A Metrological Study of Autoradiographs from Two-Dimensional Gel Electrophoresis

Odile Valliron,1,2 Ivan Lefkovits,3 Philippe Garderet,1 and Charles Steinberg3

Samples prepared from a single batch of labeled cells were subjected to two-dimensional gel electrophoresis and autoradiography. Three factors were varied: total quantity of protein, quantity of labeled protein, and exposure time. The mean background absorbance of the film remained identical (about 0.5 A) for all the treated series, whatever the exposure time and whether or not there were unlabeled proteins in the sample. Hence any spot with a peak A of the same order of magnitude can be seen. The standard deviation was about 0.05 A. Thus, the measurement precision was 2.5% of full scale for digitalization over 0 to 2 A. We derived experimental calibration curves, which are neither linear nor logarithmic because of the film response and which can be used on randomly chosen spots.

The principal object of analyzing two-dimensional (2D) gels with computers is to reduce the data from images of spots to a list of the positions of the spots and the amount of protein associated with each one (1). Metrological study must be pursued to determine with precision what can be found from what we measure and what we know a priori (2). For the present study, samples prepared from a single batch of radiolabeled cells were subjected to 2D gel electrophoresis and autoradiography. Three factors were varied: total quantity of protein, quantity of labeled protein, and exposure time. We first studied artefacts such as background film transparency and effect of the presence in the sample of unlabeled proteins or free tracer. Then we worked out a calibration curve for several spots. These experimental curves were then compared with theoretical calculations. Finally, these curves were used to measure the amount of labeled protein in spots that had not been used in the calibration.

Materials and Methods

Procedures

Labeled cells. Murine cell line BW was propagated in RPMI 1640 medium (Gibco) by inoculating at a density of 105/mL and diluting when the density reached 5 × 106/mL. For labeling with [35S]methionine, cells were pelleted from normal medium, resuspended in methionine-free medium (containing dialyzed and filtered bovine serum, 50 mL/L), and allowed to starve for 2 h at 37 °C. Then 50 μCi of [35S]methionine was added per milliliter, and after 18 h the cells were harvested and pelleted in a Sarstedt tube, each pellet containing not more than 106 cells. The medium was aspirated, but some residual non-incorporated [35S]methionine remained in the pellet (about 1% of the original volume). The cells were lysed in a non-ionic detergent as described elsewhere (3).

Isoelectric focusing. Isoelectric focusing (IEF) from pH 2.5 to pH 9 was done as described in detail elsewhere (3). The sample is applied to the basic end of the prefocused IEF gradient. Even with precautions that the applied protein remains in the solution (high urea concentration), it is possible that some proteins might not enter the gel. It is also possible that some material might leave the gel at the basic end during focusing. Owing to electroendosmosis, prolonged isoelectric focusing results in a shift of the basic end of the pH gradient, and some proteins move out of focus and might be lost (2).

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis. These gels, 17 × 17 cm, were run as described elsewhere (3). In this dimension, only a negligible fraction of protein material fails to leave the IEF "spaghetti" gel. Furthermore, no low-M, material runs beyond the bromphenol blue front. Thus, there is little if any loss during this procedure.

Autoradiography. Table 1 summarizes the gels and autoradiographs we used for this study. In all cases Kodak XAR-5 film was used. To give control films on which there are no spots, we ran gels 187 and 189 without labeled cells, but the extracts used with gel 189 contained 25 μCi of free [35S]methionine. The other gels led to autoradiographs with protein spots. Films from gels 192 and 196 were difficult to use because of unsatisfactory IEF separation. Films from gel 181 have a spot pattern different from the others, probably because, during transfer from the first to the second dimension, the "spaghetti" gel broke into three parts and seems not to have been correctly put on the plates for the second separation.

Digitalization. We digitalized films with an Optronics rotary scanner, using a 100-μm grid. Each point yields a one-byte (eight-bit) integer, so that 256 gray levels are possible. Depending on the machine setting, these 256 levels are spread over 0–2 or 0–3 A. The resulting image has only

Table 1. Autoradiographic Films Used in This Study

<table>
<thead>
<tr>
<th>Gel no.</th>
<th>Cell number (×10^-5)</th>
<th>Labeled cells normalized</th>
<th>Exposure time, days*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Labeled Unlabeled</td>
<td></td>
<td></td>
</tr>
<tr>
<td>189b</td>
<td>0 500</td>
<td>0</td>
<td>B1189 B3189 B7189</td>
</tr>
<tr>
<td>187</td>
<td>0 500</td>
<td>0</td>
<td>B1187 B3187 B7187</td>
</tr>
<tr>
<td>184</td>
<td>5 0</td>
<td>1</td>
<td>B1184 B3184 B7184</td>
</tr>
<tr>
<td>183</td>
<td>15 0</td>
<td>3</td>
<td>B1183 B3183 B7183</td>
</tr>
<tr>
<td>182</td>
<td>50 0</td>
<td>10</td>
<td>B1182 B3182 B7182</td>
</tr>
<tr>
<td>181</td>
<td>150 0</td>
<td>30</td>
<td>B1181 B3181 B7181</td>
</tr>
<tr>
<td>196</td>
<td>500 0</td>
<td>100</td>
<td>B1196 B3196 B7196</td>
</tr>
<tr>
<td>192</td>
<td>1500 0</td>
<td>300</td>
<td>B1192 B3192 B7192</td>
</tr>
<tr>
<td>186</td>
<td>375 500</td>
<td>75</td>
<td>B1186 B3186 B7186</td>
</tr>
<tr>
<td>185</td>
<td>750 500</td>
<td>150</td>
<td>B1185 B3185 B7185</td>
</tr>
<tr>
<td>200</td>
<td>15 000 500</td>
<td>300</td>
<td>B1200 B3100 B7200</td>
</tr>
</tbody>
</table>

*Films identified by individual number. b Gel containing free [35S]methionine.

1 Laboratoire d'Electronique et de Technologie de l'Informatique, Centre d'Etudes Nucléaires de Grenoble, F-38041 Grenoble, France.
2 U217 INSERM, Centre d'Etudes Nucléaires de Grenoble 85X, F-38041 Grenoble, France.
3 Basel Institute for Immunology, Grenzacherstrasse 487, CH-4058 Basel, Switzerland. (The Basel Institute was founded and is sponsored by F. Hoffmann-La Roche & Co. Ltd., Basel, Switzerland.)

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1024 pixels in each dimension, because the area that can be scanned is limited by a window in our densitometer. The central zone of the gels was always used. By using a dummy digitalization with no film in the scanner window, we verified that the scanner measures zero absorbance over the whole window.

Background

Control films and zones without spots in other films are not completely transparent. Figure 1 shows histograms of background gray levels from two control gels. The mean gray level varies from 54 to 60, which corresponds to absorbances from 0.42 to 0.47 when digitalizing over 0–2 Å. These values are independent of exposure time and of the presence or absence of ³⁵S)methionine in the sample. The standard deviation varies from three to six gray levels; thus the two last bits of the measurement are not significant.

Profiles and values obtained from zones without spots on other gels give similar results. The mean background on an autoradiograph, which is independent of both film exposure and sample quantity, is between 0.4 and 0.5 Å. This background represents 20% to 25% of the maximum when digitalizing over 0–2 Å, and 13% to 17% over 0–3 Å.

Figure 2 shows a representative profile of a line across a part of a film where there are no spots. Note that the baseline does not vary. That is, the variation in the pixel gray level seems to consist of high-frequency noise. Furthermore, the mean background gray level remains invariant whatever the image zone.

Figure 3 shows a histogram of gray levels from a gel with spots. Since most of the gel surface is not occupied by spots, these histograms are dominated by background. The part of the histogram corresponding to the spots appears as a shoulder at higher gray levels, because small spots are more numerous than larger ones. Some spots reach saturation (gray level 255). The shoulder was much less pronounced for another film (not shown) from a gel that was loaded with 150 times less labeled material than the one in Figure 3. When the arithmetic mean and standard deviation of the histogram are calculated for the 13 levels centered around the mode, these parameters are similar to those calculated from the background alone. We conclude that the presence of labeled proteins does not increase the general background.

Calibration Curves

Principles. Figure 4, a schematic representation, shows how it is possible to pass from a gray level measured experimentally on an image pixel to the relative quantity of labeled protein in the gel. Curve 1 gives the relation between the measured gray level and the absorbance of the pixel. Curve 2 shows the film response, which is directly

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Fig. 1. Background gray levels from control gels

From left to right, the peaks are from films no. B1187, B1189, B3187, B3189 (see Table 1). The first two are from gels exposed for one day; the last two are from the same set of gels, but exposed for three days. Both gels were run with a lysate of 5 × 10⁸ unlabeled cells; 5 μCi of free ³⁵S)methionine was added to the cell lysate applied to gel no. 189 (peaks 2 and 4). Mean gray level for the four peaks: 59, 55, 60, and 55. SD for these peaks: 5.1, 5.7, 4.9, and 3.9

Fig. 2. Representative profile of a line across a part of a film where there are no spots

Fig. 3. Gray levels from a gel with spots

The gel was run with a lysate of 75 × 10⁶ labeled cells mixed with 50 × 10⁶ unlabeled cells (film no. B1185, see Table 1). Mean gray level: 56 (SD 3.5)
related to the radioactivity, which in turn is proportional to the protein on the gel (curve 3). We consider here only the protein quantity at the end of electrophoresis and not at the beginning of the experiment because, as mentioned above, there may be some protein loss during electrophoresis.

It is usually assumed that spots are orthogonal bidimensional "gaussian" or "normally" distributed, with amplitude A and standard deviations $S_a$ and $S_b$ (1, 4). It is not known how $S_a$ and $S_b$ behave as the protein quantity increases. We shall assume that the surface area of the spot base does not change and that the amplitude alone increases.

If the film response is proportional to radioactivity, the amplitude increases logarithmically with increasing protein quantity. In fact, the film response is not linear, so we have studied the behavior of the amplitude with increasing exposure for several spots with different visual intensities.

Because of the background and the absence of preliminary filtration, it is difficult to define spot outlines with precision. We integrate over a constant area centered on the spot maximum. The magnitude of this integral will depend on the number of pixels under the integral.

**Measurements and Results**

Figure 5a shows curves for maximum absorbance at the center vs quantity of protein, measured on four spots (B, D, H, and G in Figure 7 below). Some curves are prepared from data from one spot (G: seven days, or B: one day); in others the means of two spots with nearly equal values are used. The curves are not completely homothetic. But when the maximal values are close, they are almost so (curves 1 and 2, maximum $\pm 2 A$; curves 4 and 5, maximum between 1 and $1.5 A$; curves 6 and 7, maximum $\pm 1 A$). That is, the shape of a curve depends on the absorbance of the spot and not on an idiosyncrasy of the spot itself or of its position on the gel. Figure 5b shows the behavior expected if the film response were ideal. By construction, the curves are homothetic, so for one absorbance we find proportional ratios (75, 150, 300). The experimental points in Figure 5a verify this law, with more or less experimental error. Thus, it is possible to use the amplitude as a measure of relative protein abundance.

Figure 6 shows the calibration curve giving the amount of protein as a function of amplitude. The integral is the sum of absorbances of the pixels over the chosen area. The amplitude for each pixel follows the curves determined in the preceding section. To add the absorbances over the whole spot amounts to taking a mean of all the curves. If we consider a smaller volume around the center of the spot, the absorbance values remain near the

![Fig. 4. Schematic representation of the metrological procedure](image)

Curve 1: measured gray level vs A. Curve 2: A vs reduced silver. Curve 3: radioactivity vs labeled protein

![Fig. 5. Protein quantification from autoradiographic spots](image)

(a) curves of amplitude (maximum OD in the center of a spot) vs. relative quantity of protein in four spots (B, D, H, G; see Figure 7) after various exposure times. (b) Theoretical curves, assuming an ideal film response. (c) Curves of absorbance integrated over 100 pixels in the center of a spot vs relative quantity of protein measured in three spots (E, G, H; see Figure 7) after various exposure times

![Fig. 6. Calibration curve for the amount of labeled protein as a function of the amplitude (maximum gray level at the center of a spot)](image)
maximum, and the curves are the same as those determined in the preceding section. The chosen surface is 10 x 10 pixels. Figure 5c shows curves of the integral (after background subtraction) as a function of relative protein quantity. The curves are made by using measurements on three spots (E, G, H; see Figure 7). In one curve a mean value for similar spots (E and G) is used; in the other, data from one spot (H). These curves are very similar to the amplitude curves. Note that the integral considered here does not include the whole spot.

Reliability of the calibration curve. We have verified that the calibration of Figure 6 is valid for spots that were not used in constructing the curve. Table 2 shows the maximum

**Table 3. Amount of Labeled Proteins Calculated from Amplitude Measurements and the Calibration Curve of Figure 6**

<table>
<thead>
<tr>
<th>Film exposure</th>
<th>Spot 1</th>
<th>Spot 2</th>
<th>Spot 3</th>
<th>Spot 4</th>
<th>Spot 5</th>
<th>Spot 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td>gl</td>
<td>lp</td>
<td>gl</td>
<td>lp</td>
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<td>lp</td>
</tr>
<tr>
<td>20</td>
<td>12</td>
<td>112</td>
<td>14</td>
<td>54</td>
<td>29</td>
<td>8</td>
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<tr>
<td>36</td>
<td>153</td>
<td>28</td>
<td>170</td>
<td>36</td>
<td>101</td>
<td>10</td>
</tr>
</tbody>
</table>

*These values are different from the ones used in the remainder of this paper.
*gl, maximum gray level (gl 255 = 2.0 A) at the spot center.
*lp, labeled protein (arbitrary units) calculated by using the calibration curve of Figure 6.

A values for each spot, and the relative amount of labeled protein. Theoretically, for a given spot the amount of labeled protein (lp) on any line of the Table should be twofold lower than on the line above. That is, lp of B7200 = 2*lp on B7185; lp of B7185 = 2*lp of B7186, etc.

The data in Table 3 come from a set of two autoradiographs from another experiment, with another set of samples but with the same type of film. The autoradiographs were made from the same gel exposed for 20 and 36 h. Theoretically, for a given spot, the amount of labeled protein should be proportional to the exposure time. In view of the measurement error (about 0.05 A), there is good agreement between theoretical expectation and the experimental values obtained by using the calibration curve.

**Discussion**

Our results allow us to draw some conclusions about the precision with which measurements can be made on autoradiographs. The mean background of the film remains identical for all the treated series, whatever the exposure time, and whether or not there are unlabeled proteins in the sample. This background is located around 0.5 A, hence any spot with a peak A of the same order of magnitude can be seen. The standard deviation is about 0.05 A. Thus, the measurement precision is 2.5% of full scale for digitalization over 0-2 A.

We derived experimental calibration curves that are neither linear nor logarithmic, because of the film response. Figure 6 shows a calibration curve giving radioactivity as a function of measured A for the films used here. We showed that such a calibration curve, which was established with a restricted set of spots, can be used on randomly chosen spots with visually different intensities and on different parts of the same film or on different films.

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**References**