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DNA sequencing by multiple capillaries that form a waveguide

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ABSTRACT

A 12 -capillary prototype electrophoresis system for DNA sequencing has been constructed. Laser illumination is introduced into an optical waveguide that is formed by an array of individual capillaries that serve both as the optical elements of the periodic array and as the channels containing sieving media for electrophoresis. A theoretical framework and experimental data will be presented to illustrate the viability of this approach.

Keywords: DNA sequencing, capillary electrophoresis, lasers, fluorescence, fiber optics

1. INTRODUCTION

Capillary electrophoresis is a widely useful analytical tool[1-6]. The small capillary core and efficient heat dissipation allow large voltage gradients and rapid separations of charged species. A particularly useful application is rapid separation of fluorescently labeled DNA molecules, which has wide applications for DNA sequencing, genetic analysis, medical diagnosis, and forensics. Typically, separations are monitored by illuminating a small region of the capillary core with a laser and measuring the induced fluorescence as molecules move through the illuminated region.

The analytical capacity of capillary separation techniques is multiplied by using many capillaries in parallel. Efficient illumination of the capillary cores together with highly sensitive detection of fluorescently emitted photons remains a desired goal for a practical diagnostics instrument. Refraction and reflections from many curved surfaces make it difficult to obtain reasonably uniform illumination and sensitive detection across many capillaries. Lu and Yeung[1] described a 25 capillary array system which achieved uniform illumination and high sensitivity by placing the closely packed capillaries in the waist region of a focussed laser beam; additionally, the capillaries were embedded in an index matching fluid for reducing reflection losses. However, their assertion that closely packed capillaries provide uniform illumination of the core regions is based on negligible refraction losses, which accumulate as the array size is increased. Quesada and Zhang[6] described an 8 channel fiber optic capillary system, which used dedicated fiber optics to achieve uniform illumination of each capillary. However, their arrangement requires a fiber optic splitter to ensure uniformity and is also restrictive in the maximum number of capillaries that can be used in the array. CCD based imaging techniques can collect data from a large number of capillaries in parallel but require careful alignment. The cost of reducing cross-talk results in a decrease in sensitivity. The fiber optic receivers in Quesada and Zhang[6] system reduce cross-talk to negligible levels, without compromising sensitivity through the use of dedicated fiber optic channels for each capillary.

In this paper we improve uniformity of illumination in the "end-fire" configuration by utilizing the refracting properties of each capillary to form an optical waveguide structure which can confine the incident laser beam to the core regions of each capillary in a modestly large array of capillaries while using low input power. Fluorescently emitted photons are detected using dedicated optical fibers as used by Quesada et al[7]. We provide experimental sequencing data for a 12 channel parallel system.

2. DESIGN OF A CAPILLARY OPTICAL WAVEGUIDE

2.1 Beam confinement

The most efficient illumination of the core of a capillary by a laser beam focussed on the capillary wall will be when the center of the beam is normal to the surface of the capillary. The fraction of the incident light that passes through the core

of the capillary will depend on several factors: the radius, r_i, of the incident laser beam and its angular convergence or divergence; the radii of the outer and inner surfaces of the capillary, r_1 and r_2, respectively; and the indices of refraction of the medium, capillary wall, and core region, n_1, n_2, and n_3, respectively (illustrated in Fig. 1).

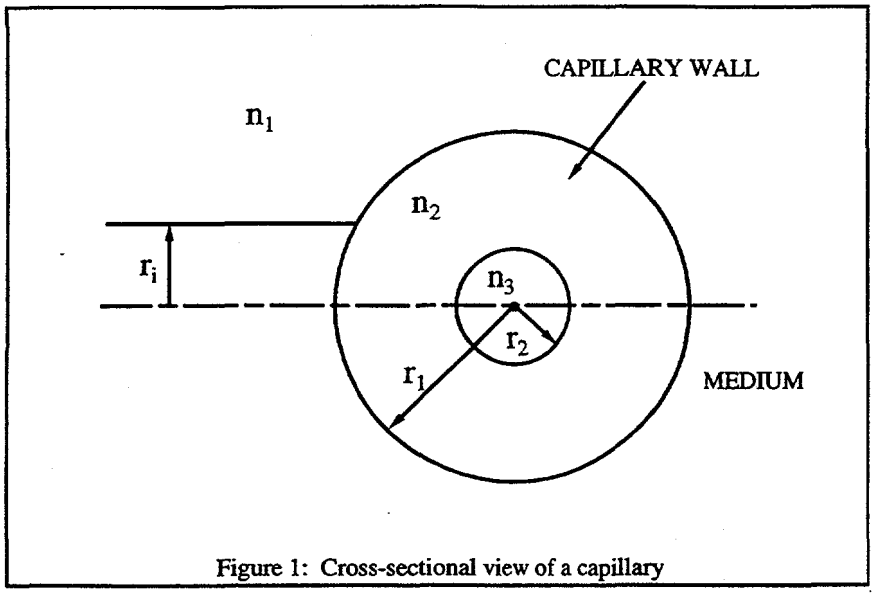

Figure 1: Cross-sectional view of a capillary

According to Snell's law, light passing from one medium into another will be bent according to the equation

$$n_i \sin(\theta_i) = n_r \sin(\theta_r) \qquad (1)$$

where n_i is the refractive index of the medium through which the incident light travels, n_r is the refractive index of the medium through which the refracted light travels, θ_i is the angle of incidence and θ_r the angle of refraction relative to the normal to the surface. Disregarding, for the moment, the small losses due to Fresnel reflection, rays that enter normal to the capillary surface will pass through the wall and core without deflection, but all other rays will be bent at each surface where the refractive index changes. Where n_1 and n_3 both differ from n_2, rays parallel to the optical axis that pass through the core will be bent at four places: 1) upon entering the capillary wall from the medium; 2) upon entering the core from the wall; 3) upon exiting the core into the wall; and 4) upon exiting the capillary wall into the medium. The maximum deflection will occur for rays that enter the capillary at the angle farthest from normal, so, if those rays pass through the core, all rays must pass through the core.

Consider a flat parallel array of identical, equally spaced capillaries illuminated with a cylindrical parallel beam of light that is centered normal to the first capillary and in the plane of the capillary array (illustrated in Fig. 2). Each capillary in the array is an equivalent optical element of four refracting surfaces. Because the capillary surfaces are cylindrical, the light will be bent only toward or away from the plane of the array; individual rays will remain parallel to a plane that is normal to the central axes of the capillaries. The conditions for confining the beam to the core of each successive capillary in the array are that 1) all of the light rays that enter the first capillary must pass through its core, and 2) the rays that exit the first capillary must illuminate the second capillary such that all the rays pass through the core of the second capillary and all other capillary cores down the array. The second condition can be only met if the combined refractive effect of the four surfaces of the capillary is not divergent and if the spacing between capillaries is appropriate. Confinement is indicated by a periodic compression and dilation of the beam as it propagates through the capillary array.

When the conditions for beam confinement are met, the refracted light will be confined to the cores of successive capillaries indefinitely, and the only losses should be those due to Fresnel reflections at the surfaces (which will be considered below). Thus, the parallel capillary array forms a waveguide. In practice, the beam emerging from the first capillary will almost always be more convergent than the collimated illuminating beam. Ray tracing predicts beam confinement with spacings as large as several capillary diameters with commercial capillaries under typical conditions of use. Theoretically, confinement should not be achieved when spacing becomes large enough that rays passing through the first capillary cross the optical axis closer to the first capillary than the midpoint between the centers of the first two capillaries, but in practice, the spacing usually must be considerably closer than this. Under conditions of confinement, the effective focal length of the combined optical elements in a parallel array of capillaries is typically a few capillary spacings long (see examples in Fig. 2).

2.2 Beam confinement in the thin lens and paraxial approximations

Another way of stating the second condition for beam confinement uses an analytical method from paraxial optics called the thin lens approximation. This analysis is presented to illustrate the physical basis for beam confinement and the relationship among relevant paramenters for successful use of capillary array optical waveguides.

DISCLAIMER

The central assumption in the thin lens approximation is, when several refracting surafces are close, we consider the intervening gaps as negligible. Thus, we only consider the refraction due to the four surfaces of a capillary and neglect their precise positions with one another. The beam confinement condition, then, in this simplified system, becomes: the sum of the refractive power of the four refracting surfaces of the capillary must be greater than or equal to 0. The refractive power, p, is a measure of the ability to bend light when light crosses a curved surface and is expressed in the paraxial approximation as:

$$p = \frac{n_i - n_r}{r} = \frac{1}{f} \qquad (2)$$

where r is the radius of curvature of the surface and f is the focal length. Positive values of p indicate convergence and negative values divergence. The above equation is for light incident on a convex surface; the sign is reversed for a concave surface. The paraxial approximation may not be very accurate for the relatively high curvatures of capillaries, but the equation shows that the absolute value of the focal length decreases with increasing difference in refractive index or decreasing radius of curvature.

The refractive index of the wall of a fused silica capillary is typically 1.46, of water 1.33 and of air 1.0. Thus, with any combination of aqueous solutions or air as the external medium or in the core, the two outer surfaces will both be convergent and the two inner surfaces both divergent to a beam illuminating from the outside. Since the radius of the core is necessarily smaller than the radius of the outside surface of the capillary, obtaining enough convergence to confine the beam within the cores of successive parallel capillaries requires that the difference in refractive index between the external medium and the wall be greater than the difference between the internal medium and the wall. The beam will not be confined if the external medium and the core have the same refractive index. Fortunately, typical uses of capillaries are in air with aqueous solution in the core, a condition that allows beam confinement over a range of different internal and external radii.

Equations derived with paraxial and thin lens approximations can estimate, with some uncertainty, whether a parallel array of capillaries of known dimensions will confine a beam; exact calculations, including determining the size and divergence of beam that could be confined, can be made using a ray tracing program (e.g. Optec III). As an example, Fig. 3 shows a comparison of the predictions by the two methods of which combinations of n_1 and r_1 would confine a parallel beam of radius $r_i = r_2$ within a parallel array of capillaries where $r_2 = 37.5$ μm, $n_2 = 1.46$, and $n_3 = 1.33$. The line shows the values of n_1 and r_1 that satisfy the equality in the beam confinement condition

$$p_1 + p_2 + p_3 + p_4 \geq 0 \qquad (3)$$

where p_1, p_2, p_3 and p_4 are the refractive powers calculated for the four surfaces of the capillary crossed by the beam, using the paraxial approximation of equation 2. Combining equations 2 and 3 gives:

$$\frac{(n_2 - n_1)}{r_1} + \frac{(n_3 - n_2)}{r_2} \geq 0 \qquad (4)$$

Combinations of values of n_1 and r_1 that lie on or below the line are predicted to confine the beam, and combinations that lie above the line are predicted not to confine the beam. The error bars give the highest values of r_1 calculated to give confinement and the lowest values calculated not to give confinement as determined by exact calculations with a ray tracing program for several different values of n_1. As expected, estimates from the paraxial approximation deviate further from the exact case the smaller the radius of curvature. Additionally, the following design rules can be ascertained from Eqn. (4).

For all conditions where both $n_2 > n_1$ and $n_2 > n_3$ (that is, the conditions typical for electrophoretic or chromatographic separations), relationship 4 can be rewritten

$$1 < \frac{r_1}{r_2} \leq \frac{(n_2 - n_1)}{(n_2 - n_3)} \qquad (5)$$

Considering the absolute physical constraint that the outer surface radius r_1 must be larger than the inner surface radius r_2, relationship 5 can be satisfied only when $n_3 > n_1$. Thus, a requirement for forming a capillary waveguide when n_1 and n_3 are both less than n_2 is that

the refractive index of the material in the core must be greater than that of the external medium. This requirement is readily understood in physical terms. Under these conditions, the two outer surfaces of a capillary will both be convergent and the two inner surfaces both divergent to a passing beam. Since the radius of the core is necessarily smaller than the radius of the outside surface of the capillary, equation 2 shows that the divergence contributed by the inner surfaces will be greater than the convergence contributed by the outer surfaces unless the difference in refractive index between the wall and the external medium is greater than the difference in refractive index between the wall and the material in the core.

Relationship 4 may also be used to consider the possibility of forming capillary waveguides where other refractive index differences across the surfaces apply, as might be encountered with exotic materials or specialized uses. Relationship 4 cannot be satisfied for any condition where both $n_2 < n_1$ and $n_2 > n_3$, because the left side of relationship 4 will always be negative. The inability to satisfy this relationship is readily understood because, under these conditions, all four capillary surfaces would be diverging and a beam could not be confined.

The opposite situation, where all four capillary surfaces would be converging, would obtain where both $n_2 > n_1$ and $n_2 < n_3$. In this case, relationship 4 will always be satisfied, because the left side of relationship 4 is always positive. Under these conditions, beam confinement will depend on the degree of convergence produced by the capillary and the spacing.

The remaining possible inequalities in refractive index across the capillary surfaces are when both $n_2 < n_1$ and $n_2 < n_3$. In this case, the two outer surfaces of a capillary will both be divergent and the two inner surfaces both convergent to a beam passing through the capillary. Since the radius of the core is necessarily smaller than the radius of the outside surface of the capillary, equation 2 shows that the convergence contributed by the inner surfaces will be greater than the divergence contributed by the outer surfaces when $n_3 \geq n_1$, and, depending on the values of r_1 and r_2, for some range of values of $n_3 < n_1$. Prediction of whether a particular beam would actually be confined under a particular set of conditions is best analyzed with the help of an exact Snell's law ray-tracing program.

Index matching across either capillary surface may also be considered in light of relationship 4. With index matching at the outer surface, that is, $n_2 = n_1$, relationship 4 is satisfied only when $n_2 \leq n_3$, that is, when the inner surfaces are index matched or converging. With index matching at the inner surface, that is, $n_2 = n_3$, relationship 4 is satisfied only when $n_2 \geq n_1$, that is, when the outer surfaces are index matched or converging.

In usual situations where capillaries are used for analysis, n_2 is greater than both n_1 and n_3, and under these conditions relationship 5 may be used as a guide to constructing planar arrays of capillaries which will support beam confinement, in much the same way as one uses a formula such as the lensmaker equation as a guide in fabricating lenses. For example, to build a capillary waveguide in air ($n_1 = 1.0$) with silica capillaries ($n_2 = 1.46$) containing dilute aqueous solutions ($n_3 \sim 1.33$), the ratio r_1/r_2 should be less than approximately $0.46/0.13 = 3.54$. For concentrated solutions of urea typically used for DNA sequencing, n_3 might be 1.4, and r_1/r_2 should be less than approximately $0.46/0.06 = 7.67$. In such a case, immersing the planar capillary array in water would increase n_1 to 1.33, thereby reducing Fresnel reflection (discussed below) while still maintaining beam confinement in capillaries where r_1/r_2 is less than approximately $0.13/0.06 = 2.17$.

2.3 Illumination of the capillary array

For most effective illumination of capillary contents, beam confinement needs to be achieved along the parallel, as well as the curved, surfaces of the cylindrical capillary. The parallel surfaces do not provide any compensation to counteract the natural divergence of the incident beam as it propagates through successive surfaces. In this situation near parallel illumination is critical for keeping the expansion of the beam to a minimum. However, the product of any particular beam radius and its divergence is a constant proportional to the wavelength of the incident light. Because of this inverse relationship, a balance between appropriate values of beam radius and divergence must be attained for useful illumination and confinement of laser beams in parallel arrays of

capillaries. In the 12-capillary apparatus described below, confinement was achieved with an IFOT that produced a beam with a radius of 10 microns and a divergence of 2° .

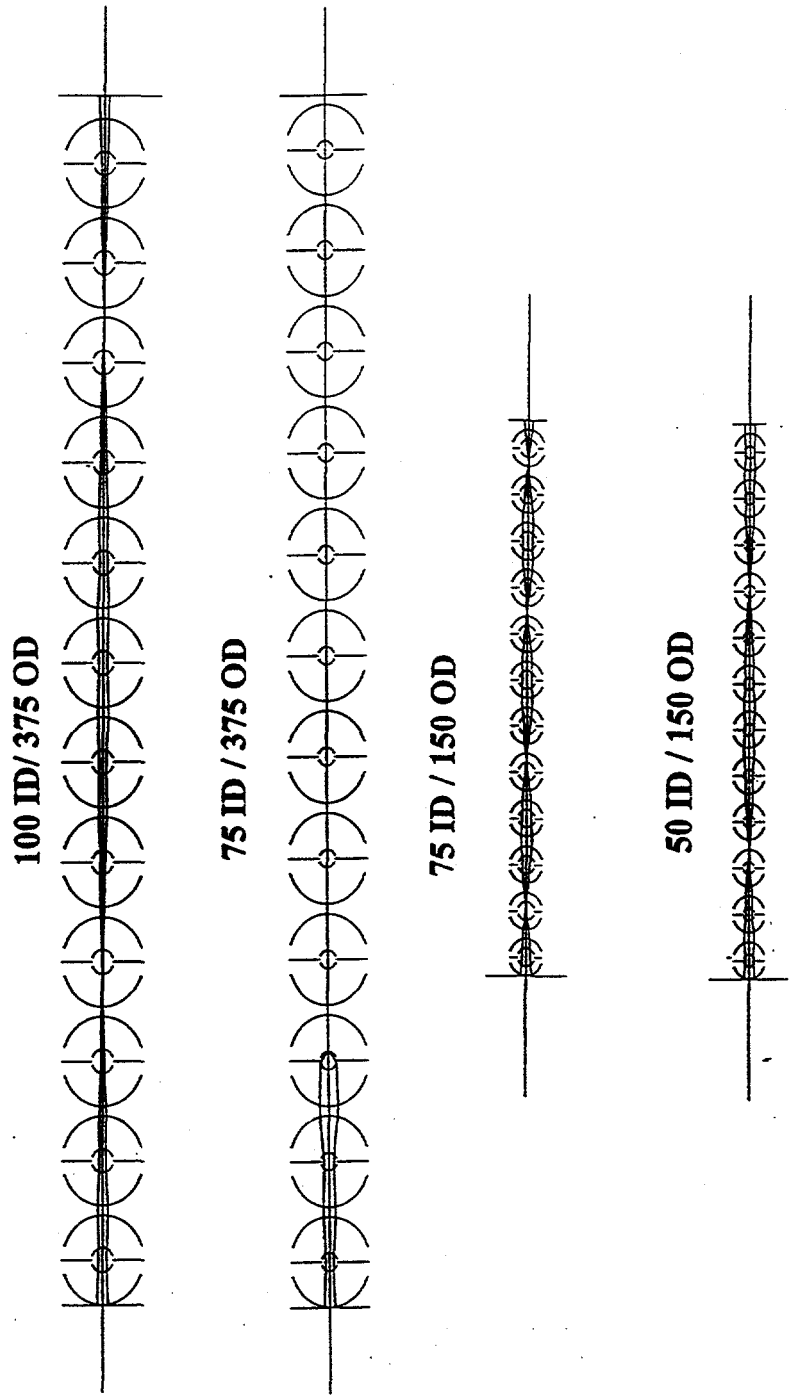

Figure 2: Ray trajectories through several commercially available capillaries

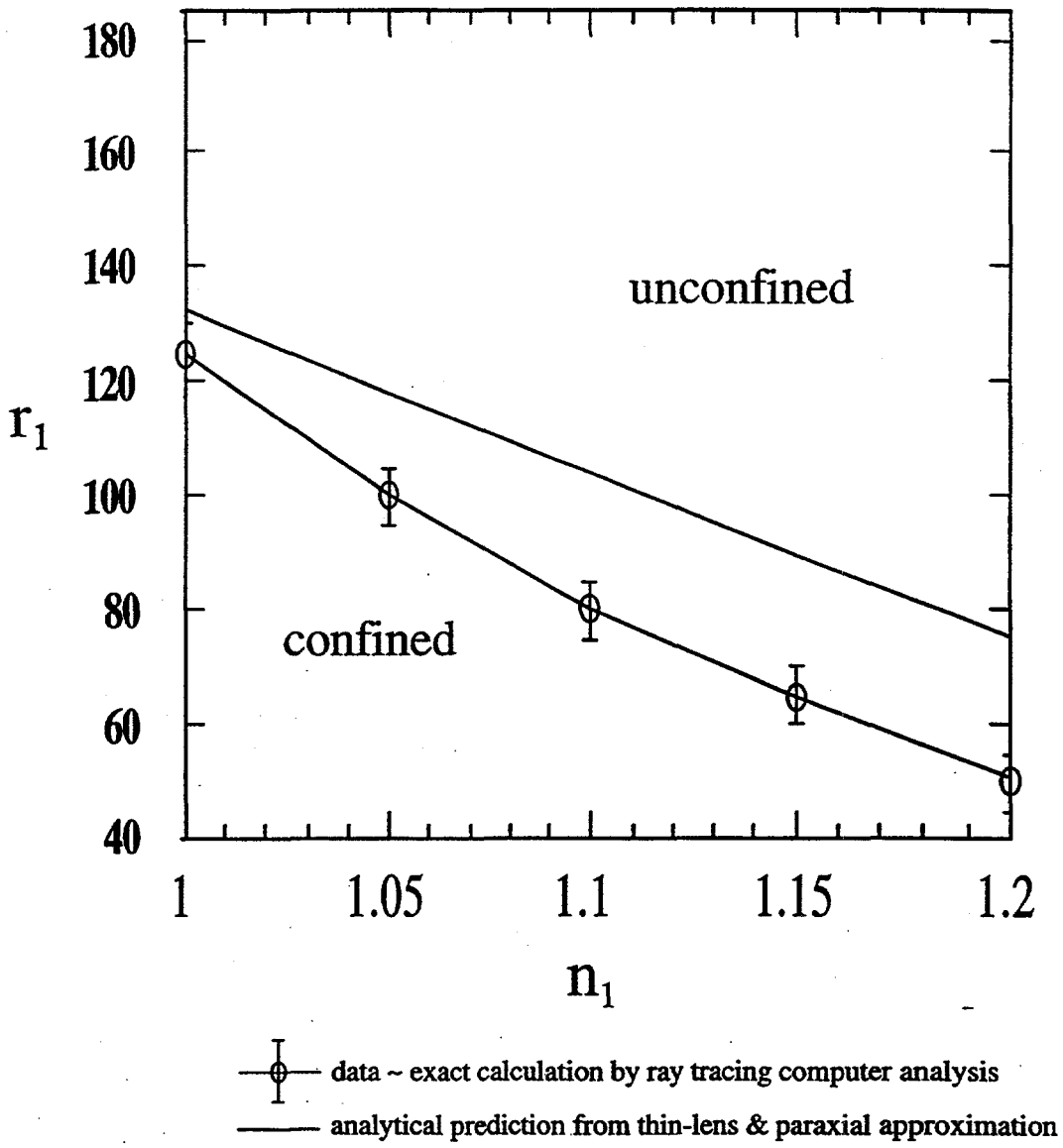

Figure 3: Confinement predicated by thin-lens and paraxial approximations or exact calculations by ray tracing computer analysis. r_1 is in microns.

2.4 Integrated Fiber Optic Transmitter (IFO)

Illumination of a multiple capillary array that forms a waveguide requires that the input laser beam satisfy a certain size requirement of r_i. A bulk optics solution using a beam expander and an appropriate converging lens can be found. However, the resulting transmission system is cumbersome and requires elaborate beam control to achieve alignment.

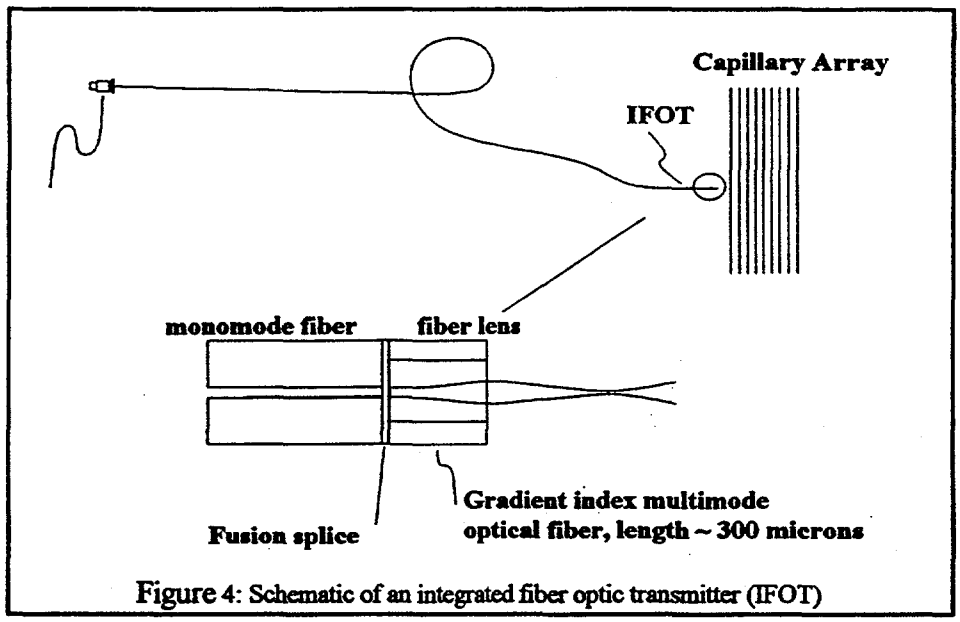

Figure 4: Schematic of an integrated fiber optic transmitter (IFOT)

An integrated fiber optic transmitter as illustrated in Fig. 4 exploits the technology of integrated fiber optics to fabricate a miniature and remote laser beam delivery system. Light from a laser is coupled to a singlemode optical fiber, the distal end of which has been fusion spliced to a short length of a gradient index multimode optical fiber. The resulting single strand of fiber is mounted into a stainless steel ferrule and polished until the desired beam radius is obtained. The IFOT has a cylindrical body of diameter 0.57 mm. and is mounted into a X-Y-Z positioner. For a detailed discussion on the design and fabrication of the IFOT refer to Khan et al[7].

2.5 Reflective losses

Under conditions of beam confinement, the only losses of light as a beam passes through the capillaries in the array will be due to Fresnel reflections at the surfaces. The fraction of the incident light reflected, R is given by

$$R = \left[\frac{n_i - n_r}{n_1 + n_r}\right]^2 \qquad (6)$$

for light that is normal to the surface. The greater the difference in refractive index across the surface and the farther the ray is from being normal to the surface, the more light will be reflected (except near the Brewster angle for illumination by light polarized parallel to the plane of incidence). Because of the high curvature in small capillaries, the fraction of light reflected will increase significantly with increase in r_i. If all of the light were normal to the surface, the fraction of incident light that emerges from the other side of a capillary, T, would be the product of the fraction transmitted at each of the four surfaces

$$T = \left[1 - \left[\frac{n_1 - n_2}{n_1 + n_2}\right]^2\right]^2 \left[1 - \left[\frac{n_2 - n_3}{n_2 + n_3}\right]^2\right]^2 \qquad (7)$$

Equation 7 will underestimate the fraction of a beam transmitted through a capillary by an amount that will depend primarily on the radius of the illuminating beam and the radii of the capillary. The fraction transmitted through a capillary with air on the outside and water on the inside, according to Eqn (7), will be 0.92725, and the fraction transmitted through 12 capillaries will be this number to the 12th power, or 0.4040.

Reflective losses can be reduced to negligible levels by minimizing the differences between the refractive indices across the surfaces. However, loss of confinement causes a much more rapid loss of illumination. Figure 5 plots the calculated illumination as a function of capillary number for a waveguide with air as the external medium and for the equivalent, nonconfining configuration with water as the external medium. Reflective losses are reduced by the lower refractive index difference when water is the external medium, but refractive losses are much greater: illumination of the 9th capillary is 2.9% of the input intensity for the unconfined beam compared with the 54.6% when the beam is confined.

2.6 Collection of fluorescent light from the capillary cores

Illumination of capillaries that form a waveguide is a simple and efficient way to deliver light to the cores of multiple capillaries. The fluorescent signal orthogonal to the illuminating beam may be imaged onto a detector through lenses, filters or prisms. Quesada and Zhang[6] demonstrated use of matched optical fiber receivers for each capillary in the array. A similar arrangement is used here such that each of the collecting optical fibers is normal to the surface of a capillary in a parallel array, with the spacing between optical fibers matched to the spacing between the capillaries. Optical fibers of high numerical aperture efficiently collect the fluorescence from each capillary, with very little cross talk between capillaries. The flexible optical fibers are convenient for delivering the collected light to a remote detecting device.

Optimum detection requires precise alignment of each collection fiber to the corresponding capillary. Independent position control of each collection fiber in the array is not practical. However, any means by which the spacing between collection fibers can be made to correspond sufficiently closely to the spacing between electrophoresis capillaries will allow all of the collection fibers to be aligned simultaneously. One way of achieving the desired alignment resolution is through the use of matched V-grooves. A block that contains V-grooves is cut normal to the axes of the grooves, producing a matched pair of V-grooved surfaces with identical spacing. The capillaries are mounted in one member of the pair and the collection fibers in the other, which guarantees that the relative alignment of the two components is nearly perfect. The collection fibers are mounted permanently, for example with epoxy, and the ends are polished. The capillaries are simply clamped in place, to allow easy replacement of individual capillaries. Alignment of the multiple V-groove fiber optic collection assembly with respect to the multiple V-groove capillary array assembly is possible with a single X-Y-Z positioner; optimum orthogonal alignment is achieved by monitoring and optimizing a spectral signature from the material in the capillary cores (e.g. The OH Raman water stretch). The cost of fabrication could be reduced by using a mold or a photomask to make the V-grooves.

3. EXPERIMENTAL RESULTS

A 12-channel working model was constructed and tested (illustrated in Fig. 6). Laser light from an Argon-ion source was coupled into a singlemode optical fiber by a 20X microscope objective. The distal end of the singlemode optical fiber was coupled to an IFOT, fabricated to deliver a Gaussian beam with a waist radius of 10 μm. The IFOT was mounted into a x-y-z positioner and located at one end of a multiple capillary array. Individual capillaries (100 μm internal diameter and 375 μm outer diameter) were threaded into a V-groove assembly and were filled with water, dye, or buffer solutions. The IFOT was aligned by observing the far-field shape of the laser beam emanating from the other side of the capillary array. Proper alignment was achieved when the far-field pattern was equivalent in size and position with the unobstructed far field laser beam pattern and the reflections above and below the plane of the capillary array were minimized. Approximately 4.5 mW of the 12 mW laser power incident on the capillary array emanated from the parallel structure, somewhat less than the 4.8 mW expected if all light were normal to the surfaces.

Twelve high numerical aperture (NA = 0.37) multimode optical fibers with core/cladding diameters of 100/140 μm were mounted into a matched V-groove fixture with epoxy and polished. The collection fiber optic array was mounted into a second x-y-z translational stage and positioned above and orthogonal to the plane of the capillary array. The collection array was aligned with the capillaries by optimizing the Raman scattering from the water molecules contained in the interior of any

one of the 12 capillaries. The alignment of all other capillary/fiber pairs was confirmed by simultaneous recording of the Raman signal from all 12 channels.

Optical signals collected from the interior of each capillary can be detected and processed using any of several techniques, ranging from dedicated photodetectors/optical filters for each collection fiber channel to the arrangement used here, where optical signals are transmitted to a spectrograph and the fluorescent spectrum from each of the 12 capillaries is detected by a CCD system. These spectral signals are repetitively read out and subsequently analyzed, for example, to give the sequence of DNA.

The 12 capillary system was tested by analyzing 12 parallel DNA sequencing reactions. The coated capillaries were filled with a 4.0% (w/v) polyacrylamide (02806, 5-6 Mdaltons, Polysciences, Inc. Warrington, PA), 3.5 M urea (BioRad, Hercules, CA), 40 mM Tris (BioRad) and 40 mM TAPS (TAPS, Fluka, Long Island, NY) solution. DNA reaction samples were simultaneously loaded by electrokinetic injection from 12 wells of a 96-titre dish. After injection, the DNA samples in the 12 wells were replaced with a running buffer (40 mM Tris, 40 mM TAPS and 3.5 M Urea). Figures 7A and 7B presents the simultaneous acquisition of the full fluorescence spectra of dye-labeled DNA sequence from all 12 capillaries as a function of time. The horizontal (long) axis indicates the time, from 19.5 to 34.2 minutes, after electrokinetic injection of the DNA samples; all pixels sharing a vertical axis represent a time slice of full spectral information obtained from all 12 capillaries in an 0.29 second interval. The vertical axis for each capillary represents the wavelength for fluorescence emission and spans the range from 475 nm to 648 nm. The samples used were aliquots from a stock 20 microliters Taq cycle sequencing reaction using dye-labeled terminators on a pGEM double-stranded DNA template. The relative spectral position of the different dye-labeled bases (C, T, A, and G) is shown along the left vertical axis for each capillary in Figures 7A and 7B. The positions of the fluorescence maxima may be used to read the individual bases directly from the figures. Capillaries 1 and 9 failed in this experiment, apparently due to a failure in loading.

The collection fiber optic array has an additional advantage that the optical signals from each capillary have distinct and separate transmission channels to the detector, thereby minimizing cross-talk between adjacent channels. The lower the cross-talk, the greater the sensitivity and accuracy of base calling. To estimate the level of cross-talk between adjacent channels, emission spectra were recorded for 5 adjacent channels before, during and after 10^{-10} M fluorescent dye was flowed through the central channel. Only 1 - 2% of the large fluorescent signal in the central channel was detected in the flanking channels and none in the outside channels (illustrated in Fig. 8).

4. CONCLUSION

A 12-channel capillary electrophoresis system, using the optical properties of the capillaries to form a waveguide, has been successfully demonstrated. Optical waveguiding reduces the incident laser power requirement. Use of matched and dedicated optical fibers provided sensitive detection of fluorescence with negligible crosstalk between channels.

5. ACKNOWLEDGMENTS

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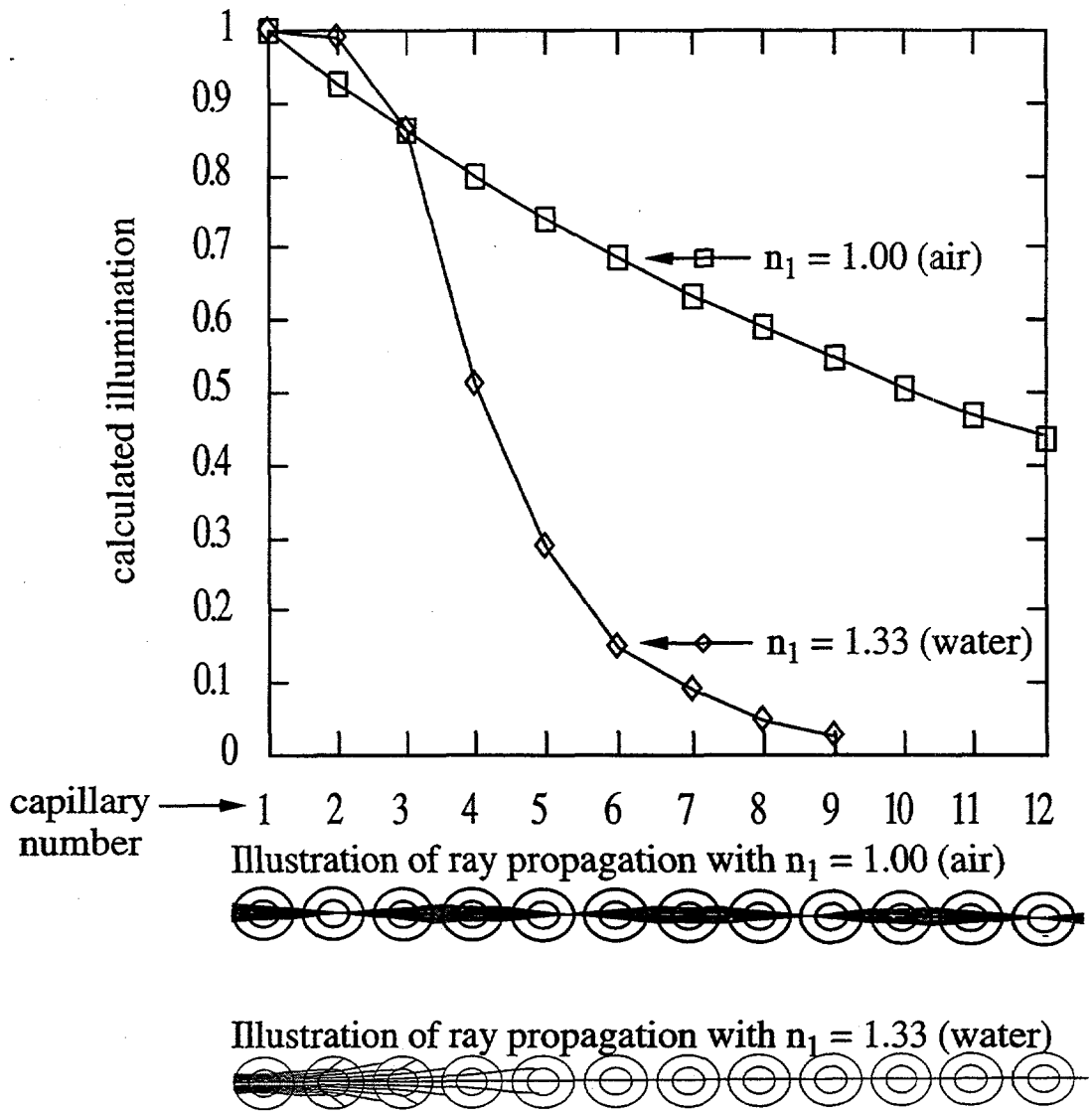

Figure 5: Theoretical illumination efficiency: Points on the curves represent both refractive and reflective transmission losses

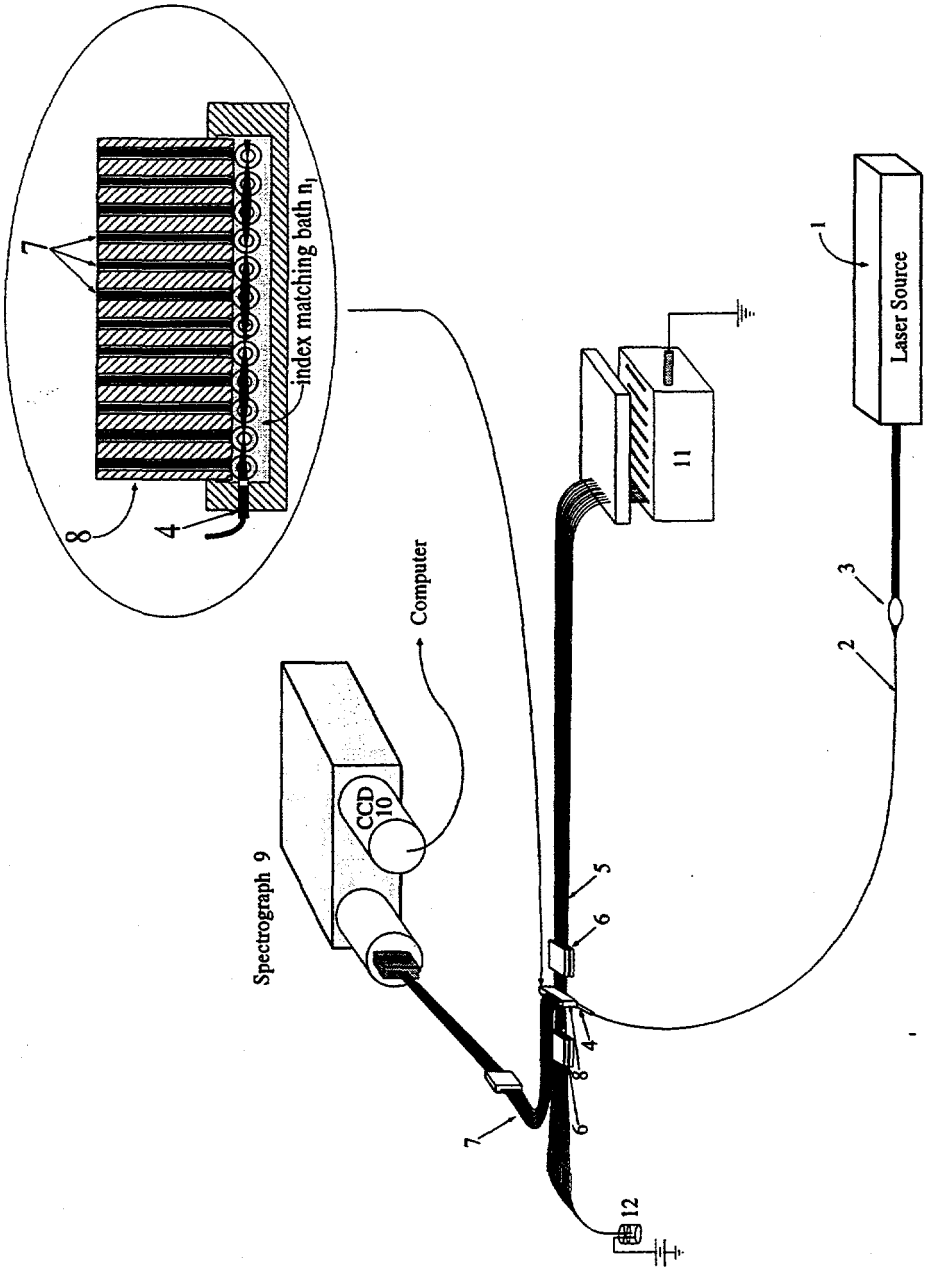

Figure 6: Schematic of a 12 channel capillary optical waveguide system: 1 - Argon-ion laser, 2 - singlemode optical fiber, 3 - launching optics, 4 - IFOT, 5 - capillaries, 6 - V-grooves, 7 - receiving optical fibers, 8 - V-grooves, 10 - CCD, 11, 12 - vessels for buffer solution.

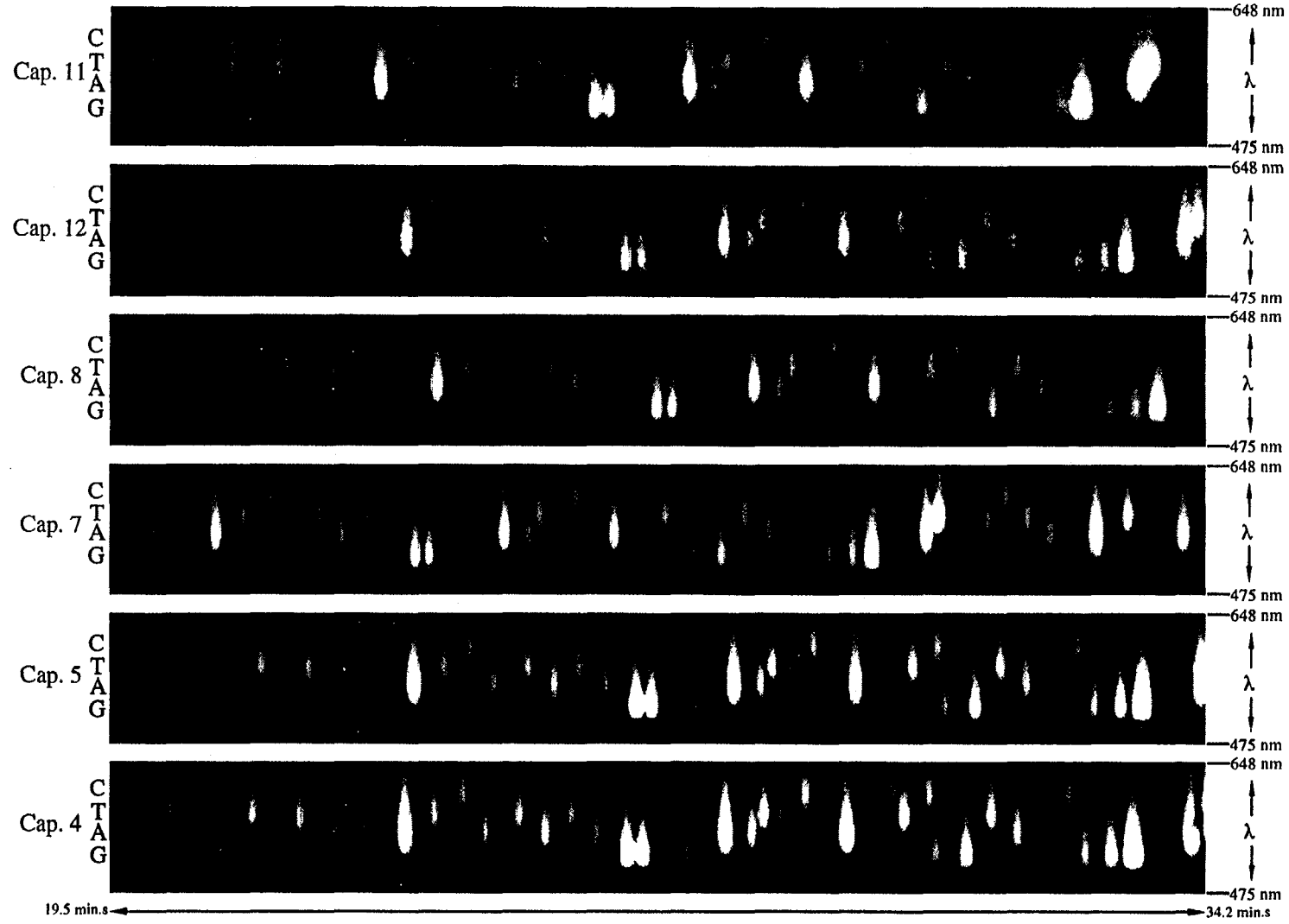

Figure 7A: Sequencing data

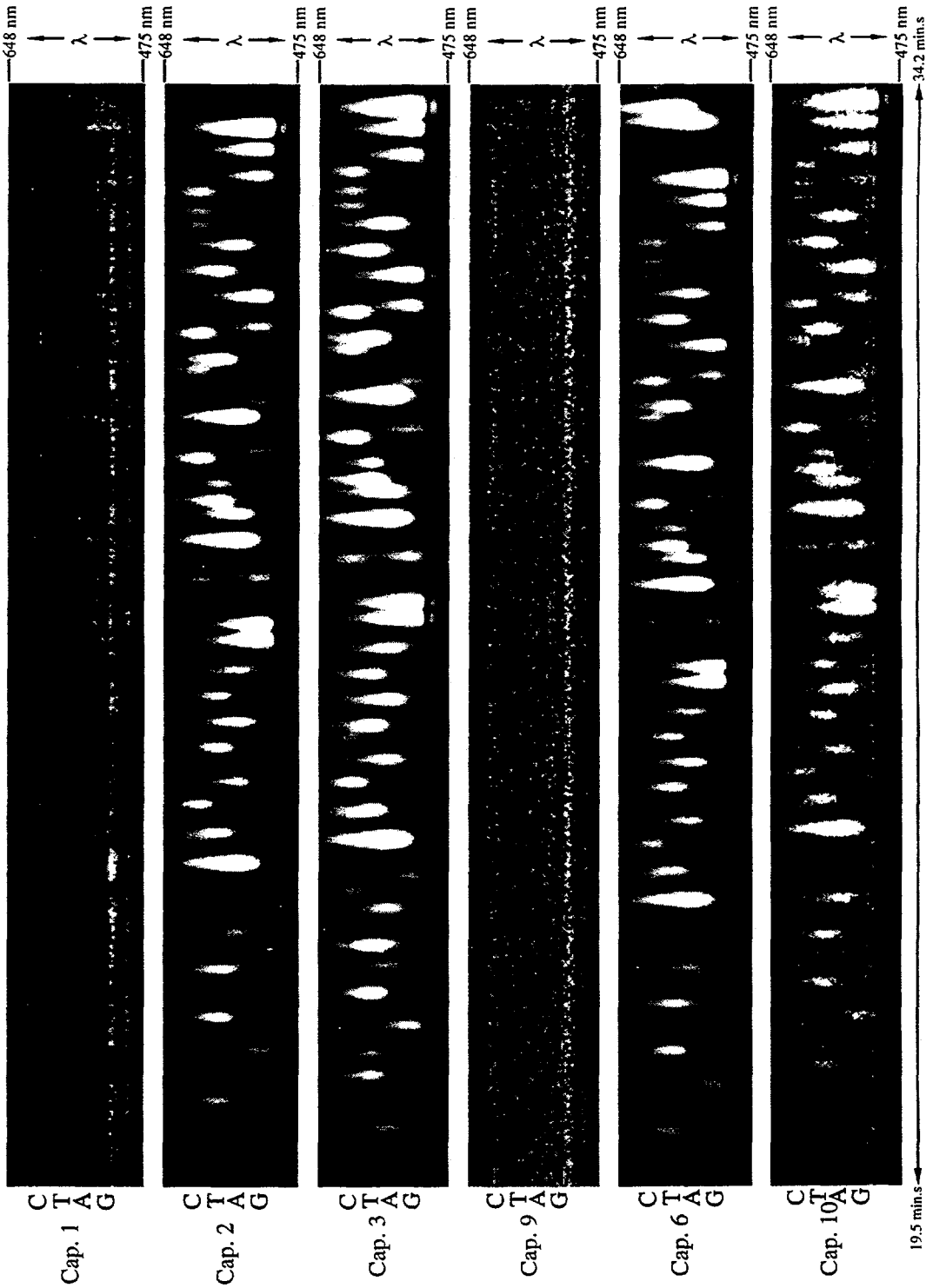

Figure 7B: Sequencing data

10^{-10} M dye flowing in capillary 6
- only water in adjacent capillaries

Only water in capillaries

Water flushed through capillary 6

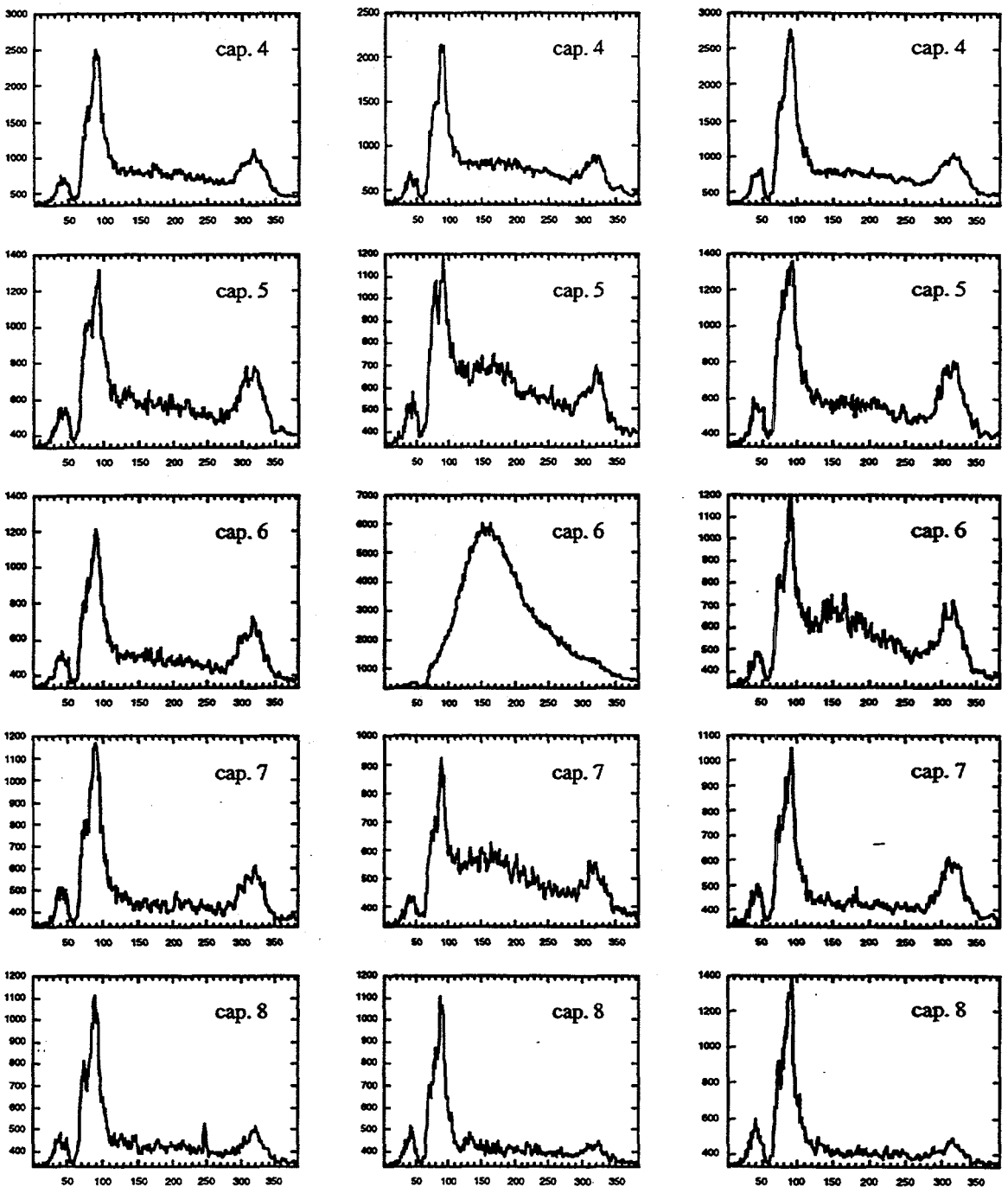

Figure 8: Measurements of cross-talk