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Numerical measures of two-dimensional gel resolution and positional reproducibility

Procedures to measure two important properties of two-dimensional electrophoresis systems have been developed. These procedures evaluate the overall resolution and positional reproducibility of two-dimensional gel patterns. Using these measures we show that various state of the art gel systems can produce patterns exhibiting resolution measures of 15 000–50 000 (yielding practical upper bounds of about 2000–6000 detectable proteins in typical cellular samples) and that positional accuracy for most spots in gel-to-gel comparisons is better than a spot width (0.5–2.0 mm). Implemented as part of a computerized image analysis system for two-dimensional gel data, these two measures can be used in the selection and optimization of gel systems, and in the selection of sets of well-resolved, well-matched spots for genetic and other quantitative sudies.

1 Introduction

Two-dimensional (2-D) gel electrophoresis (in this case isoelectric focusing in 9 M urea, 2 % NP-40 followed by electrophoresis in sodium dodecyl sulfate [1–3]) yields the highest resolution of any technique presently available for the separation of proteins, allowing thousands of polypeptides to be resolved under favorable circumstances. During the evolution of the technique, in numerous laboratories and for a variety of applications, many attempts have been made to optimize resolution and reproducibility. The ISO-DALT system described earlier [2–5] allows sets of 10, 20, or 40 analyses to be performed in parallel, yielding patterns on which more than a thousand proteins can be counted and which can be intercompared with confidence. With the advent of computer based systems for quantitating and comparing such two-dimensional patterns [6–17], it has become possible to examine objectively the properties of sets of patterns with an eye to better optimization and improved data reliability. In this paper we present methods by which two important properties of 2-D gels systems can be measured, the overall resolution of the 2-D gel patterns produced and the positional reproducibility among patterns. Resolution is an important factor in determining how many gene products can be detected (under ideal conditions) and positional reproducibility is the key to high identification accuracy.

2 Methods and materials

Two-dimensional gel electrophoresis was carried out using the 7″ × 7″, 8″ × 10″, or 12″ × 12″ ISO-DALT systems [2–5]. Isoelectric focusing gels contained 90 % 3.5–10 + 10 % 2.5–4 Ampholines (LKB) and second dimension slab gels were 10–20 % T gradients. Total cellular protein of human monocytes, lymphoblastoid cells (GM607), lymphocytes, or human fibroblasts (GM1386) were labeled with ^{35}S-methionine and the cells were solubilized directly in 9 M urea, 2 % NP-40, 2 % Ampholines, 1 % mercaptoethanol. Gels used in the positional reproducibility study were run using aliquots of a single pooled sample, with first and second dimension separations each performed as single self-consistent batches (*i. e.*, simultaneous separation in the same device). Proteins were visualized by autoradiography using Kodak XAR-2 film.

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Abbreviations: 2-D: Two-dimensional; FWHM: Full width at half maximum; TYCHO: Software analysis system

3 Computer methods and results

The problems of gel resolution and spot positional reproducibility will be considered separately below. Refer to Table 1 for definitions and formulae not given in the text.

3.1 Measurement of gel resolution

The resolution measure described here is intended to quantify the ability of a 2-D system to separate protein gene products. It is not intended to show how many spots are actually on a gel but rather to indicate the ability of the gel to separate different spots under ideal conditions. The resolution measure is designed to be easy to calculate, easy to understand, and to correspond to our intuitive feelings about resolution. It is also reasonably insensitive to the amount of sample loaded, and involves no assumptions about the physical chemistry of the separation system.

Perhaps the most suitable measure would be one in which the area of the gel is divided by the "size" of a typical spot. An appropriate and simple definition of spot size is A_i as defined in Table 1. This definition of size is independent of the overall density of a spot (provided the gel is not greatly overloaded or, in the case of autoradiographs, the film is not blatantly overexposed). Also defined is $A(x, y)$, which is an estimate of the size of a typical spot in a region around (x, y). It is well known that spot size varies systematically with the apparent molecular weight. Fig. 1 shows portions of a typical gel and illustrates the differences between spots at the top (higher molecular weights) and bottom portions of a typical gel. Fig. 2 illustrates the variation of the measured size as a function of both x and y for another typical pattern. In general, spot size depends negligibly on the position of the spot along the x-axis (isoelectric point), but varies by a factor of three or more from the top to the bottom of the gel (along the sodium dodecyl sulfate dimension). The spots' full width at half maximum (FWHM) measurements change from about 0.5 to 2.0 mm (from top to bottom) on gel patterns produced using the ISO-DALT system and analyzed for spot size and shape using the gaussian fitting approach of the TYCHO computer analysis

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Table 1. Definition of variables

(x_i, y_i)	The position coordinates of spot i measured in 100 μm pixel units. The x-axis is along the direction of increasing pH, and the y-axis is along the direction of decreasing apparent molecular weight (from the top to the bottom of the pattern). A superscript is added when it is necessary to distinguish between a master (m) or object (o) pattern.
(u_i, v_i)	The full widths at half maximum (FWHM) for spot i along the x and y axes. A superscript o or m may also be used to distinguish between a master or object pattern.
A_i	The "size" of spot i, defined to be $A_i = u_i \cdot v_i$
$A(x, y)$	An estimate of spot size in the region around (x, y).
d_i	The misregistration distance for spot i, defined as follows: $d_i = [(x_i^o - x_i^m)^2 + (y_i^o - y_i^m)^2]^{1/2}$
$\hat{d}_i$	The normalized misregistration distance for spot i, defined as follows: $\hat{d}_i = \left\{ \left[\frac{(x_i^o - x_i^m)}{(u_i^o + u_i^m)/2} \right]^2 + \left[\frac{(y_i^o - y_i^m)}{(v_i^o + v_i^m)/2} \right]^2 \right\}^{1/2}$
$\hat{d}_i^x$	The normalized misregistration distance along the x-axis, defined as follows: $\hat{d}_i^x = \frac{(x_i^o - x_i^m)}{(u_i^o + u_i^m)/2}$
$\hat{d}_i^y$	The normalized misregistration distance along the y-axis, defined as follows: $\hat{d}_i^y = \frac{(y_i^o - y_i^m)}{(v_i^o + v_i^m)/2}$

system [6, 7]. The effect of the variation in spot size is taken into account by defining the resolution measure as an integral over the useful area of the gel:

$$\Phi = \int\int (1/A(x,y))\, dx\, dy \quad (1)$$

The above definition is further simplified by fitting a straight line to the size *vs.* y data such that $A(x, y)$ is replaced by

$$A(x,y) = a + b \times y \quad (2)$$

Spots so small in amplitude that their size is likely to be ill-defined are excluded from the calculation. Spots whose maxima are beyond the range of the scanner are also excluded. A linear model was chosen, as the point scatter from the observed samples was enough to show that using higher order terms would be meaningless. Eq. (1) then becomes

$$\Phi = \int_{x_L}^{x_H} \int_{y_L}^{y_H} \frac{1}{a + b \times y}\, dx\, dy, \quad (3)$$

where (x_L, y_L) is the upper left corner of the useful area of the gel and (x_H, y_H) is the lower right corner.

This integral can be evaluated to yield

$$\Phi = \frac{(x_H - x_L)}{b} \ln \frac{a + b \times y_H}{a + b \times y_L} \quad (4)$$

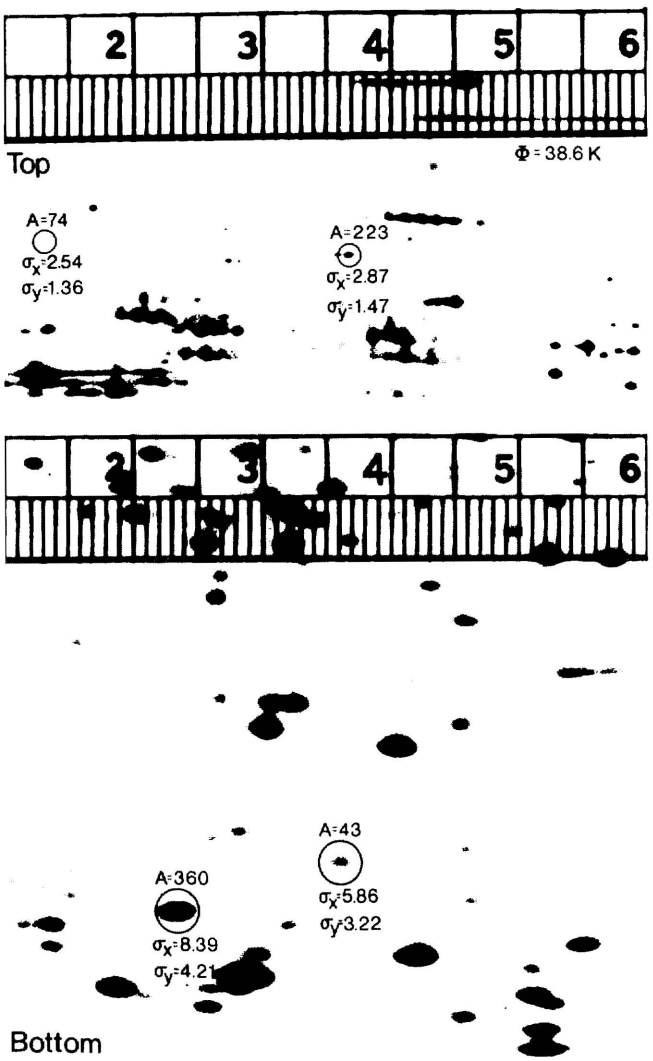

Figure 1. Sections from the top and bottom of an autoradiograph of a 2-D gel separation of proteins from a sample consisting of human monocytes. A centimeter scale is included for reference purposes. The symbols, A, σ_x, and σ_y are the parameters of the gaussian models for the indicated spots. A is the amplitude and σ_x and σ_y are the full widths at half maximum divided by 2.35 along the x and y axes, respectively. Dense spots (with high amplitudes) are slightly larger than light spots in the same gel region, but the variation is much smaller than that between top and bottom.

In the present paper, the widths and hence the sizes are evaluated from the list of spot parameters routinely calculated in the analysis of 2-D patterns by the Tycho I gel analysis system. Depending on the particular features of the separation, and especially on the gel size, values of Φ ranging from 15 000 to 35 000 are obtained routinely. In other analysis systems, the resolution could be approximated by scanning a few typical spots at the top and the bottom of the gel and estimating the slope parameter b. Eq. (4) could then be used without having to analyze the gel completely. Choosing a non-linear model for the size distribution would have precluded such estimates of Φ without a full analysis of the pattern.

It is of interest to visualize the appearance of a gel pattern with differing values of the resolution measure. Fig. 3 shows a panel

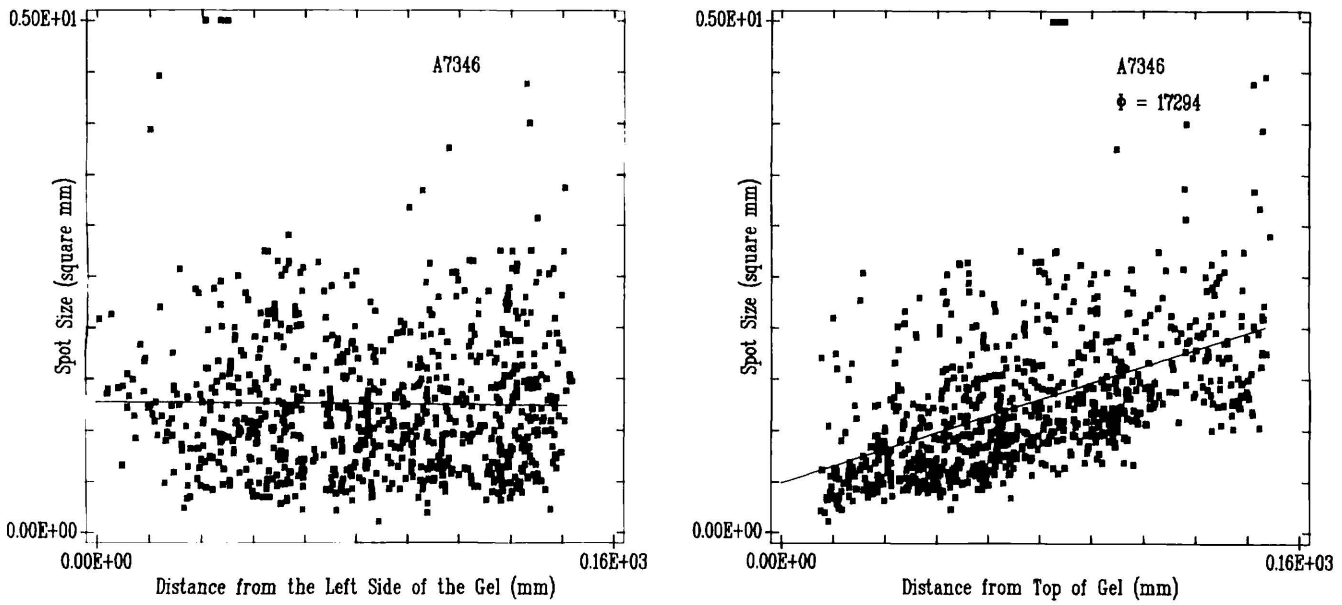

Figure 2. The size of each spot plotted as a function of its x and y position in the pattern for a typical gel (A7346), in this case from peripheral human leukocytes. The line in each frame represents a linear least-squares fit to the data.

of four views of the same pattern with various resolutions. Each picture was computed from the same gaussian model which was obtained by analyzing an autoradiograph of a gel of a lymphoblastoid cell line (GM607). In all but the upper left frame, the pattern was degraded by multiplying the x and y widths of each spot by the degradation factor shown in each panel. For each degraded pattern, the spot amplitudes were divided by one half of the degradation factor in order that the intense spots should not overwhelm the lighter ones and obscure the results. Comparison of the patterns with actual 2-D gel pictures published in a variety of journals indicates that different experimental procedures in general use produce gels differing in resolution by at least a factor of 10.

3.2 Positional reproducibility

Spots on a two-dimensional gel pattern are typically associated with spots on another pattern according to their position on the gel. Both the experimenter doing manual comparisons and the computer doing automated and semi-automated comparisons perform preliminary registration operations involving translation and rotation as well as global and/or local stretching. Direct comparison of uncompensated patterns will almost always show large "errors" [18], which are generally removable with suitable stretching algorithms. Thus, it is not required that gels be perfectly superimposible in order that spots from one pattern can be correctly associated with spots from another. Knowledge of the relative positional reproducibility of the spots on a set of gels is important in order to assess how reliably the spots are identified. Because of the elastic distortions associated with acrylamide gels, the positional reproducibility must be measured after the registration algorithms have been applied. In this sense, the reproducibility measurements must consider the gel-running system and the computer analysis routines as a combination, and must use internal consistency to assess accuracy of spot identification.

The purpose of this section is to explain the protocol by which reproducibility is assessed and to discuss the information which is obtained. We are interested in knowing how well registered a set of patterns is in reference to an experiment master pattern after the stretching algorithms have been applied. In routine operation, each pattern in the set is processed by the computer so that it is generally in registration with the experiment master pattern [6, 8], and identifications (matches) are made during and after the stretching process based primarily on proximity. The (x, y) position of each spot on a pattern is then compared with the position of the corresponding spot on the experiment master pattern (provided a match exists). Statistics are compiled on the distance between these two positions, for all matched spots. Statistics are also produced for the entire set of gels under study.

For the present study, we have compared a set of gels produced using aliquots of a single sample (to remove any inter-sample differences) and have modified the typical procedure described above in one important respect. The existence of a match (associating a spot on an experimental gel with one on the master gel pattern) has an influence on the final position of the spot in the stretched object pattern, since the existence of the match influences the iterative stretching procedure to bring the spot even closer into registration. Because of this, the final statistics appear better than they really should. A "jackknife" procedure is adopted to avoid this defect. A preliminary registration and identification pass is made on each pattern of the set to establish a set of matches with spots in the experiment master pattern. A special version of the master pattern is then created by randomly omitting a few spots. The corresponding spots in each object pattern are thus "floating" and should not be matched to anything in the experiment master pattern. Each object pattern is then stretched into registration with the modified master pattern (using the algorithms described in [8]) but compared with the unmodified master pattern. Statistics are recorded for only those spots in the object patterns that were originally matched to an omitted spot in the experiment master system. Thus the positional accuracy measurements are not improved by the presence of a match for the spot that is being measured. It may happen, of course, that a spot finds a new match when its "true" match is omitted from

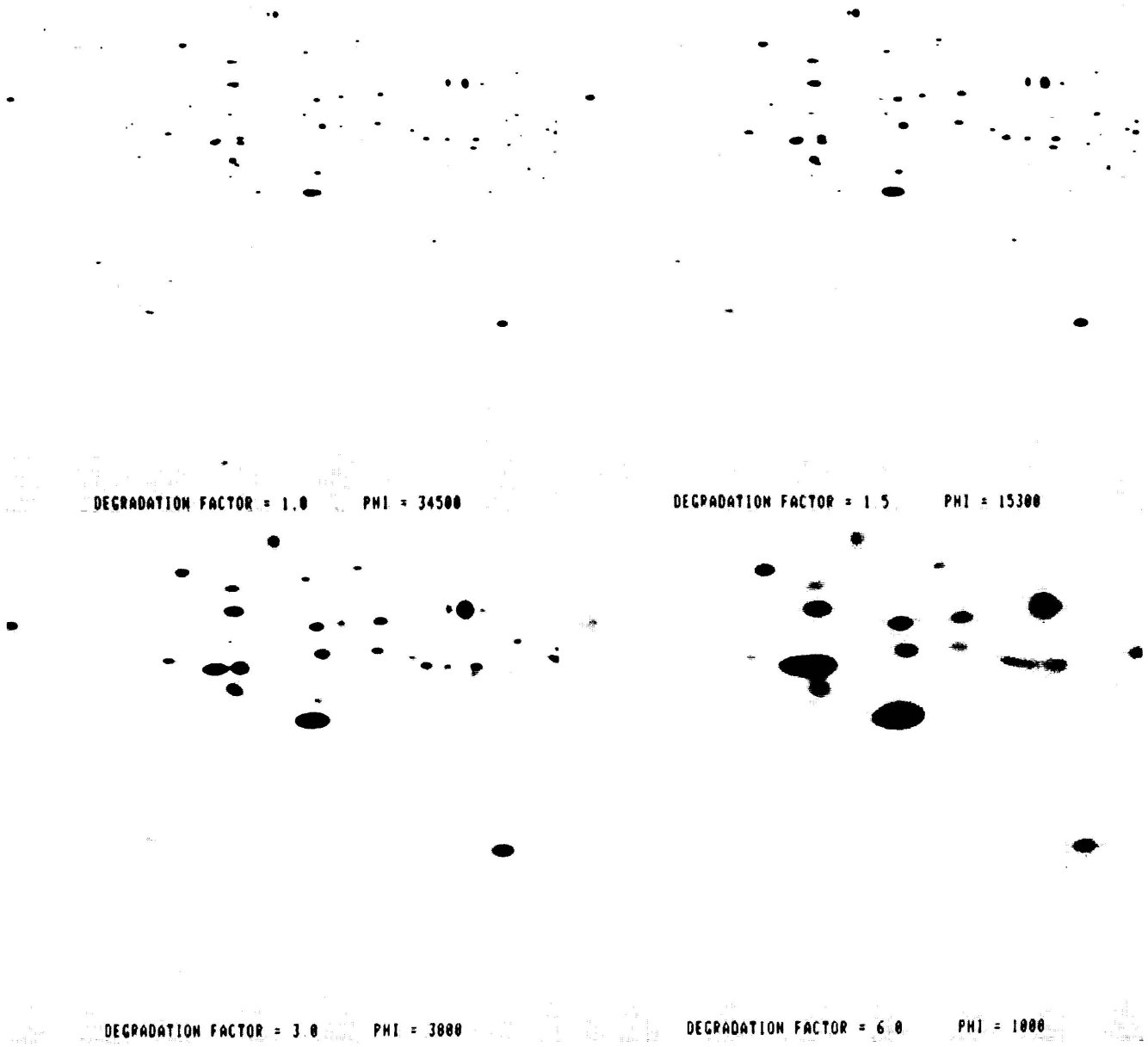

Figure 3. The effect of pattern degradation on the resolution measurement Φ. The upper left frame shows the undegraded pattern (the result of an analysis of an 8″ × 10″ gel of GM607 lymphoblastoid cells). The other three frames are degraded by multiplying the spot widths by the degradation factor displayed in each frame. Resulting resolutions calculated for each degraded image are also shown.

the master system. The presence of such incorrect matches may influence the registration in a minor way, but it is not likely that they will improve the measured accuracy. In any case, matching is part of the registration process, and the influence of wrong matches is part of the measurement. Typically 10 % of the spots are randomly selected for omission from the experiment master pattern and the process is repeated in such a way that each spot is omitted once and only once, *i. e.*, the calculation is performed ten times.

The identification numbers of spots in the object system that switched identifications (*i. e.*, became incorrect) when their counterparts in the master system were omitted are recorded for further inspection. This information is valuable for the selection of protein sets for genetic screening purposes, as these are the spots which could be misidentified if their counterpart in another gel were not present.

Four statistics are recorded for each match of a spot in the master pattern with its counterpart in an object pattern. These are the misregistration distance d_i, the normalized misregistration distance $\hat{d}_i$, and the normalized x and y misregistration distances, $\hat{d}_i^x$ and $\hat{d}_i^y$ (see Table 1).

The results of each individual analysis can be summarized and presented in a variety of ways. The average, standard deviation, maximum, and the 90 and 95 % points of the cumulative distribution of each of the above variables are printed to provide a quick overall check which is often sufficient when the patterns are in close registration. Values of these statistics exceeding certain predetermined thresholds may indicate that serious registration problems are present and that further analysis is prudent. Histograms of these variables can be plotted, if desired. However, one of the most revealing presentations is made by constructing a "registration accuracy pat-

tern" where the x and y widths in the 2-D gaussian models of the object pattern spots are replaced by the x and y misregistration errors, respectively. For visual purposes, the values of the x and y widths are not allowed to fall below a predetermined minimum value. The resulting pattern can be visually inspected for regions of imprecise registration and possible problems; large spots in such a display are spots with large registration errors. After the registration of each of the object patterns has been assessed, a separate program is run to summarize the statistics and histograms for the entire set of object patterns.

3.3 Reproducibility assessment examples

A set of sixteen autoradiographs (identical samples, gels from a single electrophoresis run) was analyzed to demonstrate the information that can be obtained from the procedure described above. The patterns were digitized using an Eikonix 785 scanner and were processed as described previously [6] to produce 2-D gaussian models. One pattern (A12621, Fig. 4) was selected to serve as an experiment master pattern to which the other fifteen patterns, called object patterns, would be compared. Fig. 5 shows the results of this analysis for three typical object patterns. The top row shows the processed images, the middle row the images calculated from the 2-D gaussian models, and the bottom row shows the registration accuracy patterns. Fig. 6 is a set of histograms for a typical individual pattern (A12639). Frames A through D are the histograms for the misregistration distance, the normalized misregistration distance, and the normalized misregistration distances for x and for y. The normalized misregistration, in which the misregistration distance for each spot is divided by the average width of the spot, gives a measure of error in "spot width" units. The annotation at the top of each histogram marks the average, 90 %, and 95 % points in the cumulative distribution function.

Figure 4. The pattern (A12621) used as the master or reference in the registration assessment example. The sample consisted of proteins labeled with [^{35}S]methionine from fibroblast line GM1386.

Two conclusions can be drawn from Fig. 5 and 6. Generally, the stretching algorithms used yield patterns closely registered to the master, with misregistration distances of the order of a fraction of a spot diameter. The errors of highest positional uncertainty occur in the sparsely populated regions near the edges of the pattern and along a vertical band near the basic (right) side of the pattern. The vertical band in the error pattern coincides with a band of imperfectly focused spots seen in the original pattern and is probably due to cathodic drift instability of the basic end of the pH gradient under the focusing conditions used.

Comparisons of the other fourteen object patterns with the master pattern gave similar results. Table 2 shows the average, 90, and 95 % points for the cumulative distributions of the normalized misregistration distance for each of the fifteen object patterns. The variation in the number of spots in each gel is primarily due to the fact that many spots were faint and were not detected in all of the patterns. All of the patterns show average normalized distances considerably less than one-half spot width and only three patterns have more than 5 % of the spots with errors exceeding one spot width. A set of misregistration histograms summarizing the entire set of fifteen object patterns is shown in Fig. 7, and an average registration accuracy pattern is shown in Fig. 8. The latter pattern allows visual inspection of misregistration errors for all the spots, and forms the basis for selection of "well-behaved" proteins.

4 Discussion

The simple resolution measure used here is both intuitive (basically a ratio of areas) and easy to calculate. It essentially measures how many spots of the size actually obtained could be packed into the pattern and still be resolved. While the result is a "hypothetical" resolution in the sense that no available real sample could produce such a number of spots, it is an accurate and important measure of the ability of the gel system to separate polypeptides. Since real protein mixtures do not exhibit uniform distributions of spots across the entire pattern, the actual number of resolvable polypeptides is less than the resolution measure indicates. As a rule of thumb (derived from experience with the 7″ × 7″ ISO-DALT system), we believe a number of proteins approximately equal to between one-sixth

Table 2. The average, 90, and 95% points of the normalized misregistration distance distribution for each of the object patterns

Gel ID	Number of spots	Average	90% Points	95% Points
A12622	434	0.24	0.43	0.55
A12623	431	0.30	0.55	0.69
A12624	419	0.34	0.63	0.95
A12625	308	0.43	0.79	1.04
A12627	285	0.42	0.80	1.01
A12629	374	0.35	0.66	0.79
A12630	343	0.36	0.65	0.81
A12631	356	0.41	0.76	0.95
A12632	331	0.39	0.73	0.87
A12633	313	0.41	0.83	0.99
A12634	360	0.34	0.58	0.90
A12635	379	0.31	0.57	0.74
A12637	392	0.41	0.77	1.02
A12638	378	0.37	0.69	0.89
A12639	409	0.26	0.51	0.63
Summary	5512	0.35	0.67	0.87

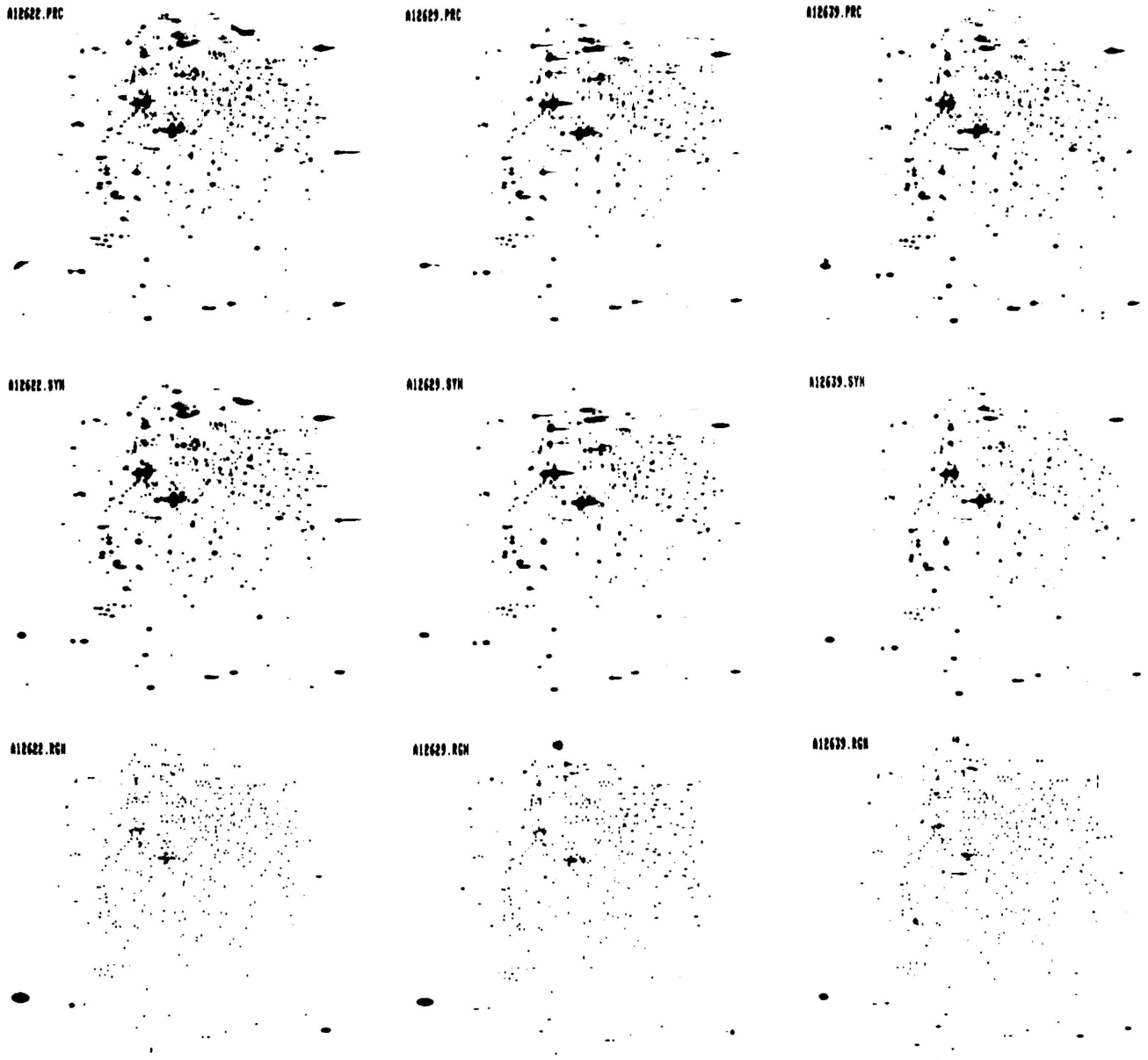

Figure 5. Patterns for three typical samples used in the registration assessment sample. The top row shows the processed images, the middle row the model images, and the bottom row the registration accuracy images.

and one-eighth of the resolution measure can be resolved in typical samples prepared from unfractionated mammalian cells. This observation is based on the number of spots actually detectable by the computer systems. Overloading, vast ovebundance of a few proteins (leading to detection problems), or the presence of only a limited number of proteins in the sample can further reduce the number of detectable proteins.

Based on an oral presentation of our approach [19], Dunn and Burghes [20] have criticized this method of estimating resolution as being too sensitive to sample loading and autoradiographic "spreading". The spreading to which they refer is primarily due to the nonlinear response characteristic of the radiographic film which can make intense spots appear to have too large a half width. The quantitation methods used in this and most other analysis systems correct for this nonlinearity, as long as the film is not saturated. Since the present method excludes the saturated (as well as the very faint) spots from the calculation of resolution, spot spreading is only a minor problem. In any case, only a small fraction of the total spots is overexposed for most of the analyses run in our laboratory.

Dunn and Burghes [20] also suggested that the total number of detected spots would be a better measure of "resolution". This number is certainly an important statistic, but it is extremely sensitive to specimen type, sample loading, exposure time, and various other factors which have little to do with the gel system itself. In most analysis systems, the number of detected "spots" can be arbitrarily increased by decreasing the detection thresholds. The result is a large spot count, primarily due to the inclusion of many spots indistinguishable from image noise. Likewise a very high resolution gel of a very pure protein would give an absurdly small resolving capacity based on the number of spots detected. We prefer to separate the problem of assessing resolution from the task of spot detection and to

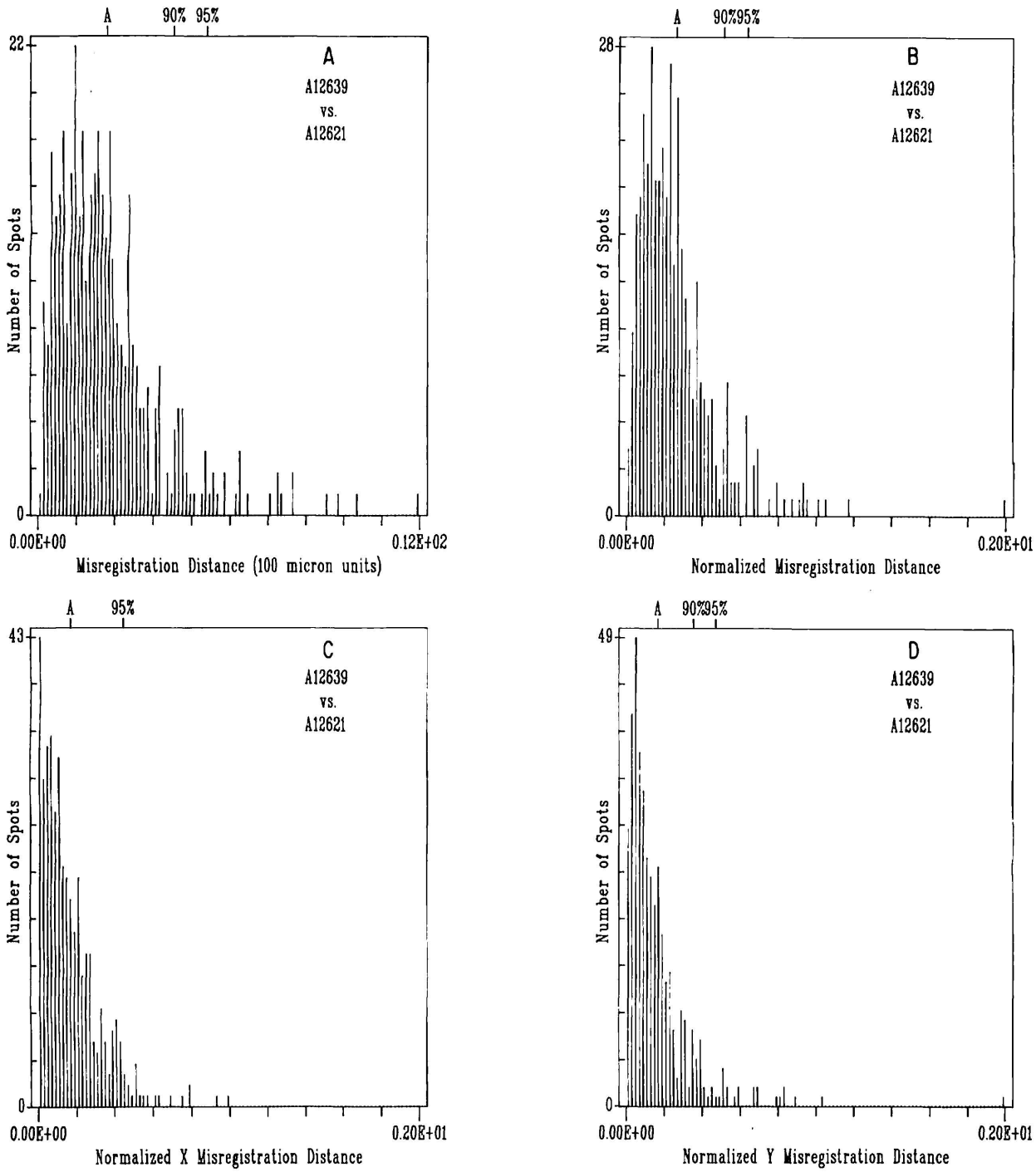

Figure 6. Histograms for assessing the registration of a typical object pattern with the master pattern shown in Fig. 4. Frame A shows the distribution of misregistration distances (in 100 μm units), frame B the normalized misregistration distances, frame C the normalized misregistration distance along the x-axis, and frame D the normalized misregistration distance along the y-axis.

make resolution effectively independent of sample type. While we do not consider the spot count to be a particularly useful indicator of gel resolution, the resolution as defined in this paper should provide a reliable upper limit to the number of spots which could be detected.

The measured size of 2-D gel spots places serious constraints on the methods which can be used to digitize the gel images. Protein spots with full widths at half maximum of less than 0.5 mm are routinely observed. Such spots would represent distributions of the order of 2, 5, or 10 scanning increments along the x or y axes for scanning step sizes of 500, 200, or 100 μm, respectively. It is apparent that scanning resolution of at least 100 μm is desirable. A 200 μm scanning increment could be used but would result in some loss of precision for the high molecular weight (small) spots. Scanning increments much larger than 200 μm appear to be unusable with gels of this type when quantitative results are required.

Given an objective resolution measure, it is possible to ask what resolution can be achieved routinely, and whether the resolution can be systematically improved. The ISO-DALT

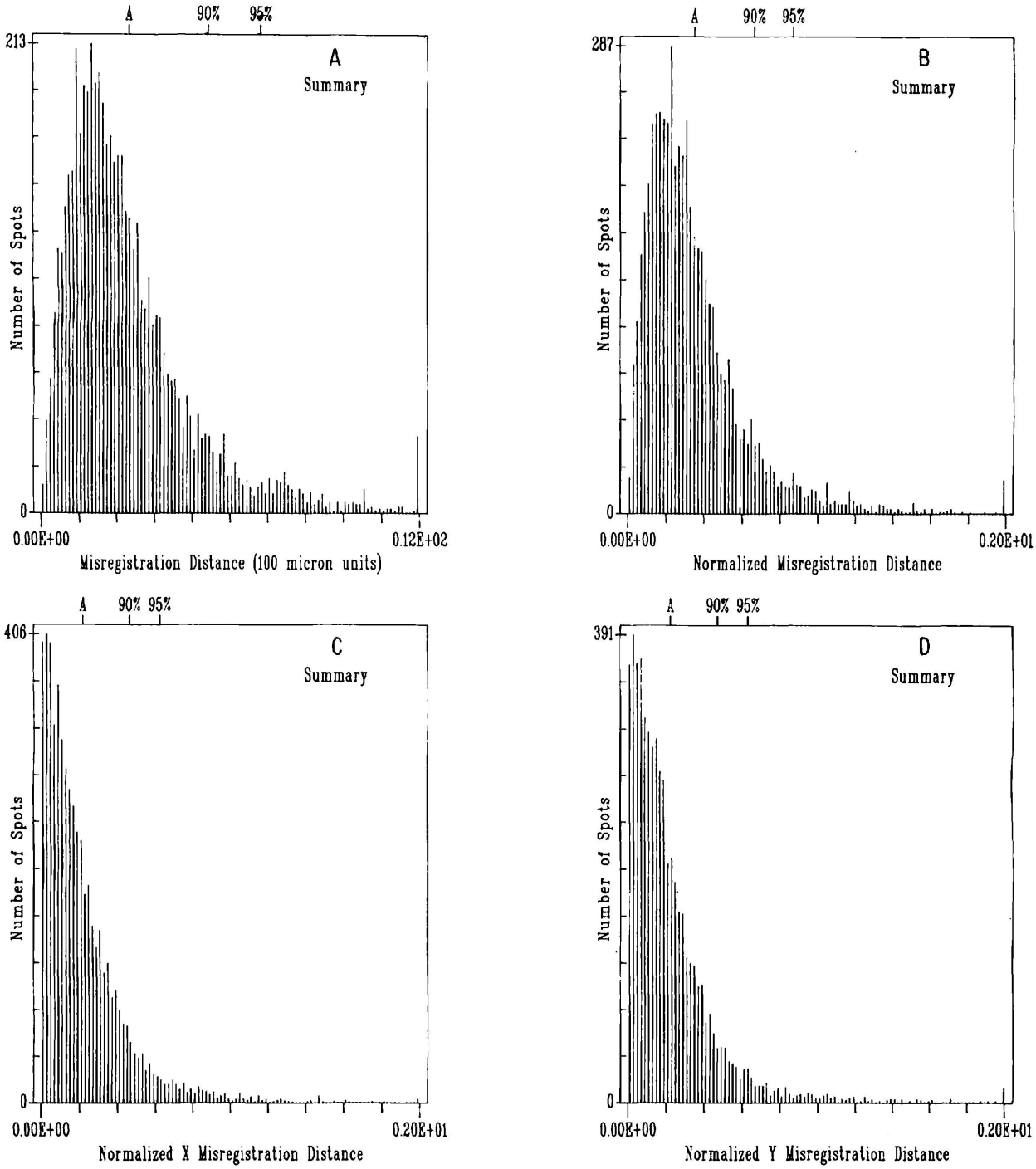

Figure 7. Histograms for assessing the registration of the entire set of fifteen object patterns with the master pattern shown in Fig. 4. Frame A shows the distribution of misregistration distances (in 100 μm units), frame B the normalized misregistration distances, frame C the normalized misregistration distance along the x-axis, and frame D the normalized misregistration distance along the y-axis.

system previously described [2–5] yields typical resolutions of $\Phi = 15\,000, 35\,000$ and $50\,000$ for the $7'' \times 7''$, $8'' \times 10''$, and $12'' \times 12''$ versions, respectively. Using the rule of thumb mentioned above, we expect these systems to be capable of resolving at least 2000, 4500 and 6000 proteins, respectively. A simple, but important aspect of resolution optimization is the increase of gel size to the largest convenient format. For reasons described in detail elsewhere (Anderson, N. L., Nance, S. L. and Anderson, N. G., manuscript in preparation), we favor an $8'' \times 10''$ format for routine use.

The procedures for assessing the positional reproducibility of sets of gels allows further optimization of the 2-D gel separation systems. The fact that the current system enables placement of 95 % of the matched spots in registration (with a reference pattern) to within the spots' full width at half maximum gives us confidence that 2-D gel patterns can be accurately compared. Our estimates of matching accuracy are, in fact, very conservative, since the spots that were "observed" in each matching run had been deleted from the respective master pattern and thus were unable to assist in guiding

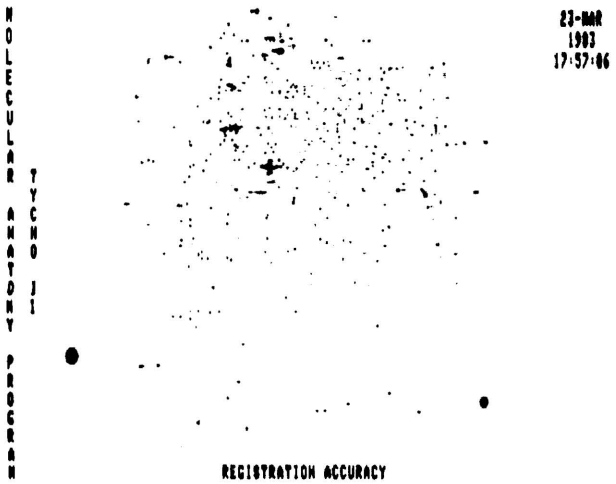

Figure 8. The registration accuracy pattern summarizing the results for the entire set of fifteen object patterns. The spot widths are the average of the widths over the object set, and the spot amplitudes and positions are from the master pattern.

themselves to the correct locations. Thus under normal matching conditions, in which most spots can find out and optimize their match in the master pattern in addition to relying on neighbors for guidance, we believe matching accuracy would be substantially better.

Our results contrast markedly with those obtained by Hurley *et al.* [18], in which very large positional uncertainties (95 % confidence limits of one-fifth to one-half total gel width, or 30 to 70 mm in the gel apparatus used), were obtained for spots shown in Fig. 3 of ref. [18]. These uncertainties appear to be on the order of 50 to 100-fold larger than our values. The difference may be due to their use of an "absolute" coordinate system (fixed to the gel) rather than the relative coordinate approach as used here, as well as to the very large diameter (6 mm) of their focusing gels and their 2-place slab-gel apparatus (necessitating multiple separate runs). Hurley *et al.* [18] showed no gel patterns so that it is impossible to assess the resolution of the gels they used. We are satisfied, however, that their values for spot positional uncertainty do not represent realistic limits for two-dimensional gels. Achievable positional accuracy is, in fact, at least 100-fold higher using the systems we have developed.

The present analysis demonstrates larger (though still small) positional errors for spots near the edge of the pattern, as expected, since these are not surrounded by neighbors. Regions of the pH gradient susceptible to drift also show systematically greater than average error. The ability to recognize the regions and spots particularly susceptible to errors allows improved, objective choices of sets of spots for genetic and other studies in which highly accurate positional comparisons are essential. The methods used in the present study detect both those spots likely to be mismatched (*i. e.*, matched to another neighboring spot if the correct spot is absent) as well as those spots that vary excessively in position between patterns. We propose to use these techniques in the analysis of mutagenized cell clones to discard from consideration the "poorly behaved" spots. For the well-behaved 90 % of spots, a matching accuracy in x (the focusing direction) of better than one FWHM (0.5 FWHM in the experiment reported here) allows direct detection of almost all charge-change mutations, since species differing by one charge are separated by more than a FWHM for almost all proteins.

Taken together, the results presented here show that 2-D electrophoretic gels have the ability to resolve large numbers of proteins (increasingly large for larger gel formats) and that the gel patterns can be matched to one another with sufficient accuracy to make feasible direct studies of genetic and regulational phenomena.

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