

Ultraviolet Native Fluorescence Detection in Capillary Electrophoresis Using a Metal Vapor NeCu Laser

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Ultraviolet (UV) excitation for laser-induced native fluorescence (LINF) detection in capillary electrophoresis (CE) offers impressive performance figures of merit when assaying peptides containing tyrosine or tryptophan residues, catecholamines, indolamines, and a number of other classes of analytes with appreciable fluorescence when excited by UV radiation. One of the largest drawbacks of native fluorescence detection schemes in CE-LINF systems has been the expense and the complexity of the lasers required for excitation in the deep UV wavelength range of 200–300 nm. An improved “turn-key” NeCu laser operating at 248.6 nm interfaced to a sheath flow-based CE system obtains a performance similar to that of large frame frequency-doubled Ar ion lasers. The detection limits for serotonin and dopamine (27 nM and 8 μ M, respectively, for \sim 3-nL injection) are similar to those obtained using a frequency-doubled Ar ion laser at 257 nm (21 nM and 8 μ M, respectively). An example of the detection of serotonin-related analytes from a single-cell electropherogram demonstrates the performance of such a system for mass-limited measurements.

Capillary electrophoresis (CE) offers a number of advantages for measuring the chemical composition of trace samples such as individual cells. It provides rapid separations, high separation efficiencies, and compatibility with small sample volumes.^{1–5} Detection in CE can be challenging owing to the small analyte band volumes and the high separation efficiency which creates temporally narrow peaks.⁶ The most sensitive detection scheme is laser-induced fluorescence (LIF), and so this mode is most commonly applied to complex biological samples such as single

cells.^{7–9} Many biomolecules have relatively low fluorescence quantum yield at common laser wavelengths and have been detected through derivatization with highly fluorescent tags.^{10–12} While the use of fluorescent probes can greatly improve limits of detection (LODs), they can suffer from limited shelf life and lack of complete specificity for the analytes and can be complicated by slow kinetics or incomplete reactions. Furthermore, derivatizations become problematic when dealing with small-volume samples (submicroliter level) or at low analyte concentration (10^{-10} – 10^{-7} M), due to decrease in the labeling efficiency and increase in background signal.^{13–16}

A large group of biological species fluoresce when excited in the ultraviolet (UV) region; therefore, UV laser excitation is an attractive alternative to derivatizing these compounds with tags that fluoresce with visible excitation. Such natively fluorescent molecules include the catecholamines, indolamines, aromatic amino acids and peptides containing them, flavins, adenosine and guanosine nucleotide analogues, and others.^{17–20} Swaile and Sepaniak²¹ first demonstrated the use of UV excitation for native fluorescence detection with detection limits of 1.4×10^{-8} M for conalbumin using a frequency-doubled Ar ion laser operating at 257 nm. Nie et al. later demonstrated the detection of a series of polycyclic hydrocarbons using the same laser with 30–150-zmol LODs.^{22,23} Yeung's laboratory improved protein detection limits

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using the 275-nm line from a large Ar laser (this line is only obtained from expensive, 440-V full-frame lasers).²⁴ Using this emission line, they described the detection of native proteins²⁵ and hemoglobin variants¹⁸ in single red blood cells, as well as insulin in single pancreatic cells.²⁶ LODs were reported as nanomolar level for tryptophan (Trp), down to 10^{-10} M for proteins containing many Trp molecules, and as low as 73 amol of insulin could be detected with signal-to noise ratio of 10. Yeung's group further applied this laser line to monitor exocytosis and release from individual mast cells,²⁷ reporting an LOD of 1.7 amol for serotonin. In addition, nucleic acid and DNA fragments were detected by the same group using a 248-nm line from a waveguide KrF laser,²⁰ with the LODs for guanosine and adenosine monophosphate at 5×10^{-8} M. Frequency-doubled Kr lasers have also been used; for example, LODs for Trp as low as 2×10^{-10} M (800 zmol at 4-nL injection) have been reported by using a frequency-doubled Kr laser operating at 284 nm.^{17,28} Besides continuous-wave lasers, several groups have looked at pulsed UV lasers for CE detection. For example, Chan and co-workers demonstrated 3.3 nM LODs for Trp with 248-nm excitation but reported a decrease in the LODs at this wavelength for bovine serum albumin and conalbumin, which contain 2 and 15 tryptophan residues.²⁹ Kok and co-workers reported low-microgram per liter regime LODs for naphthalenesulfonate species with a frequency-doubled XeCl excimer dye laser working at 280 or 325 nm.³⁰ Recently the Gooijer group investigated a frequency-quadrupled Nd:YAG laser emitting at 266 nm for CE-LIF analysis of polycyclic aromatic hydrocarbon metabolites³¹ and obtained LODs in the microgram per liter range; the Issaq group described another application, detection of Trp-containing proteins, peptides, and related indoles at the nanomolar level.³² Some of lasers have been adapted for single-cell studies and are relatively easy to operate.^{19,33,34} However, the greatest challenge to this approach has been the lack of available deep UV lasers and the high cost of those that are available so that these techniques have not gained widespread acceptance. Natively fluorescence molecules can also be detected using visible or infrared lasers using multiphoton excitation, and the Shear group reported impressive mass detection limits when coupling this detection mode with CE,³⁵⁻³⁷ however, such systems also require sophisticated lasers and are not yet common.

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We report here the use of a new hollow cathode metal vapor laser (NeCu laser) for LINF detection in CE. The performance of the CE-LINF system with this new “turnkey” laser working at 248.6 nm is compared with prior LINF detection schemes based on a frequency-doubled Ar ion laser working at 257 nm, which requires considerably more effort and costs more than 1 order of magnitude more. It is these disadvantages, rather than performance, that have limited the applicability of UV-LINF detection in CE. We also demonstrate the ability of this 248.6-nm excitation source to be used with single-neuron assays.

EXPERIMENTAL SECTION

Reagents. The electrophoresis running buffer was borate (50 mM, pH 8.7) prepared using 3.0 g of boric acid (H_3BO_3 ; Sigma, St. Louis, MO), and 9.2 g of sodium borate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$; Sigma) in 1.00 L of ultrapure water (Milli-Q filtration system; Millipore, Bedford, MA). The sheath fluid was of the same composition as the electrophoresis buffer. All standards were obtained from Sigma and were reagent quality or better.

Chemical abbreviations: tryptophan (Trp), tryptamine (TrpA), 5-hydroxytryptamine (5-HT or serotonin), *N*-acetylserotonin (NAS), 5-hydroxyindole-3-acetic acid (5-HIAA), melatonin (MEL), tyrosine (Tyr), dopamine (DA), octopamine (OA), epinephrine (E), 5,6,7,8-tetrahydrobiopterin (THB), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), β -nicotinamide adenine dinucleotide (NADH), and β -nicotinamide adenine dinucleotide phosphate (NADPH).

Cell Isolation. *Pleurobranchaea californica* (200–500 g) were obtained from Sea-life Supply (Sand City, CA) and kept in artificial seawater at 12–14 °C until use. The procedure for cell isolation procedure has been described previously.^{19,38} Briefly, single metacerebral cells (MCCs) were dissected from *P. californica* under cold anesthesia in molluscan physiological saline. The MCC was isolated under a stereomicroscope using microscissors and glass micropipets. Within 1 min, the neuron was placed in the microvial and homogenized by the combined action of tungsten needle manipulation and hypoosmotic buffer damage. The samples were either diluted to 360 nL and injected directly into the CE system for analysis or immediately frozen on dry ice for storage and analyzed within 4 h.³⁹

Electrophoresis System and Data Processing. The laboratory-assembled CE system has been described in detail previously.^{19,38} An 800-mm-long, 50- μm -i.d., 150- μm -o.d. untreated fused-silica capillary (Polymicro Technologies, Phoenix, AZ) was employed. A home-built nanovial autosampler ideally suited for single-cell assay was used for sampling. Sample injections were performed electrokinetically at 2.1 kV (current $\sim 2.4 \mu\text{A}$) for 10.0 s from a 360-nL stainless steel microvial, resulting in ~ 3 nL of sample injected into the capillary. The separation voltage was maintained at 21 kV (current $\sim 25 \mu\text{A}$). The detection end of the capillary was directed into a laboratory-assembled sheath flow cell (a $1.0 \times 1.0 \times 20$ mm quartz cuvette) with the linear sheath flow velocity at ~ 0.5 mm/s.

Excitation of the core stream in the sheath flow cell was provided by one of two lasers. The first was the previously

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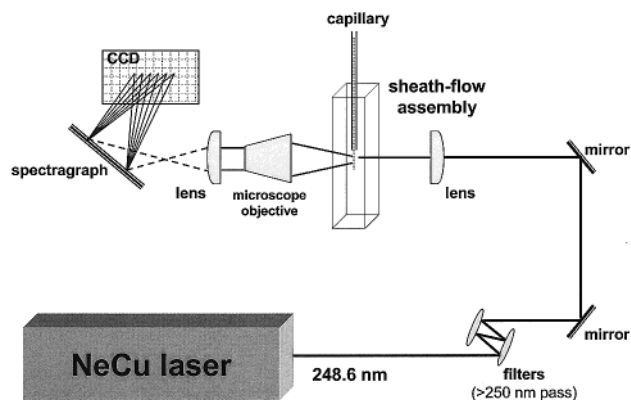


Figure 1. Schematic diagram of the LINF detection system utilizing a NeCu laser.

described frequency-doubled, liquid-cooled Ar ion laser (Innova 300 FReD; Coherent, Palo Alto, CA) operating at 257 nm.^{19,34,38} Approximately 0.5 mW was directed into the sheath flow cell and focused to a spot ~ 1 mm below the capillary outlet with a 20-mm-focal length quartz spherical plano-convex lens (Spindler and Hoyer Inc., Medford, MA). The second laser incorporated was a newly introduced NeCu laser (PSI NeCu 60; Photon Systems, Covina, CA) operating at 248.6 nm available complete for approximately \$10 000. A schematic diagram of the instrumental setup is shown in Figure 1. Two reflective edge filters (250-nm long pass) facing each other with a spacing of ~ 1.5 in. were located in front of the laser and the laser beam was introduced at $\sim 10^\circ$. The filter pair was used to eliminate or suppress tube plasma lines. After four reflections, the spectrally pure 248.6-nm light exits the filter pair and is directed to the quartz spherical plano-convex lens described above. Unless specified elsewhere, the laser was set at a pulse width of 30 μ s, repetition rate of 122 Hz, drive current of 25 A, and buss voltage of 650 V. An average power of ~ 0.6 mW (pulse peak power of ~ 150 mW) was focused to a spot ~ 1 mm below the capillary outlet inside of the sheath flow cell.

The collection optics were orthogonal to the excitation beam and consisted of a $15\times$ all-reflective microscope objective (Opticon, Billerica, MA) and a 30-mm-focal length quartz spherical plano-convex lens focusing the fluorescence emission to a $f/2.2$ CP 140 imaging spectrograph (Instruments SA, Edison, NJ), and the emission information over the 260–710-nm wavelength range was focused across the face of a liquid nitrogen-cooled $1,024 \times 256$ detector array, scientific CCD (E2V 15–11; Essex, U.K.). The wavelength-resolved CE data were processed and viewed in MATLAB (the Mathworks, Natick, MA) on a personal computer.

RESULTS AND DISCUSSION

The primary goal of the present study is to determine the feasibility of a turnkey metal vapor laser for LINF detection coupled to CE. As shown in Table 1, these NeCu lasers can lase at a variety of deep UV wavelengths in the 200–300-nm range with suitable optics; yet they have a size, weight, and power consumption similar to He–Ne lasers.

To provide a valid comparison to prior work, the same CE system was used with a frequency-doubled Ar ion laser operating at 257 nm. While the frequency-doubled Ar laser is spectrally clean, the NeCu laser, like many other lasers, has a number of incoherent emission lines (data not shown) and a broad-band tube

Table 1. Comparison of Laser Specifications

laser type	frequency-doubled Ar ion laser	NeCu laser
wavelengths (nm)	229, 238, 244, 248, 252, 257	248.6, 252.9, 259, ^a 272 ^b
input power from line (W)	15 000	50
laser head size (in.)	$8 \times 6 \times 48$	$4 \times 6 \times 26$
power supply size (in.)	$18 \times 12 \times 24$	$4 \times 8 \times 12$
cooling	water heat exchanger	free air convection

^a A group of lines including 257.1, 259.1, 259.9, and 260.0 nm. ^b A group of lines including 270.3, 271.8, 272.2, and 274.1 nm.

glow. These must be eliminated prior to focusing the output onto the sheath flow cell, as this would contribute to the fluorescence background and degrade LODs. Figure 1 shows the optical diagram of the CE-LIF system with the NeCu laser as the excitation source with an edge filter pair to remove the spectral background.

A mixture of 17 standards was detected by the NeCu system after CE separation. Figure 2 is the wavelength-resolved electropherogram of this standard mixture. Clearly, many of the important biological compounds including 5-HT, DA, their metabolites, NAS, 5-HIAA, E, and cofactors FAD, FMN, NADH, and NADPH are detected with 248.6-nm excitation.

One can select the pulse repetition rate and the tube power (by controlling the bus voltage and drive current to regulate the pulse power) to optimize the system performance. Table 2 shows the relative figures of merit for this NeCu laser working with different laser parameters. Data are presented as the relative LOD difference based on a setting of 25 A, 650 V, and 122 Hz (default settings). With higher pulse repetition rates and thus higher average powers (from ~ 0.6 mW at 122 Hz to ~ 0.9 mW at 200 Hz on the detection spot), the performance was slightly better for more photostable compounds such as NAS, DA, and 5-HIAA but worse for other compounds, particularly 5-HT. The LODs for 5-HT decreased by 80% at 200 Hz, likely caused by saturation, photodegradation, or both. Lower repetition rates did not increase sensitivity (data not shown). A bus voltage of 650 V was the optimal voltage although slightly better performance (5 and 12% for TrpA and NAS, respectively) was achieved at 750 V. We found that the LODs of all compounds improved with higher drive current (a higher pulse peak power), at least up to 30 A. However, working at higher drive currents is reported to decrease the lifetime of this laser, so that for all experiments, the intermediate settings have been used. Since the laser has operated without a decrease in output power during all experiments described in this report, no data on tube lifetime have been obtained.

To further characterize the suitability of the new LINF detection system for cellular studies, figures of merit for this system were directly compared to the same CE-LINF system using the Ar ion laser working at 257 nm. Table 3 shows the LODs for seven compounds using both systems. For Trp and the indolamines, the performance was slightly poorer (30–200%) using the new NeCu laser. This difference can be reduced at higher drive currents (see Table 2). LODs for DA were essentially the same, 7.8 and 7.6 μ M, respectively. However, the detection ability for Tyr was more than 4 times worse.

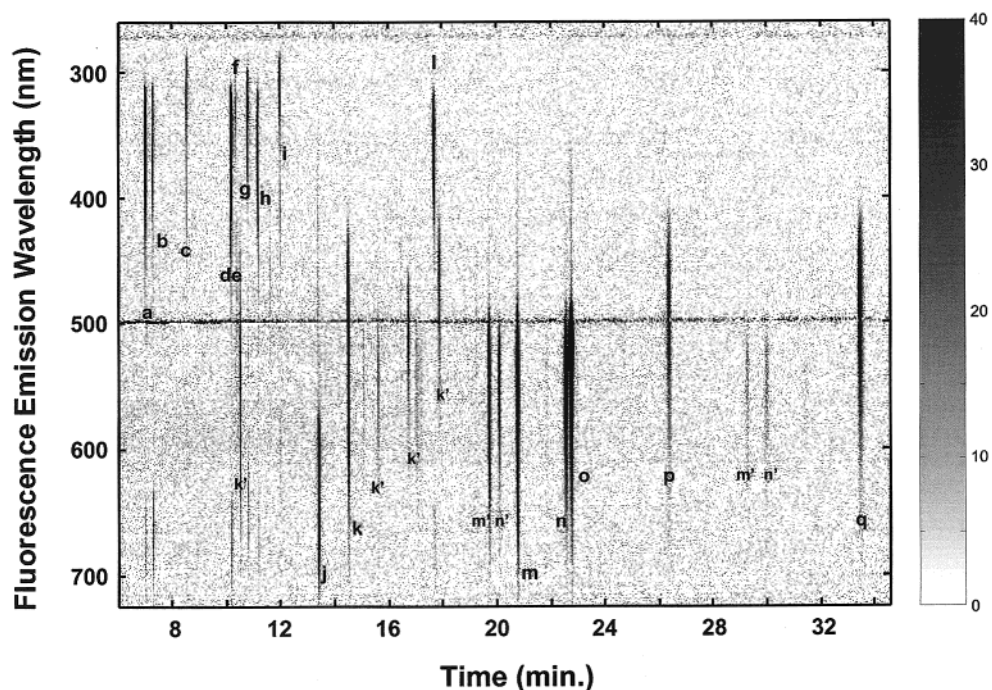


Figure 2. Wavelength-resolved fluorescence electropherogram of a mixture of standards where the y -axis shows the emission spectra wavelength and the gray scale shows the intensity of the fluorescence emission (with the scale bar to the right of the electropherogram). The two horizontal lines are H₂O Raman line (weaker) and second-order laser line (stronger) at 273 and 497 nm, respectively. Peak assignments: (a) 6.2 μ M TrpA; (b) 4.1 μ M 5-HT; (c) 15 μ M OA; (d) 3.1 μ M NAS; (e) 4.2 μ M MEL; (f) 97 μ M DA; (g) 130 μ M E; (h) 3.9 μ M Trp; (i) 28 μ M Tyr; (j) 0.23 μ M sulforhodamine 101; (k) 2.0 μ M THB; (l) 7.0 μ M 5-HIAA; (m) 8.0 μ M FMN; (n) 11 μ M FAD; (o) 0.93 μ M fluorescein; (p) 50 μ M NADH; (q) 95 μ M NADPH. The k' , m' , and n' are minor impurity peaks for k , m , and n standards, respectively.

Table 2. Relative Performance^a for Laser Operating Parameters

parameter	TrpA	5-HT	NAS	DA	Trp	5-HIAA	
repetition rate	150 Hz	3	60	-21	-39	9	-4
	200 Hz	6	84	-32	-56	44	-20
buss voltage	500 V	57	30	29	76	48	56
	750 V	-5	1	-12	16	15	8
drive current	15 A	240	260	250	560	240	200
	35 A	-30	-21	-20	-7	-14	-16

^a For all parameters, the value reported is $100(d - d_0)/d_0$, where d is the LOD at the specified settings and d_0 is the LOD at the default settings for the indicated compound. All unspecified parameters are at the default settings which include a drive current of 25 A, buss voltage of 650 V, and pulse repetition rate of 122 Hz. Thus, better performance compared to default settings is a negative number, and poorer performance is a positive number.

To demonstrate the applicability of this system to separate and detect a real-world sample, individual MCC neurons from the marine mollusk *P. californica* have been dissected and injected. As shown in Figure 3, this neuron contained significant 5-HT; the amino acids Trp and Tyr are also detected. The broad band between 14 and 20 min at \sim 330 nm is from a series of unresolved proteins containing Tyr and Trp residues. It appears the NeCu laser using 248.6-nm excitation is an exciting new alternative source for single-cell CE-LINF assay of 5-HT, DA, and related compounds.

CONCLUSIONS

We have demonstrated the feasibility of the NeCu laser working at 248.6 nm for CE-LINF detection. The detection limits

Table 3. Comparison of LODs (nM) for Selected Compounds

laser type	metal vapor NeCu laser (248.6 nm)		frequency-doubled Ar ion laser (257 nm)	
	mean LOD \pm STD ^a	n	mean LOD \pm STD ^a	n
TrpA	19 \pm 4	9	10 \pm 1	5
5-HT	27 \pm 4	9	21 \pm 5	8
NAS	43 \pm 7	9	22 \pm 3	8
DA	7800 \pm 900	9	7600 \pm 1000	5
Trp	63 \pm 5	8	24 \pm 3	8
Tyr	2100 \pm 500	6	390 \pm 70	6
5-HIAA	170 \pm 30	8	110 \pm 20	8

^a STD, standard deviation based on σ_{n-1} .

for 5-HT and DA are comparable to those obtained from the frequency-doubled Ar ion laser working at 257 nm. Assays of important amines at the single-cell level have also been demonstrated. Particularly intriguing is the availability of similar metal vapor lasers emitting at a wide range of deep UV wavelengths. Optimizing excitation wavelength is important; for example, using 284-nm excitation,²⁸ the LODs for indoles were more than 100-fold better than with 257-nm excitation using a similar sheath flow detection system, likely due to decreased absorption, poorer photostability, and a higher spectral background at 257 nm. The 291.8-nm excitation provided by a HeAu laser is expected to improve the LODs for 5-HT and indolamines (excitation maximum at 298 nm) beyond the current low-nanomolar levels achieved by other LIF systems and large-frame lasers.¹⁹ Using currently available CE-LIF systems, the LODs reported for catecholamines have been \sim 100-fold worse than for indolamines.^{17,33,38} The

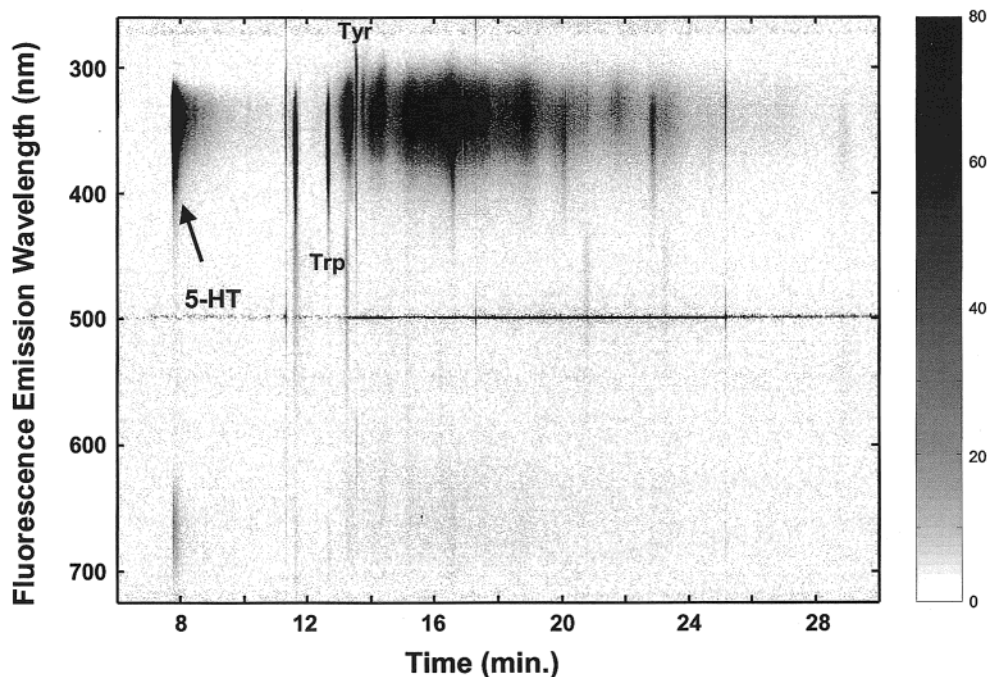


Figure 3. Wavelength-resolved fluorescence electropherogram of a MCC neuron (470- μm diameter) from *P. californica* showing 5-HT, Trp, and Tyr.

availability of a 272-nm line from the NeCu laser, closely matching the excitation maximum of Tyr and catecholamines in the 270–280-nm range, may greatly improve catecholamine measurements. In addition, a deeper UV line at 224.3 nm provided by HeAg lasers may provide more sensitive detection of catecholamines because it accesses the larger cross section $S_0 \rightarrow S_2$ transition⁴⁰ and provides greater isolation between the excitation and emission wavelengths. These advantages, however, may be offset by reduced photostability. Overall, the availability of inexpensive, rugged, and small-size metal vapor lasers that are as easy to use as a He–Ne laser promises to greatly expand the application of UV-LINF detection for CE and should allow the development of

dedicated UV-LINF systems optimized for particular classes of molecules.

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