial (see below). Within 1 min, a neuron could be easily homogenized by the combined action of tungsten needle manipulation and hypoosmotic buffer damage. The samples were either diluted to 300 nl and placed directly into the capillary electrophoresis system for analysis or immediately frozen on dry ice for storage. In control experiments, no significant differences were observed between freshly isolated and frozen samples. Many single-cell samples suffered evaporative/sublimation losses, and some had become variably dried before analysis; in this case, the cellular tissue was manually remixed with ultrapure water to resuspend/redissolve the sample and running buffer salts to ~300 nl. The single neurons were studied and analyzed blind to reduce bias; after analyte peaks were identified and quantitated, the identities and sizes of the cells were revealed.

Electrophoresis System

The CE system was assembled in our laboratory with a 850 mm of 50 μm i.d./140 μm o.d. untreated fused silica capillary (Polymicro Technologies, Tucson, AZ) and was similar to a previously described system (Fuller and Sweedler, 1996). A servo-type linear actuator (model 850B-HS; Newport, Irvine, CA) supported the injection tip of the capillary and positioned it vertically over a rotary stage (model 495; Newport) that was used to move samples and buffers into position under the capillary. Both stages were controlled by a programmable motion controller (model PMC-200P; Newport). With the actuators, the capillary was positioned to ±2 μm vertically and ±4 μm in the sample radius for use with machined microvials. Injections were made from a stainless steel 316 disk, which contained both 110 μl wells and microvial supports. Two different types of microvials were machined from stainless steel to fit snugly into the microvial supports: one type consisted of a 1 mm deep, 0.5 mm i.d., ~200 nl cylindrical well and was used for limited-volume samples; the other type contained a 0.4 mm deep cone, ~300 nl, and was used for single-cell manipulation and injections.

The detection end of the capillary was directed into a laboratory-assembled sheath-flow assembly (Timperman et al., 1995). The capillary was centered into a 1 mm × 1 mm × 20 mm quartz cuvette (NSG Precision Cells, Farmingdale, NY) serving as a sheath-flow chamber. Sheath flow was generated by hydrodynamic pressure from a buffer-filled reservoir elevated above the height-matched injection wells and sheath outlet reservoir. Linear sheath-flow velocity was 0.95 ± 0.05 mm/s. The sheath outlet reservoir was electrically connected to a 30 kV power supply (Glassman) for electrophoresis between the grounded injection disk and the sheath-flow cell; total noncapillary voltage drop was estimated at <0.01%.

Excitation of the core stream in the sheath-flow cell was provided by a frequency-doubled, liquid-cooled argon-ion laser (Innova 300 FrED; Coherent, Palo Alto, CA) operating at 257 nm. Approximately 0.8 mW were directed into the sheath-flow cell and focused to a spot 0.5–1.0 mm below the capillary outlet with a 20 mm focal length quartz spherical concave lens (Spindler and Hoyer, Medford, MA). Collection optics were orthogonal to the excitation beam and consisted of a 15× all-reflective microscope objective (Opticon, Billerica, MA) and a 30 mm focal length quartz spherical concave lens focusing the fluorescence emission onto a 300 μm slit attached to an f/2.2 CP140 imaging spectrograph (Instruments SA, Edison, NJ). A 1,024 × 256 detector-array, liquid nitrogen-cooled, scientific CCD (EEV15–11; Essex, UK) was mounted at the focal plane of the imaging spectrograph and was controlled by an AT200 controller card (Photometrics, Tucson, AZ). This CCD is overcoated with a phosphor down-converter (Metachrome II; Photometrics) and has a nearly linear wavelength response from 200–900 nm. While the CCD operated without a shutter and was oriented to permit subarray readout and binning while preserving 1,024 pixel wavelength information over the 220–670 nm or 260–710 nm wavelength range focused across the CCD face, the spectral data presented here was acquired with 2 × 256 binning (~500 wavelength elements). CCD data was 16 bit and was read out at 50 kHz/pixel. The CCD spectrograph was wavelength calibrated with the 10 most intense lines from a Hg(Ne) pen-lamp (Oriel, Stratford, CT). Focusing and optimization were completed with PMIS image-processing software (Photometrics).

Computer software written in C with both LabWindows V3.1 (National Instruments, Austin, TX) and Microsoft C++ V7.00 (Microsoft,

Redmond, VA) controlled all aspects of the injection, separation, data acquisition, and data storage. Briefly, programming code was installed into the CCD's digital signal processor, which when triggered would transfer a predefined readout array to the AT200 controller card. The trigger was programmed automatically and was generated by an A/D timing card (AT-MIO-16F; National Instruments). Spectral data returned from the CCD after each read were briefly manipulated and stored directly onto hard disk. Data acquisition was set to record and store complete spectra at 2 Hz.

Capillary Electrophoresis Analysis of Standards

Before analysis, the CE system was operated at 25 kV with ~50 mM borate buffer for at least 30 min for equilibration. The separation conditions in ~50 mM borate were chosen based on the electromigration and isolation of 5-HT from other interfering molecules. Injections were performed electrokinetically at 2.5 kV for 10 s ($I = 2.4$ μA), and separations were performed at 25 kV ($I = 25$ μA). Control injections performed from the 110 μl wells and the two types of microvials gave identical results.

Standard calibration curves of at least five points for each analyte were generated for analytes at varying concentrations. The concentration where the best-fit line (linear least squares) for the calibration curve crossed the 3σ (noise) intensity value was taken to be the limit of detection. Alternatively, for low concentration injections, a single electropherogram would be used to compute a limit of detection (3σ) based on peak height and calculation of root-mean-square noise in the baseline before and/or after the peak. Injections of individual analytes provided the data required for the electromigratory and spectral identification of standard compounds from mixtures. Data acquisition parameters and analysis parameters for cell/tissue samples were identical to those followed for the standards by capillary electrophoresis as described above.

Data Processing

Wavelength-resolved CE data was processed first with custom despiking and background-subtraction algorithms and then processed and viewed in MATLAB (The MathWorks, Natick, MA) on an IBM RS/6000 workstation. Signal-to-noise ratios, quantitation, and reproducibility were determined according to standard techniques. Analysis of calibration standards was straightforward due to the presence of single-analyte peaks originating from and returning to the baseline. Limits of detection and quantitation values for each analyte were computed by numerically integrating intensity values over optimally determined, analyte-dependent wavelength ranges to generate a postacquisition, filter-based electropherogram. Intensities from single-cell data were similarly wavelength integrated to permit rapid interpretation of electrophoresis data, although full spectral information was also used to verify all identified analytes. Analysis of single cells was performed blind.

Peak heights in cellular samples were determined by subtracting temporally broad, superimposed wavelength-integrated background fluorescence values from those in the target peak. Once a peak was determined to be correctly identified with one of the standards, comparison of maximal peak intensity to an identically prepared standard curve provided concentration information about the analyte contained in the sample well or microvial. In the case of single-cell analysis, the number of moles in the entire conical microvial volume was calculated and assumed to originate from within the single cell under analysis. The volume of that cell was then calculated based on stereomicroscopic sizing of the cell before analysis and spherical volume calculations.

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