A Comparison of Scanning and Two-Wavelength Microspectrophotometry

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ABSTRACT

Because of the absence of suitable standards, microspectrophotometry suffers from a lack of objective verification. An approach to this problem is suggested which is based on the comparison of results obtained when different techniques or instruments are applied to the same objects. The application of this approach to scanning versus two-wavelength photometry has been justified by the widely different bases of these two methods. A group of ascites tumor cells stained with gallocyanin-chrome alum was measured successively with both methods and a high degree of proportionality between the two sets of results was found. This suggests that the two methods measure the same quality of the cells within a standard deviation of 3.39 per cent. This degree of correlation is a verification of the accuracy of both of the methods and shows that either one is suitable for resolving differences in stain content between cell nuclei of the order of 10 per cent.

The lack of suitable standards in microspectrophotometry has hampered both the interpretation of biological measurements and the evaluation of different types of instrumentation. It is common practice, in lieu of a standard, to define instrument performance in terms of reproducibility or the supposed constancy of such things as the amount of Feulgen stain in non-dividing cells. Instead of relying entirely on these parameters, we felt that much can be gained by testing instruments against each other. This approach is particularly fruitful when the instruments are based on contrasting principles.

The methods of measurement which we have compared were both designed to overcome major errors in microspectrophotometry, in particular that from inhomogeneous distribution of absorbing material. The scanning technique relies on a summation of measurements through a small moving aperture. In the scanning apparatus used for this study (1), the area of the aperture is less than the theoretical optimal resolution of the microscope. Of the sources of error peculiar to scanning methods, those due to the specimen being out of focus or to areas of high optical density within the specimen are dealt with by flattening the cell with a special condenser (2). The random errors introduced by instrument noise are minimized by repeated measurements of the same object.

The two-wavelength method (3, 4) is an empirical extension based on the equations for a "two-compartment" type of distribution. The validity of this extension for known distributions has been treated theoretically (3, 4) and has been tested by photometry (5). The difference between the scanning and the two-wavelength methods can be summarized by stating that, in contrast to the scanning method, the two-wavelength method does not require the object to be in sharp focus and does not involve electronic circuits for logarithmic transformation and integration of the output signal; however the two-wavelength method is an approximation, and is vulnerable to both minor variations in the absorption spectrum of the chromophore and to non-uniformity of the field of illumination. Both methods imply that inhomogeneity of distribution of the chromophore below the level of resolution of the light microscope will not affect the measurements. But here, too, the methods can be contrasted, since non-resolvable heterogeneity would affect the two-wavelength

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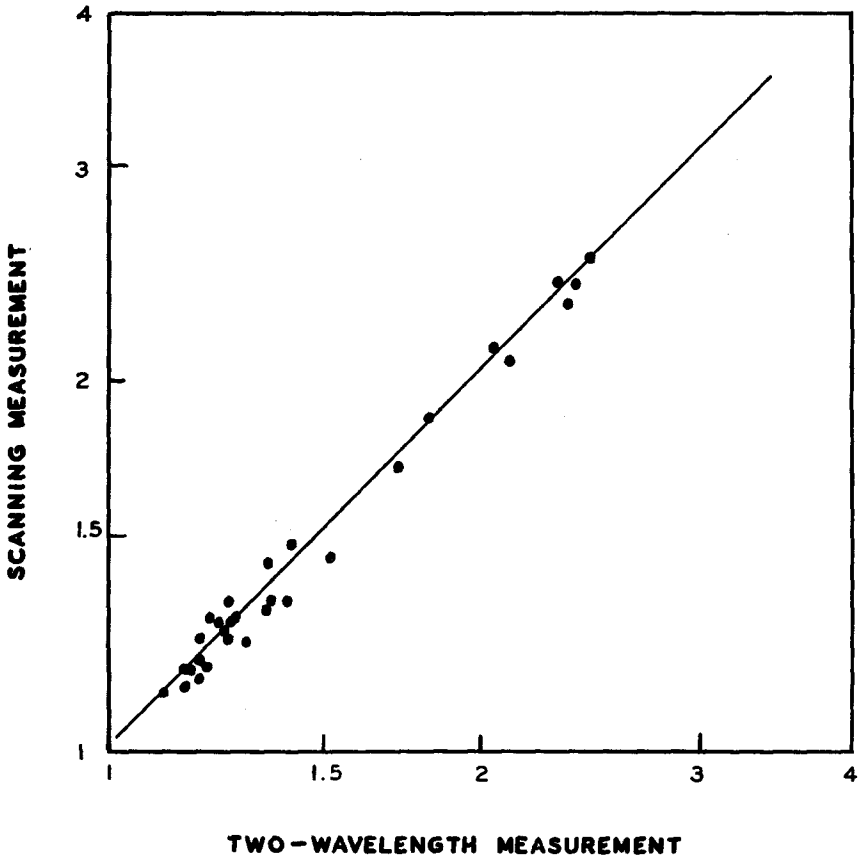

FIG. 1. A plot of the amount of gallocyanin-chrome alum stain in individual nuclei of an Ehrlich ascites tumor measured by scanning (ordinate) and two-wavelength (abscissa) microspectrophotometry. Number of cells = 30; Mean of the ratios of scanning: two-wavelength measurements = 1.017; Standard Deviation of these ratios = 0.0345; or per cent Standard Deviation = 3.39.

method when an appropriate pair of wavelengths is chosen by measuring absorption spectra from apparently homogeneous distributions in the object, whereas the heterogeneity would influence the scanning method for each successive part of the object imaged within the scanning aperture. For this reason, the magnitude of error which might be introduced by this phenomenon would be different for the two methods. A direct assessment of the significance of this effect on the two-wavelength method can be made by comparing the measured ratios of absorbance obtained from apparently homogeneous areas which differ in chromophore content. With the materials so far studied, these ratios have been agreeably constant, indicating that non-resolvable heterogeneity has not been a significant problem for the two-wavelength method.

Methods

Ascites cells from the mouse Ehrlich tumor were smeared onto coverslips, dried in air, and fixed in absolute methanol. The cells were then exposed to ribonuclease for 3 hours at 37°C., and were stained by gallocyanin-chrome alum according to Stenram's modification (6). The stained preparations were dehydrated in alcohol, washed in xylene, and then mounted in a medium of matching refractive index (consisting of immersion oil and α-bromonaphthalene). Photographs of several areas of the smear were taken, and a representative group of thirty cells, including several cells in various stages of mitosis, was chosen. Each cell was identified by number for successive measurement by the methods of photometry.

The two-wavelength measurements at 560 and 478 mμ were done on the instrument and with the precautions previously described (5). Each set of readings was taken in duplicate. The coverslip was then mailed to

London from Cambridge with the identifying photograph. There it was washed in xylene, rehydrated, and mounted in glycerol. This change in medium was intended to facilitate crushing; however, only a few of the cells included in this series had a residual thickness large enough to make crushing necessary. The series of cells was then measured by the scanning method, taking an average of three readings per cell at a wavelength of 546 mμ. There was a lapse of 3 days between the two types of measurement.

In view of the resistance of gallocyanin-chrome alum to extraction and its observed stability for periods as long as 1 year (M.L.M.), the remounting of the coverslip and the lapse of time between readings were not causes for concern.

RESULTS

The relationship between the scanning and the two-wavelength measurements for each of the thirty cells is given in Fig. 1. The two scales are logarithmic and are in unrelated arbitrary units. A slope of one was obtained for the line best fitting the points, indicating that the measurements obtained with one instrument were directly proportional to those obtained with the other. In addition, the figure demonstrates the small amount of scatter of the points about this line. The statistical parameters stated in the legend were calculated from the ratios of the two types of readings for each cell. They confirm the visual impressions gained from the figure, and give a 95 per cent confidence limit that the reading obtained with one instrument will be within 7 per cent of the reading obtained with the other. The bimodal distribution of the values along either of the coordinates of the figure is consistent with the expected behavior of DNA in a population of dividing cells (7).

DISCUSSION

These measurements indicate that there is a high degree of proportionality between the results obtained by the two methods of photometry. Since it is unlikely that two such different methods would give the same answers by chance, this correlation gives strong support to the validity of both techniques. Considering the wide morphological variation characteristic of these tumor cells (including the several metaphase figures), this conclusion indicates that distributional error can be minimized by either the scanning or the two-wavelength method. This is not only true for the gross heterogeneity resolvable by the light microscope, but evidently applies as well to the hypothetical effects of sub-resolvable molecular orientation.

Once the degree of correlation between methods in the manner we have described has been established, a realistic estimate of accuracy is then available for both methods. It is now apparent that a measured difference of less than 10 per cent between two nuclei can be considered significant with either method. Although this throws no light on the biological relevance of such differences in stain content, this important problem can be studied by using the same principle of comparison in experiments which contrast different histochemical methods applied to the same cells.

It is strongly recommended that inter-comparisons become common practice to safeguard the accuracy of measurements in cytophotometry and overcome the limitations arising from a lack of adequate standard of reference. This is particularly relevant to new instruments and techniques, and represents but a small fraction of the effort usually required for experiments in this field.

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