Characterizing Spectrally Diverse Biological Chromophores Using Capillary Electrophoresis with Multiphoton-Excited Fluorescence

Michael L. Gostkowski, J. Bridget McDoniel, Jing Wei, Theodore E. Curey, and Jason B. Shear*

Contribution from the Department of Chemistry and Biochemistry, University of Texas, Austin, Texas 78712

Received August 7, 1997[⊗]

Abstract: Minute quantities of native cellular fluorophores can be quantitatively assayed using ultraviolet fluorescence detection with microcolumn separations, but spectral diversity of biological chromophores imposes serious limitations on the use of this strategy to investigate biological components. We present an approach for rapid characterization of picoliter samples containing dissimilar cellular fluorophores—including amino acids, monoamine neurotransmitters, flavins, and pyridine nucleotides—using multiphoton excited fluorescence detection coupled to capillary electrophoresis separations. In this highly versatile approach, biological fluorophores are excited through the nearly simultaneous absorption of different numbers of low-energy photons. Because spectrally distinct species all can be excited with a single, long-wavelength laser source, fluorescence throughout the ultraviolet and visible regions can be detected efficiently with extremely low background. Samples containing serotonin, melatonin, FAD, and NADH can be reproducibly analyzed in 5-μm and 2-μm i.d. channels. Detection limits in 5-μm capillaries range from 350 zmols (38 nM) for FAD to 27 amols (1.0 μM) for serotonin. Use of 2-μm channels is shown to improve the mass detection limit for serotonin approximately as the decrease in capillary cross-sectional area (LOD $\approx$ 4 amol), and further reductions in mass detection limits are projected for analysis with even smaller diameter channels that better match the submicron size of the diffraction-limited multiphoton focal spot.

Introduction

Microcolumn separation techniques provide unique capabilities for chemically profiling minute quantities of biological material, including single cells, cytoplasmic samples, and individual secretory granules.[1−4] The experimentally simplest of these approaches, capillary electrophoresis (CE), accommodates sample volumes from the subfemtoliter to the nanoliter scale and can analyze complex mixtures of cations, neutrals, and anions within several minutes.[5−7] Laser-induced fluorescence detection has been used with CE to measure attomole amounts of unlabeled proteins and monoamine neurotransmitters derived from the deep-UV excitable fluorescent amino acids, tyrosine and tryptophan.[2,8−10] In contrast to immunohistochemical techniques, direct analysis of natively fluorescent species offers the possibility for probing the chemistry of viable cells and for quantitative determination of chemical concentrations in biological samples.

Measurements capable of correlating the fluorescence of various peptides, proteins, and monoamine neurotransmitters to levels of redox cofactors in a cell could provide important insights into energy metabolism, biosynthesis, and neurotransmitter regulation. The monoamine neurotransmitters, for example, are degraded at the outer mitochondrial membrane by monoamine oxidase (MAO). This oxidative deamination of neurotransmitters is accompanied by reduction of flavin adenine dinucleotide (FAD), a covalently bound MAO cofactor.[11,12] It has been suggested that the rate of neurotransmitter deamination could be influenced strongly by the overall respiratory state of a cell (which would affect the recycling rate of FAD) and that MAO-mediated oxidation of neurotransmitters could affect the general electron transport activity in mitochondria.[13,14] A number of redox cofactors—including the oxidized flavins/flavoproteins and the reduced nicotinamide nucleotides—can be monitored at low levels using native fluorescence detection.[15] These species, however, are spectroscopically quite distinct from tyrosine/tryptophan analogs and from one another; as a consequence, it has not been feasible to use native fluorescence as a sensitive assay for diverse biological molecules.

The excitation spectra of three representative biological fluorophores, 5-hydroxytryptamine (serotonin, an indole neurotransmitter), nicotinamide adenine dinucleotide (NADH, a pyridine nucleotide redox cofactor), and FAD (a redox cofactor), demonstrate the severe compromises one would make in exciting

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1997.

(1) Jankowski, J. A.; Tracht, S.; Sweedler, J. V. *Trends Anal. Chem.* **1995**, *14*, 170−176.
(2) Lillard, S. J.; Yeung, E. S.; McCloskey, M. A. *Anal. Chem.* **1996**, *68*, 2897−2904.
(3) Oates, M. D.; Cooper, B. R.; Jorgenson, J. W. *Anal. Chem.* **1990**, *62*, 1573−1577.
(4) Olefirowicz, T. M.; Ewing, A. G. *Anal. Chem.* **1990**, *62*, 1872−1876.
(5) Chiu, D. T.; et al. *Anal. Chem.* **1997**, *69*, 1801−1807.
(6) Monnig, C. A.; Kennedy, R. T. *Anal. Chem.* **1994**, *66*, 280R−314R.
(7) Sudor, J.; Novotny, M. V. *Anal. Chem.* **1995**, *67*, 4205−4209.
(8) Chang, H.-T.; Yeung, E. S. *Anal. Chem.* **1995**, *67*, 1079−1083.
(9) Timperman, A. T.; Oldenburg, K. E.; Sweedler, J. V. *Anal. Chem.* **1995**, *67*, 3421−3426.
(10) Chang, H.-T.; Yeung, E. S. *Anal. Chem.* **1993**, *65*, 2947−2951.
(11) Walker, W. H.; Kearney, E. B.; Seng, R. L.; Singer, T. P. *Eur. J. Biochem.* **1971**, *24*, 328−331.
(12) Kearney, E. B. et al., *Eur. J. Biochem.* **1971**, *24*, 321−327.
(13) Medvedev, A. E.; Gorkin, V. Z. *Biog. Amines* **1992**, *8*, 323−337.
(14) Smith, G. S.; Reid, R. A. *Biochem. J.* **1978**, *176*, 1011−1014.
(15) Xue, Q; Yeung, E. S. *Nature* **1995**, *373*, 681−683.

S0002-7863(97)02742-X CCC: $15.00
Published on Web 01/14/1998

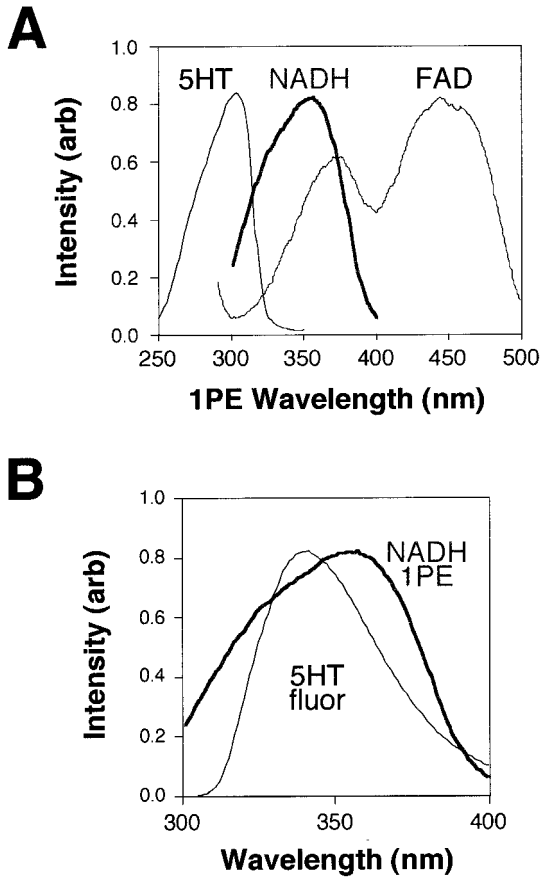

Figure 1. (A) One-photon excitation (1PE) spectra of three representative biological fluorophores, serotonin (5HT), nicotinamide adenine dinucleotide (NADH), and flavin adenine dinucleotide (FAD), using native fluorescence detection. (B) Use of more than one excitation source to analyze such samples complicated by the large spectral overlap of fluorescence (*fluor*) from short wavelength fluorophores (e.g., 5HT) with the normal excitation wavelengths for other fluorophores (e.g., NADH).

these spectrally diverse species with a single laser wavelength (Figure 1A). At no spectral region can all species be adequately excited. Alternatively, fluorescence from these species could be excited using several laser lines, but ubiquitous laser background throughout the UV and blue regions of the spectrum would greatly limit detectability: the emission spectrum of serotonin coincides to a great extent with the excitation spectrum of NADH (Figure 1B), and the emission spectrum of NADH substantially overlaps the excitation spectrum of FAD.

To overcome these spectroscopic limitations, we have developed a new approach for examining the composition of biological mixtures in which native fluorophores are fractionated by capillary electrophoresis and then are excited to fluoresce by two or more near-infrared photons. Because the photon energy of the excitation source is sufficiently small, transitions requiring substantially different energies can be excited through absorption of different numbers of quanta. Moreover, spectral overlap of absorption and emission spectra of various components does not interfere with efficient detection because all species are excited with a single, long-wavelength source.

This highly versatile technique—capillary separations coupled to multiphoton-excited (MPE) fluorescence detection—in general requires that all photons are absorbed within the uncertainty times of virtual states (∼femtoseconds), thereby achieving electronic excitation without passing through real states of intermediate energies. Thus, when ground-state depletion and photobleaching are insignificant, the fluorescence generated from a sample is proportional to I^n, where I is the instantaneous excitation intensity and n is the number of photons needed for excitation. Efficient multiphoton excitation is readily achieved using the tightly focused output from a mode-locked laser source, such as the femtosecond titanium:sapphire (Ti:S) laser.[16] In addition to the normal temporal "focusing" inherent to a mode-locked Ti:S (in these experiments, ∼100 fs pulses are produced once every 13 ns), the laser beam can be spatially focused to spot sizes of $<1\ \mu$m in diameter. As a result, a Ti:S laser operating at an average power output of 100 mW can produce instantaneous intensities of $>10^{12}$ W cm^{-2} at the focal point, a level high enough to generate efficient MPE of a wide range of chromophores without inducing dielectric breakdown of water.[17] In a recent report, Song et al. demonstrated the use of a Ti:S system to probe coumarine laser dyes fractionated with capillary electrophoresis.[18]

The two-photon excitation (2PE) spectra of flavins and pyridine nucleotides and the three-photon excitation (3PE) spectra of indoles and catecholamines have been characterized previously.[16,19] All species were found to have substantial fluorescence action cross sections in the wavelength range 710—750 nm. Thus, a single near-IR laser "line" centered at 740 nm (∼1.7 eV photon^{-1}) can generate 2PE of near-UV chromophores by depositing a total of ∼3.4 eV per molecule and 3PE of deep-UV chromophores by supplying ∼5.0 eV per molecule. In previous work, these cross-section data were used as guides for measuring 3PE cellular autofluorescence of indoles in cultured cells with laser scanning microscopy.[20] Cellular content of proteins and serotonin could be monitored within functioning cells, but unlike capillary electrophoresis analyses, such in situ measurements had no capacity to distinguish compounds with *similar* spectroscopic properties. Serotonin, for example, could not be differentiated from other related indoles, including the MAO-catalyzed deamination product, 5-hydroxyindoleacetic acid (HIAA).

Experimental Section

Chemicals. Redox cofactors (FAD and NADH) and indoles (melatonin and serotonin creatine sulfate salt) were purchased from Sigma (St. Louis, MO), and phosphate sodium salts (dibasic and monobasic monohydrate) were purchased from E. Merck Science (Gibbstown, NJ). Yeast samples were obtained locally. Water was purified using a Milli-Q reagent water system (Millipore).

Laser System. A Coherent Mira 900F femtosecond mode-locked Ti:S laser system was used for all studies. A Coherent Innova 310 multiline argon ion laser served as the optical pump source for the Ti:S, which pulses with a repetition rate of ∼76 MHz and has a relative rms power noise of ∼1%. When operating at 740 nm (fwhm ≈ 10 nm), the Mira produces individual pulses that have a temporal width of ∼100—150 fs. The output of the Ti:S laser was attenuated using a half-wave plate/polarizer pair and was adjusted to ∼90—110 mW (average power at the sample) for all experiments.

Coupling Capillary Electrophoresis and Multiphoton Excitation. A number of design innovations were necessary to efficiently couple MPE fluorescence with capillary separations. In these studies, a high numerical aperture (NA) microscope objective was used to focus the

(16) Xu, C.; Zipfel, W.; Shear, J. B.; Williams, R. M.; Webb, W. W. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 10763—10768.
(17) Stuart, B. C.; Feit, M. D.; Rubenchik, A. M.; Shore, B. W.; Perry, M. D. *Phys. Rev. Lett.* **1995**, *74*, 2248—2251.
(18) Song, J. M.; Inoue, T.; Kawazumi, H.; Ogawa, T. *J. Chromatogr. A* **1997**, *765*, 315—319.
(19) Maiti, S.; Shear, J. B.; Webb, W. W. *Biophys. J.* **1996**, *70* (Part 2 of 2), A210.
(20) Maiti, S.; Shear, J. B.; Zipfel, W. R.; Williams, R. M.; Webb, W. W. *Science* **1997**, *275*, 530—532.

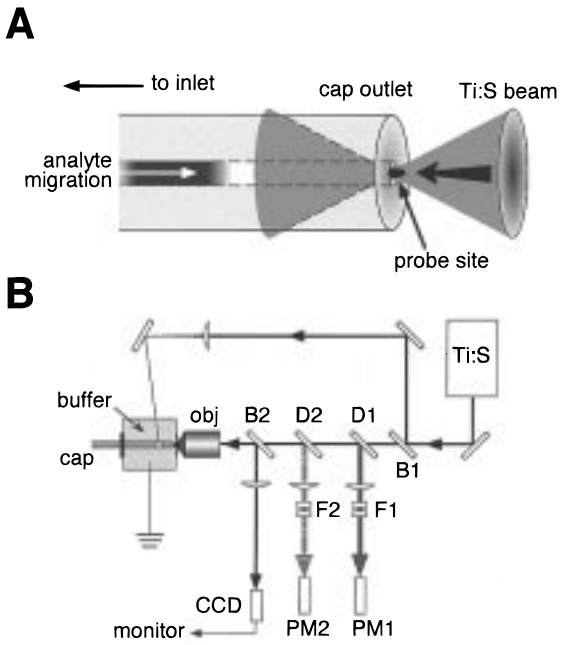

Figure 2. Multiphoton-excited (MPE) fluorescence detector for capillary separations. (A) A high numerical aperture microscope objective focusing near-infrared Ti:S light at the capillary (*cap*) outlet, with the optical axis collinear with the capillary axis. (B) The overall detection scheme showing relative position of the objective (obj), beam splitters (B1 and B2), dichroic mirrors (D1 and D2), filters (F1 and F2), and photomultiplier tubes (PM1 and PM2).

Ti:S laser beam, producing a diffraction-limited radial beam waist radius ($1/e^2$ intensity level on the central diffraction spot) of ~0.25 μm and a ($1/e^2$) axial dimension of ~0.9 μm. The dimensions of MPE probe volumes can be estimated by squaring (for 2PE) or cubing (for 3PE) the intensity distribution of the excitation light. Because rectangular (flat-wall) CE capillaries are not commercially available with inner diameters smaller than ~50 μm, circular cross section capillaries (5-μm or 2-μm i.d./360-μm o.d.) that better match the MPE probe volumes were used. To avoid intense scatter and optical aberrations caused by focusing tightly through curved capillary wall surfaces, we designed an end-column detection geometry in which the laser beam focus was positioned at the outlet aperture of the capillary channel, with the axis of the excitation light collinear with the capillary axis (Figure 2A). Fractionated compounds thus intersect the MPE probe site as they migrate through the channel aperture, before diffusing into the bulk solution of the outlet reservoir.

The overall detection system is shown in Figure 2B. In this arrangement, the Ti:S beam is directed through a series of beam splitters and dichroic mirrors into the back aperture of the microscope objective (Zeis Fluar, 1.3 NA oil immersion, infinity corrected). The objective focuses the light through a coverslip a short distance (~10−100 μm) into a modified plastic cuvette that serves as the outlet electrolyte reservoir (held at electrical ground). The separation capillary enters the cuvette through a septum on one side and terminates at the beam focus near the opposing side of the cuvette. The cuvette is secured to a three-axis (*XYZ*) translation stage driven with differential micrometers (resolution better than 0.1 μm). In addition, the capillary can be positioned relative to the cuvette using a *Z*-axis (optical axis) translation stage and a gimbal (two-axis) tilt control.

Visualization of the capillary outlet is necessary for rapid alignment, and it is not practical in our system to provide wide-field illumination through the microscope objective. As a result, we designed an alternate means for illuminating the capillary tip. In our approach, a portion of the Ti:S light is split off by a beam splitter (B1) and is focused with a lens onto a thin spot of epoxy on the capillary (within the outlet reservoir). The epoxy scatters some light at a small angle relative to the capillary axis, thereby coupling the light into the fused silica capillary walls so that it propagates to the outlet by total internal reflection. Light radiating from the capillary outlet is collected by the microscope objective, split off by a beam splitter (B2), and imaged onto a CCD video camera. As shown in Figure 3 A, the channel aperture appears as a dark circle surrounded by the bright outlet face of the capillary.

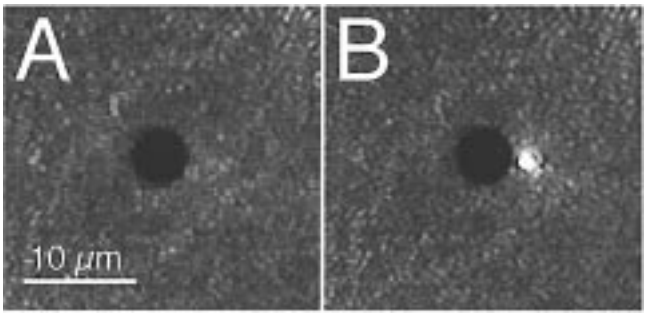

Figure 3. Video image of the capillary outlet and multiphoton focal site. End-on images of the capillary outlet show the MPE probe volume aligned with the channel aperture (A) and slightly displaced from the aperture (B).

When the image of the outlet face is in focus, the MPE focal spot lies in the same plane as the capillary face (normal to the optical axis), though not necessarily at the same *XY* position as the channel aperture. In Figure 3B, the reflection of the focal spot is seen slightly displaced from the channel aperture. When the capillary is translated so that the MPE probe site overlies the aperture, this reflection is not seen (Figure 3A). Alignment of the outlet aperture with the submicron MPE probe site typically is stable for 1 h or more before repositioning is required. The beam splitter B2 used to image the capillary tip is removed before making fluorescence measurements.

Fluorescence Measurements. Fluorescence from analytes migrating through the aperture is collected by the excitation objective and is reflected at 90° using dichroic mirrors at two separate locations (Figure 2B). Ultraviolet fluorescence is reflected by a dichroic mirror (D2) (Chroma technology, 400DCXR) which passes wavelengths greater than ~430 nm and reflects light between ~280 and ~380 nm. Two ultraviolet band-pass filters (F2) (Barr Associates, $\lambda_{max} = 330$ nm) are used to further isolate UV fluorescence, and the signal is measured using a photomultiplier tube (PM2) (Hammatsu, HC 125-02-UV) connected to a two-channel photon counter (Stanford Research Systems, SR400). The signal from this first detector is referred to as the *UV channel*. Visible fluorescence at wavelengths shorter than 620 nm (transmitted through the UV dichroic) is reflected using dichroic mirror (D1) (Chroma technology, 625DCXR). A colored glass filter (F1) (Schott Glass, BG18) and 2 cm of 1 M $CuSO_4$ solution are used to isolate visible emission from residual UV fluorescence and laser scatter. The signal from this *visible channel* is measured with a second photomultiplier (PM1) (Hammatsu, HC 125-02) connected to the photon counter. The two channels of the photon counter transfer fluorescence data through a GPIB interface to a Macintosh running LabView-based software. A single electrophoresis separation thus yields two data traces—one for UV-emitting molecules and one for visible-emitting molecules.

Capillary Electrophoresis Experiments. CE separations of standards were performed in both 5-μm and 2-μm i.d. capillaries (Polymicro, Phoenix, AZ). All separations used 2 mM phosphate buffer, pH ≈ 5.8, as the running buffer. Solutions were filtered through 0.2-μm pore cellulose acetate membranes (Osmonics, Livermore, CA). For CE-MPE analysis of commerical yeast samples (*Saccharomyces cerevisiae*), dry yeast pellets were suspended in a sucrose buffer at 37 °C for several hours, then pelleted and rinsed. The resulting cellular components then were diluted approximately 3-fold in a medium containing 25 mM Tris-hydrocholoride, 2 mM EDTA, and a small amount of β-mercaptoethanol. Cells were disrupted by vortexing with glass beads, a 1% SDS solution was added to the sample, and large fragments were removed by centrifugation and filtered before analysis. Samples from the resulting solution were injected onto the separation capillary without additional dilution.

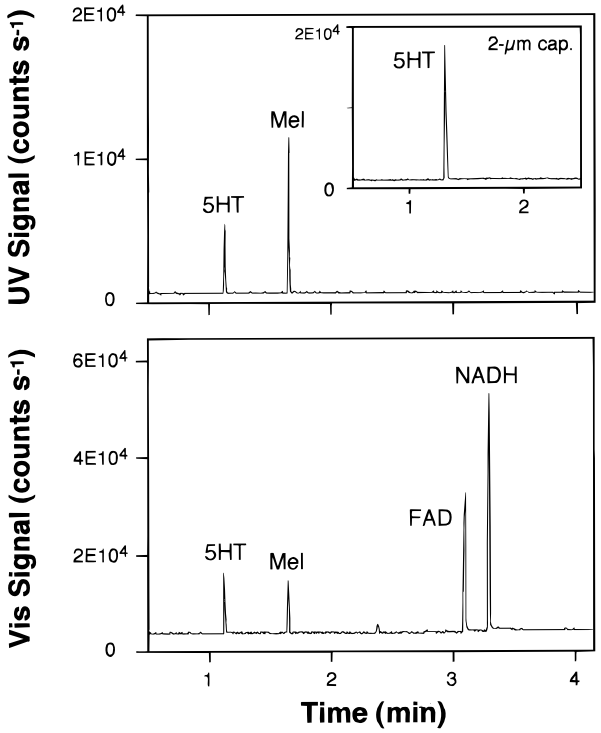

Figure 4. Capillary electrophoresis (CE) separation with two- and three-photon excited fluorescence detection of biological fluorophores. The UV-channel electropherogram (top trace) measures 5HT and melatonin (Mel) using three-photon excited fluorescence, while the visible channel electropherogram (bottom trace, same electrophoresis separation) shows two-photon excited fluorescence of FAD and NADH. Separations were performed in a ~34-cm capillary with a field of ~590 V cm^{-1}. Sample mixtures were injected electrokinetically for 3 s at ~59 V cm^{-1}, which introduced subfemtomole amounts of all species into the capillary for analysis. Concentrations of analytes were 5 μM (FAD), 25 μM (NADH), and 75 μM (5HT and Mel). Inset: Detection of 5HT after capillary electrophoresis in a 2-μm diameter capillary. Analysis was performed in a capillary ~32 cm long using a field of ~625 V cm^{-1}. 5HT (150 μM) was injected electrokinetically for 3 s at ~65 V cm^{-1}.

Results and Discussion

The ability of this system to characterize biological fluorophores with diverse chemical and/or spectral properties was examined by fractionating a mixture of two indoles, serotonin and melatonin, and two redox cofactors, FAD and NADH. Figure 4 shows the electrophoresis data traces produced using a field strength of ~590 V cm^{-1} to separate the four species. Serotonin and melatonin fluoresce maximally at ~340−360 nm and, consequently, appear as the two fluorescence peaks in the UV channel. The slow-migrating species FAD (maximum fluorescence at ~525 nm) and NADH (maximum fluorescence at ~465 nm) are detected in the visible channel (lower electropherogram). Also apparent in the visible channel are two fast-migrating peaks that clearly correspond to serotonin and melatonin (same migration times as in the UV channel). These indole peaks are observed in the visible channel because a small amount of indole emission penetrates the visible-channel filters and, more significantly, because multiphoton excitation of indoles can generate visible emitting photoproducts.[21]

The photon dependence of excitation was verified in cuvette (bulk solution) measurements (data not shown). As expected, at relatively low powers, the UV signals for serotonin and melatonin scaled as the cube of the laser intensity and the visible signals for FAD and NADH scaled as the square of the laser intensity. The visible signal for serotonin showed a more complicated dependence on excitation intensity. At high powers, serotonin visible emission scaled as $I^5 - I^6$, likely indicating three- and four-photon induced photochemical reaction followed by two-photon excited fluorescence of the photochemical reaction product.[21] At the powers used in the electrophoresis studies, ground-state depletion and photobleaching were significant for all species.

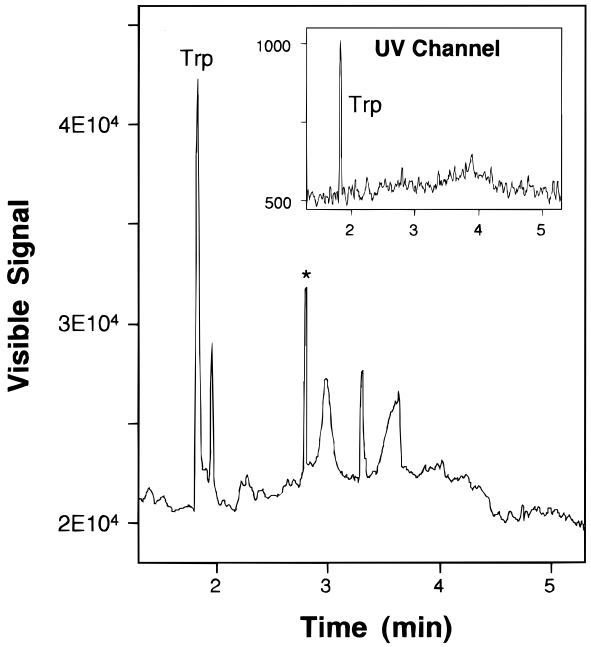

Figure 5. CE-MPE fluorescence detection of a complex biological sample. Commercial dried baker's yeast (*Saccharomyces cerevisiae*) was suspended in a sucrose buffer at 37 °C for several hours, then pelleted and rinsed. Cells then were disrupted by vortexing with glass beads, a 1% SDS solution was added, and large fragments were removed by centrifugation and filtering through 0.2-μm cellulose acetate membranes. CE conditions were similar to those in Figure 4; a 2-μm i.d. separation capillary was used for analysis. The spike marked with an asterisk in the visible channel electropherogram is an irreproducible (random) spike.

Figure 5 demonstrates the ability of this CE-MPE fluorescence system to fractionate and detect components in complex biological samples. A sample containing several picoliters of diluted baker's yeast homogenate was separated electrophoretically, yielding visible and UV-channel (inset) electropherograms. In both channels, the peak just before 2 min is tentatively identified as tryptophan (Trp) by spiking the sample with a tryptophan standard. The visible channel shows a number of reproducible peaks, possibly produced by various oxidized flavoproteins (no peaks comigrated with NADH, FAD, or FMN). In addition to the tryptophan peak, the UV channel shows a reproducible broad increase in the baseline, potentially caused by many unresolved tryptophan-containing proteins.

Estimated mass limits of detection (LODs) for CE-MPE fluorescence analyses were determined from the concentration limits (based on a signal-to-noise ratio of 3) and the injection volumes. Sample volumes were estimated from the voltage and duration of injections, the nominal capillary cross sectional area, and the experimentally determined electrophoretic mobilities. Calculated 5-μm capillary injection volumes ranged from 9 to 26 pL for the different fluorophores, depending on electrophoretic mobility. From Figure 4, detection limits calculated

(21) Shear, J. B.; Xu, C.; Webb, W. W. *Photochem. Photobiol.* **1997**, *65*, 931−936.

for UV responses were 36 amol (1.4 μM) for serotonin and 10 amol (550 nM) for melatonin. At the excitation intensities used in this separation, the redox cofactors showed considerably better detectability, with LODs of 980 zmol (110 nM) for NADH and 350 zmol (38 nM) for FAD. Because the dependence of fluorescence on laser intensity is distinct for each analyte, it is not possible to optimize detection limits for all species simultaneously. At the expense of somewhat lower sensitivities for FAD, NADH, and melatonin, the LOD for serotonin could be improved to 27 amols (1.0 μM) (electropherogram not shown). The visible channel signals for serotonin and melatonin do not scale linearly with concentration in the phosphate buffer system;[21] thus, detection limits have not been calculated from these peaks.

Background sources in standard UV fluorescence measurements—including intense Rayleigh scatter and emission from lenses and filters—do not interfere substantially with detection. Because the excitation light is centered at ~740 nm, laser scatter is easily filtered from the much shorter wavelength fluorescence; scattered light that does strike the detector photocathode is converted to background counts with poor efficiency because of low PMT quantum efficiency for photon energies less than ~2 eV. In addition, intensities necessary to produce two- or three-photon excitation are reached only within solution (i.e., near the beam focus), so MPE fluorescence of optics does not take place. The primary factor limiting detectability in these studies is fluorophore photostability. In earlier work, it was observed that multiphoton excitation of serotonin is at least as likely to produce permanent photobleaching as it is to yield fluorescence (i.e., fewer than one fluorescence photon can be obtained per indole molecule).[22] FAD and NADH appear to be more photostable than melatonin and serotonin and consequently, can be detected at somewhat lower levels.

Multiphoton excitation volumes are necessarily small when using standard mode-locked laser sources. By using lower NA focusing optics than were employed in this work, it would be possible to increase the MPE probe volume size to some extent; however, substantial increases in focal volume size eventually would decrease the rate of MPE to the point that fluorophore molecules would not be adequately probed during their residence time in the beam focus. Because of this limitation on the size of a MPE probe volume, separation capillaries ideally should have inner diameters <0.5 μm to ensure that an analyte molecule migrating from the capillary intersects the probe site. When using 5-μm i.d. capillaries, the $1/e^2$ beam waist radius is approximately 10-fold smaller than the capillary channel radius, a size mismatch that allows most of an analyte band (>95%) to diffuse into the bulk solution of the outlet reservoir without intersecting the MPE probe volume. The severity of this problem is ameliorated somewhat by the use of very high laser intensities, which produces larger, partially flattened excitation profiles. In addition, because the 2PE probe volume is somewhat larger than the 3PE volume, FAD and NADH are more efficiently probed than are serotonin and melatonin.

Analysis of serotonin in 2-μm i.d. capillaries has yielded the expected ~6-fold improvement in mass detection limits (LOD $\approx$ 4 amol/1 μM; Figure 4 inset)—approximately matching the best reported value for single-photon (UV-excited) detection of serotonin.[2] This improvement in mass detection limits relies on more efficient overlap of the probe volume with the outlet aperture of the capillary. Because the cross-sectional area of the 2-μm capillary is still substantially *larger* than the MPE focal spot, the reduction in capillary diameter does not affect peak height. However, the expected 6-fold gain in mass LODs is not observed in all cases because of a larger UV background associated with some 2-μm capillaries. The origin of this effect is currently under investigation in our laboratory. For capillaries that maintain low background levels, use of submicron channels should yield detection limits for the biological fluorophores in the low- to high-zeptomole range.

System reproducibility on a given day was as low as 9−10% relative standard deviation (peak area; $N = 7$ or 8) for separations performed using either the 5-μm or 2-μm capillaries (5-s injections at 2 kV; 0.25-s photon counting bin size), indicating that mechanical drift does not significantly affect precision for capillaries of these diameters. However, variability is somewhat greater than that reported for CE analyses with single-photon excited fluorescence.[23,24] The relatively large rms noise in the laser output and the high-order dependence of fluorescence signal on laser intensity for MPE likely contribute to measurement imprecision. Thus, the use of a noise-suppressing electrooptical device (placed between the laser source and the detection system) should improve reproducibility. The dynamic range in these experiments is constrained by the limits of detection and photon counting saturation. On the basis of serotonin UV response, the linear working range extends from the low-micromolar to low-millimolar range ($R \geq 0.99$).

Conclusions

These studies demonstrate the potential for investigating a wide array of native biological fluorophores using capillary separations coupled to MPE fluorescence detection. This highly sensitive and versatile approach for probing cellular fluorophores should prove useful in single cell analyses to correlate levels of various peptides, proteins, and monoamine neurotransmitters with their metabolic precursors and breakdown products and with the redox cofactors that mediate metabolic activity. The CE-MPE mass detection limit (<1 amol) for NADH analysis in 5-μm capillaries exceeds the best literature values we were able to locate,[25] despite the large size mismatch of the MPE focal spot and the channel aperture size. Although serotonin exhibits relatively poor photostability when subjected to multiphoton excitation at 740 nm, CE-MPE analysis of serotonin in smaller (2-μm i.d.) channels provides low-attomole detection limits—approximately equal to the best reported level when using UV excitation. By better matching channel diameters to the submicron diffraction-limited multiphoton focal spot, nanomolar to micromolar levels of native fluorophores should yield measurable signals in samples as small as 100 fL, providing a means to rapidly characterize the zeptochemistry of individual cells.

Acknowledgment. Support from the Office of Naval Research (Grant No. N00014-97-1-0494) and the Welch Foundation (Grant No. F-1331) is gratefully acknowledged. We thank S. Webber and T. Cohen for assistance in acquiring the FAD excitation spectrum and the lab of D. Appling for advice on yeast homogenization.

JA9727427

(22) Xu, C.; Shear, J. B.; Williams, R. M.; Maiti, S.; Webb, W. W. Unpublished data.

(23) Wu, N.; Sweedler, J. V.; Lin, M. *J. Chromatogr. B* **1994**, *654*, 185−191.

(24) Mank, A. J. G.; Yeung, E. S. *J. Chromatogr. A* **1995**, *708*, 309−321.

(25) Wang, Z.; Yeung, E. S. *J. Chromatogr. B* **1997**, *695*, 59−65.