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High-Resolution Separation and Accurate Size Determination in Pulsed-Field Gel Electrophoresis of DNA. 3. Effect of Electrical Field Shape†

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ABSTRACT: The resolution of pulsed-field gel electrophoresis is dramatically affected by the number and configuration of the electrodes used, because these alter the shape of the applied electrical fields. Here we present calculations and experiments on the effect of electrode position in one of the most commonly used pulsed-field gel electrophoresis configurations. The goal was to explore which aspects of the electrical field shape correlate with improved electrophoretic resolution. The most critical variable appears to be the angle between the alternate electrical fields. The most effective electrode configurations yield angles of more than 110°. A continually increasing angle between the fields produces band sharpening that greatly enhances the resolution.

In pulsed-field gel electrophoresis (PFG) DNA molecules moving in an agarose gel are forced to change their direction of migration, periodically, by alterations in the applied electrical field (Schwartz et al., 1983; Schwartz & Cantor, 1984; Carle & Olson, 1984). In the accompanying papers, we show that the pulse time and the electrical field strength can be adjusted to tune the size range of effective PFG resolution (Mathew et al., 1988a,b). It has been apparent from the very first PFG experiments that the shape of the electrical field also strongly influenced the separation pattern achieved by PFG (Schwartz & Cantor, 1984). In these early studies best results were obtained by using electrical fields that contained field gradients such as those that are generated when the positive and negative electrodes are of very different lengths (i.e., by using inhomogeneous fields). The effect of these field gradients in improving resolution was only partially understood. More recent studies have revealed that large DNAs can be resolved by PFG without field gradients when large angles between alternating applied fields are used (Carle et al., 1986; Cantor et al., 1986; Chu et al., 1986; Anand, 1986; Gemmill et al., 1987; Serwer, 1987; Southern et al., 1987).

In an attempt to optimize the quality of PFG separations and to provide a more rational basis for understanding the PFG phenomenon, we have calculated the electrical field shapes in some experimental electrode configurations that appear to provide generally excellent PFG results on a wide range of DNA sizes. Our goal was to identify the critical aspects of field shape responsible for high-resolution separations.

Materials and Methods

DNA Samples. Yeast chromosomal DNAs were prepared as described previously (Schwartz & Cantor, 1984), except that the DNA concentrations were typically 10 μg/mL. Details of the preparation of bacteriophage λ DNA concatemers are given elsewhere (Smith et al., 1986a,b; Mathew et al., 1988a). The sizes of all these DNAs are known (Mathew et al., 1988a).

PFG Electrophoresis. All of the results presented were obtained on horizontal submarine PFG apparatus using arrays of platinum point electrodes normal to the gel surface and connected through diodes to the power supply as described

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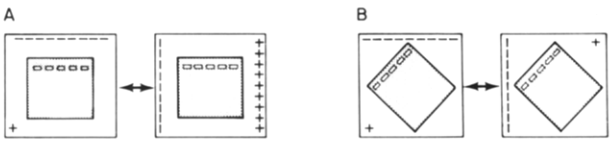

FIGURE 1: Schematic illustration of two general types of electrode configurations used in PFG. Each plus and minus sign indicates a single point cathode or anode, respectively. Shaded area is the agarose gel; rectangles indicate sample wells. (A) The two alternate electrode arrays used in the single inhomogeneous field mode; (B) The two alternate electrode arrays used in the double inhomogeneous field mode.

previously (Schwartz & Cantor, 1984; Smith et al., 1986a). In this way when individual electrodes are not energized, they cannot conduct in the plane of the gel, and so they cannot distort the electrical field produced by the other electrodes. Square apparatus ranging from 20 to 55 cm were used. The buffer was continuously circulated above and below the gel surface. Water from a thermostated bath was passed through heat-exchanging coils placed beneath the gel surface to maintain a constant temperature during the run. All gels were 20 cm square, 1% agarose, centered within the apparatus at a 45° angle to the sides except in the 20-cm apparatus, which just enclosed the gel. Running conditions were 1 × TBE buffer (100 mM Tris, 100 mM borate, and 0.2 mM EDTA), 15 °C, and 10-V applied potential/cm of box. The gel concentration and running conditions were chosen as a compromise between resolution and running speed (Mathew et al., 1988a).

One anomaly that has been noticed consistently with all PFG apparatus using point electrodes is the disappearance of the electrodes with extensive use. This is detected first as a thinning of the electrode wire, and it is particularly severe for the anodes. The use of thicker platinum wire or multiply wound electrodes slows the effect but does not eliminate it. Experiments have shown that neither the gel nor the DNA samples play any role. The effect is presumably due to platinum electrochemistry with components of the TBE buffer, possibly driven by the unusually high current densities employed at the electrode surfaces.

Calculations. Solutions to Laplace's equation with appropriate boundary conditions were calculated numerically by using a 19 by 19 grid of cells in Lotus 1-2-3, run on either an IBM PC or an HP 110. With typical boundary conditions, 400–500 iterations yielded solutions that showed less than 0.1% variation with an additional iteration. Field strengths, directions, and gradients were also computed by using Lotus 1-2-3 for point by point numerical differentiation.

Results and Discussion

Variation of PFG Electrode Geometries. Early PFG separations employed two different types of experimental geometries (Figure 1). In the single inhomogeneous mode, the electrical fields alternate parallel and perpendicular to a square agarose gel, with samples loaded near one edge of the gel in the conventional manner (Figure 1A). The north–south field is generated from a linear array of cathodes to a single corner anode. The east–west field is generated from two linear arrays of electrodes oriented as shown in Figure 1A. In the single inhomogeneous field configuration the overall geometry is asymmetric. The net direction of motion of the DNA molecules is determined by adjusting the relative applied voltages of the two alternate fields. In practice, a north–south (inhomogeneous) voltage about 3 times the east–west (homogeneous) voltage leads to molecular trajectories at roughly 45° to the edges of the gel, from upper left to lower right in Figure 1A.

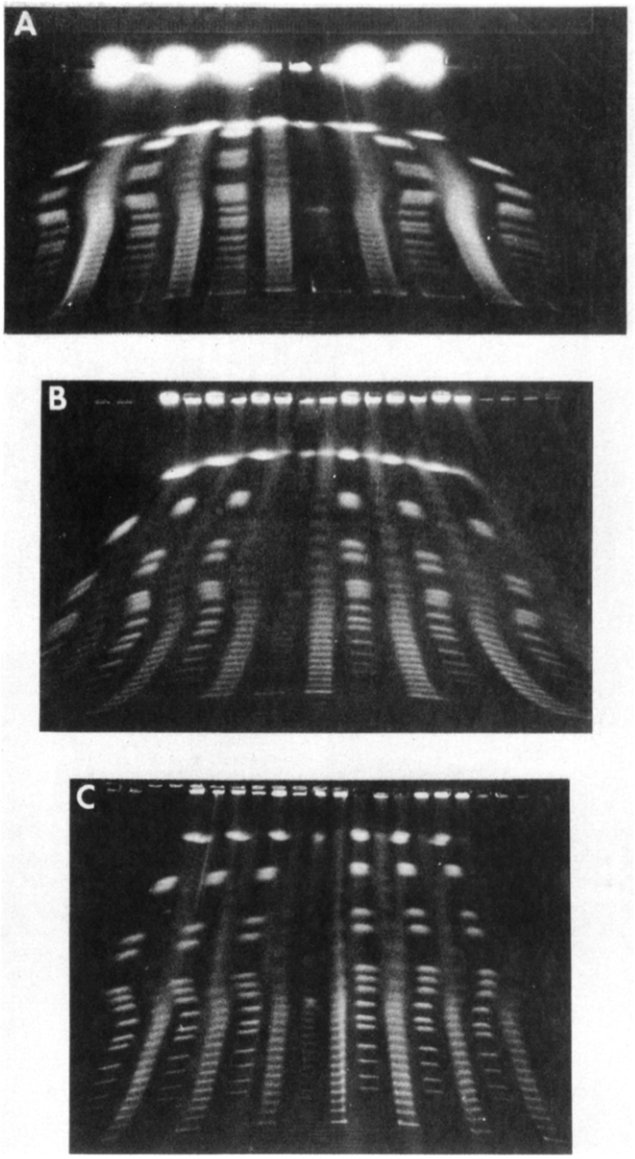

FIGURE 2: Effect of apparatus size on PFG performance. In all cases the samples alternate in adjacent lanes between *Saccharomyces cerevisiae*, strain CF14, chromosomal DNAs and λ cI_{857} DNA concatemers, except for the center lane which is λ vir DNA concatemers. The running conditions have been adjusted to provide near optimal resolution of a comparable DNA size range. All samples were run by using double inhomogeneous field configurations equivalent to those illustrated in Figure 4, in 1.2% agarose at 15 °C. (A) 20-cm apparatus using 200 V at a 120-s pulse time for 24 h; (B) 33-cm apparatus using 165 V at a 325-s pulse time for 170 h; (C) 55-cm apparatus using 500 V at a 120-s pulse time for 80 h.

In the single inhomogeneous configuration, the width of each sample band broadens only slightly as DNAs migrate. Thus, a large number of samples can be applied side by side. The resolution, particularly for samples nearest the diagonal, is quite good. However, the resolution is different in different lanes, and the actual mobilities of particular bands also vary nonlinearly. This makes it difficult to compare, quantitatively, data from different runs or even different samples in the same run.

A much more symmetrical pattern of separation is provided by the double inhomogeneous configuration, which also has been called orthogonal field alternating gel electrophoresis, or OFAGE (Carle & Olson, 1984). Here both north–south and east–west electrical fields are generated by a linear cathode array and a point anode. The two anodes may be at diagonally opposite corners of the square apparatus or may be shifted

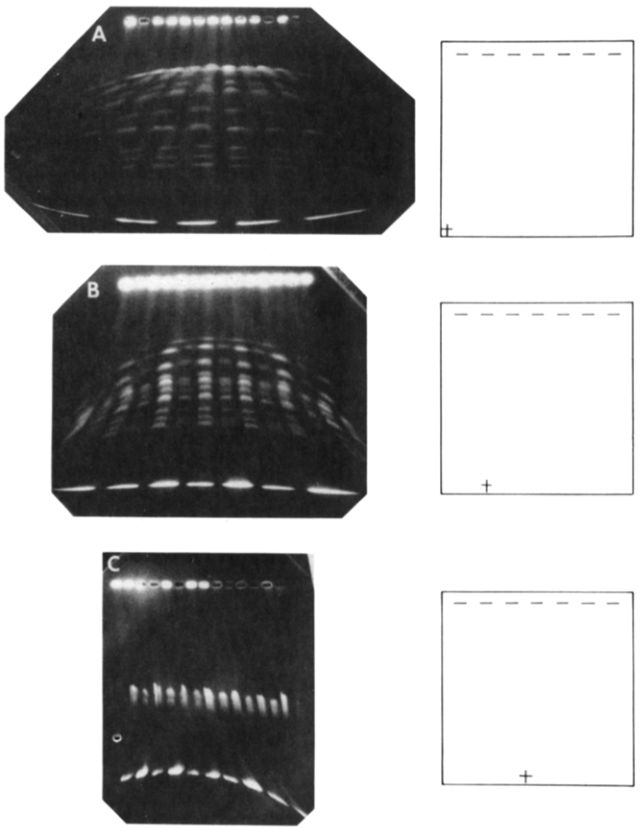

FIGURE 3: Effect of electrical field shape on PFG performance. The double inhomogeneous field configuration was used, with the electrodes placed as indicated. The samples in alternate lanes are *S. cerevisiae* chromosomal DNAs from strains D273 and DBY782, which range in size from 200 kb to 2.5 Mb. In all cases a 100-s pulse time at 300 V was used for 40 h in a 20-cm apparatus at 13 °C using 1% agarose.

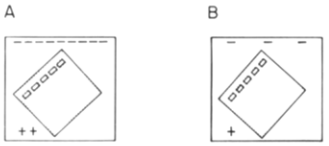

FIGURE 4: Two electrode configurations that provide consistently excellent double inhomogeneous PFG performance throughout a wide range of pulse times and electrical field strengths. (A) Double anode and a dense array of cathodes; (B) single anode with only three cathodes.

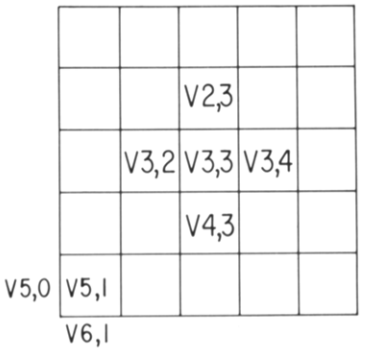

FIGURE 5: Schematic illustration of the array of cells used to obtain numerical solutions of Laplace's equations to yield the distribution of electrical potential (voltage difference) in different PFG apparatus. The outer layer establishes the boundary conditions, as shown here in the corner.

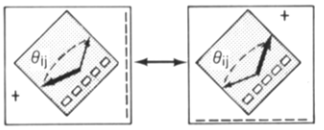

FIGURE 6: Definition of the angle between the alternate electrical fields, shown by the bold and light arrows, respectively.

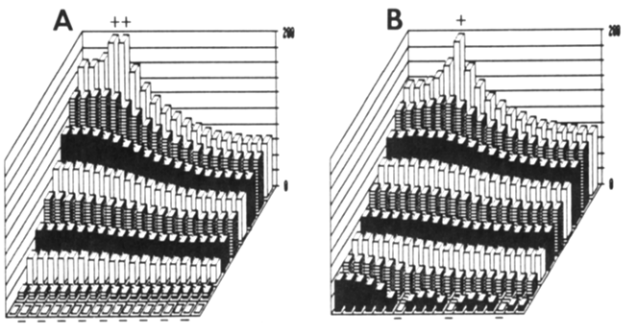

FIGURE 7: Calculated electrical potentials (A, B) for the electrode configurations illustrated in Figure 4, parts A and B, respectively, and indicated by plus and minus signs. The voltage range was set arbitrarily from 0 to 200 V, but the calculated results will scale linearly for any applied voltage difference.

away from these corners as shown in Figure 1B. However, their positions are always chosen so that the alternate fields result in a net reflection along the diagonal of the apparatus, from upper left to lower right in Figure 1B. Samples are loaded perpendicular to this diagonal and migrate in a net direction along the diagonal. In most apparatus, a square agarose running gel is placed at a 45° angle to maximize the usable area for separations.

In a typical successful double inhomogeneous configuration the resolution is quite good and is very similar in all the lanes. The overall migration of corresponding bands in adjacent lanes is nearly the same. Thus, samples appear to run almost in straight lines, and this greatly facilitates quantitative comparisons. The symmetry of the electrical field shapes means that the same applied voltage can be used for both alternate fields. It is common practice to photograph the gel along the diagonal, perpendicular to the initial row of samples, so that one has the appearance of straight bands such as obtained in conventional DNA electrophoresis. In typical double inhomogeneous configurations, the samples tend to spread laterally in a direction perpendicular to the net migration. This limits the number of samples that can be applied to the gel. In practice, the sample wells must be located far from the corners of the box to achieve the best separations. Samples also tend to stop migrating once they progress beyond a critical point in the gel. Thus, the fraction of the gel surface usable for separation is relatively limited.

Both of these difficulties can be surmounted by using larger PFG apparatus. Figure 2 shows the result of PFG separations in three different-sized apparatus, with the field geometry and nominal field strength held constant and pulse times adjusted to provide for comparable overall separation patterns. The larger apparatus clearly provides higher overall resolution. This is due, in part, to the longer distances accessible in the larger apparatus. Since there is no significant diffusion of large DNAs, the longer the running distance, the better the final resolution. However, we will show subsequently that a significant cause of the improved resolution in larger boxes is the shape of the electrical field in the regions of the apparatus containing the running gel.

The convenience and aesthetic appearance of the double inhomogeneous configuration have led to its use in most recent PFG experiments. However, the quality of the separations obtained is quite sensitive to the placement of the electrodes, particularly the point anodes (Figure 3). The best resolution

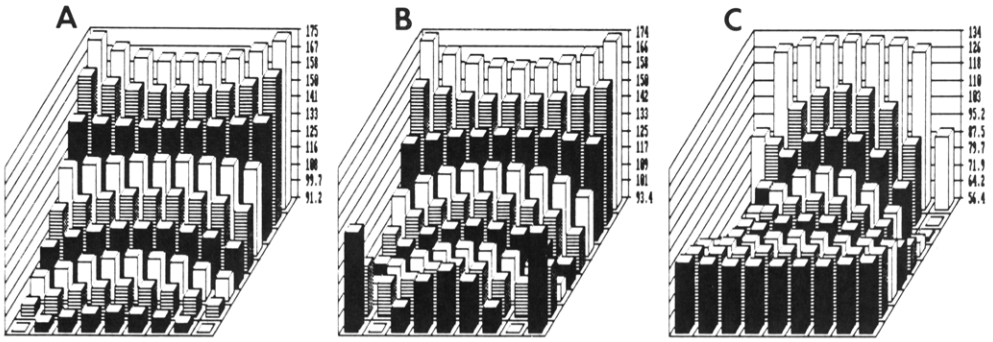

FIGURE 8: Calculated angle between the two alternate electrical field directions (A, B) for the two electrode configurations illustrated in Figure 4, parts A and B, respectively, and (C) for the configuration that generated the results illustrated in Figure 3C.

is generally obtained by placing the anode one-sixth to one-fourth of the way in from the corners of the box. The length of the linear cathode array is less critical, but resolution can often be improved by shortening this across from the anode, as shown in Figure 4B.

Two particular configurations that are generally reliable and are not overly sensitive to minor changes in electrode position are illustrated in Figure 4. One uses two nearby point anodes instead of one. This has the practical advantage that the current carried by each point anode is reduced, and the electrodes last longer (see Materials and Methods). In the other configuration, only three point cathodes are used. This causes slight distortion of the electrical fields near the electrodes, but it improves the overall separation pattern somewhat.

Calculated Field Shapes for PFG Experimental Geometries. To try to rationalize the effects described above and to determine the key features of electrical field shape critical for high-resolution separations, we have calculated the electrical fields present in a variety of different PFG box sizes and electrode configurations. The calculations involved solving Laplace's equation numerically as illustrated schematically in Figure 5. The square PFG experimental apparatus was modeled as an array of 19 × 19 cells. The outer layer of cells served to represent the plastic boundary of the gel box, while the remaining 17 × 17 array represented the agarose gel or the surrounding buffer. Since the conductivities of buffer and agarose are quite similar, the exact fraction of the box occupied by agarose should be irrelevant.

Electrodes were represented by keeping the potential of the corresponding cell constant. The effect of nonconducting plastic walls was accounted for by setting the potential of each cell in the outer layer to be the same as the potential of the cell immediately interior. For the example shown in Figure 5, we would set

$$V_{5,0} = V_{5,1} = V_{6,1} \tag{1}$$

where V_{ij} is the potential in the ijth cell. In this way there will be no potential change as one moves into the plastic and thus no electrical field component normal to the boundaries of the conducting region.

The potential in each cell was computed as the average of the four neighboring cells. For the example shown in Figure 5 the potential is

$$V_{3,3} = \tfrac{1}{4}(V_{3,2} + V_{2,3} + V_{3,4} + V_{4,3}) \tag{2}$$

Once a sufficient number of iterations produced stable solutions to Laplace's equation, the electrical fields and field gradients were calculated by the average point differences between adjacent pairs of cells. Thus, the horizontal and vertical field components are given by

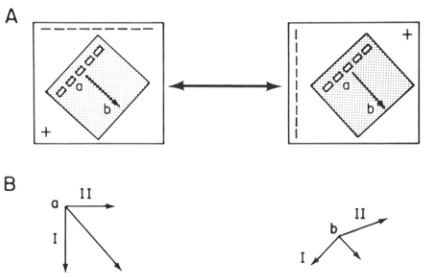

FIGURE 9: Schematic illustration of how an angle gradient leads to a net electrical field gradient along the diagonal of the gel. (A) The two alternate electrode configurations; (B) electrical fields generated by each configuration at points a and b and the resultant net field along the diagonal.

$$E_{\mathrm{h}i,j} = \tfrac{1}{2}(V_{i,j+1} - V_{i,j-1}) \tag{3A}$$

$$E_{\mathrm{v}i,j} = \tfrac{1}{2}(V_{i+1,j} - V_{i-1,j}) \tag{3B}$$

This was computed separately for each of the two alternate electrical fields, and then the angle between the fields could be computed simply as

$$\theta_{i,j} = \arctan\,(E_{\mathrm{v}i,j}/E_{\mathrm{h}i,j}) - \arctan\,(E'_{\mathrm{v}i,j}/E'_{\mathrm{h}i,j}) \tag{4}$$

where E represents a field component during one phase of the alternating cycle and E' represents that component during the other phase. The physical meaning of the angle $\theta_{i,j}$ is shown schematically in Figure 6.

We also computed the net field parallel to the overall migration direction (along the diagonal of the gel) and perpendicular to this direction. These fields are the average of the two alternate applied fields. In addition, another parameter that was examined was the field gradient. In two dimensions this is a 2 by 2 tensor with components like $\mathrm{d}Ex/\mathrm{d}y$. However, we found it convenient to work with just two averaged components. We calculated the average field gradient parallel and perpendicular to the net direction of motion along the diagonal of the gel. These averages provide the net gradient generated by the two alternate fields. Such calculations were performed for numerous experimental geometries.

The electrical potential computed for the two electrode configurations of Figure 4 is shown in Figure 7. It can be seen that, except in the immediate area of an electrode, there is a smooth variation of potential with distance. It is difficult to display the actual electrical field shapes that result from such potential surfaces. However, the field will be proportional to the slope of the potential and oriented along the direction of maximum slope at each point. With this in mind one can gain a fairly good impression of the field shapes and directions by careful inspection of Figure 7. It is clear that, in most regions of the gel, the field tends to point toward the positive

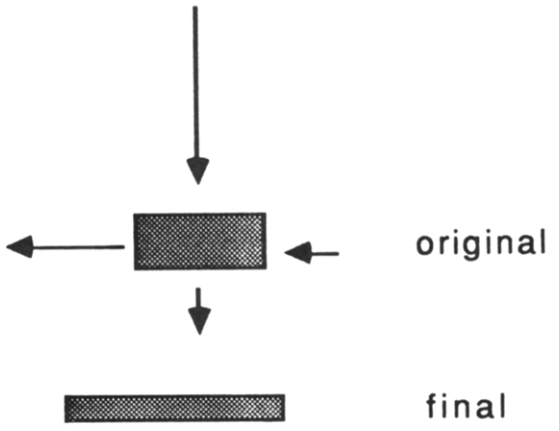

FIGURE 10: Proposed mechanism for band sharpening and stretching produced by electrical field gradients. The original bandshape is that seen near the origin of the gel, while the final shape is that after extensive electrophoresis. The arrows show the average electrophoretic mobility of DNA at the four edges of the band. The differences are highly exaggerated.

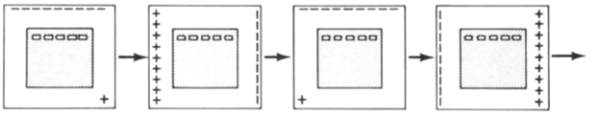

FIGURE 11: Electrode configurations used, successively, in a four-pulse PFG experiment.

electrode. In one case, the computed results for the potential were compared with direct measurements made by placing a probe electrode at various positions in the gel. The results were in excellent agreement with the calculations and gave us confidence that we could compare calculations with PFG performance to assess which parameters of the electrical fields were predominantly responsible for high-resolution separations.

The most critical shape characteristic is apparently the angle between the two alternate fields. Figure 8 illustrates this angle for three experimental geometries. Two correspond to the electrode configurations shown in Figure 4, experimental geometries that give near optimal PFG performance. The third has point anodes located at the middle of the sides of the box, a configuration that produces extremely poor resolution like the example in Figure 3. (To aid the reader, what is shown in Figure 8 is not the entire gel box but just the square agarose running surface aligned at 45° to the edge of the box.)

Inspection of Figure 8 reveals two major differences between field characteristics that give good performance and those that give poor performance. The angle between the alternate fields is always greater than 90° where good resolution is observed. In cases where there is excellent resolution, field angles typically range from 110 to 150°. In contrast, for the example where poor resolution was seen, the field angles range from 45 to 110°. One possible explanation is that when the alternate fields differ in direction insufficiently, the DNA molecules cease reorienting in response to each field switch and simply align along the resultant of the two separate fields. This then leads to what is effectively normal electrophoresis in a constant field, and since reorientation plays no significant role, no size separations occur. An alternative explanation is provided by the switchback model, where angles greater than 90° are required in order to ensure that the leading and trailing ends of the DNA strand reverse with each field alternation (Southern et al., 1987).

A second feature of fields that provide good separations is that the angle between the alternate fields keeps increasing as one moves along the diagonal in the direction of net molecular motion. The effect of this is a continuous decrease in the net field strength along the diagonal, as shown schematically in Figure 9. The resulting field gradient means that for any DNA band of finite width the front will move slower than the back, and so every band will self-sharpen. One unfortunate consequence of the electrical field shapes that produce an angle gradient is that they also tend to produce a field gradient perpendicular to the diagonal. This causes each sample to thin out along the perpendicular to the diagonal, as shown schematically in Figure 10. This explains the band spreading seen in almost all double inhomogeneous field configurations.

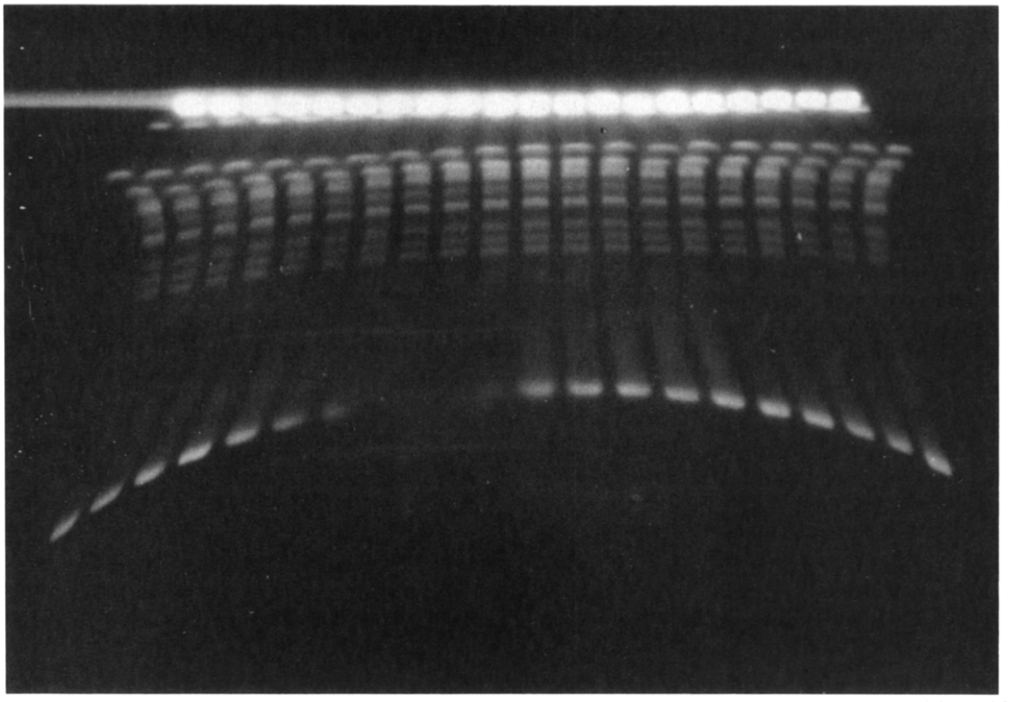

FIGURE 12: Separation of *S. cerevisiae* chromosomal DNAs using the four-pulse sequence shown in Figure 11 with a 120-s pulse time for 42 h.

The calculations illustrated in Figures 7 and 8 are valid for any size PFG apparatus. With a constant 20-cm gel, the effect of increasing the size of the apparatus is to shrink the applicable area of the grid of calculated data from the entire array for a 33-cm box to less than the central half for a 55-cm box. Inspection of the figures shows that the result of this will be mainly to provide more gradually changing fields and more smoothly increasing angles as the box size increases. In particular, the calculations show that loading the samples too close to the edge of the gel produces bad performance because the field angles there are too small and the angle gradient can even be increasing rather than decreasing, which will lead to band broadening. These characteristics, plus the larger separation length described earlier, presumably account for the improved performance of larger apparatus.

Near the center of the gel surface depicted in Figure 8, there is essentially little or no variation in angle for short distances along the diagonal. Thus, there is only a negligible net field gradient parallel to the direction of DNA motion. In spite of this, excellent PFG separations are observed in this region. This implies that the main role played by the inhomogeneous electrical fields used in typical PFG apparatus is to create angles of greater than 90°. The use of point anodes, appropriately situated, ensures this. Note that while the electrodes are nominally orthogonal in the double inhomogeneous PFG configuration, the resulting electrical fields are not orthogonal. Hence, the term OFAGE for this apparatus is a misnomer and is best avoided.

The angle gradients generated in the electrode configurations illustrated in Figure 4 serve to sharpen DNA bands. This is desirable and improves PFG resolution. However, it seems clear that the molecular weight dependence of DNA mobility in PFG electrophoresis will depend little, if at all, on whether a field gradient is present. This can be confirmed by performing PFG in apparatus where a constant angle of greater than 90° is maintained in the absence of an angle gradient. Several types of such apparatus have been described including voltage ramp (Cantor et al., 1986), voltage clamp (Chu et al., 1986), and various rotating gel or electrode configurations (Serwer, 1987; Gemmill et al., 1987; Southern et al., 1987). These give excellent size separations and show the same pulse-time dependence of the molecular weight separation range as conventional PFG. However, in our experience, the DNA bands are broader without the angle gradients, although the samples migrate in straight lanes.

The insight gained by comparing field calculations with experimental results explains why a number of well-intended attempts to improve the design of PFG apparatus did not succeed. For example, Figure 11 shows a scheme for using a program of four different fields instead of two. The idea was to alternate between single inhomogeneous mode, with DNA moving first to the left and then to the right so that the overall motion would be vertical. In principle, this would give straight bands and allow a large number of samples to be run simultaneously on an ordinary square gel. Figure 12 shows a typical gel run using the four field program. It is apparent that the bands indeed run rather straight, but the overall resolution is modest. Only 8 or 9 bands of yeast chromosomal DNA were resolved in a 36-cm apparatus, whereas 14 bands could be seen from one of the same strains in the standard double inhomogeneous mode in the same apparatus. The reason behind this poor performance is evident when one computes the angles between sets of adjacent pulses. While some angles are large, as in typical single inhomogeneous mode, others are of less than 90°.

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