J. Cell Sci. 39, 235–245 (1979)
Printed in Great Britain © *Company of Biologists Limited 1979*

COMBINED MICROSPECTROPHOTOMETRY AND AUTOMATED QUANTITATIVE AUTORADIOGRAPHY APPLIED TO THE ANALYSIS OF THE PLANT CELL CYCLE

ALAN R. GOULD

Genetics Department, Research School of Biological Sciences, Australian National University, Canberra, A.C.T. 2601, Australia

SUMMARY

Two methods, which relate grain number to cell cycle phase in Feulgen-stained autoradiographic preparations, have been developed and compared. Both methods automate grain number estimations, one by taking integrated absorbance measurements at different wavelengths, the other by measuring absorption at a single wavelength before and after chemical removal of silver grains. With tritium-labelled tobacco mosaic virus as a probe, a quantitative analysis has been made of the binding of virus particles to plant protoplasts in different compartments of the DNA replication and partition cycle. The preliminary results indicate that the quantity of virus bound by protoplasts is related to their cell cycle phase. Whilst in this case, the methods have been used with plant cells, both techniques are equally applicable to animal cells.

INTRODUCTION

The localization of particular cellular events or activities to specific phases of the cell cycle often involves sequential sampling of a synchronized cell population. The additive effects produced by cell synchrony act as a 'biological amplifier' because all cells in the population express division cycle markers in concert. This allows the combination of biochemical techniques and bulk sampling, to monitor the appearance, disappearance or fluctuation of enzyme activities or other markers, while the whole cell population traverses the cycle in step. Similarly, the most common technique for the measurement of phase durations in the cell cycle, the fraction of labelled mitoses (FLM) experiment, also relies on a time-course approach, although in this case with asynchronous populations (Quastler & Sherman, 1959). A great deal of work is involved in such multi-sampling approaches to cell cycle characterization and an alternative, which combines autoradiography and Feulgen microspectrophotometry of a single sample after flash labelling with tritiated thymidine, has been developed by Mak (1965). This combined method does no more than assign average durations for residence in the G_1, S, G_2 and mitotic compartments. The FLM analysis and Mak's method have been successfully applied to plant cell suspension cultures (Gould, Bayliss & Street, 1974) and sequential sampling of synchronized plant cell populations has also yielded information concerning markers in the plant cell cycle (King, Cox, Fowler & Street, 1974; Gould & Street, 1975; Street, Gould & King, 1976). However,

if Mak's approach is modified by the use of quantitative autoradiography and radiolabelled probes other than tritiated thymidine, it becomes possible to position and quantify events in the cell cycle without the need to synchronize or sequentially sample large populations of cells.

Two methods have been devised which not only automate autoradiographic grain counting over Feulgen-stained cells challenged with radioactive probes, but simultaneously position cells in the G_1-S-G_2-mitosis sequence by their DNA content. In a trial application, the new techniques have revealed that the capacity of certain plant cell protoplasts to bind tobacco mosaic virus is cell cycle related.

MATERIALS AND METHODS

All cell samples used in this work were derived from asynchronously dividing suspension cultures of *Crepis capillaris* or *Nicotiana sylvestris*. Whole cells were fixed in fresh 3:1 (ethanol-acetic acid); protoplasts of *N. sylvestris* were fixed in a 75 % solution of 3:1 in water, osmotically buffered with 11 % sorbitol. Fixed whole cells were rehydrated by repeated washing in distilled water and, after hydrochloric acid hydrolysis and Feulgen staining, were squashed in 45 % acetic acid on subbed slides. Coverslips were removed by the dry ice method of Conger & Fairchild (1953) and the slides were stored in water before application of autoradiographic film. Fixed protoplasts were spun down and resuspended in fresh 3:1 to dilute out the sorbitol in the original fixative. After repeated spinning down and resuspension in distilled water, protoplasts were Feulgen stained and resuspended in 25 % acetic acid. Drops of this suspension were placed on subbed slides and allowed to air dry.

Kodak A.R. 10 stripping film was used for all autoradiograph preparations. Exposures were made in light-tight boxes at 4 °C. In all cases the tracer used was tritium. Exposed autoradiographs were developed with Kodak D19 at 20 °C for 5 min and fixed in Ilford Hypam. Visual grain counting was done using the same Zeiss microscope and oil-immersion objective (N.A. 1·3) used for all absorbance measurements. Each cell or standard area was counted 5 times and background counts were taken close to cell samples and on standard slides with no label. Background counts were averaged and subtracted from counts taken over cells or standard sources.

A standard tritium reference source of poly-*n*-butyl methacrylate (Radiochemical Centre, Amersham, U.K.) with a specific activity of 50 μCi g^{-1} was sectioned on a Reichert Om U3 ultramicrotome to 1 or 5 μm and sections were dried down onto slides before application of stripping film.

Removal of silver grains from exposed and developed autoradiographs was achieved by soaking slides in freshly made up Farmer's Reducer reagent:

Solution A: 7·5 % (w/v) potassium ferricyanide,

Solution B: 24 % (w/v) sodium thiosulphate,

1 part solution A plus 4 parts solution B plus 27 parts distilled water. Four minutes' soak in this solution removed all silver grains from the emulsion and left it clear. Feulgen-stained nuclei appeared unaffected by this treatment as evidenced by their unchanged absorption spectrum after removal of silver grains.

Integrated absorbance of specimens was measured at various wavelengths using a Zeiss 02 photometer system interfaced to a Digital PDP 12 computer and the system was operated in conjunction with the APAMOS, MODIFIED program (Automatic Photometric Analysis of Microscopic Objects by Scanning). The scanning motion of the motor-driven stage sets up the measurement raster in contrast to the flying spot/static sample approach used by the Vickers M85 machines. APAMOS MODIFIED contains the routines 'Draw a map' and 'Find and Adjust' which will retain and relocate the co-ordinates of objects on slides relative to an origin specified by the operator. Mapping cell positions in Feulgen-stained autoradiograph preparations has allowed the relocation of previously labelled cells for absorbance measurements after removal of silver grains.

RESULTS

Two strategies were envisioned which would, in principle, allow the separation of absorption due to silver grains, and that due to Feulgen-stained nuclei in micro-autoradiographs of tritium-labelled cells. One method relies on absorption spectra differences for grain and Feulgen dye, the other on the possibility of eliminating grain absorption with a photographic density-reducing reagent. Both approaches require information concerning the absorbance characteristics of silver grains, developed autoradiograph emulsion and Feulgen-stained nuclei.

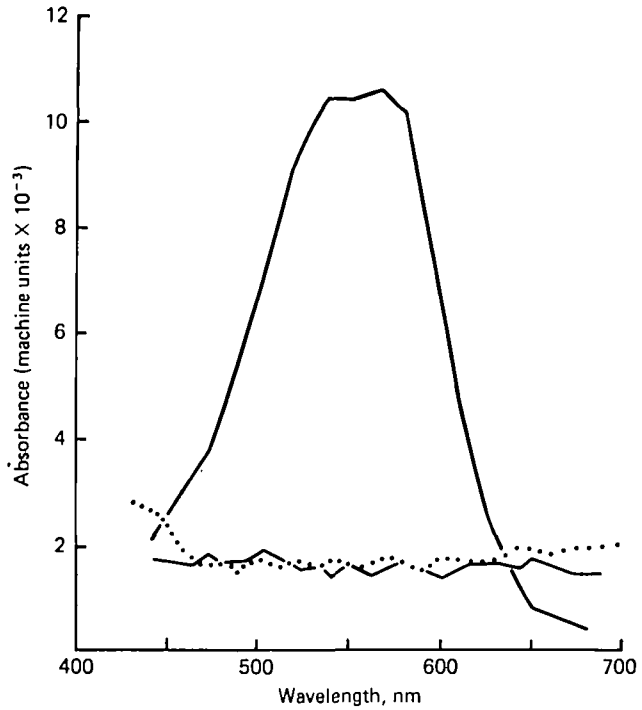

Fig. 1. Absorption spectrum peak of Feulgen-stained nucleus of *C. capillaris* compared with the flat spectral response of silver grains in developed A.R. 10 emulsion. The dotted line represents absorbance of silver grains obtained by varying detector sensitivity. Note the marked increase in apparent absorption at the blue end of the spectrum as compared with the solid line obtained by varying light-source intensity.

Preliminary investigations into the spectral response of developed Kodak A.R. 10 emulsion exposed either by tritiated poly-*n*-butyl methacrylate or by cells labelled with tritiated thymidine, confirmed previous observations (Goldstein & Williams, 1971) that there is an approximately 30% increase in absorbance at the blue end of the spectrum and no discernible absorbance peak in the visible range (Fig. 1). The very flat response of emulsion with silver grains between 470 and 670 nm, extending as it does across the absorption maximum of Feulgen-stained nuclei, suggests that it should be possible to estimate grain number and DNA content of cells by a

2-wavelength method. In addition, Feulgen-stained nuclei and developed A.R. 10 emulsion show unchanged absorption spectra after treatment with Farmer's Reducer as described in the Materials and methods section. This means that it should be possible to estimate grain number and DNA content of cells by integrated absorption measurements before and after removal of silver grains from Feulgen-stained autoradiographs.

An important sensitivity response of the microspectrophotometer system was noted during trial runs. At the extreme ends of the spectral range used (i.e. < 450 nm or > 650 nm) it was possible to set the background for measurement either by adjusting the intensity of the light source or by altering the sensitivity of the detector. However, these 2 alternatives gave different absorption readings at the above-mentioned spectral extremes (Fig. 1). More consistent results were obtained by varying light intensity, rather than detector sensitivity, to obtain background settings. Thus for measurements

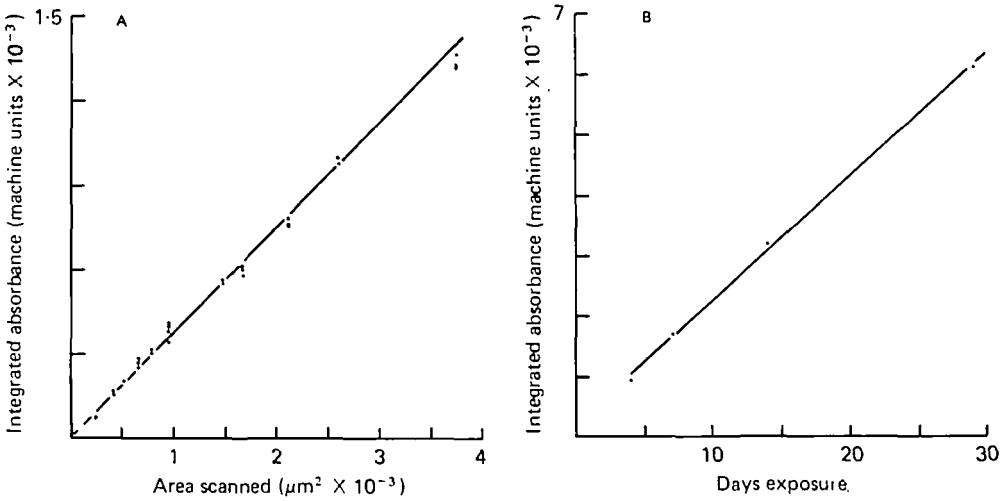

Fig. 2.A, Regression of integrated absorbance at 570 nm against area scanned over A.R. 10 emulsion exposed to tritiated poly-*n*-butyl methacrylate.

B, Regression of integrated absorbance at 570 nm of a standard area of A.R. 10 against days of exposure to tritium standard sources. Each point represents the mean of 5 measurements.

In both cases section thicknesses were carefully controlled in order to obtain comparable autoradiographic efficiencies (Falk & King, 1963).

of many cells at 2 wavelengths, every cell was measured at the first wavelength (background being set by fine adjustment of detector sensitivity) and subsequently relocated and remeasured at a new wavelength with only minor sensitivity adjustments.

Two methods, independent of visual grain counting, were used to check the correlation between machine values (integrated absorbance) and grain number. First A.R. 10 emulsion was exposed for 16 days to 1-μm sections of tritiated butyl methacrylate and then developed and fixed. Areas of exposed emulsion were scanned on the Zeiss photometer at 570 nm. Fig. 2A demonstrates the very close correlation between machine reading and area scanned, which equals grain number in the case of the

homogeneous exposure obtained from the tritium sources. Very similar readings and correlations were obtained when the grains were scanned at 470 and 670 nm. Secondly, A.R. 10 was exposed for up to 29 days to 5-μm sections of the standard tritium source before development and fixation; 5-μm sections were used to satisfy the criterion of 'infinite thickness' (Ritzen, 1967) whereby increase in section thickness will not increase β-particle emission from the surface. In Fig. 2B the very close correlation between grain number (i.e. exposure) and machine values obtained by scanning a standard area on different autoradiographs is illustrated. A visual estimation of grain number in the 29-day-exposed emulsion produced a count of about 0·25 grains/μm^2, appreciably lower than the upper value of 1·5 grains/μm^2 quoted by Goldstein & Williams (1971) to ensure direct proportionality between exposure and integrated absorbance. Machine values within the range represented by the data in Fig. 2B can therefore be expected to provide a good measure of grain number. Assuming an emission of 5×10^3 β particles/cm^2/min from the surface of a 5-μm section of the tritium standard source with sp. act. 50 μCi g^{-1}, a density of 0·25 grains/μm^2 in the 29-day exposure (Fig. 2B) represents an efficiency of approximately 0·12 grains per emergent electron. This value compares well with a value of 0·29 grains per emergent electron quoted by Ada *et al.* (1966) for Ilford L_4 emulsion, taking note that those authors rate L_4 twice as efficient as Kodak A.R. 10.

These results confirm the confidence of earlier workers in using integrated absorbance as a valid estimator of grain number in unstained autoradiographs. On this basis the correlation between visual grain count and integrated absorbance at different wavelengths over Feulgen-stained, radiolabelled cells was tested. Three wavelengths were selected with reference to Fig. 1; 570 nm (green) because this is close to the Feulgen absorption maximum, 470 nm (blue) and 670 nm (red) because absorption due to grain density is relatively constant over this range, whereas Feulgen absorption is very low at these extremes.

Fig. 3A illustrates regressions of visual grain count against integrated absorbance at these 3 wavelengths obtained by scanning 40 tritium-labelled, Feulgen-stained cells of *C. capillaris* in an autoradiograph preparation exposed for 10 days. As might be expected, the correlation coefficient obtained with the 570 nm (green) measurements is very low ($\sim$ 0·2) because the Feulgen-stained nuclei contribute very significantly to total absorption at this wavelength. Machine values at 470 nm (blue), where Feulgen absorption is low (Fig. 1) show a much better correlation ($\sim$ 0·88) with visual grain count, whilst at 670 nm (red), the correlation is very good ($\sim$ 0·97). Thus the first strategy of exploiting the different spectral characteristics of silver grains and Feulgen-stained nuclei produces good estimations of grain number. Unfortunately, as will be discussed later, it does not always identify cell cycle phase unambiguously because of the occlusion of nuclei by silver grains. The second approach, where absorption due to grain is eliminated, also yields good results, as shown in Fig 3B. The same 40 cells measured by the 2-wavelength method, were relocated by the APAMOS 'Find and Adjust' option and scanned at 570 nm before and after treatment with Farmer's Reducer. The regression of visual grain count against the fall in absorbance due to grain removal is good (0·96) and the absence of grain

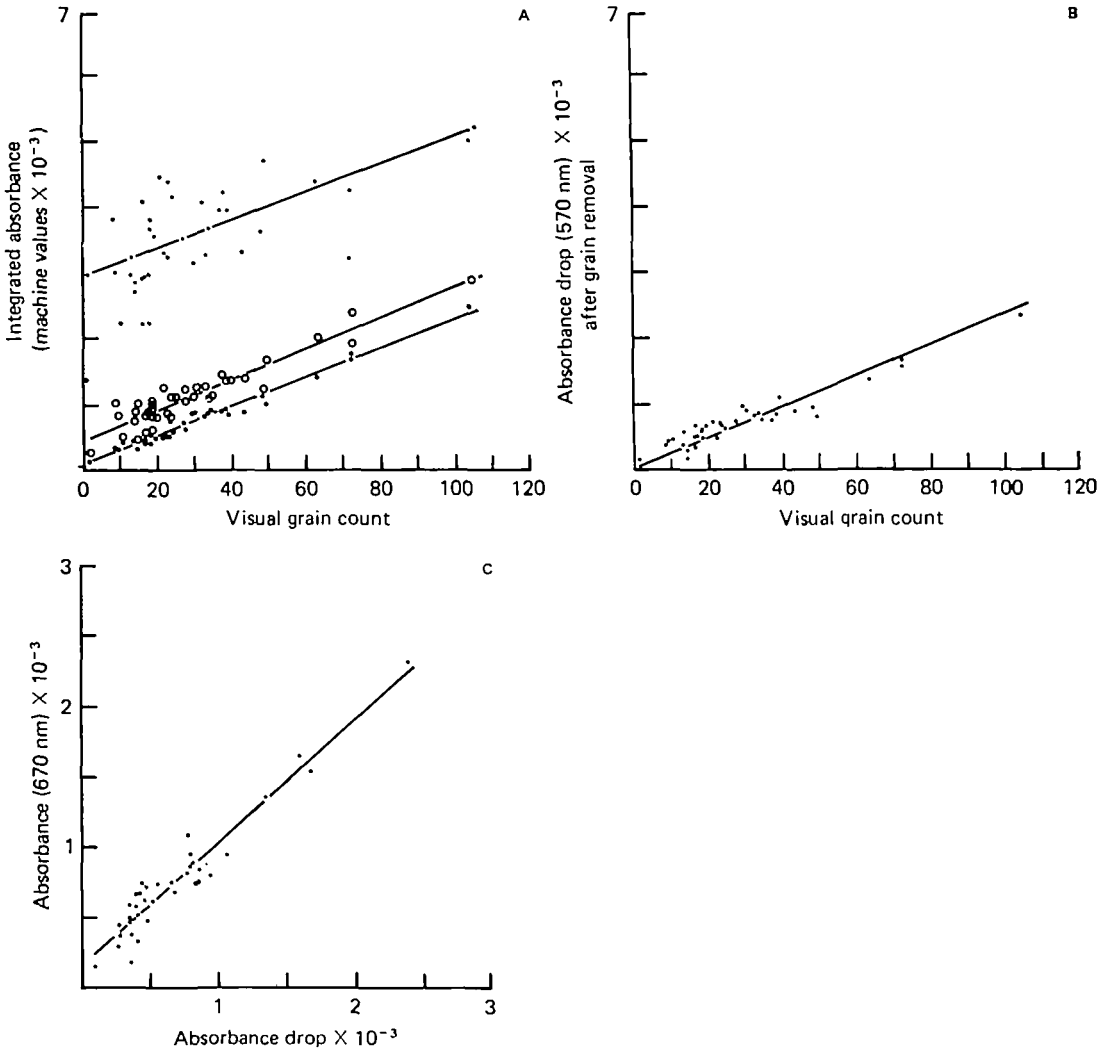

Fig. 3.A, Regression of integrated absorbance at 570 nm (●, top line), 470 nm (○, middle line), and 670 nm (●, bottom line), against visual grain count over 40 cells of *C. capillaris* labelled with tritiated thymidine. Correlation coefficients at 570 = 0·222, at 470 nm = 0·885, at 670 nm = 0·968.

B, Regression of the decrease in integrated absorbance at 570 nm after removal of silver grains against visual grain count, plotted on the same scale and for the same 40 cells as in A. Correlation coefficient = 0·961.

C, Regression of absorbance at 670 nm against absorbance drop at 570 nm after removal of silver grains for the same cells as in A. Correlation coefficient = 0·929.

allows accurate measurement of Feulgen DNA content, and therefore cell cycle phase.

The 2 approaches are summarized in Table 1 and the regression of red (670 nm) measurements against drop in absorption after grain removal (Fig. 3C) gives a good correlation coefficient which indicates that both methods measure the same thing, namely absorption due to silver grains. In other words, the 2 estimators of grain

number correlate well. The 2 estimators of Feulgen-DNA content correlate well at low grain numbers per cell but poorly at high grain numbers (regression not shown, see Discussion).

The availability in this laboratory of protoplasts derived from *Nicotiana sylvestris* and a kind gift of tritium-labelled tobacco mosaic virus (TMV, courtesy of Dr A. J. Gibbs) has allowed a useful comparative test of the 2 methods. Protoplasts isolated from an asynchronously dividing suspension culture of *N. sylvestris* were incubated

Table 1. *Summary of the 2 methods employed*

Method	Grain number estimator	Feulgen-DNA estimator
Two-wavelength: green (570 nm), red (670 nm)	Red absorbance	Green absorbance minus red absorbance
Grain removal	Drop in green absorbance due to grain removal	Green absorbance after grain removal

for 10 min with polyornithine and tritiated TMV, and then fixed. Fig. 4 illustrates data obtained by the grain-removal method from a single Feulgen-stained, autoradiograph preparation of protoplasts. The binding of TMV, measured automatically as grain number per protoplast, clearly shows quantitative variation throughout the cell cycle. *S*-phase protoplasts, identified by their intermediate DNA content between the G_1 and G_2 peaks (Fig. 4B), do not show the high levels of binding achieved by other interphase protoplasts. The specifically virological implications of such interaction between virus and protoplasts will be discussed elsewhere; these data are presented here to illustrate the potential of this experimental approach. The 2-wavelength method applied to the same protoplasts gave less easily interpretable results, especially with high grain numbers where occlusion of the Feulgen-stained nucleus becomes significant (see Discussion). Visual grain counting confirms the data obtained automatically, and it is worth noting how much more work would be involved in deriving data demonstrating differential virus binding using synchronized cultures and biochemical assays. Additionally, such a bulk-sampling approach would certainly not uncover the variability of virus binding at the single cell level revealed in Fig. 4A.

DISCUSSION

In a comparative evaluation of visual grain counting, reflectance microscopy, gross absorbance measurements and flying spot microdensitometry, Goldstein & Williams (1971) noted that in densitometric work separate assessment of absorbance due to silver grains and to dye is feasible if the absorption spectra of these 2 components differ sufficiently. The 2-wavelength method described here utilizes such differential absorption and produces machine values which correlate well with visual grain counts.

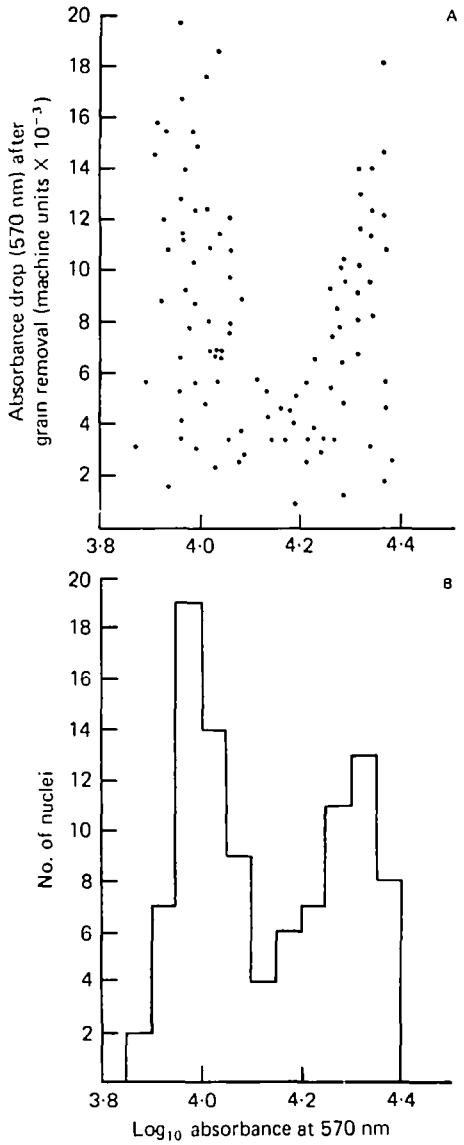

Fig. 4.A, Plot of absorbance drop at 570 nm after removal of silver grains, against the logarithm of Feulgen absorbance at 570 nm for 100 protoplasts of *N. sylvestris*. After 10-min incubation with 4 μg ml^{-1} polyornithine and tobacco mosaic virus labelled with tritiated uridine, protoplasts were fixed and stained for preparation of autoradiographs.

B, Frequency histogram showing the distribution of the 100 protoplasts in the cell division cycle. The first and second peaks represent G_1 and G_2 cells respectively, nuclei falling between the peaks are in S-phase.

However, stained autoradiographic specimens can be expected to produce high levels of light scatter and the apparent absorption of such specimens will represent a summation of 'true' or diffuse absorption due to the dye, and a 'specular' absorption due to the discrete, opaque silver grains. Obviously the accuracy with which the

DNA content of a nucleus can be measured in an autoradiographic preparation, will depend upon the degree of occlusion of Feulgen dye caused by silver grains. Thus with the 2-wavelength method, total grain number and grain distribution within a cell will affect the confidence with which that cell can be positioned in the G_1, S or G_2 compartments. Occlusion errors of this kind will be less significant if the grains appear over the cytoplasm (as in the TMV-binding experiment of Fig. 4), than in a situation where label is concentrated over the nucleus. Although the removal of silver grains in the Farmer's Reducer method allows accurate determination of Feulgen-DNA content, the machine value for grain number is obtained by subtraction of the Feulgen absorption from the reading obtained at the same wavelength (570 nm) before grain removal. Occlusion effects in this case presumably cause an underestimation of grain (specular) absorption. Whilst logically a more accurate system would involve 3 measurements per cell: (1) absorption at 670 nm with grain; (2) absorption at 670 nm after grain removal. Subtract (2) from (1) (to correct for slight Feulgen absorbance in the red) for grain estimation; and (3) absorption at 570 nm for cell cycle status, it must be remembered that rapidity is a major benefit of automated grain counting. In this sense, 3 scans per cell approaches the point of diminishing returns, especially with large cells which require extended scan patterns. Some of the largest protoplasts measured in the trial analysis required a 50×50 μm raster to accommodate them, which takes about 150 s for a complete scan. However, the increased scan area required to enclose large cells means that at the upper limit acceptable for grain density, more grains per cell can be counted without loss of proportionality between exposure and integrated absorbance. Thus with larger cells the meaningful range for automated estimation of grains per cell is increased.

The range of grain number over which the methods have been tested is narrow (up to 120 grains per cell) and is much lower than that which Goldstein & Williams (1971) refer to as 'relatively low grain concentrations' where the relationships between absorbance, exposure and grain count deviate from linearity by only a very small amount. The straight line relationship in Fig. 2B demonstrates that, in unstained material, meaningful automatic estimation of grain number can be made at densities far in excess of 120 grains per cell. Even so, the errors arising from occlusion, discussed above, preclude the use of such high densities unless label is confined to the cytoplasm and only an insignificant overlay of nuclei by grains occurs. If a very wide range of grain numbers occurs in an autoradiograph it is possible, in principle, to use a scale of exposure times, coupled with polymeric methacrylate standards of different specific activities, to relate absolute activities measured on different slides (Ritzen, 1967). Thus, if a fraction of a cell population appears very heavily labelled in a conventionally exposed autoradiograph, then a shorter exposure time, with a methacrylate standard of higher than usual specific activity, will give lower grain densities and less occlusion of the nucleus.

Three limitations inherent in the 2 methods outlined in this paper must be noted. Firstly, whilst truly kinetic or time-course experiments involving cell cycles can distinguish between early, mid- and late- G_1, S or G_2, the use of DNA content to define the status of a cell at interphase cannot be so precise, at least in G_1 and G_2.

There is no change in DNA content in the G_1 and G_2 phases and so subdivision of these compartments cannot be realized by Feulgen microspectrophotometry. *S*-phase can be more accurately analysed because replication takes place at this time and the mitotic phases, of course, are cytologically distinct. Secondly, the Farmer's Reducer method would be tedious, almost to the point of being impossible to use, with a microspectrophotometer system lacking an automatic mapping facility similar to the APAMOS 'Find and Adjust' routine. However the 2-wavelength method does not require relocation of cells and can therefore be used with machines such as the Vickers M85 scanning microdensitometer which does not have a motorized microscope stage. Thirdly, the problems associated with objects of variable or complex geometry, where autoradiographic efficiency will vary from area to area, cannot be dismissed (Perry, 1964). For meaningful quantitative comparisons correction factors must be applied to grain counts (Ada *et al.* 1966). There is no *a priori* reason to think that in Fig. 4A, the *S*-phase protoplasts, which show low grain numbers, do so because of lowered autoradiographic efficiency. This possibility has been checked by drying unlabelled protoplast preparations down onto tritiated methacrylate sections and estimating grain number in emulsion areas over the cytoplasm of different cells. In this case no significant differences could be found in grain counts over cytoplasm of cells in different cell cycle phases (as identified by Feulgen-DNA content), although the absolute efficiencies were of course lower than the value of 0·12 grains per emergent electron quoted before.

In general, the more rapid 2-wavelength method has been less successful than the grain-removal technique for positioning cells in the division cycle, but more accurate than grain removal for grain number estimation. In choosing between the 2 methods, the type of cell being measured will dictate which is the most suitable, the critical characteristics being DNA content (absolute Feulgen dye intensity), the ratio of apparent nuclear to cytoplasmic area, and the number of grains per cell.

I am indebted to Karin Harrison for her expert technical assistance, to Joanne Hughes for sectioning the butyl methacrylate tritium source and to Professor Bernard John for reading the manuscript.

REFERENCES

ADA, G. L., HUMPHREY, J. H., ASKONAS, B. A., MCDEVITT, H. O. & NOSSAL, G. J. V. (1966). Correlation of grain counts with radioactivity (^{125}I and tritium) in autoradiography. *Expl Cell Res.* **41**, 557–572.

CONGER, A. D. & FAIRCHILD, L. M. (1953). A quick freeze method for making smear slides permanent. *Stain Technol.* **28**, 281–283.

FALK, G. J. & KING, R. C. (1965). Radioautographic efficiency for tritium as a function of section thickness. *Radiat. Res.* **20**, 466–470.

GOLDSTEIN, D. J. & WILLIAMS, M. A. (1971). Quantitative autoradiography: an evaluation of visual grain counting, reflectance microscopy, gross absorbance measurements and flying-spot microdensitometry. *J. Microscopy* **94**, 215–239.

GOULD, A. R., BAYLISS, M. W. & STREET, H. E. (1974). Studies on the growth in culture of plant cells. XVII. Analysis of the cell cycle of asynchronously dividing *Acer pseudoplatanus* L. cells in suspension culture. *J. exp. Bot.* **25**, 468–478.

GOULD, A. R. & STREET, H. E. (1975). Kinetic aspects of synchrony in suspension cultures of *Acer pseudoplatanus* L. *J. Cell Sci.* **17**, 337–348.

KING, P. J., COX, B. J., FOWLER, M. W. & STREET, H. E. (1974). Metabolic events in synchronised cell cultures of *Acer pseudoplatanus* L. *Planta* **117**, 109–122.

MAK, S. (1965). Mammalian cell cycle analysis using microspectrophotometry combined with autoradiography. *Expl Cell Res.* **39**, 286–308.

PERRY, R. P. (1964). Quantitative autoradiography. In *Methods in Cell Physiology*, vol. 1 (ed. D. M. Prescott), pp. 305–326. New York & London: Academic Press.

QUASTLER, H. & SHERMAN, F. G. (1959). Cell population kinetics in the intestinal epithelium of the mouse. *Expl Cell Res.* **17**, 420–438.

RITZEN, M. (1967). A method for the autoradiographic determination of absolute specific radioactivity in cells. *Expl Cell Res.* **45**, 250–252.

STREET, H. E., GOULD, A. R. & KING, J. (1976). Nitrogen assimilation and protein synthesis in plant cell cultures. In *Perspectives in Experimental Biology*, vol. 2, Botany (ed. N. Sunderland), pp. 337–356. Oxford and New York: Pergamon.

(*Received* 9 *April* 1979)