A "High-Performance" 2D Gel Scanner

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We describe the design principles of a photometric flatbed scanner with a scan area of 250 × 250 mm², a dynamic range of 4000 gray levels, a signal/noise ratio of 2000/1, a step size of 0.1 mm, and a step frequency of 2000 steps per second. It is controlled by a microcomputer and used to acquire data for quantitative evaluation of gels or chromatograms. The performance of the instrument is demonstrated by the data obtained by scanning gratings, gray step filters, and high-resolution electropherograms.

For photometric evaluation or establishing a data base, digital image processing is an essential part of a system for two-dimensional biochemical analyses. Video or solid-state cameras and drum scanners are commercially available devices for acquiring the image data. The use of drum scanners (1) is restricted to samples on photographic or radiographic film; direct measurements on wet gels are impractical. Gels and films can be imaged by video cameras (e.g., 2–4). Here data acquisition is fast, but impeded by a small dynamic range, a low signal-noise ratio, and stray light due to the illumination of the whole field of view. Nonlinearities of the target or the illumination have to be corrected by postprocessing.

A mechanical flatbed scanner allows the removal, or at least the reduction, of these difficulties, but at the expense of scanning speed. Instruments of this type are commercially available as microdensitometers (Joyce-Loebl), scanning microphotometers (Leitz, Zeiss), or modified line scanners (Helena Laboratories, Hirschmann, LKB). Our investigations (5–7) have shown that the performance of such instruments can be improved considerably, even at speeds higher than usual. Based on that experience an instrument has been built in cooperation with Carl Zeiss, Oberkochen, F.R.G., and supported by the German Ministry for Research and Technology BMFT under contract MMT26. Its technical details are described in the following.

Mechanical and Optical Components

The most conspicuous part is the "black box" of Figure 1, a light-tight case 900 mm wide, 800 mm deep, and 230 mm high. The size is primarily determined by the size and the travel of the specimen-tray. The box houses the scanning stage and the optics for illumination, observation, and measurement. The XY-scanner GV88 and its controller TL17 are mass-produced articles of Micro Controle, Vitry, France. Both directions have a step size of 0.1 mm, a step frequency of up to 2000 steps per s, and a travel of 250 mm. Therefore, 200 mm can be scanned within 1 s; yet a meandering scan of an area 200 mm by 200 mm lasts for 2000 s (33.3 min) plus 400 s (7 min) for decelerating, reversing, and accelerating at the ends of the lines. The scanning stage is designed for automatic machining and accommodates heavy loads.

Two kinds of specimen-trays are used. Both have a clear area of 250 × 250 mm. One has a glass bottom to carry wet gels; the other accepts rigid samples, which are held in place by a magnetic bar. Ruled paper on top of the box (see Figure 1) allows one to determine the coordinates of an area of interest. The tray is inserted into the case through a slit behind a sliding door and correctly fixed by magnets on the scanning stage. During measurements, the sliding door must be closed, to exclude extraneous stray light.

Figure 2 is a diagram of the optical system for transmitted illumination. Long-distance lenses are used to allow 5-mm-thick samples on 1-mm glass. The wavelengths are selected by filters. The condenser Zeiss LD-Epiplan 4/0.1 images the measuring diaphragm onto the specimen. Its focus knob is underneath the black box. For a two-dimensional scan with a step size of 0.1 mm, the diaphragm is a circle with a diameter of 1.0 mm. Its image on the specimen has a diameter of 0.25 mm, which is approximately 2.44 times the step size. This important parameter is often neglected in practice. The sampling theorem requires that the highest frequency in the signal is below one half of the sampling frequency. Assuming a step size of 0.1 mm and a wavelength (λ) of 570 nm, Abbe’s formula 2kλ = 4π · nA/λ for the limit of resolution would require a numerical aperture nA = 10π · 0.000570/4π = 0.0014, which is impractical. In fact the illuminating aperture, 0.1, is 70 times larger. Therefore, band limitation has to be ensured by the measuring diaphragm. Linge et al. (7) have shown that a circle (not a square) with a diameter of 2 to 2.44 times the step size provides minimal distortion of the phase or the magnitude in the Fourier spectrum of the digital image. For one-dimensional scans the optimum diaphragm is a slit width equal to twice the step size.

The objective is a Zeiss Neofluar 6.3/0.2. Its focus knob is visible at the center of Figure 1. The numerical aperture is higher than in the illumination and even allows the collection of a considerable part of the light which was strewn within the specimen. Eyepieces facilitate the observation for checking position or focus. If the reflector underneath the eyepieces is removed, all light is directed to a photomultiplier.
er Hamamatsu R446. Neither the specimen nor the light source is sharply imaged onto the photocathode. An intermediate position was found to give better reproducibility. The optical system is a specialized microscope optimized for photometry, not for observation. The light source for transmitted illumination is a halogen-filament lamp, 12 V/100 W.

An epi-illumination insert to measure fluorescent gels or chromatograms fits between the objective Neofluar and the removable reflector. Figure 3 shows this device at the scanner; Figure 4 illustrates its elements. Its light source is a mercury arc lamp, Osram HBO 100W/2. All essential parts of the microscope are regular or modified components of Zeiss microscopes.

Electronic Components

Figure 2 also depicts a block diagram of the electronic components and their interconnections. The scanner is controlled by a laboratory computer MNC/DECLAB-23 (Digital Equipment Corp.) based on an LSI 11/23. A digital output module controls the scanning stage via its controller TL17, a digital input module reads the output of a tracker ball for manual control of the scanning stage and reads switches that indicate end-of-travel positions. The photometric signal enters the computer via a programmable preamplifier and a 12-bit analog-to-digital converter.

Two measures improve the quality of the photometric signal. One is monitoring the light source by a photodiode with an integrated amplifier (Centronic OSI 5J). About 5% of the illuminating beam is deflected by a glass plate behind the measuring diaphragm, so as to illuminate the photodiode through a heat-absorbing filter. The amplified output of the photomultiplier is divided by the low-pass filtered output of the photodiode, to compensate for fluctuations of the light source. The analog divider (Analog Devices 435K) acts like stabilizing the light source by a factor of 4 to 5 for transmitted light and 10 for epi-illumination. Details are discussed in ref. 5.
sets a limit of 4096 different levels for the recorded signal. To quantify the actual performance, we calculated the signal/noise ratio in four experiments each comprising 65,536 samples; it was 1160 for intensity data or 2589 for square roots of intensity measured at a rate of 2000 samples per second without moving the scanning stage. With a moving scanning stage and a glass plate as the object the signal/noise ratio was 501 for intensities or 1081 for square roots of intensities. Therefore, in practice the resolution comes close to the technical limit of 12 bits.

The photometric resolution was tested by scanning a Kodak gray step filter at a frequency of 2000 steps per second. Figure 5a shows the optical densities calculated from the recorded intensities of one line. High densities are degraded by noise, but one should keep in mind that the difference of 0.3 between the densities 3.6 and 3.3 is caused by just one step in intensity. Calculating the absorbances from the average intensities of adjacent lines reduces the quantization error considerably (Figure 5b). On using a wedged gray glass we found the deviation from linearity to be <0.005 for absorbances up to 1.0.

The spatial resolution was tested by scanning a Heidenhain grating of chromium bars on glass. The width of the bars varies from 1.25 to 100 line pairs per millimeter. (A "line pair" means a clear and an opaque bar of equal width.) Figure 6 shows a plot of the data scanned with a step size of 0.1 mm at a step frequency of 2000 steps per second. At 5.0 line pairs per millimeter the contrast is reduced to zero, indicating the limit of resolution in the normal mode of operation. For quantitative purposes, two line pairs per millimeter should be regarded as the practical limit of resolution. But there is a software option for a high-resolution mode, which allows several samples (e.g., eight) to be taken between two steps. Figure 7 is plot of the data scanned at a step frequency of 400 steps per second and a sampling frequency of 3200 readings per second. It demonstrates the resolution of 12.5 line pairs per millimeter with unreduced contrast and a resolution of 25 line pairs per millimeter with 50% contrast in the high-resolution mode, which is applicable to one dimension only. Figure 8 shows a densitometric high-resolution scan along 25 mm of an ultrathin isoelectric focusing of crude fungal pentosanase as described in ref. 8, where photographs and conventional scans of that gel are published.

Discussion

The progress in two-dimensional electrophoresis as a diagnostic tool requires reliable data for automatic evaluation or quantification. The scanner presented here has two specific merits for such applications. It represents the state of the art in the field, with the number of bits of the digital-to-analog and analog-to-digital converters being the same as in any instrument of this type. The high-density scanning appropriate for the analysis of ultrathin gels is sufficient for most applications. The resolution of two-dimensional gels is limited by the thickness of the gel, and this is therefore not a limiting factor.

![Fig. 3. Epi-illuminator, with high-pressure arc lamp OSRAM HBO 100W/2 inserted between the objective and the removable reflector below the eyepieces.](image)

![Fig. 4. Diagram of the elements of the modified Zeiss epi-illuminator IV FL. Telan system 1.1, 1.2 with lateral entrance 1.3 of illumination. Excitation filter 2.1 (G936), dichroic reflector 2.2 (FT935), and barrier filter 2.3 (LP420). Reference beam with beam splitter 3.1, imaging lens 3.2, and hybrid silicon photodiode with preamplifier 3.3 (CENTRONIC OSI SJ). Heat absorbing filter 4 (KG1), measuring diaphragm 5, three-element collector 6, and mercury arc lamp 6 (OSRAM HBO 100W/2).](image)

For further improvements the compensated signal passes an analog square-rooter and an anti-aliasing filter before it enters the programmable amplifier and the analog-to-digital converter. The extraction of the square root is a consequence of the Poisson distribution of photoelectrons. In the absence of other sources of noise, a signal S proportional to radiation intensity is composed of an ideal signal S0 and a noise term according to the formula S = S0 + 2N √S0, where the statistical parameter 2N has zero mean and unit standard deviation. By extracting the square root one gets as a very good approximation √S = √S0 + N, which states that the noise is independent of the signal and thus can be reduced by linear filters, if the signal is the square root of intensity. In addition, the signal/noise ratio √S0/N is twice as good as for intensities where it is S0/2N √S0 = √S0/2N. The applicability of the Poisson model in microphotometry is shown and more details are given in ref. 6. The square root is extracted by a feedback divider (Analog Devices 435K). Its error relative to a numerical result was found to be ±0.2% for absorbances up to A = 1, ±0.7% for absorbances up to A = 2, ±1.6% for absorbances up to A = 3, and ±2.0% for the range up to A = 4.

Performance Tests

The resolution of 12 bits of the analog-to-digital converter

![Fig. 5. Absorbances of a Kodak gray step filter, calculated from intensities measured at a wavelength of 589 nm, a step size of 0.1 mm and a scanning frequency of 2000 steps per second.](image)
Fig. 6. Plot of absorbance of a Heidenhain grating with chromium bars of various widths on glass.
A section with 1.25 to 5.0 line pairs per millimeter was scanned at a wavelength of 589 nm, a step size of 0.1 mm, and a step frequency of 2000 steps per second.

Fig. 7. Same as Fig. 6, but here a section with 12.5 to 50 line pairs per millimeter was scanned in the high-resolution mode with an effective step size of 0.0125 mm and a scanning frequency of 3200 samples per second.
At 12.5 line pairs per millimeter the measured amplitude is the same as for 1.25 line pairs per millimeter. The size of the measuring diaphragm in the plane of the grating was 0.025 mm × 0.1 mm.

...of the art in data acquisition for digital images by using an optimum measuring diaphragm to ensure band-limited optical signals and by extracting the square root of intensities to allow optimal noise reduction by linear filters. Neither feature is yet available in commercial instruments. Secondly, the essential parts of the scanner are available on the market, or modifications of such items. Therefore, the instrument can be built by institutes having access to mechanical and electronic workshops.

The compensation of fluctuations in the light source is not a new principle, but it is necessary to provide a photometric resolution of more than 1000 levels in intensity at a rate of 2000 samples per second. The speed may be low and the spatial and photometric resolution high for routine applications. It is, however, a suitable instrument for research work, because it accepts films, gels, and chromatograms and permits measurements of absorbance, transmittance, or fluorescence in transmitted light or with epi-illumination. Because of the measuring principle there is no influence of inhomogeneous illumination or local variances in the sensitivity of the sensor on the measured data, and stray light is reduced to a practical minimum.

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References