The Two-Wavelength Method of Microspectrophotometry

III. An Extension Based on Photographic Color Transparencies*

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ABSTRACT

The two-wavelength method of microspectrophotometry corrects for distributional error and measures the amount of absorbing material by taking advantage of certain spectral characteristics of the specimen. Under certain circumstances, such as the absorption of nucleic acids in the ultraviolet and of black or multiple stains in the visible, the spectral characteristics are not suitable for the application of the method. To circumvent this, a photomicrograph of the object is taken with monochromatic light of a suitable wavelength. A second plate is exposed as a contact print of the photomicrograph and is developed in the presence of a coupling agent. After bleaching and fixation, the positive appears as a monochromatic color transparency. Two-wavelength analysis of such a transparency can be made in terms of the new color. The measurements will be free of distributional error and can be equated to the original object. The necessary formulae are derived, and a method which has proven suitable for color development is given. The photographic and the direct two-wavelength method were found to give equivalent results when both were used on the same series of liver nuclei. The application of the photographic method to ultraviolet absorption has been demonstrated. The new method is potentially applicable to other types of photographic densitometry involving heterogeneous images.

Effective as it may be for the photometry of certain types of objects, the two-wavelength method (2–4) has certain limitations imposed by its own logical structure. These limitations involve the spectral requirements of the method, and can be grouped in three categories.

First, the chromophore to be measured, or its packages when these are the basic unit in which the chromophore appears, must be available somewhere in the specimen in a random distribution suitable for the determination of a spectal absorption curve. Unless one makes the unwarranted assumption that the choice of wavelengths can be made from another population with attributes similar to the one being measured, certain materials which are consistently heterogeneous must be excluded.

Second, the chromophore must have an absorption curve with sufficient contrast to supply the 1:2 ratio of absorbances necessary for the method. This requirement excludes grey stains.

Third, there must be only one independent chromophore, as two or more chromophores with overlapping absorption spectra will not provide constant ratios of absorbance throughout the object. This rules out compound stains, and the use of metachromatic dyes. Material containing a single dye will be invalidated, if there is sufficient background color to the unstained object. The protein peak adjacent to that of the nucleic acids precludes the measurement of the nucleic acids in the ultraviolet region.

A modification of the two-wavelength method has been devised which circumvents these limita-

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tions and greatly extends the applicability of the method. The principle of the new method is the photographic reproduction of the object using one wavelength, and the conversion of this image into a colored transparency. Two-wavelength analysis of the transparency based on the absorption characteristics of the dye introduced during development may then be referred back to the object, thus avoiding interference by the characteristics of the object itself. This will be referred to as the "photographic," in contrast to the "direct" two-wavelength method.

Theory:

The photographic two-wavelength method is based on three suppositions.

1. The photographic image formed by monochromatic light transmitted through an object quantitatively records the distribution and amount of absorption in the object. This, the basis of conventional photographic densitometry, is usually expressed in terms of the H-D response of the emulsion (1). When the density of the photograph, defined as the negative logarithm of the transmission, is plotted against the logarithm of the exposure, the result is a sigmoid curve. The central section of this curve will be an almost straight line whose slope (γ) and extent are characteristics of the emulsion and the method of development. When light falling on the emulsion has been transmitted through an absorbing object, the exposure will be modified by the transmission of the object. If the incident light was uniform, the relative intensities of the transmitted and incident light for the object and its background are equivalent to exposure. Transmission, in this sense, can be normalized on the abscissa of the H-D grid. The negative density of the various parts of the photographic image will then be proportional to the absorbances of the corresponding parts of the object. The retention of the distributional qualities of the object will be a function of the resolution of the reproduction, and is the essence of good photomicrography.

2. The black and white photographic negative can be accurately reproduced as a positive with the image now rendered in a single color. The making of a photographic positive does not affect the validity of the quantitative representation, provided the requirements are again met for uniform illumination, correct exposure, uniform processing, etc. The simplest approach to making a positive colored transparency is by contact printing, followed by development in the presence of a suitable coupling agent. The conversion of Ag^+ ions to insoluble silver is associated with the oxidation of the coupling agent to a colored derivative which is bound in the emulsion *in situ* with the silver. Subsequent bleaching of the silver and its removal with hypo leaves the dye unaffected. The result is a positive with the dye distributed in the same manner as the original (but now absent) silver grains. The quantitative reliability of the transformation, including the residual constancy of grain-size, can be assumed if the H-D curve of the positive—as determined with monochromatic light—is analogous in shape to that of its negative.

3. The amount of color in the positive, and hence the amount of chromophore in the object, can be determined by a two-wavelength analysis of the positive. The arguments for this are identical to those developed by Ornstein and Patau for the direct method. When the measurements are made on the basis of wavelengths defined from homogeneous areas of the photograph, the results will be in terms of grain packages. A more exacting, but not more useful, basis for dye measurement would result if the wavelengths were defined from an area of truly random (non-granular) dye distribution (such as one produced in the emulsion by a chemical—in contrast to a photographic—method). Since the amount of dye per grain in any one emulsion is constant and the desired parameter is the number of grains per unit area, the two methods of expressing the results are equivalent.

The formulae for the photographic two-wavelength method are based on the measurement of photographic transmission between areas which (*a*) are all on the straight portion of the H-D curve, and (*b*) correspond to areas of the object which were subjected to a uniform intensity of monochromatic light whose transmission affected the exposure. The subscript o refers to the object in terms of the wavelength used to expose the negative. The subscript p refers to the photographic positive (or transparency), with the γ and density measurements taken at a single wavelength (*a*). According to the H-D relationship, for a homogeneous area, B_i, the photographic density, D_i, can be expressed as

$$D_i = \gamma \log \frac{1}{T_{io}},$$

in which T is the transmission. For a series of n homogeneous areas which include the entire object,

$$\sum_1^n D_i = \gamma \sum_1^n \log \frac{1}{T_{io}}, \text{ or } \sum_1^n B_{ip} D_i = \gamma M^2 \sum_1^n B_{io} \log \frac{1}{T_{io}}.$$

M is the magnification of the photograph. The Beer-Lambert law introduces k, the absorptivity, and defines the amount of chromophore in the object as

$$m_{to} = \frac{1}{k_o} \sum_1^n B_{io} \log \frac{1}{T_{io}}.$$

Therefore,

$$m_{to} = \frac{1}{k_o \gamma M^2} \sum_1^n B_{ip} D_i.$$

Referring again to the Beer-Lambert law, the photographic parameters are given as

$$\sum_1^n B_{ip} D_i = m_{tp} k_p.$$

With the two-wavelength equations (4),

$$m_{tp} = \frac{B_p L_{ap} C_p}{k_{ap} \ln 10},$$

in which C is a correction factor, L is the light loss, or $(1 - T)$, and the two wavelengths a and b are chosen so that $2 k_{ap} = k_{bp}$.

$$m_{to} = \frac{B_p L_{ap} C_p}{k_o \gamma_a M^2 \ln 10}.$$

Thus the total amount of chromophore in the object can be determined from parameters of the positive, the only other factor being the absorptivity (k_o) of the object for the wavelength at which the negative was taken. For relative measurements—and most of the reported measurements in microspectrophotometry are relative—k_o can be ignored, and the determination involves only the measurement of the positive.

The Preparation of Transparencies:

The photographic two-wavelength method was tested by comparison with the direct method, using identical objects with each. A smear preparation of isolated rat liver cells was used. The cells were fixed by freeze-substitution, digested with RNase, stained with gallocyanin-chrome alum, and mounted in an oil of matching refractive index. A field containing about 70 cells was illuminated by a sodium lamp and photographed at a magnification of 135 times. The lamp required filters (Ilford No. 808, mercury yellow, and No. 606, spectrum yellow) to reduce the output as viewed by a spectroscope to the two sodium lines at 5893 A. The optical components were an objective of N.A. 0.45, a four times eyepiece, and an immersion condenser set to a N.A. of 0.25. Ilford R 20 quarter plate was chosen because of its sensitivity to yellow light, and its straight line response over a relatively wide range of exposure. In order to minimize glare, the area of the image was defined by a field-stop, and was two-thirds of the available area of the plate. Using the transmitted light from an empty field, a photographic step wedge was contact printed next to the image of the cells. By trial and error, the exposure of the cells and their background was adjusted to give densities on the linear portion of the plate response, and the wedge was exposed to give densities which encompassed those of the image. The plate was developed in I.D.2 (Ilford) for 2 minutes at room temperature with continuous agitation. It was

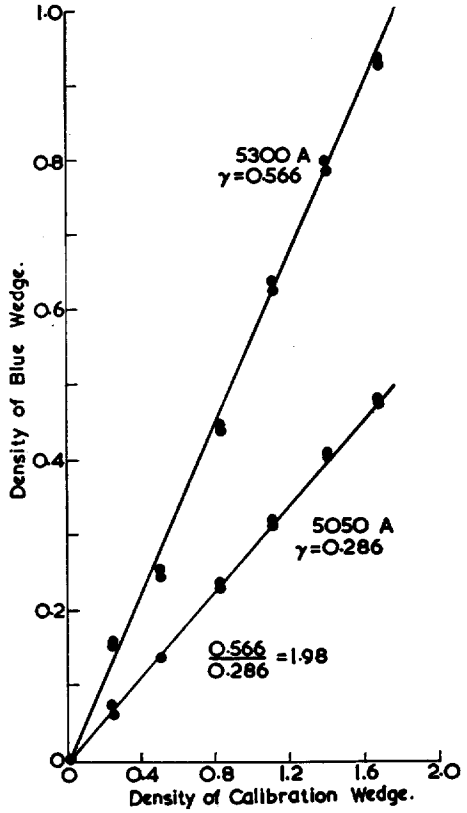

FIG. 1. H-D curves of the blue transparency. The straightness of the lines indicates the reliability of the dye-coupling process.

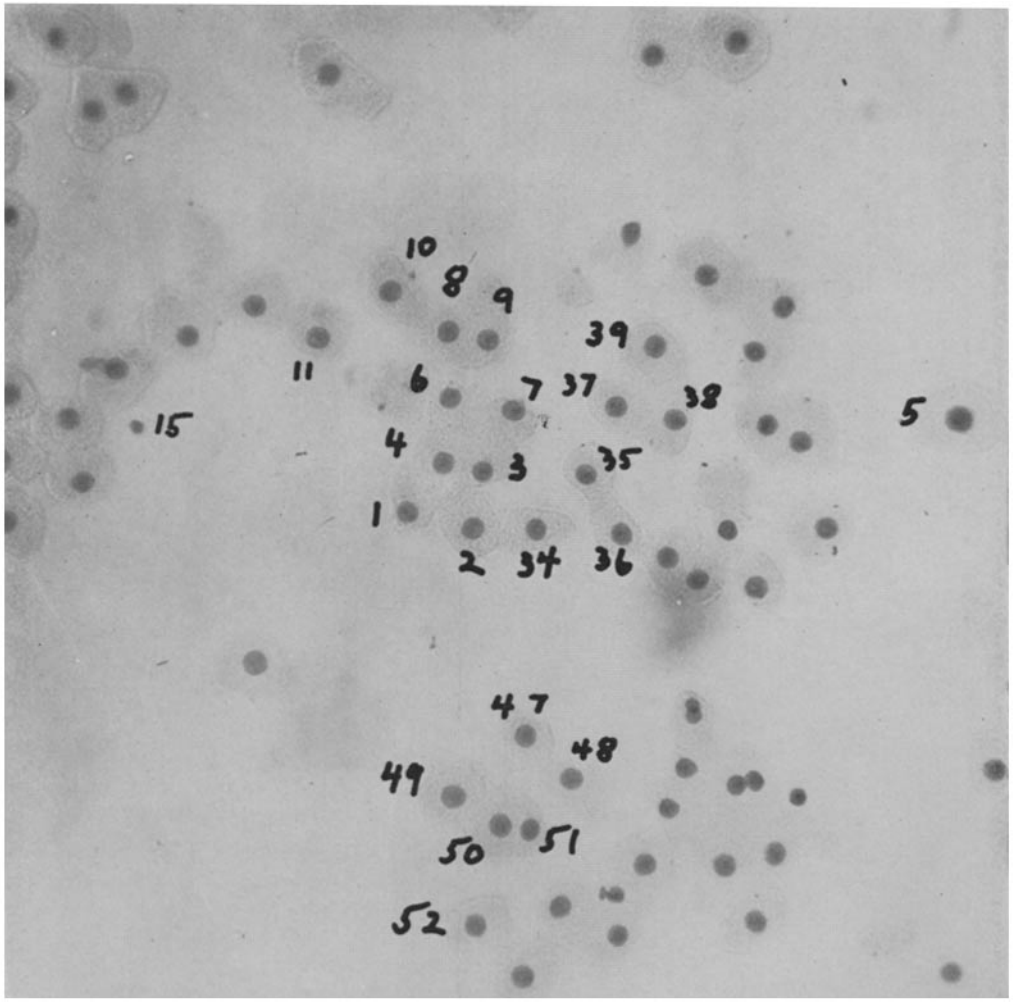

FIG. 2. The liver cells used to compare the two methods of photometry. Apart from 5 and 15, the numbered nuclei were the ones used for the test series. Magnification, 300. N.A., 0.45.

fixed and dried in a routine manner. The wedge reproduced on this negative gave a linear response of density.

The positive was made by contact-printing the negative on to Ilford N 25 plate. White light with a uniform distribution was obtained from a photographic enlarger by raising it to its maximum height, masking the field to give an appropriate area of illumination, and partially closing the iris diaphragm. The two plates were pressed together with their emulsions in contact and were exposed with the negative towards the light source. The positive was developed according to the following formula:

(*a*) Sodium carbonate (decahydrate)	80 gm.
(*b*) 2:4-dichlor-α-naphthol	2 gm.
Ascorbic acid	100 mg.
(*c*) Potassium bromide	2 gm.
Sodium sulfite	5 gm.
N:*N*-diethylparaphenylenediamine sulfite	2 gm.

Dissolve (*a*) in 400 ml. of distilled water, and add (*b*) dissolved in 100 ml. of methyl alcohol. Dissolve (*c*) in water, add it to the above mixture, and make up to 1 liter final volume with water. On standing, the solution becomes progressively blue in color, and there is a decrease in the γ obtained. Stock solutions have been replaced after a week or two. A bleach solution consisted of 5 gm. of potassium ferricyanide and 10 gm. of potassium bromide per 100 ml. of water. The fixative was 20 per cent sodium thiosulfite in water.

Development for 6 to 9 minutes at 20°C. was carried out in open trays with continuous agitation. A wash in running water for a minimum of 2 minutes was inter-

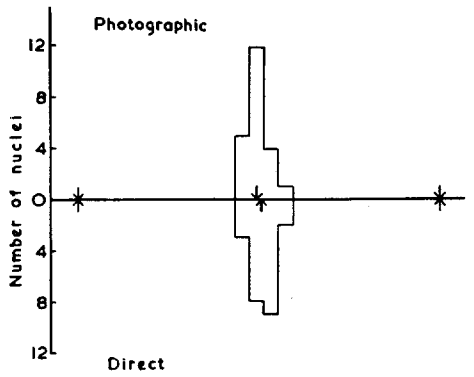

FIG. 3. A histogram of the results obtained with the two methods. The amount of chromophore per nucleus has been plotted on a logarithmic scale along the abscissa. The units are arbitrary, but are common to both methods. The arrows within the bars indicate the means for each series, and the arrows to each side indicate the halving and doubling values for the combined mean. Each bar represents a 5 per cent increment in the amount of chromophore.

spersed between each step of the procedure. The usual routine involved development, fixation, bleaching, and then a second exposure to fixative; but equivalent results have been obtained by following development with bleaching and then ending with a single fixation. The entire routine must be carried out with safelight precautions, since an acid fixing bath is contraindicated by the dye. The resulting plates have a clear blue color, with the dye deposited in discrete microscopic grains.

The Photometry:

The details of the object are more effectively rendered when the photographic reproduction is macroscopic in size. For the present purpose, it was advantageous to measure the transparencies on the same instrument used for measuring the object. Therefore, the microspectrophotometer was modified to permit plates to be read without the magnification of the objective. This was achieved by inserting the plate into the optical path immediately below the eyepiece in the plane of focus of the objective. The tube of the microscope was replaced by a table which could be adjusted in height. It had a central glass-covered perforation. The details are shown in the first paper in this series (2). A plate lying on the table will appear in focus through the upper viewing system, and the limiting diaphragm functions as it does for direct readings.

In Fig. 1, the wedge density of the blue plate has been plotted against the density of the calibration wedge. The two lines depicted were representative of the family of straight lines obtained by measuring the blue wedge at different wavelengths. These lines all passed through the same intercept on the graph. Since each step of the wedge represented a uniform density, the γ served as a composite absorbance and was convenient for the selection of appropriate wavelengths. Thus, the slopes of the two lines which are shown in the figure indicated that their wavelengths were adequate for two-wavelength measurements. The values obtained for γ reproduced to within 1 per cent when tested on the same plate.

A series of twenty-two nuclei was chosen; each nucleus was identified with a number, as shown in Fig. 2. The amount of chromophore corresponding to each nucleus was determined twice. A routine analogous to the one described by Patau was used; the nuclei were fitted closely within the photometric field, and were measured at the wavelengths shown in Fig. 1. The blank fields were always taken immediately adjacent to the cell.

The direct two-wavelength measurements were made after relocating the same cells on the original slide. The wavelengths 4800 and 5700 A with absorbances in the ratio of 1:2 were defined from several apparently homogeneous nuclei. The wavelength 5893 A was found to give an absorbance 1.92 times that at 4800 A. Using the same technique of measurement, the series was again read twice.

Since the final projected magnification of the image in both methods was identical, the calcula-

TABLE I

Statistical Analysis of Photographic and Direct Two-Wavelength Methods

	Direct method	Photographic method	Combined data
Mean, *arbitrary units*	1.487	1.460	1.473
S.D. of replication, (*per cent*)	3.0	4.6	4.4
S.D. of means for each nucleus, (*per cent*)	3.8	4.6	4.2
F test	3.19*	2.06‡	3.53‡
S.D. of nuclei, (*per cent*)	3.2	3.3	3.5

S.D. refers to standard deviation as per cent of mean.

The F test was done on the variance of means/variance of replication. * is significant to less than 1 per cent, and ‡ is significant to 5 per cent (5).

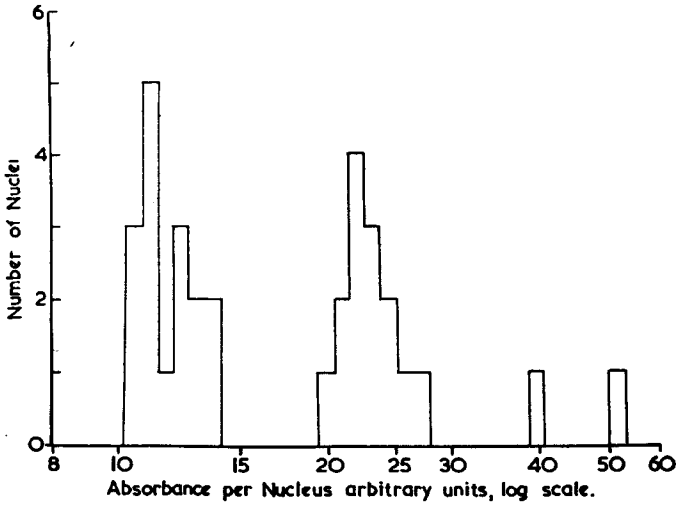

FIG. 4. A histogram of the results obtained from ultraviolet photomicrographs with the photographic two-wavelength method. The amount of chromophore has been determined for 32 nuclei from three plates. The smear preparation consisted of isolated rat liver nuclei, fixed by freeze-substitution and digested with RNase.

tions were simplified by the omission of M. In addition, with the known ratio of k_o to k_{ao} (1.92), the results of the two methods were stated in the same arbitrary units.

These results have been graphed as a histogram in Fig. 3, and their statistical parameters are given in Table I (5). The means of the two series were not significantly different ($t = 0.86$; $P > 0.4$). In both cases, analysis of variance indicated that the two readings for each nucleus were sufficient to resolve the differences between nuclei (F test). This was also shown to be true when the four readings for each nucleus, the photographic and the direct, were pooled together, indicating that the two methods were seeing the same variation. The photographic method had a slightly greater standard deviation than the direct method. The photographic readings obtained from nucleus 5 and 15, respectively, were 2.12 and 0.45 times the mean of the remaining nuclei. These were not part of the measured series, but they probably represent multiples of dye content to each side of the peak containing the bulk of the cells.

Preliminary Ultraviolet Results:

The photographic method has been tested on ultraviolet photomicrographs taken at 2536 A. Isolated nuclei from freshly homogenized rat liver were fixed by freeze-substitution, digested with RNase, and mounted in glycerin. The negatives obtained from a series of cells were printed as transparencies and read by the photographic two-wavelength method. The pooled results from three such transparencies are shown in Fig. 4. The values correspond to roughly 30 per cent more than would be predicted from chemical measurements on similar material; they are given in arbitrary units because the correction for non-specific light loss has not yet been made. Eventually, these results will be compared to parallel estimates obtained with a recording densitometer.

DISCUSSION

With his original description of the two-wavelength method, Ornstein suggested another way of avoiding distributional error (3). This was based on a photographic reproduction of the object, followed by an analysis of the photograph for its silver content. Although cumbersome, this was effectively a grain-counting procedure. In a sense, the photographic two-wavelength method carries the argument one step further by converting the silver to a convenient dye. Again, the dye can be eluted and measured directly; but it is advantageous to analyse it *in situ* without dissection of the photograph.

A blue dye was chosen for the transparency to match the performance characteristics of the microspectrophotometer. Fortunately, the first method of color development which was tried proved successful. Although none of the multitude of other procedures have been examined, the

requirements for a suitable method can be generalized. The final reaction product must be deposited in the emulsion with precise localization, good stoichiometry, and a pure color. Such things as fading, metachromasia, fogging, and contamination with secondary dyes must be avoided assiduously. If possible, the method should be straightforward and applicable to conventional emulsions.

The direct two-wavelength method is little concerned with resolution, but its photographic counterpart will correct for distributional error only when the distribution is faithfully rendered in the image. The success of the method depends on the balance between the dimensions of the heterogeneous structures in the object and the effective resolution of the final photograph. A number of compromises may be necessary. For instance, the optical resolution may have to be curtailed in order to have sufficient depth of focus to include the entire object; or the magnification may be reduced to give an adequate sample of objects on a single plate.

The photographic two-wavelength method is readily applied to ultraviolet microscopy. The illumination is usually monochromatic, and the available constants for nucleic acids give an estimate of k_o. The application to visible microscopy requires a line-source or an effective monochromator. The evaluation of k_o would be entirely analogous to the procedure with one-wavelength photometry. Black stains can be measured by assuming infinite absorptivity, but it is important to be certain that the object meets the requirements for uniform packaging. By choosing wavelengths which correspond to the absorption peaks of the component chromophores, multiple stains can be resolved and measured. The treatment of a specimen which is measured by the photographic method because of heterogeneity is necessarily relative, and the photomicrographs can be made at any one of a variety of wavelengths. A microspectrophotometer is not essential for the method; it should be possible to adapt a conventional spectrophotometer to the reading of photographs.

When a specimen is suitable for either method, there are certain advantages to using the direct two-wavelength method. It is independent of focus and resolution, and is relatively insensitive to glare (4). The random errors associated with the photographic process are avoided, as is the time spent preparing the transparencies. However, once the plates are made, the measurements can be made with equal speed with either method, and the selection of wavelengths is more rapid and dependable on the photographs.

In its broadest sense, the photographic two-wavelength method is a method of densitometry. Its applications are therefore not limited to photomicrography. It can be considered for any problem in which the measurement of photographic density is required in material which is heterogeneous. Some of the possible applications that come to mind are electron micrography, x-ray micrography, x-ray crystallography, x-ray photography, and autoradiography. It is hoped that this demonstration of the application of the method to microspectrophotometry will stimulate others to develop it in allied fields.

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