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Journal of Chromatography B, 695 (1997) 85–92

JOURNAL OF CHROMATOGRAPHY B

On-the-fly fluorescence lifetime detection of labeled DNA primers

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Abstract

The first application of frequency-domain, on-the-fly fluorescence lifetime detection to the detection, identification and resolution of fluorescent labeled oligonucleotide primers in capillary electrophoresis is presented. These studies, which are directed towards the development of four-decay DNA sequencing, employed two common sequencing primers, SP6 and M13/pUC. The primers were each tagged by a derivative of either fluorescein or BODIPY. Fluorescence emission maxima and lifetimes of the free dyes and the labeled primers were determined both in batch mode and on-the-fly in capillary electrophoresis. Fluorescence intensity and lifetime electropherograms were extracted from dynamic lifetime data that were acquired at 0.1-s intervals during the CE separation. Lifetimes were recovered using either conventional non-linear least-squares analysis or the self-modeling maximum entropy method, which does not require prior knowledge of the system. Based on migration time and fluorescence lifetime, peaks could be detected and identified, and co-eluting peaks could be resolved. Interference from background impurities and scattered light was greatly reduced by the combination of physical separation and lifetime resolution.

Keywords: On-the-fly detection; Fluorescence lifetime; DNA

1. Introduction

Fluorescence lifetime is under investigation for on-the-fly detection of capillary electrophoresis (CE) separated DNA fragments, for application to four-decay detection in DNA sequencing. In a four-decay scheme, fluorescence lifetime would be detected instead of color in a single-lane separation of labeled DNA fragments. There are several reasons for investigating lifetime detection for sequencing as well as more general CE applications. In contrast to the analog nature of intensity or spectral measurements, fluorescence lifetime is an intrinsic, concentration-independent characteristic of a compound, affording 'digital' detection in which the lifetime of the compound is observed as soon as the concentration of the compound exceeds its detection limit. Moreover, since the lifetime of a compound is generally governed by the simple, exact relationships of first-order kinetics, deviations from the expected decay behavior will indicate matrix effects or other sources of inaccuracy that might go unnoticed in simple spectroscopic detection.

Fluorescence lifetime detection can be achieved using either time-domain or frequency-domain techniques [1]. The time domain uses pulsed excitation to generate a decay curve, from which the fluorescence lifetime profile is extracted. The frequency domain uses continuous (cw) excitation that is intensity modulated at a high (MHz–GHz) frequency, producing a time-dependent intensity signal that is comprised of a sinusoidal (a.c.) component superimposed on a time-independent (d.c.) intensity component. The fluorescence emission is phase-

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PII S0378-4347(97)00102-3

shifted and demodulated relative to the exciting light and the fluorescence lifetime can be calculated independently from the phase-shift ϕ and the modulation ratio m.

Frequency domain technology has been shown to provide unprecedented speed for on-the-fly detection and lifetime resolution in HPLC [2,3] and CE [4]. Continuous, on-the-fly fluorescence lifetime detection is achieved by using a state-of-the-art, commercial instrument that incorporates multiharmonic Fourier transform (MHF) technology to allow measurements of phase and modulation simultaneously at many modulation frequencies. An entire frequency response, which contains all of the information required to resolve the signal into the contributing lifetimes and their fractional intensity contributions, can be acquired on the order of milliseconds, providing many points per CE peak. This is a major advantage over time-domain detection, which generally must assume monoexponential decay for on-the-fly detection even when the actual decay is more complex [5,6]. Fluorescence intensity is simultaneously derived from the same measurement to produce a lifetime–intensity electropherogram in no more time than is required for an intensity-based electropherogram alone.

In this work, we evaluate fluorescence lifetime detection using the CE–MHF system for on-the-fly detection, identification and resolution of labeled DNA. Two common primers, M13/pUC reverse primer and SP6 primer, were investigated. Both primers were labeled with either fluorescein (FL) or BODIPY-FL C_3 succimidyl ester (BODIPY) at the 5′ end. These four primers, which were purchased ready-made at low cost, provided an excellent opportunity to demonstrate the power of the combined CE–MHF technique for analysis of real samples containing labeled DNA fragments.

2. Experimental

2.1. Sample preparation for batch mode study

The labeled primers were purchased from Molecular Probes. They included M13/pUC reverse primer (d(CAGGAAACAGCTATGACC)) and SP6 primer (d(CGATTTAGGTGACACTATAG)), each labeled with either fluorescein (FL) or BODIPY-FL C_3 succimidyl ester (BD) at the 5′ end. The four labeled primers (M13-FL, M13-BD, SP6-FL and SP6-BD) were solubilized in 100 mM Tris buffer (pH 8.6) to yield a concentration of 0.25 AU per ml (approximately 1 μM) in the batch studies or 0.4 AU per ml (approximately 2 μM) in the CE studies. Mixtures were prepared to have equal intensity contributions from each component.

2.2. CE separation

Separations were performed on a Beckman P/ACE 5000 which was equipped with a CE–MS interface to provide an external power supply. A ssDNA separation kit (eCAP ssDNA 100-R, Beckman, Fullerton, CA, USA) provided the DNA capillary column, a replaceable gel and a Tris–borate buffer with 7 M urea. The electrokinetic injection mode on the CE was used to introduce samples into the gel-filled capillary column (100 mm I.D.). The total column length was 48 cm and the distance from inlet to detection window was 40 cm. A constant voltage of −15 kV was used to drive the analyte from the inlet to the window for the lifetime measurement. Experiments were performed at room temperature.

2.3. Lifetime measurements and OFLD

An MHF fluorescence lifetime instrument (Model 4850 MHF, Spectronics Instruments, Rochester, MN, USA) was used for batch lifetime measurements as well as on-the-fly lifetime detection, for which it was interfaced to the CE as previously described [4]. In the present work, an air-cooled Ar^+ laser (Model 543R-AP-A01, Omnichrome, Chino, CA, USA) provided 100 mW excitation at 488 nm. Emission was selected through a 515-nm long pass filter. A 488-nm holographic filter (Notch-Plus, Kaiser Optical Systems, Ann Arbor, MI, USA) was added in the emission beam to greatly reduce the contribution from scattered laser light, which allowed the use of the 515-nm long pass filter instead of the 550-nm long pass filter used in the previous work. In addition, a 10× microscope objective was added in the emission path to improve collection of the emitted light. A cross-correlation frequency of 10 Hz was used in the lifetime measurements, resulting in

10 lifetime measurements per second. An IBM 486DX33 computer was used to collect the lifetime data.

2.4. Data analysis

In OFLD, both fluorescence intensity and lifetime are recovered from the dynamic MHF data to simultaneously provide intensity and lifetime electropherograms. The fluorescence intensity was recovered from the dynamic MHF data using an in-house program [2]. The lifetime data were analyzed using either conventional non-linear least squares (NLLS) or the maximum entropy method (MEM). Using NLLS, the data were fitted to an a priori model in which the lifetimes were either allowed to float or fixed to predetermined values. The recovered lifetime profile contains the lifetimes and the fractional contribution of each lifetime component to the total intensity at that point in the electropherogram. Contributions from scattered light (Rayleigh and Raman) can be resolved by adding a very short lifetime component to the model, since scattered light has an effective lifetime of zero on the time-scale of fluorescence emission.

MEM is a self-modeling method which does not require prior knowledge or assumptions about the fluorescence decay of the sample. It is therefore able to indicate the presence of impurities, matrix effects and background through the unbiased recovery of a complete lifetime profile. MEM has proven to be a valuable tool for recovery of fluorescence lifetime distributions from frequency-domain data [7–12] and its effectiveness for analysis of OFLD data in CE has been demonstrated [4].

In the present work, batch lifetimes were recovered using MEM only while the OFLD lifetime data were analyzed using both MEM and NLLS. The MEM analysis used a lifetime window of 0.1–150 ns that was divided into 200 lifetime cells. In the MEM lifetime electropherograms, each lifetime profile was multiplied by the intensity at that point as previously described [4]. The effect of this weighting scheme is to reduce the contributions from random background noise in baseline regions of the electropherogram where the recovered lifetimes are meaningless, since lifetime is undefined and recovered profiles are meaningless in the absence of detectable signal.

3. Results and discussion

3.1. Batch studies

The emission spectra of the attached dyes (not shown) were independent of the primer to which they were attached. The FL labeled primers exhibited a broad emission band (490–620 nm) with a maximum around 525 nm, while the BD-labeled primers exhibited a narrower emission band (495–580 nm) with a maximum around 514 nm. The FL labeled primers both had lifetimes of 4.1 ns, while the lifetimes of SP6-BD and M13-BD were 5.4 ns and 6.2 ns, respectively, reflecting the dependence of the BODIPY lifetime on its surrounding microenvironment. Using MEM analysis, it was not possible to consistently resolve the contributions of the two dyes in batch mixtures of the two M13 primers or of the two SP6 primers. This is most likely due to the similarity of the lifetimes compounded by sample impurities and heterogeneity of the labeled primers themselves.

3.2. Intensity electropherograms of individual primers

The intensity electropherograms, extracted from the MHF kinetic lifetime data, of each sample are shown in Fig. 1. The samples all show multiple peaks except SP6-FL, indicating that they contain more than homogeneous, labeled primer. The additional components may be fluorescent impurities or labeled DNA fragments of multiple lengths. Conformational heterogeneity of the attached dyes may also occur. The information from the intensity electropherogram is insufficient to identify the peaks. Using OFLD, the fluorescence lifetime of each peak can be obtained to assist in peak identification and resolution.

3.3. Data analysis in OFLD

In the absence of detectable intensity in baseline regions between peaks, 'lifetimes' are recovered which are meaningless and which vary according to data analysis, lifetime reference, etc. This is because lifetime, unlike intensity which approaches zero in the absence of signal, is undefined in the absence of

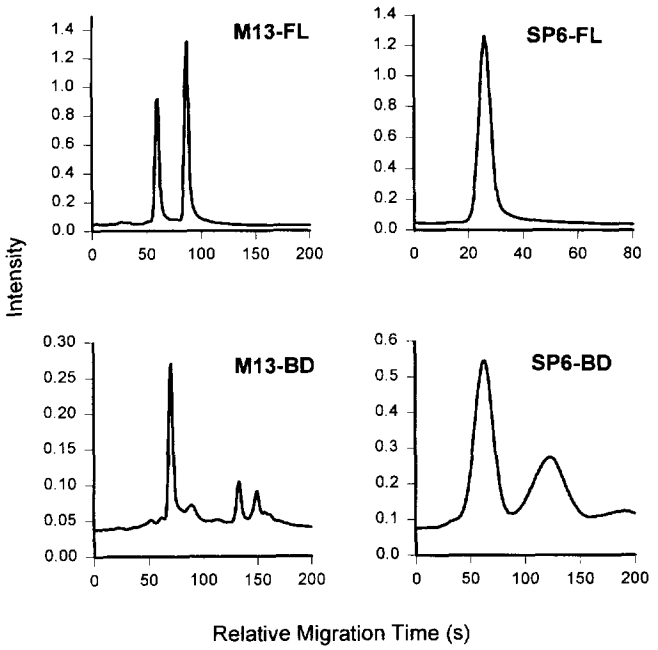


Fig. 1. Fluorescence intensity electropherogram of individual labeled primers.

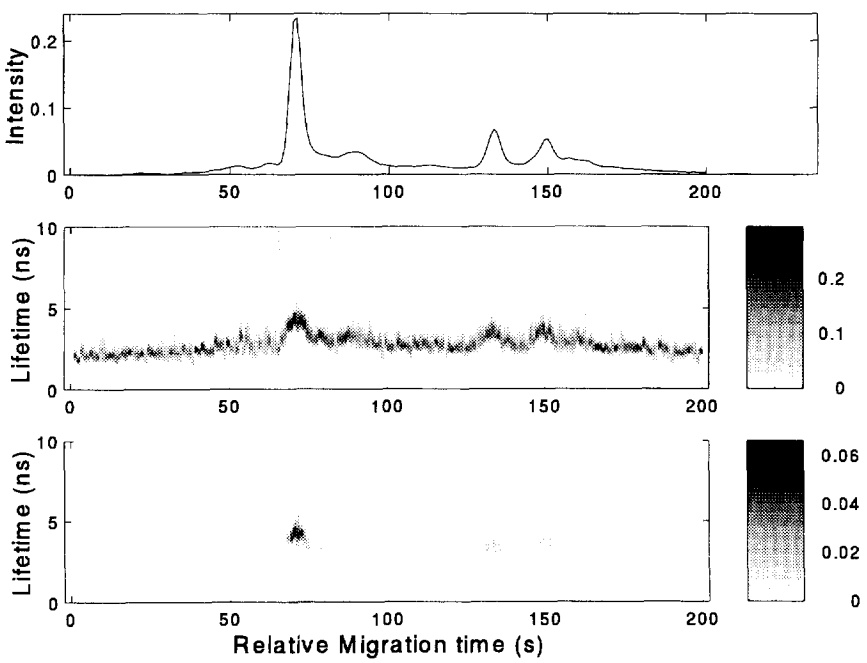


Fig. 2. Intensity (top) and lifetime (middle and bottom) electropherograms of M13-BD. The bottom electropherogram was obtained by multiplying the fractional intensities in the middle electropherogram by the total intensities in the top electropherogram to weight the lifetime contributions according to the total intensity at that point.

signal. In order to minimize the appearance of lifetime noise in the presentation of the electropherograms, the fractional intensities in the lifetime distributions recovered by MEM are multiplied by the total intensity at that point in the electropherogram [4]. Fig. 2 contrasts the lifetime electropherogram of M13-BD before and after this intensity adjustment. After intensity adjustment, it is much easier to discern the meaningful lifetimes across the electropherogram. For M13-BD, there is clearly only one significant peak with a lifetime corresponding to that of BD.

Fig. 3 shows the lifetime electropherograms of the M13-FL/SP6-BD mixture recovered from NLLS analysis using one- and two-component models. For the one-component model (top), the recovered lifetimes were 2.4 and 2.8 ns for M13-FL and SP6-BD, respectively, which were much shorter than the expected values. For the two-component model (bottom) in which one component was fixed to a very short lifetime (0.02 ns) to account for scattered light, recovered lifetimes corresponded to the expected values of 4 ns for M13-FL and 5 ns for SP6-BD. Again, the lifetimes between peaks have no meaning and vary systematically according to analysis conditions.

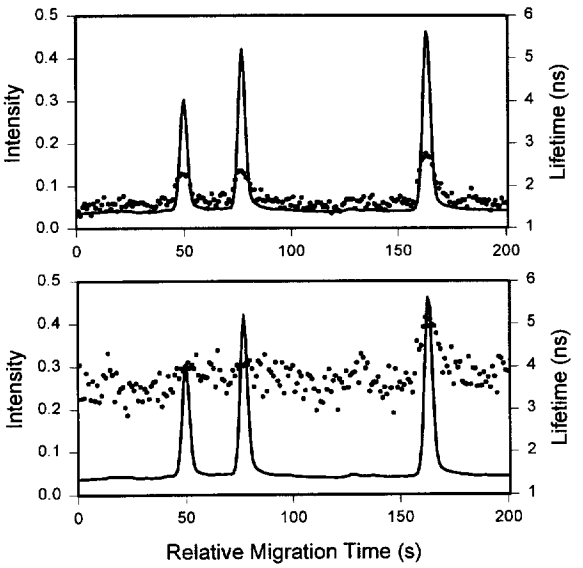


Fig. 3. Intensity (solid line) and lifetime (solid circles) electropherograms of M13-FL/SP6-BD mixture. Lifetimes were recovered from NLLS fits to a one-component model (top) or two-component model in which the second component (not shown) is fixed to a very short lifetime to account for scattered light (bottom). The one-component fit gives incorrect lifetimes of 2.4 ns for M13-FL and 2.6 ns for SP6-BD. The two-component fit recovers reasonable lifetimes of about 4 ns for M13-FL and 5 ns for SP6-BD. Based on lifetime results, the first two peaks in the intensity electropherogram are from the M13-FL sample and the third peak is from the SP6-BD sample. The lifetimes recovered between peaks are meaningless noise recovered in the absence of detectable signal.

3.4. Indication and resolution of scattered background

As shown in Fig. 3, interference from scattered light is a particular nuisance in on-capillary detection in CE. The scattered light signal depends upon the optical alignment of the instrument and the configuration of the optical elements. Even after scattered light has been minimized through optimization of the instrumental configuration and the use of optical components such as the holographic filter used in this work, it is unlikely that scattered light background can be entirely eliminated from the detected signal. Fortunately, lifetime analysis provides a powerful tool to resolve the scattered light background, which has an effective lifetime of zero on the ns time-scale, from the fluorescence signal. Fig. 4 (top) shows the manifestation of scattered light in raw MHF phase and modulation data taken at a single point on the electropherogram of SP6-BD. Contributions from scattered light become increasingly important as modulation frequency increases due to the inverse relationship between frequency and lifetime. Also, due to their different dependencies on lifetime, the phase response is weighted towards shorter-lived decays while the modulation response is weighted towards longer-lived decays. Thus, in the frequency response of SP6-BD, the phase curve, and to a lesser extent the modulation curve, falls off substantially at high frequencies due to the high scattered light background. Fig. 4 (bottom) shows the ability of MEM analysis to accurately extract the fluorescence signal from the scattered light background in the lifetime electropherogram of SP6-BD.

These results show that OFLD is able to accurately recover lifetimes of the eluting primers even in the presence of large scattered light background,

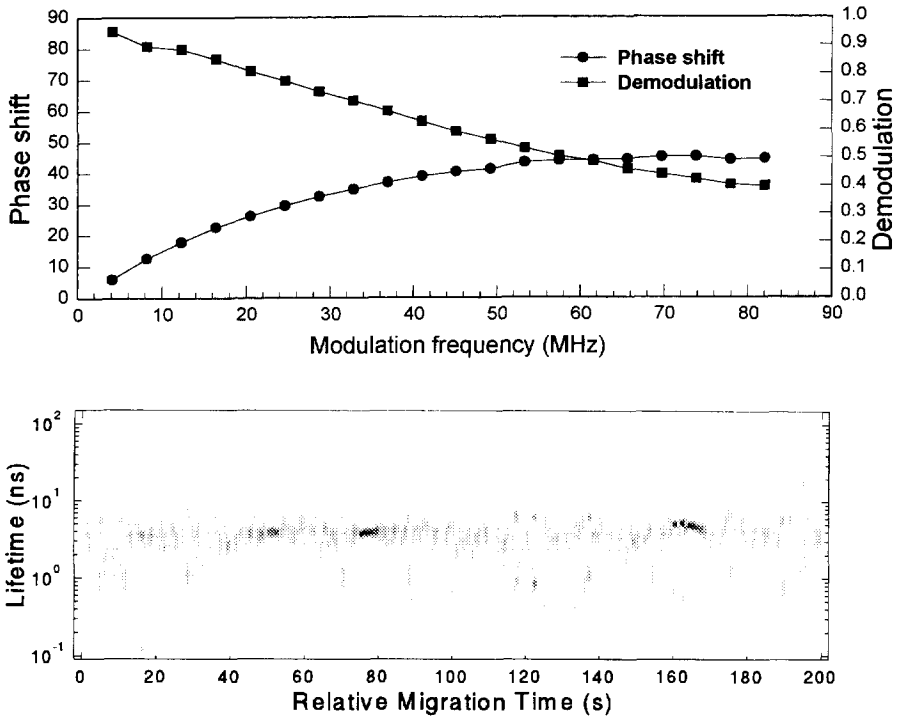


Fig. 4. MHF frequency response (top) and fluorescence lifetime electropherogram of M13-FL/SP6-BD mixture (bottom) showing strong background light in the form of Rayleigh scattering. The MHF frequency response is a 1 s segment of the ten 0.1 s increments from the peak around 165 s relative migration time. The existence of background light is indicated by the recovery of very short lifetime components as well as the plateau of the phase curve at high frequencies in the MHF frequency response.

using either MEM analysis or NLLS analysis in which a fixed, very short lifetime component is added to the fitting model to account for scattered light.

3.5. OFLD of binary and quaternary mixtures of labeled primers

Fig. 5 shows the intensity and lifetime electropherograms obtained using OFLD and MEM analysis. The first peak, which elutes near 40 s on the relative time scale, can be identified as FL-labeled primer based on the recovered lifetime of ≈4 ns. The second major peak, which elutes at approximately 65 s, is actually co-eluting peaks which could not be differentiated in the intensity electropherogram. The lifetime electropherogram shows that the lifetime changes across this peak, becoming shorter as the peaks elute. The first peak is therefore due to BD-labeled primer while the second is due to FL-labeled primer. This is consistent with the intensity electropherograms of the individual primers (Fig. 1). M13-FL has two major peaks, the second of which overlaps with the slightly slower M13-BD peak. The minor peaks at longer migration times are from the impurities in the M13-BD sample. The other binary mixtures of primers were also studied, with similarly successful results.

Fig. 6 shows the intensity and lifetime electropherograms of a quaternary mixture of the primers. Based on migration order and lifetime, all of the major peaks could be identified. According to the lifetime results, these peaks are identified to be FL, BD, FL, BD and FL. Since M13 migrates faster then SP6 due its smaller mass, the peaks can then be

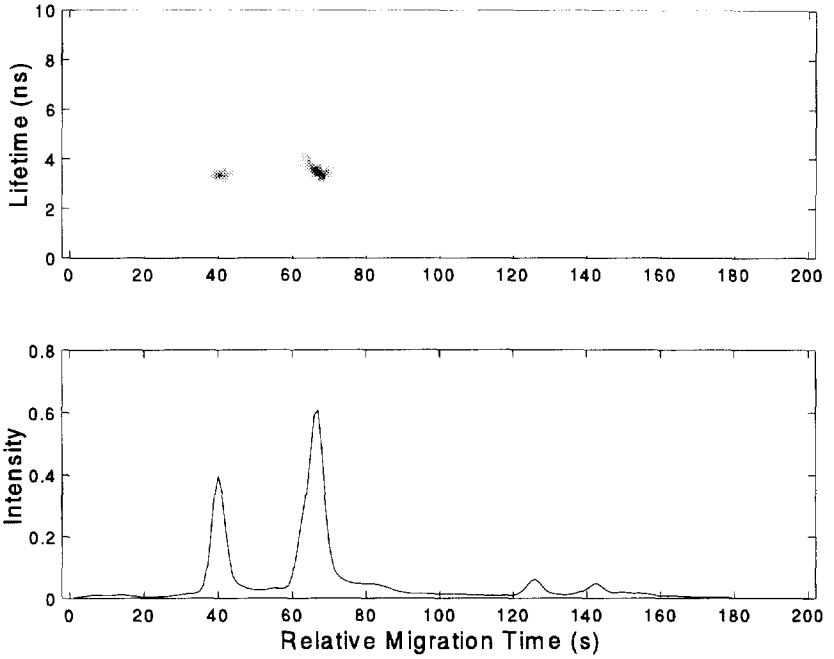


Fig. 5. Fluorescence intensity and lifetime electropherograms of M13-FL/M13-BD mixture showing a co-eluting peak. Lifetimes were recovered from MEM analysis.

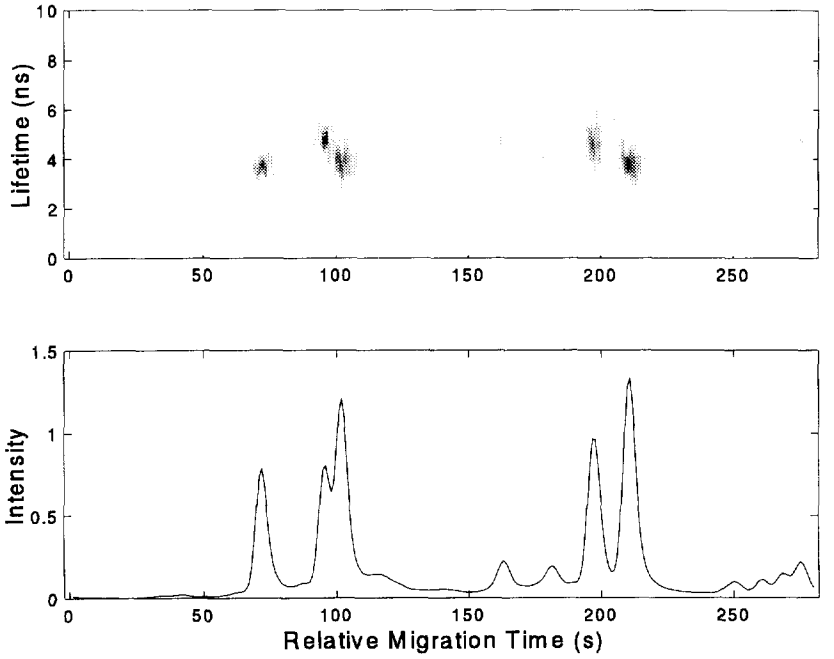
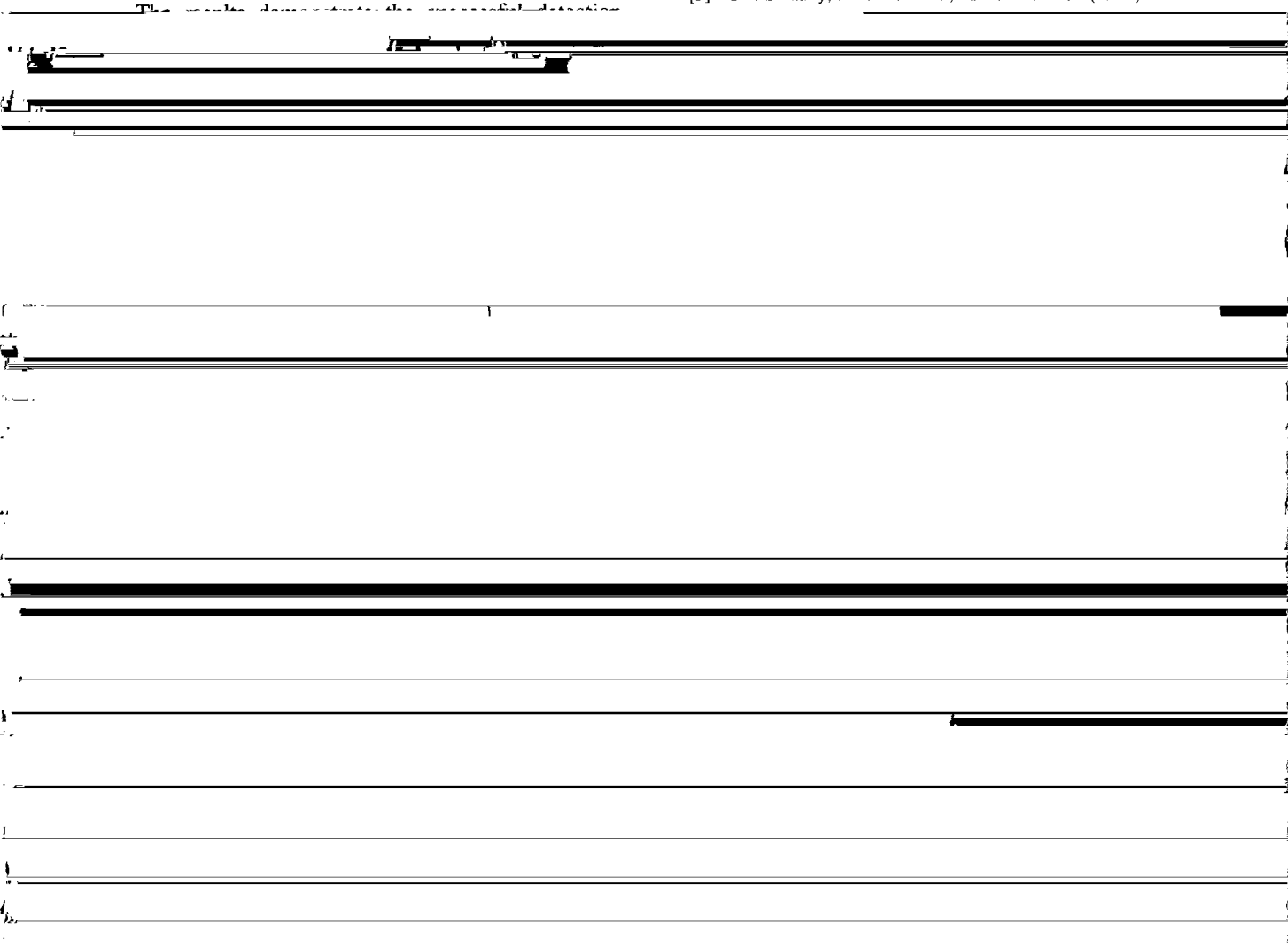


Fig. 6. Fluorescence intensity and lifetime electropherograms of M13-FL/M13-BD/SP6-FL/SP6-BD mixture. Lifetimes were recovered from MEM analysis. The peaks are identified, in order of migration, as M13-FL, M13-BD, M13-FL, SP6-BD and SP6-FL.

identified, in migration order, as M13-FL, M13-BD, M13-FL, SP6-BD and SP6-FL.

4. Conclusions



and identification of fluorescent-labeled DNA primers using OFLD. Regions of peak overlap, which were not signalled in the intensity electropherograms, were not only indicated but also resolved by OFLD. Data analysis using the self-modeling MEM is particularly valuable for minimizing interference from scattered light and recovering, without prior knowledge, accurate lifetimes of the labeled primers.

Acknowledgments

References

[1] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Plenum, New York, 1983.
[2] M.B. Smalley, J.M. Shaver, L.B. McGown, Anal. Chem. 65 (1993) 3466.
[3] M.B. Smalley, L.B. McGown, Anal. Chem. 67 (1995) 1371.
[5] D.J. Desilets, P.T. Kissinger, F.E. Lytle, Anal. Chem. 59 (1987) 1830.
[6] S.A. Soper, B.L. LeGendre Jr., D.C. Williams, Anal. Chem. 67 (1995) 4358.
[7] A.K. Livesey, J.C. Brochon, Biophys. J. 52 (1987) 693.
[8] J.C. Brochon, A.K. Livesey, Chem. Phys. Lett. 174 (1990) 517.
[9] L.B. McGown, S.L. Hemmingsen, J.M. Shaver, Appl. Spectrosc. 49 (1995) 60.
[10] J.M. Shaver, L.B. McGown, Anal. Chem. 68 (1996) 9.
[11] J.M. Shaver, L.B. McGown, Anal. Chem. 68 (1996) 611.
[12] R. Swaminathan, N. Periasamy, Proc. Indian. Acad. Sci (Chem. Sci.) 108 (1996) 39.