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Real-Time Fluorescence Imaging in Analytical Chemistry

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ABSTRACT

A detection system for capillary electroseparation methods based on fluorescence imaging has been developed. In capillary electrophoresis (CE) the detection unit is normally placed near the outlet part of the fused silica column where a window is opened in the coating and the fluorescence is recorded over a short distance to maintain a high resolution. Our method employs fluorescence imaging of the whole column during separation of various samples. The column is positioned in a straight holder and the outer protective coating of the column is removed to get optical access to the sample. An excimer/dye laser is used for excitation of the sample and the fluorescence is recorded with an image-intensified CCD detector and displayed in real-time. The CCD detector is read out with a rate of about 5 frames per second and the corresponding full fluorescence line profiles along the column are displayed. Thus, full electropherogram are displayed showing the propagation and gradual separation of the sample fractions. The main advantage of this method is that parameters such as sample concentrations, diffusion, wall interaction and sample-to-sample interaction can be studied in real-time over the full length of the column, which is crucial for efficient system optimisation. Among several applications, isoelectric focusing, isotachophoresis and enzyme-substrate interactions can be mentioned. Methods for increasing the collection efficiency, such as fiber optic arrays, have been investigated as well as different methods for computer-assisted signal integration and filtering. A fiber array consisting of 500 optical quartz fibers has been constructed that give a substantial improvement of the optical collection efficiency.

Key Words: capillary electrophoresis, chromatography, electrochromatography, laser-induced fluorescence, fluorescence imaging, CCD

INTRODUCTION

Availability of pure substances for determining the structure and function of the enormous array of components encountered in biological system has always been a challenge in analytical biochemistry. The fact that a single cell may contain several thousand proteins, hundreds of RNA's, multiple DNA and polysaccharide components makes the isolation of any single macromolecular species a challenge.[1] For this reason separation technology and advancement of biochemistry have been intimately linked from ultracentrifugation (The Swedberg, 30th), electrophoresis (Tiselius & Hjerten, 40th), gas chromatography GC (Martin & Synge ,40th) gelfiltration chromatography (Porath & Flodin, 50th) to high performance liquid chromatography HPLC (Kirkland & Majors, 70th). Unfortunately, many of the analytical techniques upon which life scientists have depended are labour intensive, slow and/or of low resolving power. A new candidate, capillary electrophoresis CE[2,3], has developed during the last decade from a promising technique to a method of choice for many applications, developing and growing in all directions.[4] A lower concentration sensitivity in comparison with liquid chromatography (LC) can be compensated by sample enrichment and preconcentration such as isotachophoresis (ITP)[5,6]. In ITP the sample is injected into the capillary in-between a leading and a terminating electrolyte. If the mobility of the sample is between that of the two electrolytes and the concentration of the leading electrolyte is higher than that of the sample, a sample concentration will occur. After the on-line preconcentration is achieved an electrophoretic separation can be initiated by switching electrolytes.

One explanation to the success of CE is that it combines the rapidity and automation capability of HPLC and the extremely high resolving power of electrophoresis. It is also possible to transfer some of the knowledge from HPLC during method development. Secondly, CE uses only minute amounts of separation media (µl-ml) and

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sample volumes (pl-nl). The extremely small sample volumes makes it possible to look into a single cell[7,8] and analyse its content of a certain species. CE also produce minute amounts of waste. The use of CE as a tool in analytical chemistry has rapidly increased in the past few years and has found important applications in analysis of metabolites, pharmaceuticals and biopolymers and will be the future work horse in this area. CE may be the tool of choice to solve such challenging tasks as to elucidate the human genome, where about one billion base pairs have to be identified.

To extract more information from CE experiments, a new approach has been proposed by our group[9]. The system employs real-time fluorescence-imaging detection[10,11] for a better understanding of the processes acting in capillary electrophoresis. Rather than exciting just one point at the end of the capillary column we have chosen to use the entire column as a detection cuvette and thus generate information during the main part of the separation with a limit of detection of $5x10^{-10}$ M for fluorescein. Many useful and unexpected findings have been revealed during our investigations performed so far. In addition, this technique was used to image the process of ITP, displaying the gradual sample concentration during the isotachophoretic phase prior to CE separation[12]. Parallel to our work, similar systems have been developed elsewhere utilising both absorption and fluorescence detection[13]. An interesting imaging technique employs a rectangular channel plate in which the fluorescence is detected by a fiber optic array perpendicular to the flow direction and where an injection capillary is scanned across the channel plate[14]. In this paper we present the principles of our detection system and discuss the advantages and problems in relation with conventional CE detectors. In particular, we will address the system detection limit and show how this can be improved. Some preliminary supporting data will also be shown. We will also show data from a few experiments. The system detection limit was determined using fluorescein at different concentrations. One example of isotachophoresis (ITP) will also be shown.

MATERIAL AND METHODS

Samples

Fluorescein standard sodium salt (internal standard, P/N 477406, Beckman Instruments, Fullerton, CA) was used at concentrations 50, 33 and 20 nM for estimation of the limit of detection. The DNA standard was an HAE III restriction digest of ϕX 174 RF DNA (gibco BRL, Div. of life Technologies, Gaithersburg, MD). The digest consisted of 11 fragments with the following sizes: 72, 118, 194, 234, 271, 281, 301, 603, 872, 1078 and 1353 base pairs. The nucleotide mixture was ethanol precipitated and redissolved in distilled water to a concentration of 40 μg/mL. Rhodamine B (base) (Janssen Chimica, Beerse, Belgium) was used for ITP and was dissolved in ethanol and diluted in 6-aminocaproic acid (EACA) (Fluka, Buchs, Switzerland) to a concentration of $\approx 10^{-7}$ M. The leading electrolyte was EACA and the terminating electrolyte was acetic acid (E. Merck, Darmstadt, Germany). For further details, see refs 10 and 12.

Electrophoretic Set-up

The CE equipment used for the ITP experiments was built in-house. A stabilised power supply (0-30 kV, Zeta-elektronik, Höör, Sweden) was used. The electrode vessels consisted of Eppendorf microfuge tubes. The capillary was fixed in a Plexiglas holder and fixed in a horizontal position, which allowed the laser light to access the capillary with the exception of stretches at each end. Injections of the analytes for ITP were made by hand with a 5 μL Hamilton micro-syringe. Fluorescein samples were introduced electrokinetically for 5 sec at 65 V/cm and analysed at 195 V/cm. DNA samples were introduced for 5 sec at 21 V/cm and separated at 157 v/cm. 100 μm inner diameter capillaries (DB-17, J&W Scientific, Folsom, CA in fluorescein experiments and ds DNA 1000, Beckman Instruments, Fullerton, CA in DNA experiments), were used. In the case of ITP, 100 μm inner diameter fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA, modified by polyacryl amide coating), were used. The total length was 20 cm, out of which 7 cm were covered by the camera. The outer protective polyamide coating on the capillary was removed according to refs 10 and 12.

Detection System

A system utilising laser-induced fluorescence imaging as a detection method was constructed. The novelty of this detection method is based on the fact that the fluorescence from samples in the capillary is imaged onto a CCD. By this technique the sample can be continuously followed through the capillary, displaying *e.g.* a separation, binding, catalysis, isoelectric focusing or a concentration process. The system set-up is shown in Fig. 1. The excitation source was an excimer laser pumped dye laser (Estonian Academy of Science, ELI-76E, Tallin, Estonia and ELTO Ltd., VL 2200, Tartu Estonia, respectively) tuneable over the entire visible region. The output of the dye laser was coupled into a 1 mm diameter non-fluorescent optical fiber. The mean power out from the optical fiber was in

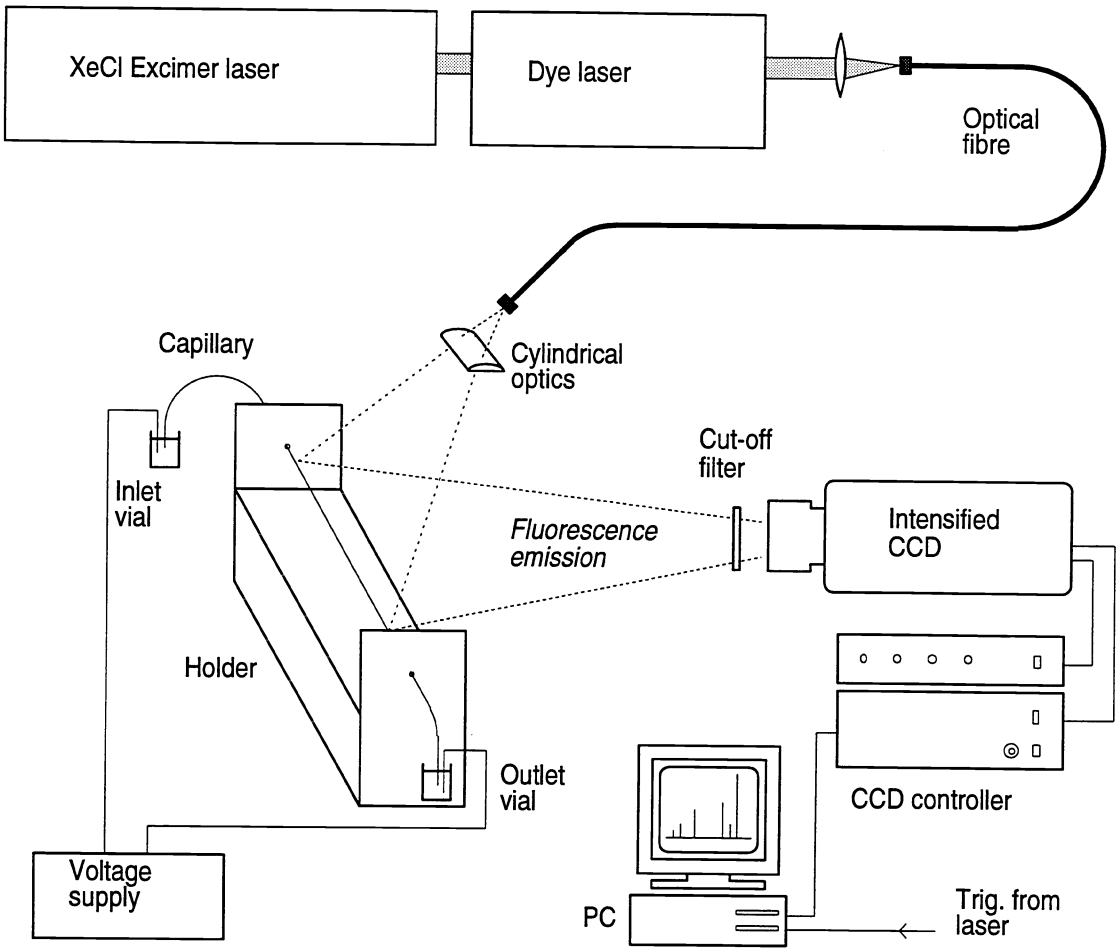

Fig. 1. Set-up for fluorescence imaging of capillary electrophoresis.

the range of 1 mW. The laser was generally operated at 5 Hz and the excitation wavelength was either 488 or 500 nm. The divergent light from the optical fiber was focused to a 10 cm linear streak onto the capillary by means of cylindrical lenses. The capillary was fixed in a straight position in a Plexiglas holder with exception to the inlet and the outlet portion that were dipped into the respective electrolyte vials. The fluorescence was collected with an image intensified CCD camera (Spectroscopy Instruments, ICCD-576E, Gilching, Germany) equipped with a 35 mm camera objective. The distance from the capillary to the CCD was about 30 cm. The CCD camera was cooled to -20°C to lower the dark current of the detector. In front of the CCD a cut-off filter (Schott, GG530, Mainz, Germany) was placed to remove stray light at the laser wavelength while transmitting the fluorescence light. The fluorescence profile across the capillary detected by the CCD was read out, digitized, stored and displayed on a PC in real-time. The principle of the recording of a fluorescence profile is shown in Fig. 2. The pixels along a horizontal streak of the CCD, in the pixels where the fluorescence is recorded, were vertically binned together and shifted downwards to the CCD shift register and the horizontal fluorescence profile was displayed as in the lower part of Fig. 2. One run typically lasted for a few minutes producing about 500-1000 consecutive fluorescence profiles. In the case of slower migrating samples the signal from up to 10 individual profiles (or scans) were added together to increase the signal-to-noise ratio.

RESULTS AND DISCUSSION

The work on a multipurpose system for fluorescence imaging in analytical chemistry, in particular, in CE has been in progress for a few years. The work is divided into two parts; the work on optimising the performance of the detection system and applications in analytical chemistry. One important parameter that has to be taken into consideration is the detection limit of the system. In order to determine the detection limit, a series of CE

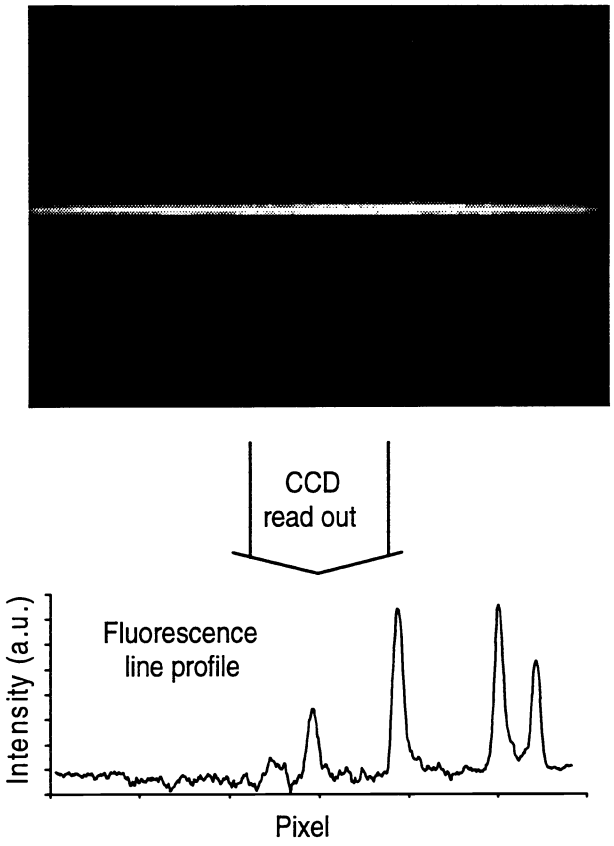

Fig. 2. Principle of data acquisition. The fluorescence from the capillary is imaged as a streak onto the CCD. The signal is binned and shifted to the shift register and read out to the PC. The fluorescence intensity as a function of position along the capillary is displayed on the PC monitor at a repetition rate of the data integration time. (Note that the image and profile displayed here are not recorded at the same time).

experiments with fluorescein were performed. The fluorescein was electrokinetically injected into the capillary. One example of such a recording is shown in Fig. 3. The time scale starts shortly after injection and a fluorescein peak, moving from left to right is followed in the figure. For clarity, only every 7th fluorescence profiles are shown in Fig. 3. The signal-to-noise (S/N) ratio was calculated from a mean value of the S/N ratio of the individual profiles in Fig. 3. Three recordings at different concentrations were made and the corresponding S/N ratios are shown in the insert in Fig 3. From this curve the limit of detection for fluorescein was estimated to be about $5x10^{-10}$. It is important to note, however, that since the

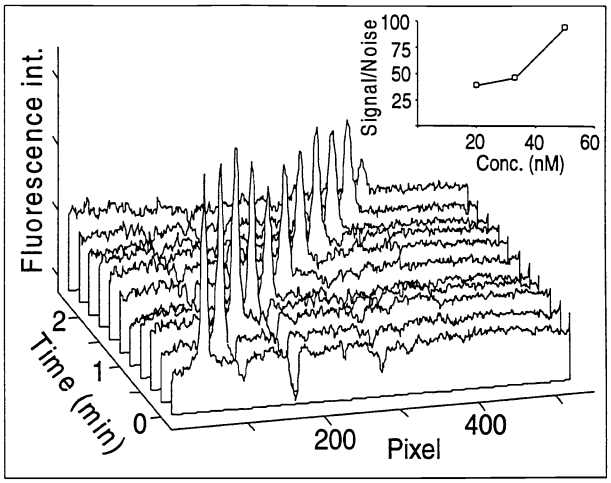

Fig. 3. Selected electropherogram from an electrophoretic run with fluorescein. The concentration was 20 nM. The electropherogram are presented as fluorescence intensity vs. pixel and are separated by 15 sec. in time. The insert shows the calculated S/N for the run in Fig. 3 and 2 additional runs (not shown here).

S/N ratio presented in the insert was a mean value, several individual profiles had a higher S/N ratio. Thus, when the full 5 min. recording is observed, *e.g.* as a video recording, even smaller concentrations are possible to detect. The fluctuation in fluorescence intensity between different electropherogram is due to fluctuations in laser pulse energy.

Real-time imaging of capillary electrophoresis gives excellent opportunities to follow a separation on column. An example of this is shown in Fig. 4a, where selected electropherogram from a DNA size ladder separation are shown. The DNA mixture (Hae III restriction digest of ϕX 174 RF DNA, lower part) was almost separated already when it entered the visualised portion of the capillary, which was placed a few cm from the inlet. It is interesting to note that a separation can be achieved in a capillary length of only a few cm. This was also confirmed with a commercial capillary electrophoresis equipment (Beckman P/ACE 2050). In Fig 4b a close-up from Fig. 4a is shown. Here, the gradual separation of one of the peaks dividing into two peaks, is shown. With the present set-up it was not possible to visualise the inlet and the first cm of the capillary although this would have in a better way illustrated the initial separation of the DNA mixture. This matter will be discussed in the next section.

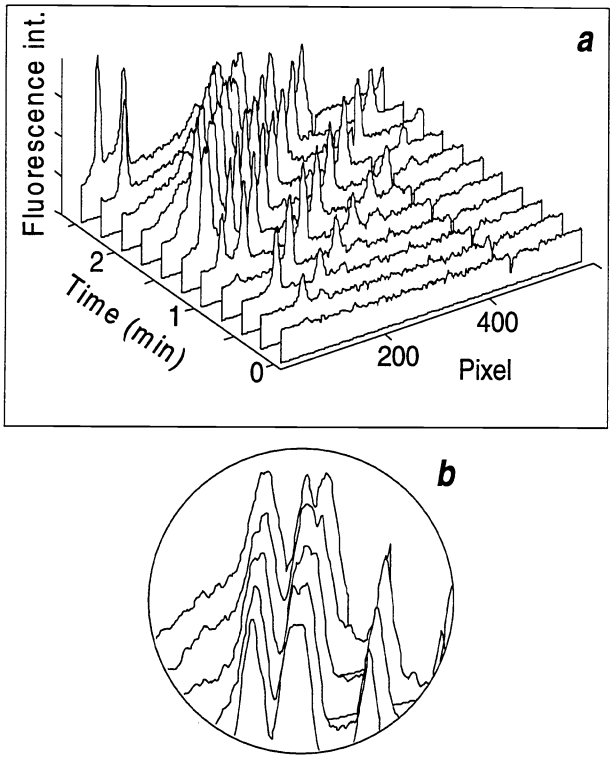

Fig. 4. (a) Fluorescence profiles from an electrophoretic separation of a DNA mixture (Hae III restriction digest of ϕ X 174 RF DNA). Each electropherogram is presented as fluorescence intensity vs. pixel and are separated by 15 sec. along the time axis. (b) Close-up from (a) showing the gradual separation of the peak first appearing at 1.25 min moving from the left to the right.

One limitation of CE is the lower concentration sensitivity compared with HPLC. This limitation can, however, be overcome by different preconcentration methods. A very powerful preconcentration method is isotachophoresis (ITP). Real-time fluorescence imaging is an excellent tool for illustrating the dynamics of ITP. An example of real-time fluorescence imaging of ITP is shown in Fig. 5. Rhodamin B was chosen as a test sample for the experiments and was dissolved in ethanol and diluted in 6-aminocaproic acid (EACA) to a concentration of $\approx 10^{-7}$ M. The leading electrolyte was EACA and the terminating electrolyte was acetic acid. As can be seen, the sample starts to accumulate in the left of the detection window. This is the location of the border between the sample zone and the terminating electrolyte. As the whole train moves from left to right, more and more Rhodamin is "collected" from the sample zone and is concentrated in the border. Note also that the fluorescence from the Rhodamin in the

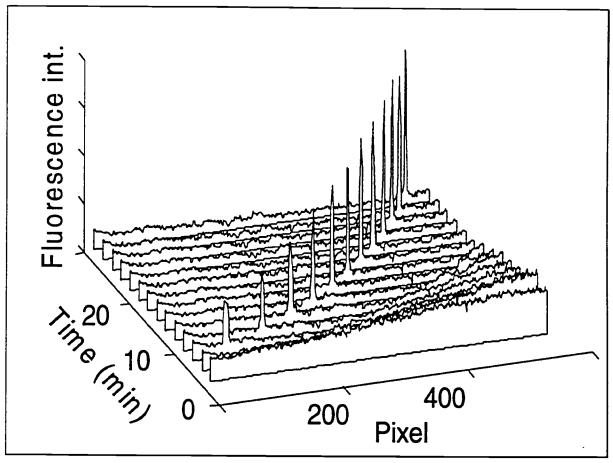

Fig. 5. Isotachophoretic preconcentration of Rhodamin B in ethanol/EACA.. The fluorescence intensity is shown as a function of position along the capillary and time after injection.

sample zone that is not yet collected can be observed as a broad continuum to the right in the first profiles at early times. The Rhodamin will eventually be collected by the peak if the ITP is allowed to proceed for long enough time. This clearly illustrates the advantage of real-time imaging as a detection method for preconcentration. If the ITP is terminated too early only a portion of the sample is collected. On the other hand, the ITP must not be terminated too late for practical reasons. With this technique we can easily determine the optimal time to terminate the ITP and initiate a CE. In this experiment we did not include the CE since it was not the object of this investigation.

SYSTEM DEVELOPMENTS

Two very important parameters for a successful electrophoresis as for all other analytical techniques, are resolution and detection limit. We have shown here, *e.g.* in Fig. 5, an excellent resolving power of the imaging system. In the ITP measurements the resolution was limited by the pixel size and number of pixels (576) per row of the CCD. In the future, a CCD camera with twice as many or even more pixel per row, will be used. This will facilitate an even better system resolution. However, it should be pointed out, that optical resolution and collection efficiency are related parameters. Hence, it is very difficult to simultaneously achieve a high resolution and an efficient collection of the fluorescence. If optics with a low f# is chosen to increase the light collection, the optical resolution will be accordingly decreased. Note that this is in analogy with the width of the detection window

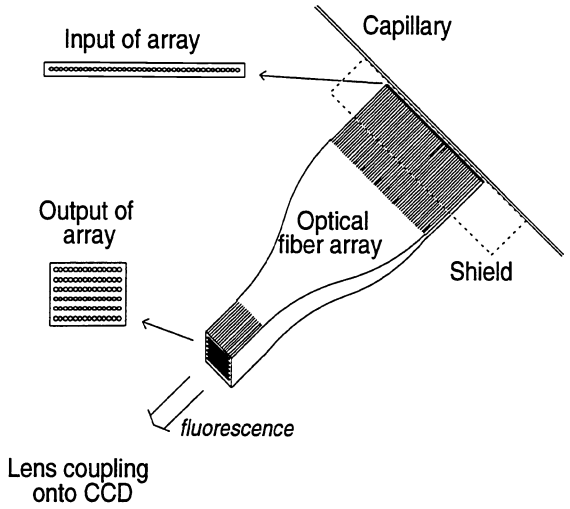

Fig. 6. Set-up of optical fiber array for fluorescence collection. The array consists of 400, 100 μ m diameter optical fibers. The fibers were arranged to a single row at the input and rearranged to six rows at the output of the array as indicated in the Fig. The fluorescence was imaged onto the CCD using two f2 lenses. A shield was positioned close to the capillary to lower the amount of autofluorescence from the fiber array.

for conventional CE. In other words, if a high collection efficiency of the optics is achieved, precautions must be taken in order not to deteriorate the optical resolution of the system.

The main challenge of the imaging system is to image a long section of the capillary. As the length of the imaged section increases, the lower the solid angle of detection has to be to avoid aberrations and, thus, a poor resolution will result. One approach to this problem is to use an array of optical fibers.

Fiber Array Construction

An optical fiber array, 50 mm wide was constructed of 400 optical fibers (100 μm core, 110 μm cladding, 125 μm coating), each 30 cm long. The fibers were glued with epoxy between two pieces of floatglass (55 mm wide, 8 mm thick, 25 mm length), which were bevelled to an angle of 20 degrees. These are used as a fixture during the polishing procedure. The output was divided into six linear groups, where each group was glued with epoxy to a microscope slide and these packets were glued on top of each other. Precautions were taken to make sure that the order of the fibers were the same at the collecting and the output sides. After the epoxy was cured, the ends of the fibers were covered with Norland Optical Adhesive no. 68. This adhesive is harder than epoxy and more suitable to polish. The collecting end is polished with the floatglass as bearer and a 20 degrees edge will occur in the middle of each fiber. A free hand polishing will smoothen the edge and the rounded shape will enhance the collection of the fluorescence light from the inner of the capillary. The output end is polished flat. The fiber array and its position in the overall set-up is shown in Fig. 6.

Data Acquisition and Results

The emission from the fluorophores in the capillary was collected with the linear fiber array. The collecting end was fixed as close as possible to the capillary and therefore it was necessary to shield the fiber array from the excitation light with a metal plate, carefully adjusted to avoid fluorescence emission from the collecting end. The output end, divided into six smaller arrays, was arranged to a quadratic configuration to produce a more compact array. The output was imaged on to the 576 x 384 pixel image-intensified CCD camera through a lens system and cut-off a filter, which prevents the scattered excitation light to enter the camera. The compact arrangement of the fiber array output facilitates the use of small diameter optics between the fiber array and the CCD camera and avoids the problem of minifying the image of the capillary. In the case of the fiber array, the CCD was binned into 6 groups producing 6 line profiles in contrast to the previous arrangement that yield single profiles for each laser shot. A complex electropherogram using the optical fiber array arrangement is shown in Fig. 7. The sample was Rhodamin B at a concentration of $1x10^{-10}$ M. Each of the six line profiles display a section of the capillary and these profiles can easily be post-processed (added together) to form a complete electropherogram.

The gain of using the fiber array arrangement is twofold. First, it yields an improvement in terms of optical collection efficiency in relation to the camera objective previously used. A theoretical calculation showed that as much as a factor of 30 can be gained by the fiber array arrangement in comparison with the camera objective approach. The result of the calculation is graphically presented in Fig. 8, where the solid angle of the optical fibre is shown as a function of distance from the capillary. A value of 1 corresponds to the collection efficiency equal to that of the camera objective arrangement. The results of our preliminary studies were also in accordance with the calculations. However, it should be pointed out here, that

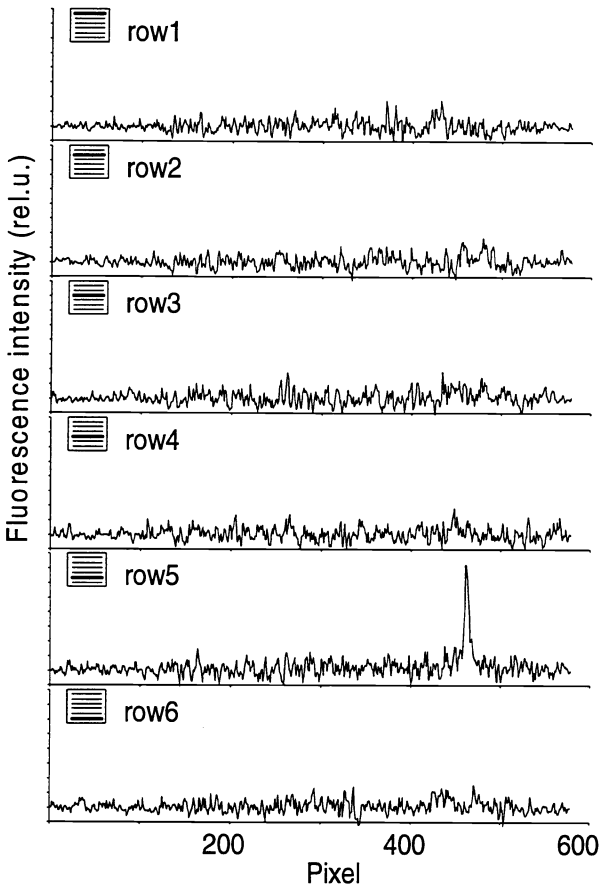

Fig. 7. Fluorescence line profiles using the fiber array arrangement. The sample was $1x10^{-10}$ M fluorescein. All six profiles were simultaneously recorded at 35 seconds post injection.

the camera objective arrangement would probably be possible to improve in terms of collection efficiency. On the other hand, if a capillary with a smaller diameter is used, the gain factor of the fiber array arrangement would also increase. Secondly, with the fiber array arrangement, light is preferentially collected from the inside of the capillary, which is the region where the fluorescence from the sample is produced. Thus, the fiber array arrangement additionally contributes to an increase of the signal-to-background fluorescence ratio. Also this has been experimentally verified.

In addition to the above mentioned improvement of the limit of detection, methods for computer assisted signal filtration and integration have been taken into consideration. It is clear, that with the knowledge of the migration speed of the samples, the individual peaks can be followed and a signal integration over a longer time period (*e.g.* 1 min.) can be employed. In such a method the

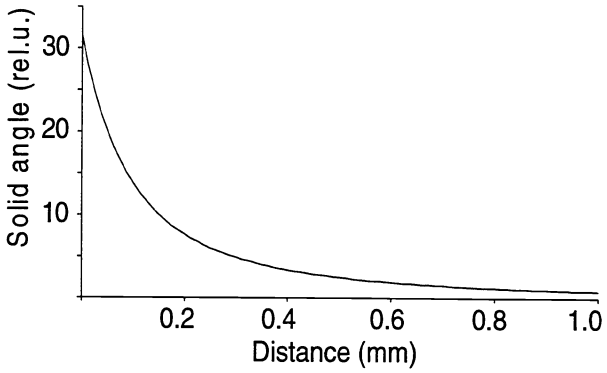

Fig. 8. Theoretical calculation of the useful solid angle in relative units (compared with the camera objective approach) as a function of distance from the capillary outer wall for the fiber array.

signal from a certain sample would be assumed to move a certain number of pixels between each recorded frame. The advantage would then be that the signal would be added for all frames, whereas stationary fluorescence background from *e.g.* the capillary and adsorbed fluorophores would cancel out, and the S/N ratio would thus be enhanced. Early results using this method suggests a substantial improvement in limit of detection.

In this paper the experimental set-up for a fluorescence imaging detector is shown. We have also shown a few examples of the use of the detector and discussed some of the advantages with this system. The strength of this methodology lies in the fact that the whole or a substantial part of the capillary may be monitored simultaneously, with applications to new system developments but maybe also for pure analytical work. In addition to the work presented here, measurements were performed with application to capillary chromatography and electrochromatography[15]. This methodology can be used for many different kinds of analytical techniques where optical access can be obtained to a column or a plate. Valuable information can be obtained from measurements of the injection site or from transition zones such as frits in packed columns and real-time imaging may be the method of choice for system optimisation in many applications.

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