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THE DISTRIBUTIONAL ERROR PROBLEM IN CYTOPHOTOMETRY

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ABSTRACT

Use of that portion of the characteristic curve of photographic film in which transmission is linear with log exposure eliminates the distributional error and thus makes possible the assessment of total mass of an inhomogeneously distributed cell constituent without scanning or measuring the cell's projected area. Measurement consists of obtaining the difference in output of a photocell receiving the total light transmitted by a photomicrograph of the cell, and a photomicrograph of the microscope field illumination against which the cell was photographed. A method of making such measurements is given, and its basis is described.

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Distributional error in cytophotometry arises as a result of inhomogeneous distribution of absorbing material in the specimen. Its origin can be visualized easily as follows: Consider a uniform sheet, one unit square, which transmits half of the light incident upon it. A light meter collecting the light passing through this sheet reads 50%. However, if this material is divided into four equal squares and these are superimposed on one another in an aperture one unit square, thus occupying a quarter of the area of the aperture, the meter reads $3/4 \times 1 + (1/4)(1/2)^4$, or 76.6%. Similarly, in microspectrophotometric assay, nonuniform distribution of cellular material leads to low estimates of the quantity of absorbing substance.¹ This error is known as the distributional error. It can be avoided in several ways, for instance: (a) by scanning the cell image (optical or photographic) with a microphotometer or densitometer provided with a suitable function-transforming and integrating system,^{2, 3} (b) by measuring the transmission of a plug through a cell or nucleus-- i. e., a cylinder through an area of the absorbing body where the distribution of absorbing material can be considered to be uniform--and correcting on the basis of an assumed geometry of absorbing material, e. g., a sphere⁴, (c) by using a crushing condenser that flattens the cellular contents⁵, (d) by using a two-wave-length method appropriate to stained

constituents,^{6, 7} or (e) by photographic methods, of which one involves a quantitative analysis of the silver in a photomicrograph,⁷ and another is presented below.

DISCUSSION

Microspectrophotometric determinations are predicated on the validity of the Beer-Bouguer law,

$$\epsilon = \log \frac{1}{\tau} = k \left(\frac{m}{a} \right),$$

which describes the exponential nature of the light-absorption process. Here τ is the optical transmission of the specimen, ϵ is the specimen extinction, and m/a is the mass per area, or superficial concentration, of absorbing material of extinction coefficient k .

In evaluating photomicrographs of cells, use has frequently been made of the low-exposure region of the characteristic curve of photographic film where the transmission t of the developed film is linear with the log of exposure E , or with extinction ϵ , i. e.,

$$t = a + b\epsilon.$$

Exposure is related to extinction as follows:

$$\epsilon = \log E_0 - \log E,$$

where E_0 is an exposure corresponding to $\epsilon = 0$. Both E and ϵ are measures of the light transmitted, either by the specimen or by a sector wheel used for film sensitometry.

The argument presented here is that, if the transmission t of the developed film is linear with the extinction ϵ of the specimen or the sector wheel, then it is possible to construct a simple photometer that integrates, without scanning, the total extinction, or mass, of a nonuniformly distributed cellular constituent. The film, in this case, performs the function

transformation made necessary by the exponential attenuation of light by the specimen. A procedure for obtaining, in register, consecutive photometer readings of a photograph of a field containing a cell, and one containing no cell, is essential to this technique because of the nonuniformity of microscope field illumination. Such a technique is described.

The linearity of film transmission t with ϵ , over a certain range, is expected from the following considerations. Assume that, at low exposure, the probability that a photon sensitizes a silver halide grain in the emulsion is not affected by previous exposure; also that we deal with a population of silver grains that require only a single photon for sensitization. Then the number of sensitized silver grains per unit area of film is

$$N = pE,$$

where p , a constant, relates exposure E to the number of photons falling on a unit area of emulsion, and also contains a term for probability of interaction between photons and silver halide grains. We further say that the transmission of light by the developed film is related to the number N of silver grains by the exponential law,

$$\ln I/I_0 = \ln t = -qN,$$

where q measures the size and opacity of the developed silver grains, and I and I_0 are respectively the attenuated and reference intensities of the photometer beam. Then

$$\ln 1/t = 2.3 \log 1/t = qpE,$$

and

$$\log \log 1/t = \log (qp/2.3) + \log E.$$

Carlson,⁸ who has proposed a method very similar to this one for the evaluation of dry mass in soft-x-ray microradiography, has pointed out that, through a limited range, $\log \log 1/t$ is linear with $-t$, which allows us to write, with r and b as constants,

$$t = r - b \log \log 1/t,$$

therefore

$$t = r - b(\log qp/2.3 + \log E).$$

Substituting from the previously stated relationship between E and ϵ , we have

$$t = [r - b(\log qp/2.3 + \log E_0)] + b\epsilon,$$

or, if the bracketed constants are expressed as one constant, a ,

$$t = a + b\epsilon.$$

The relationship pointed out by Carlson can be verified to hold for a range of t of 25 to 60% with a slope, b , of 0.82. It would be surprising to find that the slope of t plotted against $\log E$ did not vary with film emulsion type and development conditions, and indeed our sensitometric measurements yielded slopes between 0.7 and 1.0 as conditions were varied. With suitable development conditions, the range of ϵ linearity can generally be made longer than 0.5. This adequately covers the ultraviolet (nucleic acid) extinction of many cells. Eastman Kodak type 103-0 spectrographic film, exposed through a sector wheel to unfiltered tungsten illumination, and tank-developed with Agfa Rodinal 1:25 at 20°C for 20 min, yielded a linear region of slope 0.95 covering a t range of 30 to 80%, and a range of ϵ of 0.53.

On this plot, none of six sensitometric points was further than .01 unit, or 1% transmission unit, from a straight line fitting all six points. Densitometry was done with a Jarrell-Ash spectrographic densitometer. Good characteristics were also obtained with a variety of films available for amateur and commercial photography with several commercially available developers.

The contention that the linearity of transmission t with extinction ϵ makes possible a non-scanning photometric assay of total mass is essentially the same as the contention that, having weight as a linear function of number of, say, pennies, one may determine the number of pennies with a balance without knowing how the pennies are stacked on the balance pan.

Formally, the argument is as follows: Let us determine the average transmission T over a frame of area F of developed film, exposed so that t is everywhere linear with ϵ . In practice, with the film described above, the exposure is made so that the density of the cell-less part of the photographic image (the background) corresponds to a transmission of about 30%; this exposure is designated E_0 so that $\epsilon = 0$ at $t = 30\%$. The frame is supposed to contain a cell image of nonuniform extinction ϵ_c , and the background to have a variable extinction ϵ_{bg} .

To determine the average transmission T we move a photocell, which collects the light passed by a small area a of the film, n times, so as to cover the frame area F . We obtain the average transmission of the cell plus the background,

$$T_{c+bg} = \frac{1}{n} \sum_{i=1}^{i=n=\frac{F}{a}} [a_i + b(\epsilon_c + \epsilon_{bg})_i] = a + \frac{b}{n} \sum \epsilon_{c_i} + \frac{b}{n} \sum \epsilon_{bg_i}$$

That ϵ_{c+bg} is equal to $\epsilon_c + \epsilon_{bg}$ follows from the logarithmic dependence of ϵ on the specimen transmission, namely

$$\epsilon_{c+bg} = \log \frac{1}{T_{c+bg}} = \log \frac{1}{T_c} \cdot \frac{1}{T_{bg}} = \log \frac{1}{T_c} + \log \frac{1}{T_{bg}} = \epsilon_c + \epsilon_{bg}$$

The necessity of assigning a transmission or extinction to each subarea of the background results from the nonuniformity of microscope field illumination.

Similarly we read a cell-less photograph of the same area of the microscope field illumination against which the cell was photographed.

We obtain

$$T_{bg} = \frac{1}{n} \sum_{i=1}^{i=n=\frac{F}{a}} [a_i + b\epsilon_{bg_i}] = a + \frac{b}{n} \sum \epsilon_{bg_i}$$

The Beer-Bouguer law is applied to the difference,

$$T_c = T_{c+bg} - T_{bg} = \frac{b}{n} \sum_{i=1}^{i=n=\frac{F}{a}} \epsilon_{c_i} = \frac{bk}{n} \sum \left(\frac{m_i}{a}\right)_i = \frac{bk}{n} \sum m_i \frac{n}{F} = \frac{kb}{F} M,$$

where M is the total mass of the absorbing constituent.

The term n for the number of divisions of the frame has dropped out, and we conclude that the measurement can be made as well with one reading of one photcell that collects, at once, all the light passed by the frame, as with n readings from a photcell that measures the light passed by n separate subareas of the frame, the latter being equivalent to scanning.

The projected area of the cell does not affect the measurement. The frame area F becomes a constant of the system, but should be chosen small to increase accuracy. In practice, the frame is a mask that excludes all the background except a small periphery around the cell image on the photograph.

PROCEDURE

A method of carrying out these operations in the laboratory is being developed. A photomicrographic exposure of the cell is made at a suitable wave length on 35-mm film. The cell is taken out of the field with the mechanical stage controls, and another photograph is taken of the microscope field illumination. The film is developed and projected with a 35-mm slide projector on an opal glass screen, the back of which is viewed by a photocell at a sufficient distance so that the position of the image on the screen does not seriously affect the photocell output. Any one of a series of frames consisting of circular openings of various diameters in cardboard rectangles can be hung over the opal glass screen so as to include a minimum of background with the cell image as seen by the photocell.

For a measurement, the photometer is zeroed with no light, and a frame is selected and hung over the opal glass screen. The system is adjusted, either at the light source or at the photocell, to give a reading of 100% when a clear (unexposed, developed) section of film, or better, when an appropriate calibrated neutral-density filter is in the projector. The latter allows fuller utilization of the photometer scale. Then readings are taken, first with a cell image in the frame, and then with a corresponding section of background (a separate photograph) in the frame. The

registering of these two consecutive images in the frame can be accomplished by means of a fiducial mark adjacent to the opal glass that indicates, say, a corner of the 35-mm frame.

From the difference of the readings, the frame constant F , and the film characteristic b , one may calculate the total extinction due to the absorbing constituent of interest, or--if the extinction coefficient k is known--the total mass.

Actually, such calculations are subject to corrections for errors due to scattering or nonspecific light loss in the specimen, glare and focusing errors in the microscope, etc. For a detailed treatment of these errors, reference is made to other discussions.⁹⁻¹¹

However, when relative rather than absolute quantities are sought, and cytophotometry is applied to the determination of amounts of absorbing constituents before and after a specific extractive procedure, some of the errors cancel, and with the correction of the distributional error, quite accurate results should be obtainable.

In the method described here, it is of course essential to control exposure rigidly. This may be accomplished to some extent by regulation of power to the microscope (monochromator) lamp, and is better accomplished by the use of an integrating photometer that watches the microscope illumination and closes the camera shutter after a given exposure. Also, it should be mentioned that, whereas the nonuniformity of the microscope field illumination is corrected for by this method, any irregularities in the illumination of the photometer system will affect the results, as also, of course, will glare in the photometer system, nonuniformities in the film, irregularities in and noncellular matter on the microscope slide,

and nonlinearity in the photometer response.

Also, a source of error arises in the departure from conditions of specular absorption in the photometer system. To the extent that light scattered from the silver grains is not eliminated from the image of the film projected on the opal glass, there will ensue (a) a difference between the film characteristic curve as determined by the photometer and by a densitometer which measures specular transmission (i. e., light passed after all absorbed and scattered light is eliminated from the beam), and (b) a position effect, related to the centering of the cell image (on the photograph) with respect to the projection lens. The latter leads to erroneously high measurements for cell images off the optical axis of the projection system.

Errors occurring for reason (a) can be avoided by either performing the densitometric determination of the film characteristic directly with the photometer, or by correcting the film characteristic curve, as determined with a densitometer, for the difference of the optical geometry of the two systems. The error (b) can be minimized by using a projection lens of low aperture, i. e., high $1/f$ value, and by centering the cell image in the 35-mm frame, and in the projector.

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