Capillary electrophoresis with wavelength-resolved laser-induced fluorescence detection

Xin Zhang · Jeffrey N. Stuart · Jonathan V. Sweedler

Abstract Capillary electrophoresis (CE) enables rapid separations with high separation efficiency and compatibility with small sample volumes. Laser-induced fluorescence detection can result in extremely low limits of detection in CE. Single-channel fluorescence detection, however, furnishes little qualitative information about a species being detected, except for its CE migration time. Use of multidimensional information often enables unambiguous identification of analytes. Combination of CE with information-rich wavelength-resolved fluorescence detection is analogous with ultraviolet–visible diode-array detection and furnishes both qualitative and quantitative chemical information about target species. This review discusses recent advances in wavelength-resolved laser-induced fluorescence detection coupled with CE, with an emphasis on instrument design.

Keywords Review · Capillary electrophoresis · Laser-induced fluorescence · Wavelength-resolved fluorescence

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<td>CCD</td>
<td>charge-coupled device</td>
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<td>CE</td>
<td>capillary electrophoresis</td>
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<tr>
<td>ICCD</td>
<td>intensified CCD</td>
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<td>IPDA</td>
<td>intensified photodiode array</td>
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<td>LCFW</td>
<td>liquid core waveguide</td>
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<td>LIF</td>
<td>laser-induced fluorescence</td>
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<td>LINF</td>
<td>laser-induced native fluorescence</td>
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<td>LOD</td>
<td>limit of detection</td>
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<td>PDA</td>
<td>photodiode array</td>
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<tr>
<td>TDI</td>
<td>time-delayed integration</td>
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<td>TIR</td>
<td>total internal reflection</td>
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<td>UV</td>
<td>ultraviolet</td>
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Chemicals

- DA: dopamine
- E: epinephrine
- FAD: flavin adenine dinucleotide
- FMN: flavin mononucleotide
- 5-HIAA: 5-hydroxyindole-3-acetic acid
- 5-HT or Serotonin: 5-hydroxytryptamine
- MEL: melatonin
- NADH: β-nicotinamide adenine dinucleotide
- NADPH: β-nicotinamide adenine dinucleotide phosphate
- NAS: n-acetyl serotonin
- OA: octopamine
- THB: 5,6,7,8-tetrahydrobiopterin
- Trp: tryptophan
- TrpA: tryptamine
- Tyr: tyrosine

Introduction

In little over two decades capillary electrophoresis (CE) has emerged as a powerful analytical technique for determining the chemical composition of mass-limited samples [1]. It enables rapid separations with high separation efficiency and compatibility with small sample volumes [2, 3, 4, 5]. CE has been used for a variety of applications, including determination of inorganic ions [6, 7], environmental [8, 9], food [10], and clinical analysis [11], peptide and protein separations [12, 13], single-cell assays [14, 15], and DNA sequencing [16, 17, 18]. The state-of-the-art of CE is addressed in several recent reviews [1, 19, 20].

The diminutive capillaries employed in CE, the concomitant small peak volumes, and the temporally narrow peaks which result from the high-efficiency separation complicate detection [21]. Detection in CE is most commonly performed on-column by use of absorbance, often diode-array, detection [22, 23]. To achieve better performance a variety of other detection methods has been developed for CE, including conductivity [24], radiochemistry [25], refractometry [26, 27], and electrochemistry.
Laser-induced fluorescence detection in CE

In 1981 Jorgenson and Lukacs [38] described the fluorescence detection of labeled amino acids separated by CE. In this pioneering work a high-pressure mercury arc lamp was used as the excitation source and fluorescence was detected by means of a standard fluorimeter. A low background and a linear relationship between source and signal intensity make fluorescence detection an inherently sensitive method. Fluorescence detection for CE commonly employs a laser as the excitation source. Lasers emit intense radiation with extremely narrow bandwidths, high spatial coherence, and negligible chromatic aberration; their output is easily focused on to the small flow channel of a capillary. Since the first introduction of LIF detection for CE by Zare et al. [39], limits of detection (LODs) have improved dramatically – detection limits for most research-grade instruments are in the zeptomole range for some fluorophores [2, 3, 4, 5]. Dovichi and co-workers developed a more sensitive LIF method that used a shear flow cuvette [40, 41] as the sample cell to reduce background fluorescence. LOD as low as six molecules of sulforhodamine 101 [42] and ~1 molecule of β-galactosidase in an enzymatic assay [43] were reported by this group. Because of its low LOD, LIF is most often applied to mass-limited biological samples.

Multichannel LIF detection in CE

Combining CE with information-rich spectroscopic detection, e.g. multichannel fluorescence detection, provides both qualitative and quantitative chemical information. This is of interest because of possible lack of reproducibility in electrophoretic migration times and the possibility of coelution of analytes in a complex sample matrix. Use of a two-dimensional system also reduces matrix interferences, thus improving signal-to-noise ratios. Compounds can therefore be identified with greater confidence by use of multi-dimensional fluorescence detection, such as wavelength-resolved, time-resolved, and polarization fluorescence detection. Wavelength-resolved fluorescence furnishes spectral information to complement migration time. Time-resolved fluorescence [44, 45, 46] involves measurement of fluorescence lifetimes, to discriminate against background impurities and to assist in analyte identification. Polarization fluorescence detection [47, 48] makes use of a molecule’s inherent rotational correlation time. This review focuses on wavelength-resolved fluorescence detection, one of the most widely established multichannel fluorescence detection schemes.

Wavelength-resolved LIF detection in CE

History

The first report of wavelength-resolved fluorescence detection in CE used a photodiode array (PDA) to produce a 4-nm-per-channel spectral distribution [49]. LOD for fluorescein was 60 fg, or 20 µmol L⁻¹. Array detectors with low-noise characteristics, e.g. charge-coupled devices (CCD) and intensified PDA (IPDA) enable a large improvement in LOD [50, 51, 52]. The first use of a CCD combined with a polychromator to achieve wavelength-resolved fluorescence in CE was reported by Cheng and coworkers [53]. In this initial demonstration the CCD was used in conventional camera mode with only a 3% duty cycle, and so detection limits were relatively poor – approximately 4 attomoles for fluorescein. In 1991 the Zare group modified the time-delayed integration (TDI) mode to read the CCD for their application [54]. Use of the TDI mode enables synchronization of the transfer of photogenerated charge with the movement of the image of the analyte band on the array. This enabled the fluorescence to be integrated over the entire time in the observation zone and resulted in a two-to-five-fold improvement in performance. LOD were improved dramatically to 20–80 zmole (13 nL injection) for fluorescein isothiocyanate-labeled amino acids. Also in 1991 a CCD-LIF system with a 94% duty cycle, which furnished high-quality spectral information, was demonstrated [55]. The concentration LOD for Joe-A oligonucleotide primer was 26 pmol L⁻¹. Several other research groups have since reported CCD- or IPDA-based detection with a variety of instrumental configurations and applications (vide infra) and CE with wavelength-resolved fluorescence detection has now been successfully commercialized. The Applied Biosystems (ABI) Prism 377 DNA sequencer is an automated instrument designed for analysis of fluorescently-labeled DNA fragments separated by capillary gel electrophoresis [56]. A spectrograph-CCD is used to achieve wavelength resolution, to enable differentiation of the different dye-labeled nucleotides. Reading of up to 900 bases with 99.8% accuracy is possible.

Instrumentation and design

Typically, fluorescence detection systems consist of three major subsystems – excitation, collection, and detection.
Choice of excitation laser source is based on the requirements of a particular application. Collection optics frequently consist of a high numerical aperture microscope objective, designed to collect the maximum amount of emitted light. Elimination of scattering and background is often achieved by use of spectral and spatial filters, end-column sheath-flow cell detection, or, more recently, in-column detection and liquid core waveguide (LCW) end-on detection (vide infra). For multiwavelength detection a wavelength selector is required; this consists either of a series of filters for preselected wavelengths or a grating or prism for detection over a range of wavelengths. Multi-element array detectors, e.g. cryogenically-cooled CCD and IPDA, typically enable multiwavelength detection, with the detector characteristics often dictating system performance. Cryogenically-cooled two-dimensional CCD cameras are integrated solid-state photometric detectors characterized by negligible dark current, high quantum efficiency, low readout noise, wide dynamic range, and the ability to bin photo-generated charges from multiple pixels [50, 51, 57]. The IPDA is a solid-state diode array coupled to an image intensifier via a fiber-optic bundle. With the image intensifier as an amplifier for photons, a gain of 3000 to 9000 is typically obtained, thus the signal-to-noise ratio of the IPDA detector is significantly higher than that of the PDA and the IPDA is therefore highly sensitive under low-light conditions [58, 59]. In comparison, the CCD has extremely low noise, generally under 5 e− and has a dark current less than 1e− per hour if properly cooled, and so is commonly used without intensification in this application [51, 57]. Although the CCD detector is reported to have significant advantages over the IPDA in terms of spectral resolution, intensity, and precision of data in the vacuum UV for laser-induced plasma spectroscopy [60], intensified detectors such as the intensified CCD (ICCD) and IPDA are suitable for time-resolved measurements with better temporal resolution [60]. For wavelength-resolved fluorescence detection in CE readout time is usually not an issue. Several different instrumental configurations are described for wavelength-resolved fluorescence detection in CE.

**CCD-based detection**

The first wavelength-resolved CE-LIF-CCD system developed by us was based on previous work by Zare’s group, and utilized an Ar/Kr mixed gas ion laser, which provided great flexibility in excitation wavelength [61]. The detection window (~2 mm section of the capillary) was illuminated axially through a cylindrical lens. The fluorescence was collected, via a collection optic assembly at a 90° angle, during the entire time the analyte band was resident in the detection window, to enhance the LIF sensitivity. Rayleigh scattering (owing to on-column detection) was removed by use of spatial filters. The combination of a high-throughput imaging spectrograph (405 grooves mm⁻¹ holographic grating) and a liquid-nitrogen-cooled CCD (1024×256 pixels, 3 Hz readout, 5×256 binning) enabled use of a 500-nm spectral window adjustable in the 300–1000 nm region, which is the widest fluorescence wavelength range collected in a wavelength-resolved CE-LIF system. LOD of 80 molecules for sulforhodamine 101 (5×10⁻¹⁴ mol L⁻¹) and 220 molecules for fluorescein (1.5×10⁻¹³ mol L⁻¹) were achieved. In one application amino acids labeled with Bodipy 503/512 C₃ and Bodipy 576/589 C₃ were differentiated on the basis of emission spectrum and migration time.

Another instrumental configuration with similar spectrograph-CCD was demonstrated in a two-laser–two-color flow cytometry study [62]. A sheath-flow cell (quartz cuvette) was used as the sample chamber. Excitation of the core stream was achieved by means of a liquid-cooled Ar⁺ laser and an ArKr⁺ laser operating at 488 nm and 568 nm, respectively. Collection optics included a microscope objective (to eliminate fluorescence from the collection optics) and a series of 500-nm high-pass and 568-nm laser-line rejection filters. The spectrograph (grating 285 grooves mm⁻¹) and CCD were oriented to enable sub-array readout and binning (3 Hz readout, 2×256 binning) while preserving 1024-pixel wavelength information over the 350–800 nm wavelength range. By use of single-particle fluorescence emission spectra, submicron particles were individually identified, sized, and distinguished.

Blaschke et al. recently built an on-column wavelength-resolved CE-LIF system using an Ar⁺ laser working at 257 nm and an intensified CCD-spectrograph to achieve wavelength resolution. Similar to previously described on-column LIF detection, the capillary was illuminated with the laser profile for a 1.5 mm length along the detection window. With an imaging spectrograph (1200 grooves mm⁻¹ grating) and an intensified CCD camera (9.9 Hz readout, 10×80 binning or 4.5 Hz, 48×40 binning), a spectral window of 105 [63] or 160 nm [64] could be selected over the 180–400 nm wavelength range. The combination of migration time and spectral information enabled simultaneous detection and quantitation of etoposide and etoposide phosphate in plasma for the first time.

An interesting and intriguing design for CE with wavelength-resolved fluorescence detection is the LCW fluorescence detector developed by Roeraade’s group [65]. The principle of total internal reflection (TIR) was used in an effort to reject more stray light and for efficient illumination and collection of light from large capillary arrays. TIR was achieved by coating the separation capillary externally with a polymer with a lower refractive index than the separation medium, thereby inducing liquid-core waveguiding so that imaging could occur at the capillary end. As shown in Fig. 1, the laser beam from a multi-line, multi-mode Ar⁺ laser was expanded to ~10 mm and then focused by means of a cylindrical lens on to the capillary array positioned at the focal line (the axial extent of the beam inside the capillary was ~25 µm). The emitted fluorescent light was guided approximately 50 mm to the end of the capillary, which was placed in a buffer chamber. The wall of the container opposite the capillary ends was a planar glass plate. By using glass filters to absorb the primary light, the capillary ends were imaged via a cam-
era objective on to a thermoelectrically-cooled CCD camera. A prism was placed between the filter and the camera to allow for color dispersion. The CCD chip (1024 TKB, 100 kHz read-out rate) was binned in hardware into superpixels (8×8 pixels large). A 10 superpixel-wide spectrum covered approximately the 480–620 nm range. The CCD was operated in camera mode with an exposure time of 0.6 s for imaging.

Some unique properties result from this instrument design. The LOD for fluorescein was 62 fmol L⁻¹, which is lower than for most sheath-flow systems (note that the mass limits of detection cannot be specified, because the injection volume is uncertain). The orthogonal geometry implies that no laser light should be detected, but in practice this is not so, because of dust, scratches on the capillary surface, and Rayleigh scattering. Yet, even this light can be spatially differentiated in this system, because of the principle of LCW. Light scattered near the periphery of the capillary exterior should propagate down the capillary toward the outer surface, whereas fluorescent light from the sample near the center of the capillary will result

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**Fig. 1** Schematic diagram of CE with end-on wavelength-resolved LCW fluorescence detection [65]. Figure reprinted with permission from Analytical Chemistry. Copyright 2000 American Chemical Society

**Fig. 2** Top view of the two-laser–two-window on-column wavelength-resolved fluorescence detection system [67]. Figure reprinted with permission from Analytical Chemistry. Copyright 1993 American Chemical Society
in a peak in the center of the CCD image. Effective stray light rejection is the most impressive accomplishment of the design, especially in comparison with other more labor-intensive designs in which sheath-flow cuvettes or scanning confocal detectors are employed. The use of interference filters and a prism for color dispersion means, however, that a highly collimated excitation source is necessary. This high-sensitivity design is simple and robust, and has recently been adapted to four-color DNA sequencing by gel electrophoresis in a ninety-one capillary array [66].

**IPDA-based detection**

Wavelength-resolved detection has also been accomplished by use of an IPDA mounted on a spectrograph. In 1993 Karger’s group [67] developed a two-laser–two-window IPDA detection method for four-color DNA sequencing in CE. As shown in Fig. 2, two different lasers (488 nm Ar⁺ laser and 543 nm He–Ne laser) were used to excite different groups of fluorophores sequentially on-column.

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**Fig. 3** (A) Schematic diagram of the sheath-flow-based CE system utilizing a frequency-doubled Ar⁺ laser for excitation and a spectrograph-CCD for wavelength-resolved fluorescence detection. (B) Wavelength-resolved fluorescence electropherogram obtained from 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 electrophogram). Excitation was at 257 nm. The two horizontal lines indicated by *arrows* are the major H₂O Raman line at ~284 nm (3654 cm⁻¹) (weaker) and the 2nd-order laser line (stronger) at 514 nm. Peak assignments: (a) 6.2 µmol L⁻¹ TrpA; (b) 4.1 µmol L⁻¹ 5-HT; (c) 15 µmol L⁻¹ OA; (d) 3.1 µmol L⁻¹ NAS; (e) 4.2 µmol L⁻¹ MEL; (f) 97 µmol L⁻¹ DA; (g) 130 µmol L⁻¹ E; (h) 3.9 µmol L⁻¹ Trp; (i) 28 µmol L⁻¹ Tyr; (j) 0.23 µmol L⁻¹ sulforhodamine 101; (k) 2.0 µmol L⁻¹ THB; (l) 7.0 µmol L⁻¹ 5-HIAA; (m) 8.0 µmol L⁻¹ FMN; (n) 11 µmol L⁻¹ FAD; (o) 0.93 µmol L⁻¹ fluorescein; (p) 50 µmol L⁻¹ NADH; (q) 95 µmol L⁻¹ NADPH. The k’, m’, and n’ are minor impurity peaks for k, m, and n standards, respectively.
Fluorescence spectra (510–700 nm and 550–700 nm, respectively) from these two windows were imaged on the separate diodes in the array (1024 channels) via a spectrograph (150 grooves mm\(^{-1}\) grating) and band-pass filters. This design introduced use of multi-window detection to optimize excitation wavelengths and minimize photobleaching. The IPDA enabled low-level detection (concentration LOD in the pmol L\(^{-1}\) range) and spectral resolution to aid dye discrimination. No further development or application using this system has, however, since been reported.

Another IPDA-based wavelength-resolved fluorescence detection system was constructed by Majidi’s group [68]. Their detection configuration was quite different from either on-column or end-column sheath-flow-based detection. Instead, a 100 µm fiber optic was inserted into a 200-µm i.d. fused-silica separation capillary and extended to the end of the detection window. Electromagnetic radiation (2nd harmonic, 532 nm) from a pulsed, Q-switched Nd:YAG laser was focused on to the fiber optic, which then supplied excitation energy for fluorescence measurements. Axial excitation of fluorescent molecules in-column avoids the common problem of light scattering (often encountered with on-column excitation), because the excitation beam does not need to pass through the capillary wall [54]. Fluorescence emission from analytes in the capillary was focused through a lens to a cooled IPDA (1024 channels) mounted on a spectrograph (600 grooves mm\(^{-1}\)). Three-hundred spectra taken every 100 ms were co-added to obtain a data point. The wavelength range covered was 140 nm. Separation and identification of rhodamine derivatives were demonstrated. Although the authors reported “unoptimized” detection limits, they are currently too high for many applications (0.26 pmol for rhodamine 590 chloride and 1.5 pmol for kiton red). In addition, the separation efficiency was poor, possibly because of the low separation voltage (500 V) or deposition of analytes on the surface of the fiber optic during the separation. It will be interesting to follow further developments which use this approach.

**Native fluorescence detection**

Perhaps the greatest benefit of wavelength-resolved fluorescence detection is laser-induced native fluorescence

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**Table 1** Summary of CE with wavelength-resolved LIF systems

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<tr>
<th>Detector</th>
<th>Detector settings</th>
<th>Detection configuration</th>
<th>Excitation laser (nm)</th>
<th>Spectral win. (nm)</th>
<th>Application</th>
<th>LOD</th>
<th>Ref.</th>
</tr>
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<tr>
<td>CCD</td>
<td>3</td>
<td>On-column</td>
<td>Ar/Kr(^{+}) (multi-line)</td>
<td>500 (300–1000)</td>
<td>Bodipy-labeled amino acids</td>
<td>50 fmol L(^{-1})</td>
<td>[61]</td>
</tr>
<tr>
<td>3</td>
<td>2×256</td>
<td>Sheath-flow</td>
<td>Ar(^{+}) (488) and Ar/Kr(^{+}) (568)</td>
<td>350–800</td>
<td>Flow cytometry</td>
<td>6000 SR 101</td>
<td>[62]</td>
</tr>
<tr>
<td>5</td>
<td>5×256</td>
<td>Freq.-d. Kr (284)</td>
<td>Freq.-d. Ar(^{+}) (257)</td>
<td>280–500</td>
<td>Peptides (Trp, Tyr)</td>
<td>20 nmol L(^{-1})</td>
<td>[71]</td>
</tr>
<tr>
<td>50 K</td>
<td>2×256</td>
<td>NeCu (248.6)</td>
<td>NeCu (248.6)</td>
<td>260–710</td>
<td>Single neuron</td>
<td>20 nmol L(^{-1})</td>
<td>[73, 74]</td>
</tr>
<tr>
<td>4.5</td>
<td>48×40</td>
<td>On-column</td>
<td>Freq.-d. Ar(^{+}) (257)</td>
<td>105 (180–400)</td>
<td>Clinical samples</td>
<td>1 µg L(^{-1})</td>
<td>[63]</td>
</tr>
<tr>
<td>9.9</td>
<td>10×80</td>
<td>On-column</td>
<td>Ar(^{+}) (multi-lines)</td>
<td>160 (180–400)</td>
<td>Clinical samples</td>
<td>100 µg L(^{-1})</td>
<td>[64]</td>
</tr>
<tr>
<td>100 K</td>
<td>10×10</td>
<td>LCW end-on</td>
<td>Ar(^{+}) (488) and He-Ne (543)</td>
<td>480–620</td>
<td>DNA sequencing</td>
<td>62 fmol L(^{-1})</td>
<td>[65]</td>
</tr>
<tr>
<td>IPDA</td>
<td>1024 channels</td>
<td>2-laser–2-win. On-column</td>
<td>KrF-excimer dye (280 or 350)</td>
<td>510–700 550–700 311–512</td>
<td>DNA sequencing</td>
<td>Low pmol L(^{-1})</td>
<td>[67]</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>On-column</td>
<td>Large frame Ar(^{+}) (275)</td>
<td>410–588</td>
<td>Environmental</td>
<td>Sub µg L(^{-1})</td>
<td>[75, 76]</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>In-column</td>
<td>Q-switched Nd:YAG (532)</td>
<td>140</td>
<td>–</td>
<td>0.26 pmol R590</td>
<td>[68]</td>
</tr>
</tbody>
</table>

(Abbreviations: 5-HT, 5-hydroxytryptamine; DA, dopamine; FL, fluorescein; Freq.-d., Frequency-doubled; gr., grooves; R590, rhodamine 590; Ref., Reference; Spcg., spectrograph; SR 101, sulforhodamine 101; win., window)
LINF) studies, because different analytes have characteristic emission spectral profiles, in contrast with the constant fluorescent emission resulting from derivatization with a single fluorescent probe. Native fluorescence detection averts the complications associated with derivatization reactions, such as limited shelf life of fluorescence probes, lack of complete specificity for the analytes, and slow kinetics or incomplete reactions. Derivatization also can be problematic when dealing with small sample volumes (sub-microliter level) or low analyte concentrations ($10^{-10}$ mol L$^{-1}$ to $10^{-7}$ mol L$^{-1}$), because of a decrease in the labeling efficiency and an increase in background signal [37, 69, 70]. As an attractive alternative to labeling compounds with tags that absorb and fluoresce in the visible region, native fluorescence detection normally requires an aromatic (fluorescent) molecule and usually requires ultraviolet (UV) laser excitation.

Taking advantage of the intrinsic fluorescence of tryptophan (Trp) and tyrosine (Tyr) we incorporated a frequency-doubled krypton laser operating at 284 nm and modified the detection assembly to achieve the first wavelength-resolved native fluorescence detection in CE [71]. Because Rayleigh scattering and capillary background fluorescence are more prominent in the UV region than in the visible, a sheath flow cell (1.0 mm × 1.0 mm quartz cuvette) was employed; with the sheath flow system, no spatial filters were required. A sheath flow cell can also function as a post-column derivatization reactor [72], or enable independent optimization of separation and detection conditions [73]. In contrast with on-column detection, observation time is limited by diffusion in the end-column sheath-flow detection cell, because analytes continue to diffuse into a larger volume after leaving the capillary. The laser was therefore focused to a tight spot inside the sheath-flow cell just below the capillary outlet. The CCD was operated under conditions similar to those in our initial arrangement – 5 Hz readout and 5 × 256 binning. Detection limits as low as $2 \times 10^{-10}$ mol L$^{-1}$ were achieved for Trp; this is among the lowest reported values for Trp by fluorescence detection in CE without derivatization.

This system was further improved for direct single-neuron analysis [74]. A schematic diagram of the instrument is shown in Fig. 3A. A frequency-doubled Ar$^+$ laser was used to provide 257 nm laser excitation. Complete fluorescence emission spectra (260–710 nm) were recorded, and interference from endogenous impurities or co-eluting substances was eliminated by selection of an appropriate wavelength range for integration, thus improving the signal-to-noise ratio. LOD ranged from low attomole to femtomole. Combination of electrophoretic migration time and fluorescence spectral information enabled simultaneous identification of more than 30 compounds in individ-

**Fig. 4** Three-dimensional electropherogram obtained from naphthalenesulfonates (NS). The structures of the nine NS are shown on the y-axis. Excitation was at 280 nm [75]. Figure reprinted with permission from Journal of Chromatography. Copyright 1997 Elsevier.
ual neurons. A typical wavelength-resolved electropherogram of selected standards is shown in Fig. 3B. This figure shows the separating power and the variety of analyte spectral profiles that can be recorded by use of this system.

Other examples of CE with wavelength-resolved native fluorescence detection include an IPDA-based system developed by Gooijer et al., who used a pulsed KrF-excimer-dye (Rhodamine 6G) laser with a frequency-doubling crystal to provide tunable UV output [75] or a modified Ar+ laser to provide 275 nm excitation [76]. Fluorescence light was collected on column via a microscope objective and focused on to a spectrograph-IPDA (600 grooves mm⁻¹ grating; IPDA 1024 channels). The spectral window was adjusted to cover different wavelength ranges corresponding to specific applications (Table 1). Figure 4 shows a three-dimensional electropherogram (spectral window 311–512 nm) of naphthalene monosulfonates, a major class of environmental pollutant. The potential of wavelength-resolved detection for identification purposes is apparent.

One of the largest drawbacks of UV excitation in wavelength-resolved fluorescence detection with CE has been the expense and the complexity of the lasers required for excitation in the far UV wavelength range of 200–300 nm. An improved “turn-key” metal-vapor NeCu laser operating at 248.6 nm was recently demonstrated for a wavelength-resolved LINF study [77]. The performance obtained was similar to that from large-frame frequency-doubled Ar+ lasers. The availability of inexpensive, rugged, and small metal-vapor lasers promises to expand substantially the application of wavelength-resolved UV-LINF detection for CE and should enable the development of dedicated systems optimized for particular classes of molecule. Concerns about the laser lifetime might, however, prevent rapid adoption of the metal-vapor lasers.

Applications

**Multi-fluorophore DNA sequencing**

DNA sequencing technologies have been under rapid development, driven by the need of the Human Genome Pro-
ject for low-cost, high-throughput sequencing. The standard method is didoxy terminator chemistry with separation of tagged DNA fragments. Typically the four A-T-C-G bases are tagged with specific fluorophores with different fluorescence properties [78, 79]. Wavelength-resolved laser-induced fluorescence detection results in high sensitivity and enables easy discrimination and unambiguous identification of the four bases, and CE affords a rapid and highly efficient separation. On the basis of analysis of the spectral characteristics of the four standard dye-labeled primers, FAM, JOE, ROX, and TAMRA, separation and identification of DNA sequencing reaction products were demonstrated by the Karger group using a two-laser–two-window CE-LIF-IPDA [67] (vide supra). Figure 5 shows an example of the four spectral channel DNA sequencing data obtained by use of this system for analysis of M13mp18. A capillary array, such as that designed by Roeraade [66], also promises the additional characteristic of high-throughput DNA sequencing.

Cellular analyses

Simultaneous detection and quantitation of small molecules, metabolites, and cofactors at the level of individual cells provide fundamental information for cellular physiologists. By using spectral information combined with CE migration time, the identification, purity, and amount of many natively fluorescent analytes in complex cell samples can be determined. On excitation at 257 nm many classes of biologically important compound (e.g. aromatic amino acids, neurotransmitters, and cofactors) emit native fluorescence. Successful single-neuron analyses of diverse aromatic monoamine neurotransmitters (indolamines and catecholamines) have been demonstrated [73, 74, 80]. LODs of 20 nmol L\(^{-1}\) for serotonin (indolamine) [74] and 120 nmol L\(^{-1}\) for dopamine (catecholamine) [73] were achieved. An example of a wavelength-resolved electropherogram of a serotonergic metacerebral neuron from *Pleurobranchaea californica* is shown in Fig. 6. The neurotransmitter, serotonin (5-HT), was detected, as were the amino acids Trp and Tyr and the cofactor 5,6,7,8-tetrahydrobiopterin (THB).

Clinical assays

In clinical settings samples are characterized by sample volumes <1 mL, biological fluid matrices (e.g. plasma, urine), a quantitative range of 3–4 orders of magnitude in concentration, and a limit of quantitation in the µg L\(^{-1}\) to ng L\(^{-1}\) range [81]. CE with wavelength-resolved fluorescence detection has proven to be a competitive analytical method for addressing these challenges. A study of salicylate compounds in urine was recently reported [82]. Thormann and colleagues distinguished between gentisic acid and a co-migrating endogenous urinary compound; this is not possible by use of single-channel fluorescence detection. This technique, in contrast with literature methods, also enabled the putative assignment of several unknown peaks. Other clinical applications – analysis of tramadol in urine [63], etoposide and etoposide phosphate in human plasma [64], and several other drug assays [81] – have been reported by Blaschke et al.

Environmental applications

The applicability of CE in environmental analysis is usually rather limited because of poor concentration detection limits in CE compared with liquid chromatography [9]. Combination of a well-designed instrument with high-sensitivity LIF detection and the impurity-discriminating method such as CE-LIF-IPDA promises the additional characteristic of high-throughput DNA sequencing.
power of wavelength-resolved native fluorescence detection, however, enables sensitive analysis of aromatic naphthalenesulfonates in river water [75]. As shown in Fig. 7, selectivity was enhanced by narrowing the emission window. The reported LOD was in the low µg L$^{-1}$ to sub-µg L$^{-1}$ range, adequate for real-world environmental monitoring.

Other biological applications

Another biological application was demonstrated by a study of the isoflavone formononetin in red clover extract [76]. Structurally related isoflavonoids and metabolites occur together in some biological fluids. On excitation at 275 nm several furnish markedly different fluorescence spectra and can be separated and distinguished by means of CE coupled to wavelength-resolved fluorescence detection. Identification and quantitation were possible at a concentration of $3 \times 10^{-5}$ mol L$^{-1}$, or 17 µg formononetin per gram red clover.

Separation diagnostics

In addition to analytical applications, the information in the fluorescence emission spectrum can yield diagnostic

Fig. 7 Electropherogram obtained from a River Elbe sample by use of on-column wavelength-resolved fluorescence detection. Excitation was at 280 nm and the emission range was (A) 311–512 nm or (B) 349–380 nm. When the smaller wavelength window is used, only the naphthalenesulfonates which do not have an OH or NH$_2$ group appear in the electropherograms [75]. Figure reprinted with permission from Journal of Chromatography. Copyright 1997 Elsevier
information on the separation. For example, monitoring of the pH inside the capillary during a separation has been achieved by use of a pH-sensitive fluorophore, carboxy SNARF-1 [83]. The fluorescence emission profile of SNARF-1 shifts at different pH, which enabled measurement of a counter-propagating pH gradient. Ionic strength, temperature, and ions can be measured by use of different fluorophores. Other diagnostic possibilities include correction of source drift and alignment problems, by use of Rayleigh and Raman scattering, and dynamic choice of wavelength range with regard to background features [52].

Conclusions and outlook

Several wavelength-resolved CE-LIF systems have been described. The instrumental configurations and applications are summarized in Table 1. Wavelength-resolved fluorescence detection is normally achieved by use of either a CCD or an IPDA mounted on a spectrograph. The main design differences in the different systems are detection-cell configurations, including on-column detection, in-column detection, sheath-flowed based end-column detection, and LCW end-on imaging. Sheath-flow detection results in the lowest LOD, whereas LCW end-on imaging provides the potential of large-scale multiplexing without greatly sacrificing sensitivity. Wavelength-resolved fluorescence detection, in providing information complimentary to migration time, has several advantages for complex samples such as biological and environmental material. With wavelength-resolved fluorescence analyte misidentification is less likely, even under changing electrophoretic conditions. One inherent limitation of fluorescence detection is the inability to identify unknown peaks without a reference compound. A combination of migration time and fluorescence emission spectra can, however, provide some information about analyte structure [75, 82]. The high sensitivity, high electrophoretic performance, and adequate spectral resolution of CE with wavelength-resolved laser-induced fluorescence detection has already been commercialized for four-color DNA sequencing, and we expect further advances in instrumentation and practical applications.

References
