A simplified method for computer analysis of autoradiograms from two-dimensional gels*

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A simple method is described for computer analysis of a discrete number of spots on autoradiograms from two-dimensional gels. The method involves digitizing the density data on an autoradiograph with a rotating drum densitometer and displaying the data on a graphics computer terminal. The software allows the operator to select the boundaries of the spots to be analyzed. The software then integrates the density of the spots and tabulates the data. Graphics options allow the operator to display a computer-generated image of the area of the film being analyzed. Accurate integration of weak or overlapping spots is accomplished by a nonlinear least squares fit of the density data to normal Gaussian curves in the x and y dimensions followed by analytical integration of the equations. Since the software is written in Fortran and the equipment required to run the programs is available in most computing centers, this technique should allow laboratories of modest resources to quantitate information from two-dimensional gels.

The technique of two-dimensional electrophoresis followed by autoradiography is often used to resolve and display complex mixtures of proteins in homogenates or subcellular fractions (1-3). It is widely appreciated that the full power of this technique will be reached only when the gel systems are standardized and computer analysis of the autoradiograms is used to compile standard coordinates and intensities for a large number of proteins (4). At least four laboratories have directed their efforts to this end and have reported methods for computerized analysis of the entire autoradiogram (3, 5-7). Other workers have described computer programs for storing, transforming, or displaying the coordinates of proteins in the two-dimensional array (8, 9).

For some applications, each of the published approaches to data analysis presents shortcomings. In our work, two-dimensional electrophoresis is used to resolve the proteins whose phosphorylation state is altered following treatment of ['P']PO₄³⁻-labeled, intact hepatocytes with hormones. Experience has shown that there are only 13 such proteins. While the computer software developed by Bossinger et al. (5) or Garrels (3) would allow quantitation of the phosphorylation changes, in practice these programs are unnecessarily complex and costly in terms of computer time, required hardware, and ancillary services. For this reason, we have developed a simplified method for computer analysis of a small number of spots on autoradiograms from two-dimensional gels. The program displays a computer-generated image of any portion of an autoradiogram on the screen of a graphics computer terminal and allows the operator to select the region of the x-ray film to be analyzed. The report describes the software and points out its advantages for quantifying the intensities of a small number of spots in a two-dimensional autoradiogram. The program is written in Fortran IV and is designed to run on equipment usually available in a university computing center. For these reasons, the method should be of general utility to laboratories that use two-dimensional electrophoresis to separate a few proteins of interest from complex mixtures.

MATERIALS AND METHODS AND RESULTS

Computer Display of the Autoradiograph—PROG-2 is used to locate, integrate, and generate an image of the density data from the autoradiogram. Its commands and their functions are listed in Table 1 (see "Materials and Methods" in the Miniprint). The sections below give examples of the use of the important commands in PROG-2 with actual data. The first step in the quantitative analysis of an autoradiograph is to locate the coordinates of the data to be integrated. This task is accomplished by having the computer display the entire autoradiograph on the Tektronix graphics terminal and then selecting the appropriate x and y coordinates needed for higher resolution images. The process of defining and resolving a series of spots is presented in Fig. 1, A-D. The boxed area encompasses four major phosphoproteins ranging in Mr = 80,000 to 56,000 (top to bottom) and isoelectric point from about 6.0 to 6.5 (left to right). Fig. 1B presents a computer generated image of the boxed section obtained by selecting x coordinates of 359-516 and y coordinates of 289-386 with the SPOT command. For this figure, the LEVELS command was used to set the plot level at 0.04 absorbance above the film background of 0.35 absorbance. Only dots were plotted in order to obtain a rapid display (10-13 s). Each dot in this image represents the optical density reading of a 0.2-mm square section of the x-ray film (pixel) with a density of 0.39 absorbance.

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The "Materials and Methods" and portions of "Results" (including Figs. 3 and 4 and Tables 1-3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD, 20014. Request Document No. 82M-562, cite the authors, and include a check or money order for $4.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Fig. 1. Computer imaging of an autoradiograph. A, a two-dimensional autoradiograph made from $^3$P-labeled cytosolic proteins isolated from glucagon-treated hepatocytes. The molecular weight dimension is vertical and the isoelectric focusing dimension is horizontal with the acidic end on the left. B, a computer-generated image of the boxed section of the autoradiograph. Each dot represents a 0.2-mm square area of film (pixel). The computer plots dots for those pixels with optical densities 0.04 or more above film background of 0.35 absorbance. C, computer image of the same film area shown in B with the plot level raised to 0.09 absorbance above background and crosses and stars plotted in place of dots for regions of higher density. D, a computer image focused on Spot 3 from C obtained by changing the $x$ and $y$ coordinates with the SPOT command. Dots are plotted for pixels with optical density values of 0.44–0.60, crosses for pixels with optical density values between 0.60 and 0.70 and stars for pixels with optical density values above 0.70.

Fig. 1D shows further use of the SPOT command to resolve and integrate the density of one of the spots in Fig. 1C, Spot 3. For this image, the $x$ and $y$ coordinates were narrowed around Spot 3 and slightly different contouring was provided using the LEVELS command, as outlined above. Note how the crosses and stars add a definite impression of density contour.

Integration of the density of the film is automatically provided for areas bounded by the square and the ellipse each time the SPOT command is used. The LEVELS command only affects the graphic output of the SPOT command; LEVELS does not affect the integration routines. Values printed with each image include: the square integral, its baseline, and the number of pixels summed; the elliptical integral, its baseline, and the number of pixels summed; the plot levels and $x$, $y$ coordinates (these details are not shown in Fig. 1). For Spot 3, these values were: square integral = 11,075 with a baseline of 0.35 absorbance and number of pixels = 1512; elliptical integral = 10,709 with a baseline of 0.35 absorbance.
and number of pixels = 1116. For well separated spots, the values of the square and elliptical integrals usually agree to within 0.5%; however, the elliptical integral more closely approximates the true shape of the spot. Moreover, if necessary, the density contributions of closely adjacent spots can be minimized by selection of the x and y coordinates to cause the neighboring spots to fall outside the area of the ellipse.

For strong, well separated spots, the above method of integration provides acceptable accuracy. However, weak spots and closely adjacent spots require the more complex methods of analysis available with PROG-3 (see below).

At this point, Spot 3 has been located, its image displayed, and its density values integrated. The operator may now save the information in one of three ways by: (a) making a permanent copy of the image and integral information displayed on the screen using the Tektronix 4631 Hardcopy Unit; (b) storing the square and elliptical integral values, their baseline, and the number of pixels integrated in a running table with the SAVE TABLE command; or (c) transferring the entire matrix of optical density data bounded by the x and y coordinates to a new disk file with the SAVE command. The new file can then be retrieved and analyzed using the more complex techniques available with PROG-3 (see below). Options (b) and (c) allow the operator to identify the stored data with numbers and names, respectively. Any combination of the three options can be chosen for each spot.

**Use of Complex Analysis to Integrate the Density of Weak or Overlapping Spots**—The above discussion presents a straightforward method of integrating spot densities. For strong spots (integrated densities greater than 200), the routines with PROG-2 are quite accurate. Most spots on a properly developed autoradiogram fall into this category. However, weak spots (integrated densities of 100-200) and closely adjacent spots (strong or weak) cannot be accurately analyzed by PROG-2. Weak spots are difficult to integrate with PROG-2 because small changes in the film background subtracted from the integral will markedly affect the final value. (See Figs. 3 and 4 and Table 3 in the Miniprint for a description of this problem.) Overlapping spots are not accurately integrated with PROG-2 because the algorithms cannot discriminate between the density contributions of adjacent spots. A third program, PROG-3, has been developed to integrate weak or overlapping spots accurately. This program uses nonlinear least squares curve fitting techniques to generate normal Gaussian curves describing the density distribution of a spot in both the x and y dimensions and then analytically integrates the equations. (See "Materials and Methods" in the Miniprint for a full description of this program.)

Fig. 2 describes the features of PROG-3 using Spot 4 of Fig. 1C as an example. Fig. 2A shows the image of Spot 4 generated with PROG-2. The integrated density of the elliptical integral from this analysis is 1301. The data in the area of the film bounded by the square is stored with the SAVE command, retrieved by PROG-3, and the density of the spot integrated. The value obtained is 1321, a result within 2% of that obtained with PROG-2. Fig. 2B shows the contour plot (one of PROG-3's plot routines) of the Gaussian curves fit to the data. The three ellipses represent constant optical densities of 0.40, 0.54, and 0.66, outer to inner, respectively. Fig. 2, C and D present the nonlinear least squares determined Gaussian curves (solid lines) and the actual data points (stars). Fig. 2C presents the distribution of the density values in the y dimension when the spot is scanned holding x constant at 475 or 478 (these x values are marked by short arrows in Fig. 2B). Note the excellent fit of the calculated curves to the actual data points. Fig. 2D presents a similar scan made in the x dimension. Scans at all other values of x and y across the spot revealed the same excellent fit (data not shown). The plots of Fig. 2, B-D are available with each use of PROG-3 and are important for determining the quality of the nonlinear least squares Gaussian fit to the experimental points.

Two important features of the algorithms used in PROG-3 provide accurate analysis of weak or overlapping spots. PROG-3 correctly integrates the density of weak spots because it determines film background as one of the fitting parameters.
required to make the spot have a Gaussian shape. This feature minimizes the background fluctuations that lead to inaccurate values for weak spots. (See Figs. 3 and 4 and Table 3 in the Miniprint for a more complete description of this function of PROG-3.) The algorithms of PROG-3 can separate the density contributions from overlapping spots because they assume that the density distribution of a given spot follows a single Gaussian distribution in the $x$ dimension and a separate single Gaussian distribution in the $y$ dimension. Thus, the algorithms will fit Gaussian curves to only one of a set of overlapping spots. The operator can direct the program to the spot of choice by proper selection of the spot boundaries when the data is stored for PROG-3 with the SAVE command. When the program is run, the algorithms ignore missing data or extraneous data from overlapping spots and provide the complete integral for a single Gaussian-shaped spot. Supporting data for this function of PROG-3 and other details about its uses can be found in the miniprint supplement.

**DISCUSSION**

While the technique of two-dimensional electrophoresis is widely used to resolve complex mixtures of proteins, quantitative analysis of the patterns obtained is not common. Among the main reasons for this situation is the large technical and financial investment needed for automatic analysis of the data in autoradiograms of two-dimensional gels (3–7). While this advanced technology is clearly required to be able to analyze and compare hundreds or thousands of spots, many laboratories use two-dimensional gels to separate a few proteins of interest from complex mixtures. The computer approaches described in this report offer many advantages that allow rapid, accurate quantitation of 10–50 spots in an autoradiogram. These advantages include simplicity, versatility, and low cost, yet the computer programs allow use of the full power of the two-dimensional technique. The obvious disadvantage of the approach is that the analysis is not automatic; operator intervention is required to select the area of the film to be analyzed. However, operator intervention also provides versatility that allows the program to be used for other purposes.

The main advantage of the software is that it is written in Fortran IV and is designed to run on a university computing center’s main frame computer (a Control Data Corporation Cyber 730 in this case). Thus, the hardware required (disk drives, tape drives, central processor, and the graphics terminal) are owned and maintained by the computer center. The speed, memory capacity, and versatility of this type of system far exceed those of the mini-computer systems often used for analyzing two-dimensional gels (5, 7). Moreover, this type of facility should be available in most university or research settings for a nominal usage fee. The high speed densitometer needed to scan the autoradiographs is perhaps less widely available. However, since the output of this unit can be stored on magnetic tape, it is quite practical to travel to an available, off-site densitometer and scan many autoradiographs in one session. The tapes can then be analyzed on-site over a longer period of time. It should be noted that, while the software described in this report was developed using one particular hardware configuration, the main advantages of the method are independent of the computer equipment used. If the three basic items of hardware are available, the programs should allow laboratories of modest resources to use the full power of the two-dimensional technique.

A second advantage of the software described in this report is that the algorithms used to integrate the density of the spots can be tailored to meet the complexity of the integration. For well separated spots of moderate to high density, the simple summing routines of PROG-2 provide rapid, accurate integration of the spot densities. Experience has shown that this mode of integration, combined with appropriate use of the BASE VALUE command to hold the subtracted background constant, can be used to integrate 80–90% of the spots in an autoradiogram. This mode of operation is very efficient because it uses little central processor time. Moreover, since the boundaries of the spots are selected interactively by the operator, the computer does not have to match or align the coordinates of spots in autoradiograms from different gels to compare two experiments. Thus, reproducibility of gel systems does not have to be as stringent as in totally computerized systems.

Weak or overlapping spots that require more complex analysis than is available with the SPOT command may be analyzed with PROG-3. This program contains algorithms that define the density distribution of a spot as Gaussian in the $x$ and $y$ dimensions and simultaneously estimate the appropriate background. In addition, since the equations do not require an entire spot for integration of density, PROG-3 is very useful for accurately integrating weak and/or overlapping spots. While the routines for PROG-3 could be used with all spots, they provide no improvement in accuracy for well separated spots of moderate to high density. Since PROG-3 requires more central processor time than PROG-2, its use is best reserved for weak or overlapping spots. The combined use of PROG-2 and PROG-3 can quickly and accurately process 10–50 spots of all types on a typical autoradiogram.

The final advantage of the software presented in this report is its versatility. Since the SPOT command of PROG-2 makes no assumptions about the area selected to be integrated, it can be used to integrate densitometric data from sources other than two-dimensional gels. As noted in the Miniprint, PROG-2 has been used to integrate direct positive transparencies made from the stained proteins on two-dimensional gels, long thin areas (1 x 15 mm) in one-dimensional gels used in nucleic acid research and autoradiographs from two-dimensional peptide maps made on thin layer cellulose plates. Other uses are certainly possible.

The authors will provide documented copies of the program to interested parties on request.

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Computer Analysis of Autoradiograms

**SPECIALIZED TUTORIAL ON**

**A Simplified Method for Computer Analysis of Autoradiograms from Two-Dimensional Gels**

by James C. Grunwald and Michael L. Johnson

**MATERIALS AND METHODS**

**Required Hardware:** The computer programs described for analyzing autoradiograms from two-dimensional gels require access to three major pieces of equipment: a high-speed densitometer, a computer capable of scanning and storing the optical density of each gel spot, and a computer terminal. The actual computer software used for the program (PROC-1) was developed and run on the Control Data Corporation Cyber 730 computer at the University of California, Los Angeles. The densitometer used was a Tektronix 4611 Hardcopy Unit. The Hardcopy Unit was interfaced via Tektronix 4611 Graphic terminal to a Tektronix 4650 Hardcopy Unit. The Hardcopy Unit was controlled and monitored via the operator keyboard and terminal.

**The Optronic Densimeter:** The Optronic Densimeter scans the film with a 200 x 300 grid and graphically displays the background and the background density at each grid point (0-2.55). Therefore, a 0.2 mm square area of the film (pixel) with an optical density of 0.150 will give a reading of 100. The automatic integrator usually measures 100 x 100 mm; translating this to 100,000 pixels per film. The primary purpose of the computer programs is to analyze the data to determine the levels of protein phosphorylation. The data described could be utilized to run a variety of different experiments.

The Optronic densimeter scans the X-ray film with a 200 x 300 grid size and graphically displays the background and the background density at each grid point (0-2.55). Therefore, a 0.2 mm square area of the film (pixel) with an optical density of 0.150 will give a reading of 100. The automatic integrator usually measures 100 x 100 mm; translating this to 100,000 pixels per film. The primary purpose of the computer programs is to analyze the data to determine the levels of protein phosphorylation. The data described could be utilized to run a variety of different experiments.

**Programs and Computational Methods:** The software for analysis of autoradiograms consists of three separate programs written in Fortran IV. These have been named PROC-1, PROC-2, and PROC-3. PROC-1 is a utility program and PROCs 2 and 3 are used to integrate the density of spots on the autoradiograms.

PROC-1 is necessary because the Optronic P-1000 scanner stores the densitometric data as 8-track magnetic tape and our CDC Cyber 730 does not allow a time sharing user to manipulate data stored on magnetic tape. Commands to the scanner must be entered on a teletypewriter or a terminal which can be used by the interactive program PROC-2. This configuration has proven useful since it allows the user to correct the data in question, selectively editing these commands and their uses, to be obtained from Table 1 (below).

The Optronic densimeter with the aid of a computer at the University of California, Los Angeles, was able to analyze all the data in an autoradiogram by generating a digital histogram of the density values within the autoradiogram. The integrated density was calculated as the total optical density of the film within the calculated background. The integrated density was calculated as the total optical density of the film within the calculated background. The integrated density was calculated as the total optical density of the film within the calculated background.

**TABLE 1 - The Commands of PROC-1**

| Command | Use |
|---------|-----|
| SPOT | Selects and integrates the region of the X-ray film to be analyzed |
| LEVELS | Selects the symbols printed for the optical density of each band |
| SAVE | Table command to save the spot number and integrated area from each analysis |
| GET | Table command to generate the integrated area |
| TABLE | Table command to generate the integrated area |

**TABLE 2:** The use of PROC-1. The 2D computer program was developed by the authors. The Table program was used to determine the density of the Optronic P-1000 scanner. The computer programs described for analyzing autoradiograms from two-dimensional gels require access to three major pieces of equipment: a high-speed densitometer, a computer capable of scanning and storing the optical density of each gel spot, and a computer terminal. The actual computer software used for the program (PROC-1) was developed and run on the Control Data Corporation Cyber 730 computer at the University of California, Los Angeles. The densitometer used was a Tektronix 4611 Hardcopy Unit. The Hardcopy Unit was interfaced via Tektronix 4611 Graphic terminal to a Tektronix 4650 Hardcopy Unit. The Hardcopy Unit was controlled and monitored via the operator keyboard and terminal. The Optronic densimeter scans the X-ray film with a 200 x 300 grid size and graphically displays the background and the background density at each grid point (0-2.55). Therefore, a 0.2 mm square area of the film (pixel) with an optical density of 0.150 will give a reading of 100. The automatic integrator usually measures 100 x 100 mm; translating this to 100,000 pixels per film. The primary purpose of the computer programs is to analyze the data to determine the levels of protein phosphorylation. The data described could be utilized to run a variety of different experiments.

The above method of background calculation works well for spots with densities of 0.05 or higher. For background densities below 0.05, the computer program was adapted to calculate the background density using a linear extrapolation. The background density was calculated as the sum of the density readings from the background and the background density at each grid point (0-2.55). Therefore, a 0.2 mm square area of the film (pixel) with an optical density of 0.150 will give a reading of 100. The automatic integrator usually measures 100 x 100 mm; translating this to 100,000 pixels per film. The primary purpose of the computer programs is to analyze the data to determine the levels of protein phosphorylation. The data described could be utilized to run a variety of different experiments.

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The main problem with integrating spots such as Spot 4 is that small changes in the background level subtracted from the integral will markedly affect the final value (see below). The magnitude of the effect of change in background level will depend on the baseline change on the peak area. For a sharp strong spot (for example Spot 2) the effect will be little to no effect, whereas that for a more diffuse spot (for example Spot 3) will be more greatly affected. However, no matter what the spot shape, the effect of a small change in background will have the greatest effect on weak spots.

The effects of small changes in the film background on the integrated density of strong and weak spots are clearly shown in Figure 4. The actual amount of deviation from the film background is expressed as a percentage. Note that the deviation from the film background for Spot 2 is 0.35% and for Spot 3 is 0.84%. These deviations are relatively small compared with the 1.5% deviation of the background. If the film background subtraction is performed for a spot that includes the peak of the spot, then the background will be affected. For example, if the spot shape is assumed to have a Gaussian shape, then the area of the spot will be calculated as the area under the curve, and the deviation of the calculated area will be calculated as the deviation of the peak from the film background.

The effect of raising the film background (see Table 3) on the integrated density of Spots 1-4 in Figure 3 is indicated in the Illustration. The position of the integrated densities of Spots 1-4 is shown at the top of Figure 4 along the X-axis by the arrowheads labeled 1-4. The solid lines represent the effect of the change in background level on the integrated density of the same spot.

Table 3 compares the use of PROC-2 and PROC-3 to integrate the four spots labeled 1-4 in Figure 3. The baseline in this area of the film is 35.72 (0.35 O.D.) measured in large blank areas of the film. The table shows the integrated densities calculated by each program, the deviations from the film background, and the percentage change in the baseline for each spot. The percentage change in the baseline is calculated as the deviation from the film background expressed as a percentage of the total area of the spot.

If PROC-2 is used for analysis and background level is held constant at 35.72 with the BASE DATA command, the integrated spot densities obtained with the SPOT command are within 3% of those obtained with PROC-3 for all 4 spots (data not shown).

Analysis of Overlapping Spots: The methods of calculation used by PROC-3 to fit Gaussian curves to the data points do not require data from the entire area of the spot, as long as the peak of the spot is in the field viewed by the program. The data points used by the program are selected by the program's routine and are selected by the user. If the program's routine selects data points that are outside the area of the spot, the program will calculate an area that is larger than the area of the spot.

If PROC-2 is used for analysis and background level is held constant at 35.72 with the BASE DATA command, the integrated spot densities obtained with the SPOT command are within 3% of those obtained with PROC-3 for all 4 spots (data not shown).