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Optical Errors in Scanning Stage Absorbance Cytophotometry.

I. Procedures for Correcting Apparent Integrated Absorbance Values for Distributional, Glare, and Diffraction Errors

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The main optical errors in the cytophotometric determination of the integrated absorbance of an ideal amplitude object are distributional error, glare error and diffraction error. Scanning reduces the magnitude of the distributional error considerably, but the remaining (residual) distributional error still may be important, depending on the shape and absorbance of the object and on the size of the smallest step of the scanning raster in relation to the object size. A theoretical relationship is derived for model objects which allows an estimation of the magnitude of this residual distributional error at various conditions of measurement. Glare is caused by optical imperfections, especially in the substage optics. Its effect is a uniform redistribution over the image plane of that fraction of the incident light that passes outside the projection of the measuring spot in the object plane. The magnitude of the resulting glare error depends on the glare fraction and on the absorbance of the object. It is shown theoretically that the local neighborhood of the measuring field contributes to the measured light intensity at the image plane. This is caused by diffraction, inherent to the wave nature of light. The resulting diffraction error, so far neglected in cytophotometry, is, like the residual distributional error, dependent on the shape and absorbance of the object and on the ratio step size to object size. The effect of this diffraction error is shown to be substantial for small and intensely stained objects like Feulgen-stained nuclei. KEY WORDS: Cytophotometry; Absorbance; Glare; Diffraction; Distributional error.

Introduction

The use of scanning cytophotometers for absorbance measurements in quantitative cytochemistry necessitates a sound knowledge of the factors influencing the accuracy and reproducibility of the measurements performed with such instruments.

In 1970 Mayall and Mendelsohn gave a general discussion of the sources of error in absorbance cytophotometry and more specifically the instrument errors encountered when using a mechanical scanner at the image plane. Next to systematic errors due to the chemical and physical processes involved in the staining and embedding procedure of the specimen, the systematic errors of the instrument were reported to be condensor aperture error, glare, chromatic error, and residual distributional error. The authors calculated the relative importance of each of these errors for the value of the integrated absorbance to be expected for three types of nuclei in model cells, all having the same amount of chromophore, but with local absorbances varying from 0.2 to 0.4 and area to circumference ratios between 0.14 and 0.38. Assuming a glare fraction of 1% over the entire object area, the total negative systematic error for the value of the integrated absorbance of these model nuclei was calculated to be between 0.7 and 6.5%, depending on the chosen parameters and on the assumptions made for the calculation. Diffraction effects were assumed to have influence only on those measuring fields actually containing the borderline of the object, causing a redistribution of light and lowering the effect of distributional error.

In a series of articles Goldstein (1970, 1971, 1975) discussed the influence of the same factors, based on theoretical as well as experimental investigations, for another type of scanning cytophotometer, the flying spot scanner. He developed a method for correcting the residual distributional error by measuring the object with various spot widths and extrapolating the values found to zero spot width. He also described a method for correcting glare errors by electronic

compensation of the light reaching the photomultiplier due to the apparent transmission of an opaque object of the same size. Such measurement of the substage glare by determination of the apparent transmission of an opaque particle has been the method generally used in cytophotometry. For image and flying spot scanners the glare correction found varies between 1–5%, depending on the measuring spot and the area of the illuminated field.

For stage scanning cytophotometers, this overall glare fraction can be kept below 1%, since only a very small cone of light illuminates the measuring area. However, in the literature so far there has been no recognition that diffraction phenomena cause a redistribution of light which, as with substage glare, depresses the measured absorbance values. This implies that in addition to the corrections for residual distributional error and for glare error, a correction for a diffraction error is necessary. Whereas the present article will be restricted to a theoretical investigation of the magnitude of the diffraction error and a reconsideration of the residual distributional error, both as functions of absorbance, measuring spot size,[1] and object size, a following communication (Duijndam et al., 1980) will deal with the application and evaluation of the theoretical correction factors on measurements made with a scanning stage cytophotometer.

Residual Distributional Error

Consider a square object with edge D and a homogeneous absorbance A_0 (transmission $T_0 = 10^{-A_0}$) in a background of absorbance zero that is scanned by a square spot with edge d ($d < D$) with step sizes S (Figure 1). Both the sides of the spot and the directions of the scanning raster are assumed to be parallel to those of the object. The expected sum of the measured absorbances for a randomly selected scanning raster is represented by the integral of the scanning raster over a square with side S of the sum of the measured absorbances of the corresponding spots not fully outside the boundaries of the object, divided by S^2. This is equivalent to $1/S^2$ times the integral of a spot center over the square with size $(D + 2d)$ in which the spot is not fully outside the boundaries of the object. The measured absorbance at a certain position of the spot is the negative 10 logarithm of the mean transmission of the object. The integral value A^1 of this parameter, now using natural logarithms, equals

$$A^1 = -(D-d)^2 \ln T_0 + 4d\left[\int_0^1 -(D-d)\ln(sT_0 + 1 - s)\right. \\ \left. + 2d\left\{\int_s^1 -\ln(1 - st + stT_0)\,dt\right\}ds\right] \quad (1)$$

[1]Since the resolution of a scanning table is limited by the smallest dimension of the scanning raster (step size) and the measuring spot size is usually adapted to cover the square determined by the step size, in the following considerations the spot size, unless specified otherwise, will be assumed to be equal to a step size of 0.5 μm.

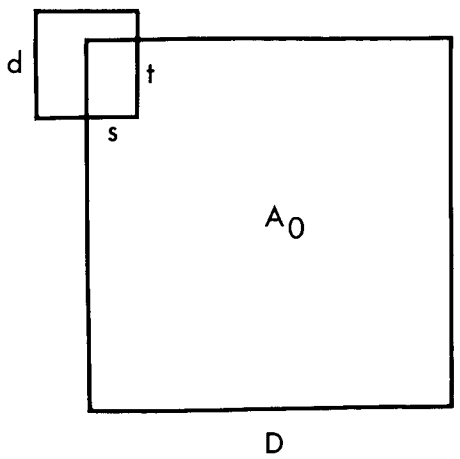


Figure 1. Theoretical model for the determination of the magnitude of the residual distributional error. A homogeneously stained object of length D with absorbance A_0 is scanned by a square measuring spot of size d.

The first part of this expression covers the area where the spot lies fully within the boundaries of the object, the second part describes the contribution of the four edges of the object, s representing the fraction of the spot edge over the object in the direction orthogonal to the considered edge, while t represents that fraction in the other direction. Equation (1), after calculation and rearrangement, equals

$$A^1 = \frac{T_0(D+d)^2 - (D-d)^2}{1 - T_0} \ln T_0 + 4Dd - 4d^2 \sum_{n=2}^{\infty} \frac{(1-T_0)^{n-1}}{n^2}$$

The expected sum of the measured absorbances equals $A^1/(S^2 \ln 10)$, so that for the expected apparent integrated absorbance EA_{app} the following equation holds:

$$EA_{app} = S^2 \frac{A^1}{(\ln 10)S^2} = \frac{(D-d)^2 - T_0(D+d)^2}{1 - T_0} A_0 \\ + \frac{4Dd - 4d^2 \sum_{n=2}^{\infty} \frac{(1-T_0)^{n-1}}{n^2}}{\ln 10} \quad (2)$$

If we compare the true integrated absorbance $A_{true} = A_0 D^2$ with the expected apparent integrated absorbance found in eq. (2), it follows that $EA_{app} = A_{true} + Bd + Cd^2$ (in which B and C represent functions of A_0 and D). It is clear that EA_{app} is a quadratic function of d and approaches A_{true} if d approaches zero.

If for nonsquare objects with heterogeneously divided local absorbances, we define the true integrated absorbance of objects scanned with circular spots as

$$\iint_{\text{object area}} (\text{absorbance})\,dx\,dy,$$

it is to be expected that the same kind of relationship between EA_{app} and A_{true} will hold, probably with other values for B and C. But because

$$A_{true} = \lim_{d \to 0} EA_{app}$$

still holds, A_{true} may be estimated by fitting a parabolic curve through the determined values for EA_{app} at various values of the spot size, and estimation of the value of this curve at zero spot size.

For measurements performed at one spot size only, eq. (2) can be used to calculate the expected error made when an object with a certain mean local absorbance is measured at a certain ratio between spot size and object size. For a number of values of this ratio and of the mean local absorbance such a calculation was performed. The results are graphically represented in Figure 2. Equation (2) only holds under the assumptions of square and homogeneously stained objects scanned by square spots. An indication of the effect of scanning inhomogeneously stained objects is given in Table 1, where an actual scan line through a stained chicken erythrocyte nucleus is compared with the scan line through the same object, but now with the same amount of chromophore homogeneously dispersed over the same area. The residual distributional errors calculated for the two cases show that the difference is relatively small.

The value for EA_{app} can be obtained exactly only when an infinite number of determinations should be performed, but it can be estimated by the mean of a finite number of measurements. The standard error of this estimate equals the

Table 1. *Dependence of the residual distributional error on the distribution of the chromophore. Comparison of a scan line through a stained chicken erythrocyte nucleus and through the same amount of chromophore homogeneously dispersed.*

Scan line through stained chicken erythrocyte nucleus		Scan line through hypothetical object with the same amount of chromophore homogeneously dispersed	
Actual absorbanee	Apparent absorbance from mean transmittance	Mean absorbance	Apparent absorbance from mean transmittance
0.01 0.61	0.205	0.00 0.923	0.252
0.61 1.19	0.810	0.923 0.923	0.923
1.19 1.38	1.280	0.923 0.923	0.923
1.38 1.30	1.341	0.923 0.923	0.923
1.30 0.91	1.063	0.923 0.923	0.923
0.91 0.15	0.381	0.923 0.923	0.923
0.15 0.00	0.069	0.923 0.00	0.252
True integrated absorbance	5.540	True integrated absorbance	5.540
Apparent integrated absorbance	5.149	Apparent integrated absorbance	5.119
Error 7.1%		Error 7.6%	

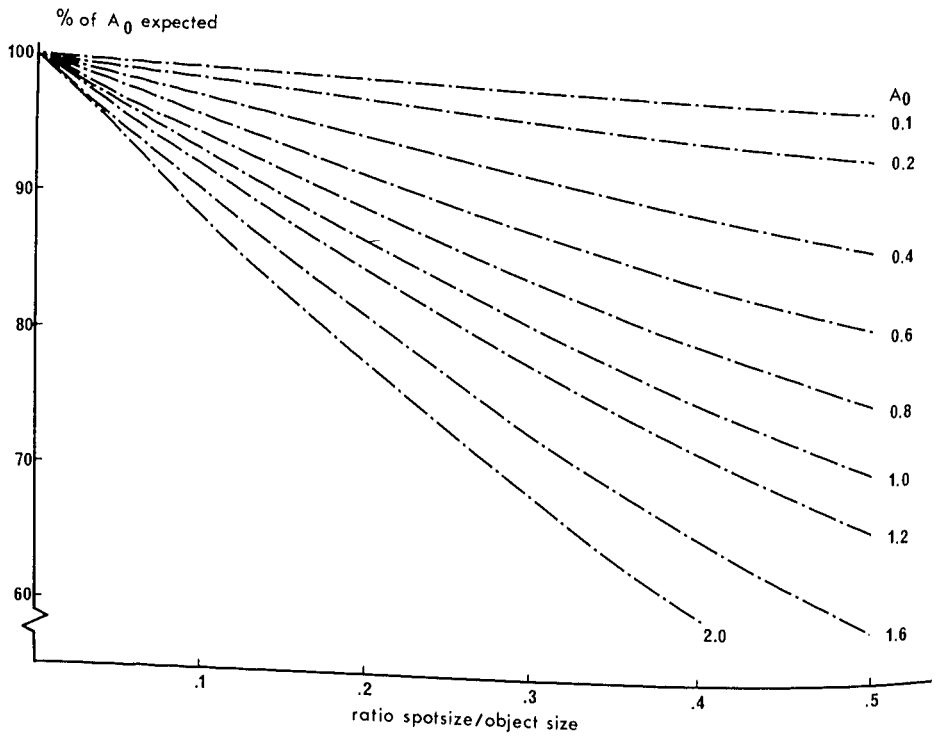


Figure 2. Calculated dependence of the residual distributional error on the ratio between spot size and object size (d/D) as a function of the mean local absorbance. Ordinate: percentage of true integrated absorbance.

standard deviation of a single observation A_{app} divided by the square root of the number of observations.

For the theoretical model described it is in principle possible to calculate the standard deviation of A_{app}, but such calculations become very tedious because this standard deviation is dependent on A_0, d/D, and S/D. When S is halved, for instance, the mean number of spots considered increases by a factor of 4 and the standard deviation will at least be halved. We therefore first consider the case where $S = d$. The theoretical maximum of A_{app} then equals A_{true} when D is a whole multiple of S and the spots cover only the object. In practice, most values of EA_{app} are relatively near to this maximum, and it can be expected that the standard deviation of A_{app} is approximately proportional to $1 - (EA_{app}/A_{true})$.

When spot size d is doubled while S is held equal to d, the quantity $1 - (EA_{app}/A_{true})$ is also approximately doubled because of the nearly linear relationship between EA_{app} and d/D (Figure 2). The standard deviation of A_{app} is therefore also approximately doubled. When, however, S is then halved (so that it equals its original value), the standard deviation is also approximately halved. Consequently, when d/D varies and S/D is kept constant, as is the case for normal measurements, the standard deviation is approximately constant. Hence the standard deviation is approximately proportional to $1 - (EA_{app}/A_{true})$ for that value of EA_{app} that is determined at $d = S$. This means that more determinations are necessary to obtain a certain standard error relative to A_{true} when either small objects (S/D high) or high absorbances are present. In practice, we are not dealing with square objects scanned by square spots that exactly cover the object. Therefore the theoretical maximum of A_{app} will not be reached and the standard deviation will even become lower than approximated for the model, although the same proportionality is still expected.

Redistribution of Light Caused by Diffraction

The microscope can be considered as a linear system performing a Fourier transform on the image resulting from the preceding lens (Goodman, 1968). The Fourier transform of an image in the object plane may be seen in the back-focal plane (Fraunhofer plane). If the image in the object plane is considered as the (complex) sum of two-dimensional sine waves with distinct values for spatial frequency, amplitude, and phase, then the intensity observed on a certain location of the back-focal plane is a measure for the amplitude of a wave, of which the spatial frequency is determined by the distance to the optical axis. This frequency increases linearly with the distance from the optical axis. The Fourier transform of the image in the back-focal plane results in the final image in the focal plane, limited by the measuring diaphragm. Because higher spatial frequencies in the original image fall outside the pupil of the object, this final image will not contain these higher frequencies, and therefore will be limited in spatial resolution.

Accordingly a point source in the object plane is imaged as a disk (Airy disk, Figure 3). The place of the first minimum is given by

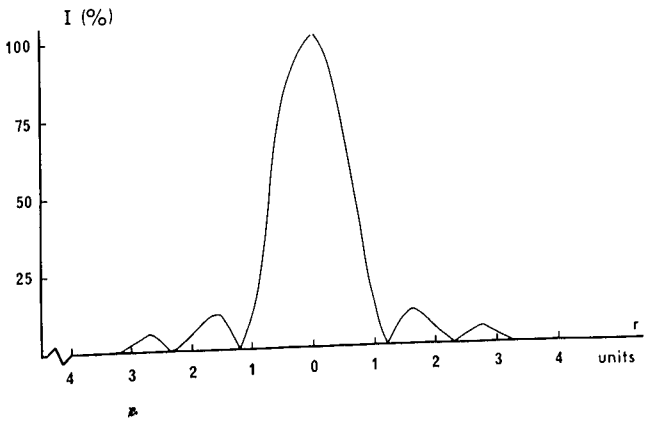
$$r_0 = 1.22\,\lambda\,\frac{d_0}{L},$$

in which λ is the wavelength of the light used, d_0 the distance from the object to the back-focal plane, and L the diameter of the objective pupil. For $\lambda = 560$ nm (pararosaniline-Schiff photometry) and $L/d_0 = 3.84$, r_0 equals 0.18 μm. If we want to determine the local intensities in the image plane due to the redistribution, depending on the radius r of the measuring spot, we have to calculate, in the manner of Sheppard and Choudhury (1977), the (complex) sum over the measuring diaphragm of the contribution of all point sources in the object plane imaged by the microscope. In Figure 4 this theoretical summation is displayed for an amplitude edge in the object plane, assuming incoherent light and right focus. Although these assumptions cannot be considered as completely realistic in practical microscopy, the procedure allows an estimation of the magnitude of the diffraction effect.

As may be judged from Figure 4 the influence of the diffraction effect on an edge results in 6% measured transmission at a distance of 0.25 μm from the edge in the object and 1% at 0.75 μm, using a spot diameter of 0.5 μm.

The effects described here find their origin entirely in the wave characteristics of light and are therefore inherent to the use of a microscope. The diffraction effect is not due to imperfections or dust in the optics and will be visible at any absorbance edge of the image in the object plane. In cytophotometry it should, therefore, be treated together with the effects of substage glare and distributional error.

Figure 3. Intensity $I(r)$ of a cross section through the center of the Airy disk as a function of the radius r; 1 unit = $\lambda d_0/L$, in our case 0.15 μm.



rected, since the highest absorbances of an object can be expected in the central area of that object. If the opaque object used for the correction has a higher perimeter to area ratio than the object, the effect of overcorrection will be enhanced. This may explain the relatively large variations in glare (between 5 and 13%) reported by Bedi and Goldstein (1976) for objects of 20–30 μm^2 and local absorbances in the range from 0.6 to 1.0.

According to our method, the determination of the substage glare fraction is performed by measuring the apparent transmission in the center of a large opaque particle. The contribution of the diffraction was derived theoretically, but also can be approached experimentally (Duijndam et al., 1980).

The method for correcting the residual distributional error described in this article is an extended version of the way in which Goldstein (1970) treated this phenomenon. The generalization made in the theoretical treatment allows estimations of the error made already from measurements of the apparent integrated absorbance performed at one spot size only. The relationship between spot size and apparent integrated absorbance was reported by Goldstein (1970) to be a linear one, although later Bedi and Goldstein (1976) pointed out a nearly linear relationship. From our theoretical treatise a quadratic relationship is expected, although for larger objects and lower absorbances the relationship approaches linearity. The assumption of linearity leads to extrapolated values at zero spot size which are estimated too low when the relationship is quadratic. Moreover, Goldstein (1970) discusses this relationship only in one direction, i.e., the direction of the scan line in the flying spot scanner. The shift to the next scan line, although small (0.4 μm), also introduces a distributional error leading to undercorrection. Although it is not stated explicitly why, Goldstein does report that his correction for residual distributional error is conservative, that is, leading to values which could be higher, but not lower. It is not clear, however, in which way Bedi and Goldstein (1976) can obtain results by application of this method that would mean a decrease in the value of the integrated absorbance after correction for residual distributional error, at least in a number of cases.

The methods described in this article to correct for residual distributional error and for diffraction error both tend to slight undercorrections, because for both methods the apparent mean local absorbance is used, accepting that this value is already lowered by the optical errors.

To obtain cytophotometric data largely free from the optical errors described the following possibilities seem promising.

In a computer-directed stage scanner, the corrections for diffraction and glare error (after separate determination of the glare fraction by measuring the apparent transmission in the center of an opaque particle), as well as the correction for the residual distributional error, could be incorporated in the scanning program to obtain directly a corrected value of the integrated absorbance.

Recently Brakenhoff et al. (1979) (cf. also Sheppard et al. (1977)), described a confocal scanning microscope with a laser beam as the light source. He reported that higher resolution and better image quality could be obtained. This instrument is largely free of diffraction and glare errors because of the coinciding illuminating and measuring diaphragms. For cytophotometric measurements it should, therefore, be an ideal instrument since only the residual distributional error remains as a systematic optical error.

A third way to obtain corrected results directly could be the combination of the recently developed computer program BICOSCAN (Van der Ploeg et al., 1979), a simultaneous two-wavelength scanning procedure, with the two-wavelength method of Patau (1952) and Ornstein (1952), provided that the chromophore spectrum is known to be constant.

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