

Technical Note

Ultraviolet native fluorescence detection in capillary electrophoresis using a novel metal-vapor NeCu laser

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Ultraviolet excitation for laser-induced native fluorescence detection in capillary electrophoresis 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 ultraviolet radiation. The largest drawback of native fluorescence detection schemes in CE/LIF 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 capillary electrophoresis system obtains similar performance to large frame frequency-doubled Ar ion lasers. The detection limits for serotonin and dopamine for a ~3 nL injection (27 nM and 7.8 μ M respectively) are similar to those obtained using a frequency-doubled Ar ion laser at 257 nm (21 nM and 7.6 μ M). 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.

Key Words: capillary electrophoresis, laser-induced fluorescence, native fluorescence, deep UV laser, serotonin, dopamine, single cell.

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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 with derivatization with highly fluorescent tags.¹⁰⁻¹² While the use of fluorescent probes can greatly improve limits of detection (LODs), they suffer from limited shelf life, 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 (< microliter level) or at low analyte concentration (10^{-9} M, sometimes at the 10^{-7} to 10^{-8} M level), because either the labeling efficiency decreases or the back-ground signal increases.¹³⁻¹⁶

As a large group of biological species fluorescence when excited in the ultraviolet (UV) region, 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 analogs, 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 the protein detection limits 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 reported nanomolar LODs for tryptophan (Trp), and down to 10^{-10} M detection limits for proteins which contain many Trp molecules. They have described the detection of native proteins²⁵ and hemoglobin variants¹⁸ in single red blood cells

using this system. 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. Lunte and coworker reported a detection limit of 1 nM for kynurenic acid (a metabolite of Trp) using a 325nm excitation beam of He-Cd laser.²⁶ Frequency-doubled Kr lasers have also been used; for example, LODs for Trp as low as 2×10^{-10} M (800 zmol) have been reported by using frequency doubled Kr laser operating at 284 nm.^{18, 27} Besides continuous wave lasers, several groups have looked at pulsed UV excimer lasers for CE detection. For example, Chan and coworkers demonstrated 3.3 nanomolar 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.²⁸ Kok and coworkers reported a low g/l regime LODs for naphthalene sulfonate species with a frequency-doubled XeCl excimer dye laser working at 280 nm or 325 nm.²⁹ Recently the Gooijer group investigated a frequency-quadrupled Nd:YAG laser emitting at 266nm for CE-LIF analysis of polycyclic aromatic hydrocarbon metabolites³⁰ and obtained LODs in the ng/ml range. Some of these lasers have been adapted for single cell studies and are relatively easy to operate.^{19, 31, 32} However, the largest problem with this approach has been the lack of available ultraviolet 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 has reported impressive mass limits of detection when coupling this detection mode with CE;³³⁻³⁵ however, such systems also require sophisticated lasers and are not commonly used.

We report here the use of a new hollow cathode metal vapor laser (NeCu laser) for laser induced native fluorescence (LINF) detection in CE. The performance of CE-LINF system with this new “turn-key” laser working at 248.6 nm are compared with prior LINF detection schemes based on a frequency-doubled Ar ion laser working at 257 nm, which require considerably more effort and cost more than an 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 boric acid (H_3BO_3 ; Sigma, St. Louis, MO), 9.2 g sodium borate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$, Sigma) in 1.00 L 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 (Trpt), 5-hydroxytryptamine (5-HT or serotonin), n-acetyl serotonin (NAS), 5-hydroxyindolacetic acid (5-HIAA), tyrosine (Tyr), dopamine (DA), octopamine (OA), epinephrine (E), 5,6,7,8-tetrahydrobiopterin (THB), flavin mononucleotide (FMN), flavin adenine dicucleotide (FAD), - nicotinamide adenine dinucleotide (NADH), - 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, 36} Briefly, single metacerebral cells (MCCs) were dissected from *Pleurobranchaea californica* under cold anesthesia in molluscan physiological saline. The MCC was isolated under a stereomicroscope using microscissors and glass micropipettes. Within a minute, 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 capillary electrophoresis system for analysis or immediately frozen on dry ice for storage.

Electrophoresis system and data processing. The laboratory-assembled CE system has been described in detail previously.^{19, 36} 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, and about 3 nL sample was 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/sec.

Excitation of the core stream in the sheath flow cell was provided by one of two lasers. The first was the previously described frequency-doubled, liquid-cooled Ar ion laser (Innova 300 FReD; Coherent, Palo Alto, CA) operating at 257 nm.^{19, 32, 36} Approximately 0.5 mW was directed into the sheath flow cell and focused to a spot ~ 1.0 mm below the capillary outlet with a 20 mm focal length quartz spherical concave lens (Spindler and Hoyer Inc., Medford, MA). The second NeCu laser (PSI NeCu 60, Photon Systems, Covina, CA) consisted of a newly introduced system (a schematic diagram of the instrumental setup is shown in figure 1). Two reflective blocking filters (>250 nm pass) facing each other with a spacing of about 1.5 inches were located in front of the laser and the laser beam was introduced in about 10 degrees. The filter pair was used to eliminate or suppress tube plasma lines. After 4 bounces, the spectrally pure 248.6 nm light exits the filter pair and is directed to the same quartz spherical concave lens. Unless specified elsewhere, the laser was set a pulse width of 30 μs , repetition rate of 122 Hz, drive current of 25A and buss voltage of 650V. An average power of ~ 0.6 mW (pulse peak power of ~ 150 mW) after two UV mirrors was focused to a spot ~ 1.0 mm below the capillary outlet inside of the sheath flow cell.

The collection optics were orthogonal to the excitation beam focusing the fluorescence emission to a f/2.2 CP 140 imaging spectrograph (Instruments SA, Edison, NJ) and the emission information over the 260-720 nm wavelength range was focused across the face of a $1,024 \times 256$ detector-array, liquid-nitrogen-cooled scientific CCD (EEV 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 major goal of the present study is to determine the feasibility of a turn-key metal vapor laser for LINF detection coupled to CE. As shown in table 1, with suitable optics, these NeCu lasers can lase at a variety of deep UV wavelengths in the 200-300 nm range; yet they have the size, weight and power consumption similar to He-Ne lasers.

In order 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 Cu vapor laser, like many other lasers, has a number of emission lines (data not shown) and broad band tube glow that must be eliminated prior to focusing the output onto the sheath flow cell as these 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 utilizing a filter pair to remove the spectral background.

A mixture of 17 standards at low concentrations 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 by 248.6 nm excitation.

One can select the pulse repetition rate, the tube power (by controlling the bus voltage and drive current to control the pulse power) to optimize the system performance. Table 2 shows the relative figures of merits of this NeCu laser working with different laser parameters. Data are presented by the relative LODs difference based on a setting of 25A, 650 V and 122Hz (default

setting). As a result, with higher pulse repetition rates, thus higher average power (from about 0.6 mW at 122 Hz to about 0.9 mW at 200 Hz on the detection spot), the performance were slightly better for more photostable compounds such as NAS, DA and 5-HIAA but worse for the others, particularly 5-HT. The LODs for 5-HT decreased by 80% at 200Hz, likely caused by saturation and /or photodegradation. Lower repetition rate did not increase sensitivity (data not shown) for the LIF detection in this system. Buss voltage of 650V was found to be the optimal voltage in this study although slightly better performances (5% and 12% for Trpt and NAS) were achieved at 750 V. For the tube drive current, we found that the LODs of all compounds studied improved with higher drive current, thus higher pulse peak power at least up to 30A. However, working at higher drive currents is reported to decrease the life-time of this laser so that for all experiments, the intermediate settings have been used. As the laser has operated during all experiments described in this report without a decrease in output power, no data on tube lifetime has been obtained.

To further characterize the suitability of the new LINF detection system for cellular studies, figures of merit of 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 of interest using both systems, including 5-HT, DA and their metabolites using these laser parameters. For Trp and indolamines, the performance was slightly poorer (30% to 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 μ M and 7.6 μ M respectively. However, the detection ability for Tyr was more than four times worse. This is not unexpected because the excitation maximum is in the 290-300 nm range for indolamines and in the 270-280 nm range for catecholamines, which are closer to 257 nm excitation. If the 272 nm optics are used for the NeCu laser, the performance of the metal vapor laser is expected to be much better for these compounds.

In order to demonstrate the applicability of this system to separate and detect a real-world sample, individual MCC neurons from the marine mollusk *Pleurobranchaea californica* have

been dissected and injected into this system for assay. As shown in figure 3, this neuron contained significant 5-HT; the amino acids Trp and Tyr are also detected. Thus, 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 for 5-HT and DA are comparable to those obtained from the frequency-doubled Ar ion laser working at 257nm. Assays of these important amines at the single-cell level are possible. Particularly intriguing is the availability of similar metal vapor lasers emitting at a wide range of deep UV wavelengths. The 291.8 nm beam provided by a HeAu laser is expected to improve the LODs for 5-HT or indolamines (excitation maximum at 298 nm), as LODs for 5-HT at low nM level has been achieved by other LIF systems and large frame lasers.¹⁹ Using currently available CE-LIF systems, the LODs reported for catecholamines have been ~100-fold worse than for indolamines.^{17, 31, 36} The availability of 271 nm line (excitation maximum of Tyr or catecholamine is in the 270-280 nm range) from the NeCu laser should greatly improve catecholamine measurements. In addition, a deeper UV line at 224.3 nm provided by HeAg lasers may provide more sensitive detection of catecholamine because it accesses the larger cross section $S_0 \rightarrow S_2$ transition³⁷ and provides greater isolation between the excitation and emission wavelength. These advantages may be offset by less photostability. Overall, the availability of inexpensive, rugged, small-size and easy-to-use metal vapor lasers that are as simple to use as a He-Ne laser promises to greatly expand the availability 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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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 [†] , 272 [‡]
Input power from line (W)	15,000	50
Laser head size (inches)	8 × 6 × 48	1.7 Dia × 26
Power supply size (inches)	18 × 12 × 24	6 × 4 × 10
Cooling	Water-heat exchanger	Free Air convection

[†] A group of lines including 257.1, 259.1, 259.9 and 260.0 nm.

[‡] A group of lines including 270.3, 271.8, 272.2 and 274.1 nm.

Table 2. Relative performance* for laser operating parameters

Parameter	Trpt	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

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

Table 3. Comparison of detection limits (nM) for selected compounds

Laser Type	Metal vapor NeCu laser (248.6 nm)		Frequency-doubled Ar ion laser (257 nm)	
	mean LOD \pm STD *	n	mean LOD \pm SEM *	n
Tryp	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

*STD: Standard Deviation based on $n-1$.

Figure Captions

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

Figure 2. Wavelength resolved fluorescence electropherogram of a mixture of standards where the y-axis shows the emission spectra wavelength and the false color 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 2nd order laser line (stronger) at 273 nm and 497 nm, respectively. Peak assignments: (a) 6.2 μM Trpt; (b) 4.1 μM 5-HT; (c) 15 μM OA; (d) 15 μM NAS; (e) 4.2 μM MEL; (f) 850 μM DA; (g) 130 μM E; (h) 3.9 μM Trp; (i) 28 μM Tyr; (j) 0.23 μM sulforhodamine 101; (k) 2.0 μM THB; (l) 7.0 μM 5-HIAA; (m) 8.0 μM FMN; (n) 11 μM FAD; (o) 0.93 μM fluorescein; (p) 50 μM NADH; (q) 95 μM NADPH.

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





