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Capillary array scanner for time-resolved detection and identification of fluorescently labelled DNA fragments

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Abstract

The first capillary array scanner for time-resolved fluorescence detection in parallel capillary electrophoresis based on semiconductor technology is described. The system consists essentially of a confocal fluorescence microscope and a *x,y*-microscope scanning stage. Fluorescence of the labelled probe molecules was excited using a short-pulse diode laser emitting at 640 nm with a repetition rate of 50 MHz. Using a single filter system the fluorescence decays of different labels were detected by an avalanche photodiode in combination with a PC plug-in card for time-correlated single-photon counting (TCSPC). The time-resolved fluorescence signals were analyzed and identified by a maximum likelihood estimator (MLE). The *x,y*-microscope scanning stage allows for discontinuous, bidirectional scanning of up to 16 capillaries in an array, resulting in longer fluorescence collection times per capillary compared to scanners working in a continuous mode. Synchronization of the alignment and measurement process were developed to allow for data acquisition without overhead. Detection limits in the subzeptomol range for different dye molecules separated in parallel capillaries have been achieved. In addition, we report on parallel time-resolved detection and separation of more than 400 bases of single base extension DNA fragments in capillary array electrophoresis. Using only semiconductor technology the presented technique represents a low-cost alternative for high throughput DNA sequencing in parallel capillaries.

Keywords: Capillary array scanner; Pulsed diode laser; Detection, electrophoresis; DNA fragments

1. Introduction

Large scale sequencing as initiated by the Human Genome Project affords new methods for high speed DNA sequencing. One of the labour intensive and time consuming steps in DNA sequencing is slab gel electrophoresis. In comparison, capillary electrophoresis provides superior speed of separation. Due to the effective heat dissipation of capillaries higher electric fields can be applied during separation. In the last years capillary electrophoresis has proved to be a very powerful technique for separation of DNA sequencing fragments generated by the Sanger dideoxynucleotide chain terminating reaction [1–5]. Generally, up to 600 bp can be separated in less than 120 min in a single capillary. Recently, Carrilho et al. [6] demonstrated DNA sequencing of up to 1000 bases within 80 min using a 2% T linear polyacrylamide gel in a capillary with an effective length of 30 cm at an electric field of 150 V/cm.

Although, the separation time is very short, the throughput of a single capillary is low compared to slab gels with parallel lanes. In order to overcome this limitation, capillary array electrophoresis with

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parallel capillaries has been developed by Mathies and co-workers [7,8]. They used a confocal fluorescence scanner in combination with an argon ion laser and conventional dye chemistry, i.e. identification upon different emission maxima of the dyes used to label the DNA fragments, and performed simultaneous DNA sequencing in 25 parallel capillaries. In the following years, much progress has been made in the development of various capillary array electrophoresis (CAE) systems. Referring to their detection scheme the array systems can be roughly divided in two main groups: (a) array systems, which use a defocussed laser beam in combination with a charge coupled device camera (CCD camera) to excite and image all capillaries simultaneously and (b) scanning systems, i.e confocal fluorescence microscopes with moving capillaries. Different imaging CAE systems have been developed, including multiple sheath flow systems [9] multiple laser focusing [10,11], on-column illumination [12] and fiber-optic array illumination [13]. Besides the 100% duty cycle of such imaging systems, there are some basic disadvantages. For efficient excitation of the samples in each capillary relatively high laser powers of 10–100 mW have to be applied. In addition, since no spatial filters are used, these systems are more sensitive to scattered light from capillary walls. Thus lower signal-to-noise ratios are obtained.

Although the duty cycle is limited by the speed of the translational stage confocal fluorescence scanners offer higher sensitivity. In addition, the speed and accuracy of confocal scanning systems has been improved by Kheterpal et al. [14]. Laser powers of less than 1 mW are sufficient to efficiently excite fluorescent probes by confocal microscopy. As an example, Kheterpal et al. [15] developed a rotating confocal fluorescence scanner, which was successfully applied for parallel DNA sequencing in up to 128 capillaries with average read lengths of more than 300 bases per capillary. Furthermore, the described scanning system is designed to handle up to 1000 parallel capillaries.

However, independent of the used method the background signal due to impurities sets detection limits, especially in the commonly used green wavelength region. Therefore excitation in the red spectral region appears to be a desirable alternative. Using recently [16,17] developed rhodamine and oxazine dyes with absorption and emission above 620 nm the gas lasers can be exchanged by small diode lasers emitting at 630–640 nm. Semiconductor lasers offer several advantages such as small size, low cost due to mass production, inherently low noise and long lifetime. For this reason, several groups started to develop time-resolved detection methods to detect and identify differently labelled DNA fragments during CGE [18–22]. More recently [23,24] the first four-dye-one-lane DNA sequencing with on-line diode laser based time-resolved fluorescence detection in capillary electrophoresis has been successfully demonstrated. Using a pattern recognition technique [25–27] the sequence information of 660 bp was determined with a probability of correct classification of $>90\%$. In addition, these results has been obtained directly from the raw data without the use of any mobility corrections that are necessary with other methods.

In this paper, we present the first diode laser-based capillary array scanner using time-resolved detection and identification of fluorescently labelled probes. The capillary array electrophoresis system is equipped with a short-pulse diode laser emitting at 640 nm with a repetition rate of 50 MHz to efficiently excite the probe molecules. As detector served an avalanche diode. These two components allow for fast and sensitive detection of new rhodamine and oxazine as well as carbocyanine dyes absorbing in the range 630–670 nm. Since the identification of the dyes is based on their characteristic fluorescence decay times no filter wheels or other spectral components have to be applied, i.e. a very simple detection device using a single bandpass filter can be used to identify differently labelled probe molecules. Using a discontinuous, bidirectional scanning mode the data of several parallel capillaries can be acquired without any overhead. Electrophoresis was carried out in eight parallel capillaries mounted on the scanning stage. The used technique permits simultaneous separation of single base extension fragments with more than 400 bases read length directly from unprocessed raw data. The obtained data demonstrate the applicability of this method for multiplex dye DNA sequencing in capillary array electrophoresis.

2. Experimental

2.1. Synthesis of sequencing primer

Chemicals were purchased from Fluka (Ulm, Germany). The oligonucleotide (5′-TGT AAA ACG ACG GCC AGT-3′) was custom-synthesised by Boehringer Mannheim GmbH (Penzberg, Germany). The modified oligonucleotide carried one amino group at the 5′-terminus for labelling with the dye. Cy5 was obtained as monofunctionalised *N*-hydroxy-succinimidyl ester from Amersham Life Science Inc. (Cleveland, Ohio, USA). The coupling reaction was carried out in 250 m*M* carbonate buffer pH 9.3 at room temperature for 2 h. The labelled oligonucleotide was purified by reversed-phase high-performance liquid chromatography (HPLC) with a gradient of triethylammoniumacetate–acetonitrile buffer. The reaction yield was 70%. The purity of the labelled primer was checked by a P/ACE System 2100 capillary electrophoresis instrument (Beckman Instruments, Inc., Fullerton, CA, USA).

2.2. Capillary preparation and electrophoresis

All chemicals for capillary and buffer preparation were purchased from Sigma (Deisenhofen, Germany). Fused-silica capillaries of 75 μm I.D. and 375 μm O.D. or 150 μm O.D. (Polymicro Technologies Incorporated, Phoenix, AZ, USA) were cut to a total length of 55 cm and fitted with a detector window by burning off a section of the polyimide coating approximately 15 cm from one end. Each capillary was initially rinsed with 0.1 *M* NaOH and water. Similar to the method described by Hjerten [28] the capillaries were pretreated with 40 μl [(γ-methacryloxy) propyl]trimethoxysilane diluted in 0.5 ml water, 0.5 ml methanol and 20 μl acetic acid and flushed with water again. The non cross-linked polyacrylamide was prepared from 4 ml aliquots of degassed acrylamide solution (5% T) containing 50 m*M* tris(hydroxymethyl)aminomethane (tris), 50 m*M* *N*-tris–(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), 2 m*M* EDTA and 7 *M* urea pH 8.4 (TTE–urea buffer). The polymerization was initiated by addition of 4 μl *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and 8 μl of a 10% (w/v) aqueous solution of ammonium persulphate (APS). The capillaries were kept in a refrigerator for at least two days until use.

The capillaries were mounted on in-house built capillary holders which were capable to fix up to 16 individual capillaries in parallel in spatially separated positions. For alignment and hysteresis tests 16 capillaries with 375 μm O.D. and 75 μm I.D. were filled with solutions of four different dyes (Cy 5, MR 121, JA 167 and MR 200-1) in TTE–urea buffer. Synthesis and structures of the rhodamine and oxazine derivatives are described in Refs. [16,29–31]. All electrophoretic separations were performed using a high voltage power supply (Heinzinger, Germany) with the cathode connected to the injection side. The samples were injected electrokinetically at 40 V/cm for 15 s. Electrophoresis was performed at a field strength of 150 V/cm using TTE–urea buffer in both reservoirs.

2.3. Cycle sequencing

The sequencing reactions were generated from M13mp18-(+) single strand DNA (Pharmacia, Freiburg, Germany) using the SequiTherm™ Long-Read™ cycle sequencing kit (Epicentre Technologies, Madison, WI, USA). Four μl of the Cy5 labelled primer (10 pmol) were mixed with 4 μl template (332 fmol), 4.5 μl water, 2.5 μl sequencing buffer, 8 μl ddTTP (0.45 m*M* ddTTP; 45 FM dNTP), 1 μl polymerase (5 units) and 1 μl DMSO (Fluka, Neu-Ulm, Germany) to give a total volume of 25 μl. Thermal cycling was performed in a MJ Research Inc. PTC-100™ cycler with heated lid (Watertown, Mass., USA) in 36 cycles (20 s at 94°C, 22 s at 55°C and 40 s at 72°C). After completion the samples were desalted by micro-dialysis on a 0.025 μm pore size type PV filter (Millipore Corp., Bedford, MA, USA) as described for the desalting of PCR products [32]. The membrane carrying the sample was floated on deionized water of HPLC grade (Fluka, Neu-Ulm, Germany) for 45 min. After recovery the sample was dried in a vacuum centrifuge (Bachofer, Reutlingen, Germany) for 15 min and dissolved in 12.5 μl formamide (Serva, Heidelberg, Germany) and 12.5 μl water. The samples were stored at −20°C until use. For electrokinetic

injection an aliquot of 4 μl was removed from the sample, heat-denatured at 95°C for 3.5 min, chilled on ice and transferred to a microvial.

Alternatively to fragments from cycle sequencing reactions the Cy 5 labelled ALFExpress Sizer 50–500 (Pharmacia/Amersham Life Science, Cleveland, Ohio, USA) was used as the sample in electrophoretic runs. Two μl of the sizer were diluted with 4 μl of TTE buffer and 3 μl of dextrane blue according to the manufacturer's protocol and heat-denatured at 95°C prior to injection.

2.4. Capillary array scanner

The confocal setup for time-resolved fluorescence detection in capillary array electrophoresis is shown in Fig. 1. The time-resolved capillary array scanner is based on an inverted microscope (Axiovert 100TV, Zeiss, Germany) equipped with a motion controller driven *X*,*Y*-microscope scanning stage (SCAN 100× 100 and MC 2000, Märzhäuser, Wetzlar, Germany). As excitation source we used a pulsed diode laser emitting at 640 nm with a repetition rate of 50 MHz. The HL6316 G laser diode (Hitachi, Japan) was driven by an Avtech AVN 4 C pulse generator (Avtech, Ottawa, Canada). Passing an excitation filter (639DF9; Omega Optics, Brattleboro, VT) the beam was directed to the microscope objective (40×, NA = 0.63, Melles Griot, Germany) by a dichroic beamsplitter (645DRLP; Omega Optics, Brattleboro, VT). The average laser power was 800 μW at the sample. The fluorescence light was collected by the same objective, filtered by a bandpass filter (675RDF50; Omega Optics, Brattleboro, VT) and a

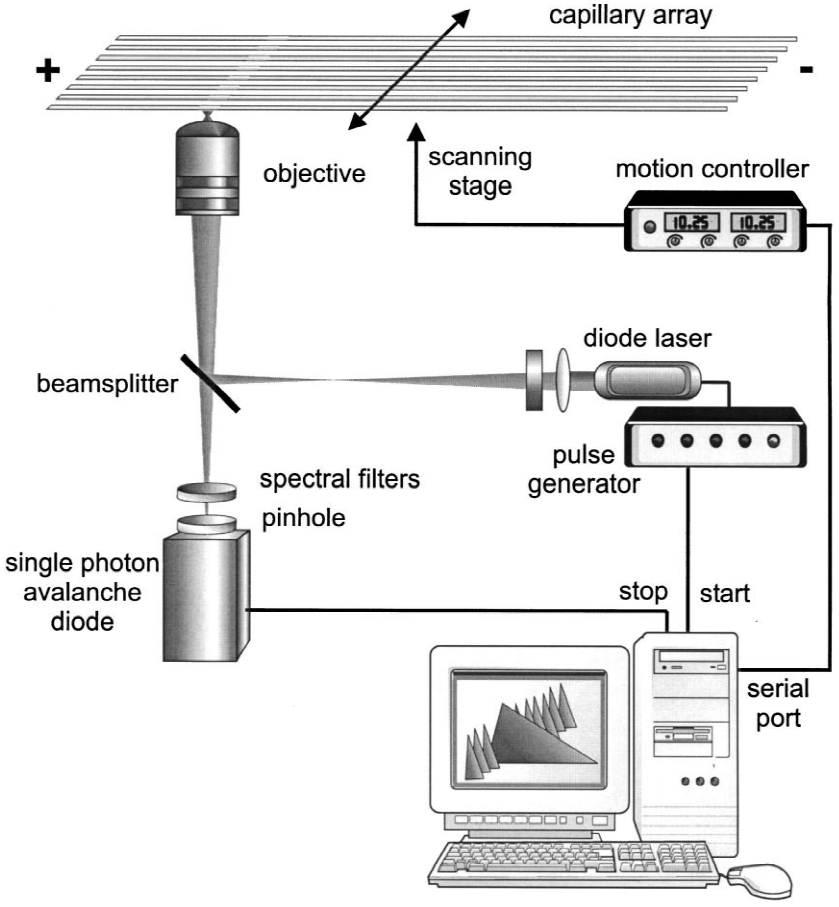

Fig. 1. Experimental setup of the capillary array scanner for time-resolved fluorescence detection.

Raman edge cut-off filter (650REFLP; Omega Optics, Brattleboro, VT) and imaged through the TV outlet on the bottom of the microscope onto a 200 μm pinhole oriented directly in front of the avalanche photodiode (SPAD, AQ131, EG&G Optoelectronics, Canada). The signal of the avalanche photodiode was fed into a time-correlated single photon counting (TCSPC) PC interface card (SPC-430, Becker & Hickl, Berlin, Germany) to acquire time-resolved data. With this card a minimum collection time of 150 μs per decay curve (64 channels) for a gap-free measurement is possible [33]. All time-resolved measurements were carried out in the reverse mode, i.e. the detector signal served as start signal, whereas the laser pulse was used as stop signal. The instrument response function of the entire system was determined to be 600 ps (FWHM). From the data of the TCSPC-card electropherograms were generated. For this, all photons of a decay curve were added up to a bin of a multichannel-scaler trace. The fluorescence decay time determination in capillary array electrophoresis was realized by a monoexponential maximum likelihood estimator (MLE) using the following relation [34]:

$$\frac{\omega}{e^{-\frac{\omega}{\tau}}-1}-\frac{k\omega}{e^{\frac{k\omega}{\tau}}}=\frac{\sum_{i=1}^{k} in_i\omega}{\sum_{i=1}^{k} n} \quad (1)$$

where ω is the width of each channel, k the number of utilized time channels, n the number of photon counts taken into account, and n_i the number of photon counts in time channel i. The parameters used to determine lifetimes are $k=20$, $\omega=0.195$ ns.

For synchronization of the scanning motion with the detection device a Windows 32® based software with Microsoft Visual C++ 5.0 running under Windows NT® was developed. The communication with the motion controller takes place via the RS-232 serial port at 19 600 bps using the built-in command language VENUS-1 of the motion controller. Triggering of the detection device and data acquisition was done through calls to a Windows 32® DLL (Becker & Hickl, Berlin, Germany). The measurement routine was completely written in C to minimize time consuming code overhead. Within the routine the Windows data storage is done in binary format without system cache to avoid arbitrarily gaining control to the operating system. Nevertheless the acquired data can be displayed online within the portions of code that are waiting for a response from the serial port. For data analysis another software package is in development, allowing the application of pattern recognition algorithms and other mass data procedures.

3. Results and discussion

3.1. Discontinuous bidirectional scanning

A capillary array scanner suitable for time-resolved identification of fluorescently labelled probes must exhibit proper alignment and scanning of the capillaries along with sensitive detection. In order to obtain high scan rates and sufficient duty cycles the motion of the scanning stage should be as quick as possible. On the other hand, the time to acquire a fluorescence decay profile (collection time) for each capillary should be as long as possible for a definite detection of the signal. Using a continuous scanning mode the collection time per capillary is controlled by the scan speed, the diameter of the capillaries and their distances, i.e. the collection time decreases with increasing scan speed. The spacing of the capillaries is limited by their outer diameter. Hence, using continuous scanning, capillaries with small outer/inner diameter ratios should be used to obtain sufficient collection times and scan rates. Unfortunately those capillaries are susceptible to mechanical breakage. Furthermore, the data overhead generated by unsynchronized continuous scanning is relatively large. For example, using capillaries with 150 μm O.D. and 75 μm I.D. arrayed in close contact to each other the same amount of data points are collected inside and outside of the capillaries, i.e. at least 50% of the collected data do not originate from the samples. In addition, due to strong scattering at the inner capillary walls the actual amount of useful data is even smaller.

To circumvent this problem, we implemented a discontinuous, bidirectional scanning mode in our system. The proper alignment of the capillaries and the collection of fluorescence signals are synchronized by a software on a personal computer. The data

collection starts as the scanning stage stops at the desired position. As the collection time ends a signal is send to the motion controller to start the alignment of the next capillary. Once the capillary is aligned properly (settling time) data acquisition is restarted. Therefore, no data overhead is generated during the measurements and the correct lane tracking of data is performed online. After each capillary is sampled once the scan direction is reversed. The next scan line starts at the last capillary of the previous line. Hence, the total time (t_{total}) for a scan line with n capillaries is given by $t_{total} = t_{collection}n + t_{settling}(n - 1)$ with an average scanrate $f = 1/t_{total}$. Thus the sampling intervalls for distinct capillaries depend on their positions in the array.

The resulting electropherograms are displayed immediately on the computer screen. Table 1 summarises the results of several scan tests obtained from parallel capillaries (375 μm O.D., 75 μm I.D., spacing 400 μm) containing solutions of fluorescent dyes. The number of capillaries and the collection time in the capillaries are varied to determine the settling time and the scan rate of the system. The settling time is longer than the 40 ms calculated from the acceleration and maximum speed of the scanning stage. This is due to the poor data transmission rate between the motion controller and the computer via the serial port. At higher transmission rates, e.g. as provided by the IEEE 488 port, the settling time would decrease significantly. In comparison a continuous scanning mode with a constant velocity of 10 mm/s and a settling time of 40 ms passes the inner diameter of the capillary in only 7.5 ms. To reach a collection time of 50 ms in each capillary the speed of motion has to be reduced more than six times resulting in a poor settling time of 267 ms. This demonstrates the superior performance of the discontinuous scanning mode which allows faster settling with longer collection times. The obtained scan rates of up to 0.5 Hz on 16 parallel capillaries (Table 1) demonstrate that the developed system can be successfully applied for high throughout biological analysis.

3.2. Time-resolved fluorescence detection

For time-resolved identification of labelled probes the fluorescent dyes used as labels have to exhibit different fluorescence decay kinetics. In order to excite the labelled probes with a single laser line and to detect them efficiently using the same filter, the dyes should show similar absorption and emission characteristics with high extinction coefficients at the excitation wavelength and high fluorescence quantum yields. The spectroscopic properties of the used fluorescent dyes in bulk solution are listed in Table 2 together with the measured fluorescence decay times and the detection limits which were achieved in the capillary array scanner (for details of dye synthesis and spectroscopy see Refs. [16,17]). The detection limits for the different dyes were calculated from measurements on diluted dye solutions in the capillary. The detection volume was estimated from the magnification of the used microscope objective (40×) and the spatial filter (200 μm pinhole) to approximately 500 femtoliters. The detection limits were determined at the point of deviation from linearity in log intensity vs. log concentration plots. The

Table 1
Scanrates at different numbers of capillaries, different collection and settling times using discontinuous bidirectional scanning. The capillaries (I.D. 75 μm, O.D. 375 μm) were spaced 400 μm and filled with an aqueous 10^{-9} *M* Cy5 solution. Excitation power at the capillary: 0.8 mW

Number of capillaries	Collection time (ms)	Settling time (ms)	Scan-rate (Hz)
4	100	85–95	1.49
4	50	95–105	2.00
4	10	100–150	2.64
8	100	85–95	0.69
8	50	95–105	0.91
8	10	100–150	1.2
16	100	85–95	0.34
16	50	95–105	0.43
16	10	100–150	0.52

Table 2
Spectroscopic characteristics of the used fluorescent dyes in aqueous 50 m*M* tris, 50 m*M* TAPS, 2 m*M* EDTA, 7 *M* urea buffer (TTE–urea buffer). τ is the fluorescence decay time measured in bulk solutions. In order to prevent reabsorption and reemission of photons concentrations $<10^{-6}$ *M* were used. τ_c denotes the fluorescence decay times measured in capillaries filled with 10^{-9} *M* solutions of the different dyes in aqueous TTE–urea buffer, respectively. Fluorescence decay times τ_c were calculated using the maximum likelihood estimator (Eq. (1)). The detection limit was determined estimating a detection volume of approximately 500 femtoliter (40× microscope objective and 200 μm pinhole in front of the avalanche photodiode)

Dye	λ_{abs} (nm)	λ_{em} (nm)	τ (ns)	τ_c (ns)	Detection limit (ymol)
JF9	631	645	3.89	3.62	150
JA167	638	662	2.91	2.97	250
MR121	666	680	2.29	2.25	200
CY5	652	670	1.21	1.26	35

achieved detection limits in the subzeptomol range (corresponding to about 20–150 dye molecules) clearly demonstrate the sensitivity of the developed system.

As can be seen in Fig. 2a, the dyes exhibit monoexponential fluorescence kinetics and their decay times are adequately long to allow the use of relatively simple instrumentation. The instrument response function of the entire system is shown in Fig. 2b. The calculated fluorescence decay times of the dyes measured in capillaries are in good agreement with the data obtained from standard measurements in bulk solution (Table 2). Recently we demonstrated the first one-lane, four-dye, time-resolved DNA sequencing using pulsed diode laser excitation at 630 nm [24]. Due to the similar absorption and emission characteristics but distinct fluorescence decay times of the used multiplex dyes (3.7, 2.9, 2.4 and 1.6 ns) classification is possible, even at low signal intensities and multiexponential fluorescence kinetics (overlapping or co-migrating fragments). Different dyes can be easily identified due to their different fluorescence kinetics by a pattern recognition technique. Using this technique we demonstrated ultrasensitive time-resolved fluorescence detection and identification of these dyes with

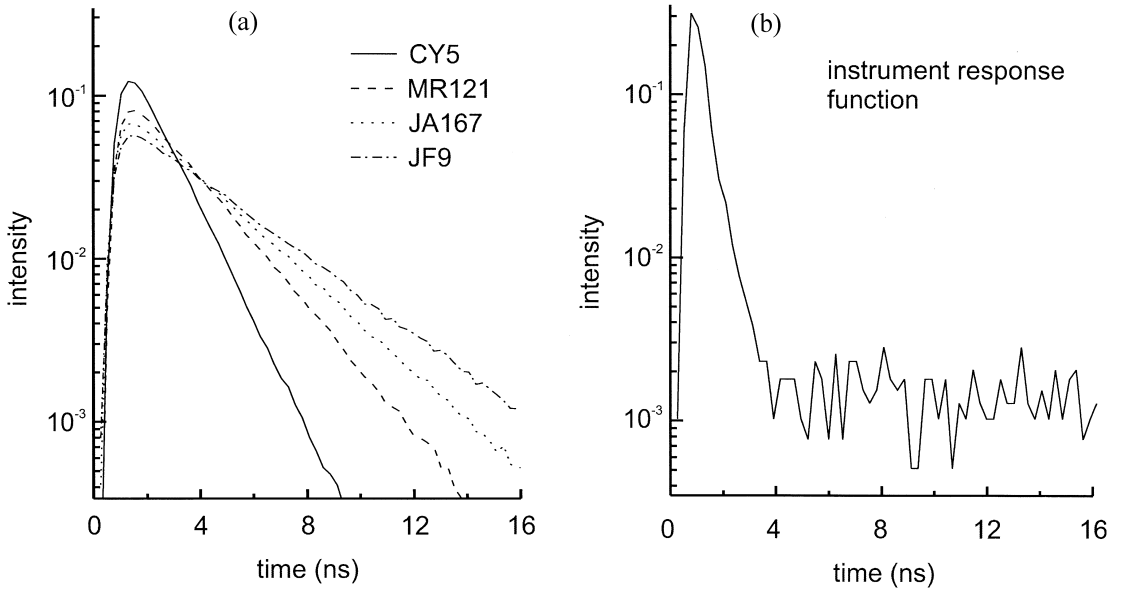

Fig. 2. (a) Normalized fluorescence decays of dyes Cy5, MR121, JA167 and JF9 measured in capillaries in TTE–urea buffer and (b) instrument response function (IRF) of the entire system.

diode laser excitation even on the single-molecule level [35,36]. Here, it should be pointed out that for the identification of different fluorescent dyes it is not necessary to collect as many photons which are necessary for an exact decay time determination using TCSPC and conventional deconvolution algorithms. Instead a much smaller number of photons is sufficient. The identification is achieved by comparing the measured decays (raw data) with the expected fluorescence decays measured in concentrated solutions with high precision (see [24–27] for further details).

3.3. Parallel electrophoretic separations

The experimental setup for capillary array electrophoresis with time-resolved fluorescence detection is shown in Fig. 1 and described in the Experimental section. Electropherograms were obtained from the time-resolved data of the TCSPC-card recorded during a run. For this, all photons of a decay curve (from each histogram) were added up to a bin and plotted versus migration time. Fig. 3a shows the raw data of an electropherogram obtained from one capillary out of four in a capillary array. The 11 fragments from the Cy5 sizer ranging from 50 to 500 bases are well resolved. With a background rate of 5 kHz signal-to-background ratios varying between 12 and 60 were achieved during electrophoresis. The capillary array scanner reached a scan rate of 1.6 Hz at a collection time of 100 ms in each capillary. For example, at this rate the 50 bp fragment is resolved with 18 scan sweeps. The final fragment passes the detector within 20 min demonstrating the performance of the capillary array scanner in rapid DNA sizing. Fig. 3b shows a 3D-plot of the same electropherogram which outlines the additional time-resolved information. In fact we detect four parameters simultaneously in our time-resolved capillary array scanner to identify a fragment in a given sample: (a) The fluorescence intensity (count rate), (b) the spectral property, (c) the fluorescence decay profile, and (d) the migration time of the compound. The measured fluorescence decay times of Cy5 labelled DNA fragments of $\tau = 1.4$ ns are in good agreement with our experimental results in multiplex dye DNA sequencing [24]. Upon covalent coupling of Cy5 to oligonucleotides the fluorescence decay time increases slightly.

In addition, we tested the electrophoretic separation efficiency of our system with DNA sequencing fragments. Single base extension fragments from a ddTTP terminated Sanger reaction using Cy5 labelled primer were injected simultaneously in an array of eight capillaries (Fig. 4). The electropherograms depict raw data without any further processing. As expected from the high spatial resolution of confocal microscopy no cross-talks between different capillaries are observed. Using a collection time of 50 ms, the system reached a scan rate of 0.9 Hz. As a peak crosses the detection region six or more sweeps are made under separation conditions which is sufficient to render the peaks. For example, the 80 bases fragment is sampled eight times within the 8.7 s bandwith and the 330 bases fragment is sampled 23 times (23.9 s bandwith). Although the separation was carried out at room temperature, which typically results in poor resolution of compressed regions, the DNA fragments are well resolved. Up to 400 bases can be easily compared to the corresponding region of the M13mp18 sequence directly from raw data. As shown in Fig. 5, the characteristic quintet and quartet sequence of the M13mp18 template around 300 bases can be called from the electropherogram as simple as the triplet at 410 bases directly from the raw data. It should be noted that data processing with peak identification algorithms would further extend the read length. In addition, heating of the capillaries would enhance the speed of separation, due to decreasing viscosity of the separation matrix. However, for time-resolved DNA sequencing four multiplex dyes with different fluorescence decay kinetics are required. In addition, the use of differently labelled DNA fragments often results in inhomogeneous electrophoretic mobilities which can seriously complicate the interpretation of the gel patterns. Therefore we optimized the electrophoretic mobilities of differently labelled DNA fragments by adjusting spacers, coupling positions and dye structures [24]. Using such an optimized primer set we achieved an accuracy of correct base identification of more than 90%, up to 660 bp in one-lane, four-dye multiplex DNA sequencing without the use of any further mobiltity correction. On the basis of these results up to 4.8 kbp sequence information can be

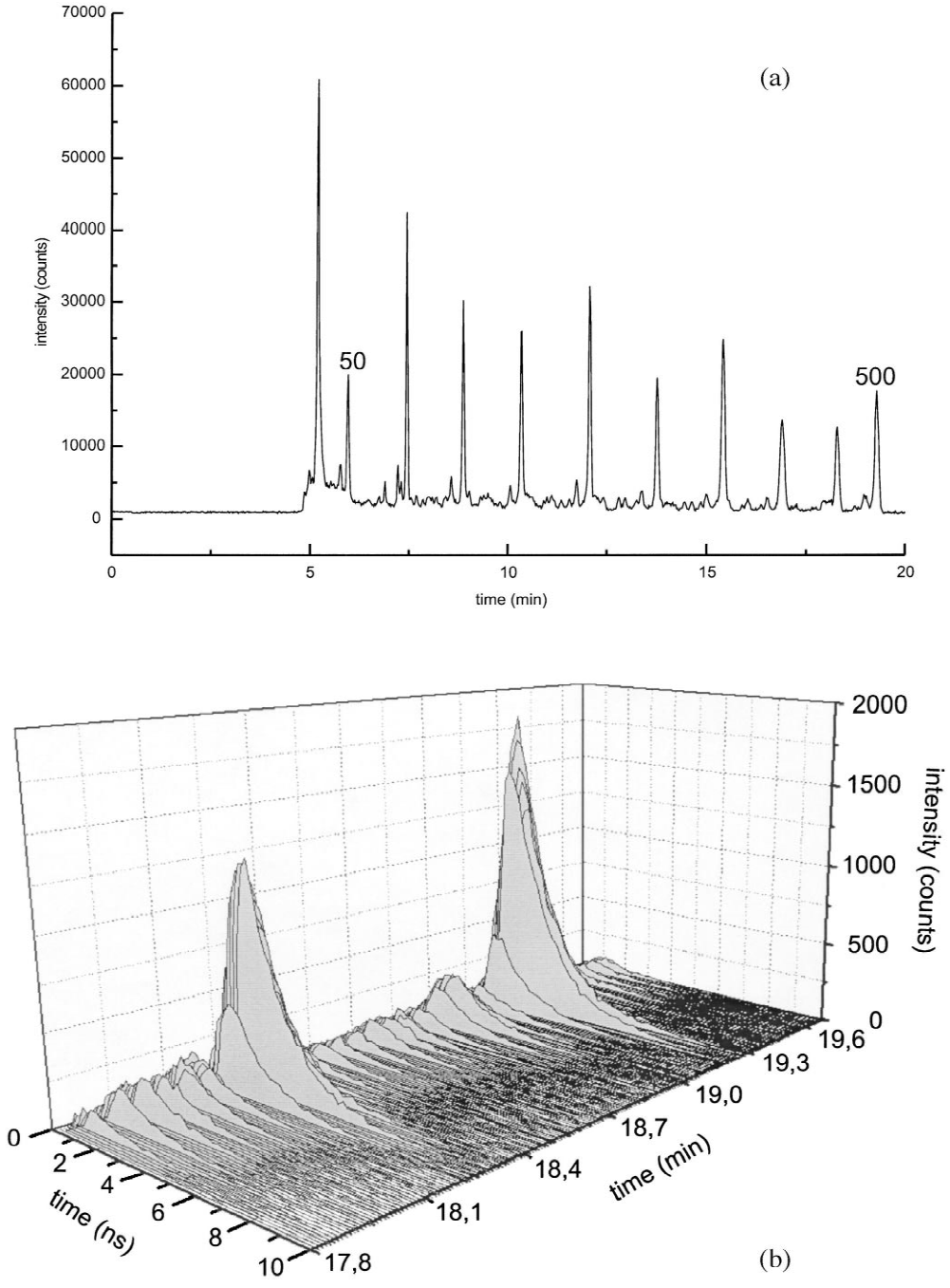

Fig. 3. (a) Electropherogram of Cy5 sizer separated simultaneously in four gel-filled capillaries ($l = 19$ cm, $l_d = 10.5$ cm, 150 μm O.D., 75 μm I.D., capillary spacing 160 μm, gel: 5% T linear polyacrylamide gel in aqueous TTE–urea buffer) at 6 kV and room temperature. (b) Expanded view as 3D-image showing the time-resolved dimension of the data. Collection time for each histogram was 100 ms.

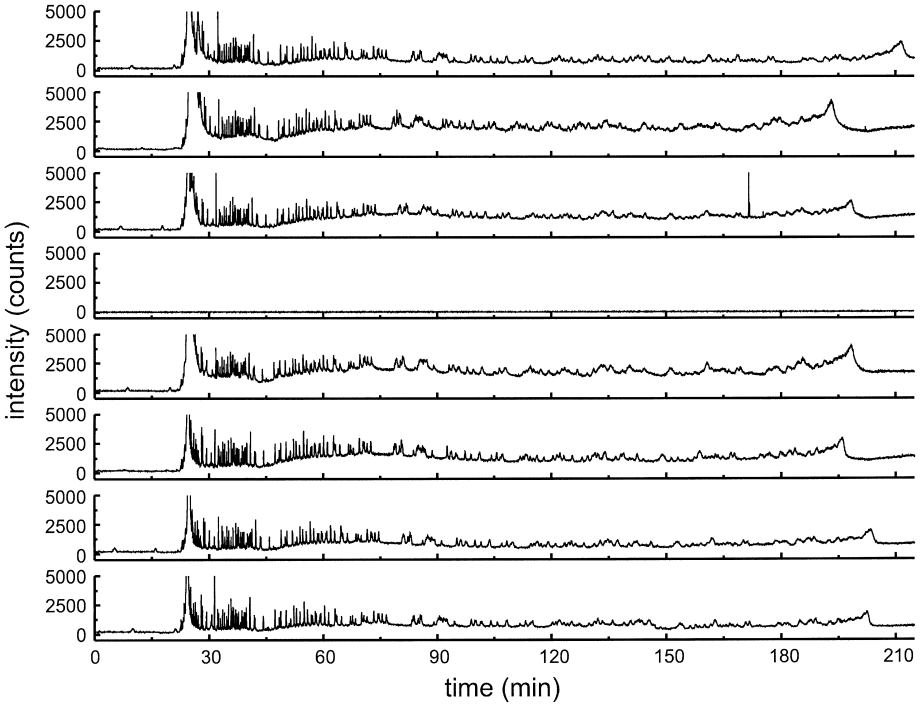

Fig. 4. Electropherograms of single base extension fragments from Sanger sequencing reactions of M13mp18(+)-strand using Cy5 labelled primer and ddTTP in eight parallel capillaries. Conditions: l=41 cm, l_d=30 cm, 375 μm O.D., 75 μm, I.D. gel: As stated in Fig. 3, 6.4 kV, injection at 40 V/cm for 15 s, collection time 50 ms. Unfortunately, the injection in one capillary failed due the current breakdown.

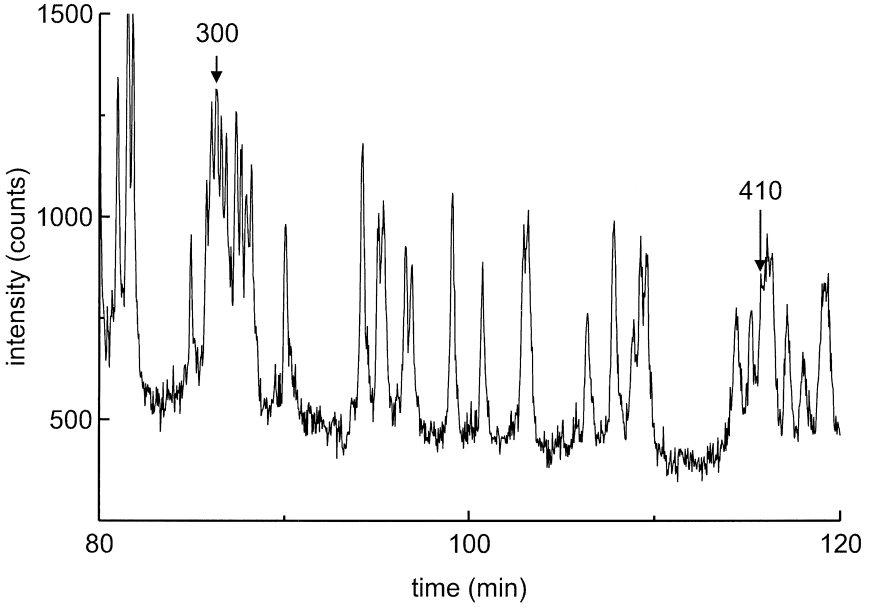

Fig. 5. Detailed view on a section of the single base extension fragments separation shown in Fig. 4. Some regions are marked with the base number as means of orientation.

obtained in a single run with a capillary array scanner using eight parallel capillaries and multiplex dye technology with time-resolved detection and identification.

4. Conclusions

We have demonstrated the first capillary array scanner for time-resolved fluorescence detection and identification using pulsed diode laser excitation at 640 nm. The discontinuous, bidirectional scanning mode minimises the settling time in relation to the fluorescence collection time, allowing longer sampling intervalls compared to continuous scanning devices. In addition the synchronisation of the alignment and measurement process does not produce any data overhead and is capable of online displaying of results. Besides high detection sensitivity the confocal detection setup offers spatial separation of the individual channels without any cross-talk. Detection limits for different dyes in the subzeptomol range corresponding to 20 to 150 detected dye molecules have been achieved. The identification of different fluorescently labelled probes in parallel capillaries by the characterisitc fluorescence decay time of the attached dyes is possible. Thus the entire system can be applied to parallel rapid sizing of labelled DNA fragments in a capillary array. The throughput of the system can be easily further improved by using differently labelled DNA fragments per capillary, i.e. four different characteristic fluorescence decay times per capillary. We have demonstrated parallel separation of single base extension fragments labelled with Cy5 in eight capillaries with a scanning rate of 0.9 Hz. The sequence determination and time-resolved identification of more than 400 bases directly from the raw data implies the applicability of this scanner for multiplex dye DNA sequencing. To enhance the number of capillaries, i.e. the throughput of the system, a more sophisticated scanning hardware has to be developed to decrease the settling time per capillary. With collection times of 10 ms the settling time should be in the order of a few milliseconds to obtain suitable scan-rates. Another very promising technique is the use of time-gated CCD cameras for time-resolved fluorescence detection. In combination with low-cost excitation sources like diode lasers such cameras would provide a 100% duty cycle in capillary array electrophoresis.

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