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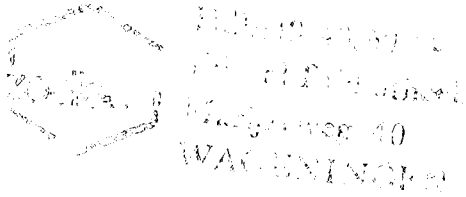

HIDACSYS: Computer Programs for Interactive Scanning Cytophotometry

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Summary. A description is given of a combination of three programs developed for computer-assisted stage scanning cytophotometry and cytofluorometry of isolated, close-lying, or touching objects in different types of microscopical preparations.

The advantages and limitations of the individual programs are discussed, as well as the local specimen conditions determining the optimal application range of each of the programs.

The applicability of these programs was investigated by determination of the integrated absorbance values of Feulgen or gallocyanin-chrome alum stained chicken erythrocytes, human leucocytes, and skin biopsy cells in imprint preparations, as well as of guinea pig peritoneal granulocytes which had been submitted to a simultaneous coupling azo dye incubation for alkaline phosphatase activity.

Introduction

During the last decade, cytophotometry is changing from an isolated research activity into a routine biomedical and laboratory capability, due to the fact that reliable and manageable stage, flying spot, or TV scanners, and – more recently – flow cytometers (cell analysers and sorters) have become available commercially.

The image-plane scanner developed by Deeley (1955) formed the basis of the Barr and Stroud GN5 (Barr and Stroud Ltd., Glasgow, Scotland), while a flying spot object-plane scanner originally designed by Smith (1967) is used in the Vickers M85 (Vickers Instrum. Ltd., YO3 7SD York, UK). The Zeiss SMP (Carl Zeiss, Oberkochen, FRG) and the Leitz MPV (Leitz GmbH, Wetzlar, FRG) instruments are provided with scanning stages of which the principle

was developed by Caspersson in 1936. Television scanning techniques are applied in the Quantimet system (Image Analysing Computers Ltd., Melbourn, Royston SG8 6EJ, Hertdordshire, UK) and the TAS apparatus manufactured by Leitz. Some of these cytophotometers are supplied in combination with a computer that controls scanning, signal sampling and processing, etc. This last type of scanners has in principle a great versatility in terms of measurement and calculation potential and—if necessary—extension to image analysis and automation programs. Nevertheless, the current commercial programs for accurate determination of integrated absorbance lack either versatility or speed when it comes to the measurement of a number of objects in routine microscopical preparations. In such preparations, areas with well-separated cells will be interspersed with areas in which the cells are lying more densely packed. These cell clusters are often important because they may contain highly informative cells, whose omission from measurement would bias the results due to non-random sampling.

In order to determine integrated absorbance values, two criteria must be satisfied: proper background correction, and accurate delimitation of the object boundary. In early cytophotometric procedures determination of the background correction could only be performed correctly when relatively large areas of free background were available around the measured cells. In areas where cells lay close together, background readings had to be taken at relatively large distances from the site of the actual absorbance measurements.

The Barr and Stroud scanning cytophotometer still suffers from this drawback: measurements for background correction have to be made in an area of identical size as that in which the object was measured. With this apparatus therefore, only cells well separated from surrounding objects can be selected for measurement. This requirement not only limits the number of cells that can be studied, but—as explained above—can also lead to distorted results.

In programs for computer-assisted scanning cytophotometers [presently available commercially: APAMOS (Zeiss) and MIDAS (Leitz)] background determinations are performed by means of an intrahistogram method (or—less accurately—by thresholding). This method has a drawback when other objects or parts thereof are present in the scanned area, since their exclusion from the integrated absorbance determination requires laborious and time-consuming interactions. Optimally, the program should assess its own ability to delimit the object and determine the background directly around it, but for close-lying cells this problem has so far only been solved on big computers (see e.g. Mendelsohn et al., 1966; Ingram and Preston, 1970; Groen, 1977).

The present report describes such a program (DIODA) written for a mini computer (PDP 11/10). This program was developed in combination with two others (FASTSCAN and ARRAYSCAN) which can be used in areas where cells are well separated or touching respectively. FASTCAN is faster but does not provide isolation facilities; the isolation with ARRAYSCAN takes more time than the DIODA program, but provides adequate *interactive* isolation facilities. The three programs were combined in the present HIDACSYS measuring system in such a way that switching from one program to any of the others can be performed readily at any time when in a given area the objects are, for instance, more (or less) closely packed than the originally chosen pro-

gram permits (or necessitates) to scan. This resulted in a program system that allows reliable and relatively rapid scanning of a representative number of cells per preparation within a reasonable period of time.

Material and Methods

Cell Preparations

Human white blood cells were sedimented under gravity from fresh heparinized venous blood. After repeated washing in a Hanks' balanced salt solution, the suspension was diluted wih that medium until the desired cell concentration was reached. If stored – for at most 8 h – the suspension was kept at 4° C. Microscopical preparations were made with the valve centrifuge (Van Duijn et al., 1977). During this procedure the cells retain a good morphology and the cellular area is larger (due to the flattening under gravity) than in air-dried preparations.

Skin imprint preparations were made from the fresh-cut surface of human skin samples taken with a 5 mm biopsy punch (Van Vloten et al., 1974).

Lymph node imprint preparations on glass slides were made from pieces of fresh-cut human lymph nodes.

Cervical cell preparations were prepared according to Beyer-Boon et al. (1977). While still wet, the microscopical preparations were first fixed with formaldehyde vapor at 50° C for 30 min, and then – to obtain better preservation of the chromatin structure – postfixed in a mixture of ethanol, chloroform, and acetic acid (6:3:1 volume parts) for 15 min at room temperature.

Chicken erythrocytes were sedimented from fresh heparinized venous blood by centrifugation for 5 min at $1500 \times g$. After repeated washing in cold Hanks' balanced salt solution, the cells were diluted with the same medium until the desired cell concentration was reached. Microscopical slides were made with the valve centrifuge.

Guinea pig leucocytes were obtained from exudates collected from the abdominal cavity of guinea pigs after the intraperitoneal injection of a hypertonic NaCl solution (Van der Ploeg and Van Duijn, 1968). Microscopical preparations – made with the valve centrifuge – were only air-dried before being stained for alkaline phosphatase activity.

Cytochemical Staining Procedures

Feulgen staining was carried out as described by Duijndam and Van Duijn (1975). Unless otherwise stated above, preparations were fixed prior to the staining procedure for 1 h at room tmperature in a freshly made mixture of methanol, formaldehyde 35% (w/v), and glacial acetic acid (85:10:5 volume parts; Böhm et al., 1968).

The Schiff reagent was prepared according to Graumann (1953) with pararosaniline ("Acridin-frei", Chroma, Stuttgart, FRG). After passing through a graded alcohol-xylene series, the preparations were mounted in Caedax (Merck AG, Darmstadt, FRG) to which Cargille oil of $n_D^{25} = 1.460$ (Cargille Lab., Cedar Grove, N.Y., USA) was added as described by Van der Ploeg et al (1974a). Cytophotometry was performed, as described in detail below, with light selected from a quartz halogen lamp with an interference filter AL 559 (60% transmittance at 559 nm, 20 nm bandwidth at 50% of peak transmittance; Schott & Gen., Mainz, FRG).

Gallocyanin-Chrome Alum Staining of DNA. The gallocyanin-chrome alum staining medium was prepared according to Mayall (1969), taking into consideration the remarks of Kiefer (1970). Gallocyanin (Standard) was purchased from Fluka AG, Buchs, Switzerland. Prior to the staining, the

microscopical preparations were fixed according to Böhm et al. (1968), and after washing treated with a ribonuclease solution prepared from bovine pancreas ribonuclease (RASE, Worthington, Biochem. Corp., Freehold, N.J., USA) in distilled water at 37° C for 3 h.

The mixture of Caedax with Cargille oil described for the Feulgen-stained preparations was applied as a mounting medium. For the cytophotometry of these preparations, the measuring light beam was selected with an AL 577 interference filter (59% transmittance at 577 nm, 40 nm bandwidth at 50% of peak transmittance; Schott).

Simultaneous Coupling Azo Dye Method for Alkaline Phosphatase Activity: Unfixed guinea pig granulocyte preparations were stained as described by Van der Ploeg and Van Duijn (1968), and cytophotometry was performed with a measuring light beam selected with the AL 577 filter.

Cytophotometry

Scanning was performed with a Cytoscan SMP (Zeiss, Oberkochen, FRG) interfaced to a PDP 11/10 computer (Digital Equipment Corporation, Maynard, Mass., USA). Movement of the microscope stage is controlled by the computer programs mentioned below via two stepping motors at a speed of 200 steps of 0.5 μm second. Measurements were taken at 0.5 μm intervals with a Neofluar 100 × /1.30 Zeiss objective lens and a circular measuring diaphragm (diameter = 0.2 mm, which – calculated back to the specimen – corresponds with 0.44 μm).

With the interference filters already mentioned, measuring beams were selected (diameter of illuminated field being 1.5–2.0 μm at the specimen level) from light emitted by a quartz halogen lamp (12 V–100 W, 7023; Philips B.V., Eindhoven, The Netherlands).

Software Tools. The three programs described below are modules in the HIDACSYS system* (Van den Broek, 1976). This system provides facilities to display measured data in simulated grey tones (Smeulders and Vosspoel, 1977) and to plot data and figures on a display or in print. HIDACSYS runs under the MINIBOSS operating system (Boleij and Hortensius, 1976). The system can control up to five scanning cytophotometers simultaneously. There are optional facilities for performing measurements in transmittance, absorbance, and fluorescence, both in the original slides and in photographic negatives.

Directly after the initiation of each step of the microscope stage, one sample is taken of a DC-300 Hz filtered light intensity signal. The programs can record 512 linearly spaced levels of light intensity, which are directly converted to logarithmic values without preliminary fitting of the intensity values into a 0 to 100% transmission scale. Because the motor-driven microscope stage shows some backlash, signal sampling in programs where the topological position of the measured values is of importance (DIODA and ARRAYSCAN) is only performed while the stage is moving from left to right (Fig. 1 A).

In the FASTSCAN program, which only measures and computes the integrated absorbance of the objects scanned and the number of object points, sampling is also done during the backward movement (Fig. 1 B). In all programs, five steps are automatically added on both sides of each scanline (where no signal sampling is done) to allow the stage to reach its regular speed and movement before measurements are taken.

Hardware Configuration. The PDP 11/10 computer is equipped with a 24 K word core memory, an LPS 11 interface, a Tektronix 4010 storage scope (Tektronix Inc., Beaverton, Oregon, USA), an ASR 33 (Teletype Corp., Skokie, Ill., USA) used as a system console, and a DU 11 line interface with modem (9600 baud).

In view of the fact that for current purposes three scanning cytophotometers are connected to this configuration in a time-sharing mode (Boleij and Hortensius, 1976), there is also a VT 50 Decscope for each of the other microscopes. These scopes offer adequate facilities for the measurement of isolated objects in preparations where only the integrated absorbance, or total fluorescence emission intensity value in combination with the number of object points is of interest. We also have such additional user-designed electronics as a scanstage interface, a photomultiplier (with filter and amplifier) connected to the A–D converter, and a joystick (with key switches) connected to a 16 bit I/O of the LPS.

* for information concerning the source programs contact A.M. Vossepoel

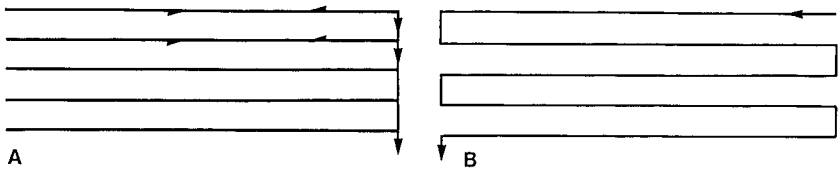

Fig. 1. Schematic representation of scanning patterns of the microscope stage in: **A** the DIODA and ARRAYSCAN programs (signal measurements are only made while stage is moving from left to right). **B** FASTSCAN program (sampling also during the reverse movement). In all three programs five extra steps are added on both sides of each horizontal line, to allow readjustment of the stabilized movement of the stage. No measurements are taken in these intervals

FASTSCAN

This program has been developed to permit measurement of the total integrated absorbance (or fluorescence emission) of individual microscopic objects lying free in a "clean" background (Fig. 2F). As the first step, a measuring frame enclosing the object under study is chosen by the operator. Delimitation of this area (about 4 times larger than the object) can be realized by use of 2-quadrant positioning (Van der Ploeg et al., 1974a) or by typing the number of steps per line and the number of lines on the terminal. To the left and right of this measuring rectangle, background zones of 3 to 5 steps are taken. The width of these zones can be decreased when there are absorbing objects lying close to the cell to be measured (Van der Ploeg et al., 1974b).

After a test run along the border of the frame, focusing of the preparation, and adjustment of the photomultiplier signal, automatic scanning is started. Measurements are taken while the stage is travelling from left to right as well as during the reverse movement (Fig. 1B). At the end of a scanline, optical density values in the two background zones are averaged to obtain a mean value for that line, and each reading on that line within the measuring field is corrected for that background value (1 ms computing time) in order to determine the number of object points.

If there is more than 10% difference between the value obtained by averaging the background light intensities measured in one scanline with that of subsequent lines, the program produces a warning message on the scope together with the advice that the scan be rejected. This also occurs at any scanpoint where the measured signal is in either under- or overflow.

The sum of the corrected absorbance values for that line is stored, and at the end of the scan, the integrated corrected absorbance of the object is calculated (200 μs). This integrated value is shown on the Tektronix scope and—if accepted by the operator—is stored in memory to be printed out and/or processed into a histogram. Together with the integrated absorbance, the scope shows the number of object points (=individual corrected absorbances above a chosen lower limit, e.g., >0.020). Computing time needed to calculate and display these figures is about 200 ms.

DIODA

The DIODA program (Vossepoel et al., 1977) was developed to measure cells or nuclei lying relatively close to each other but without really touching (Fig. 2D). A measuring rectangle is chosen as described above, taking into consideration that it must be large enough to provide extra measuring points next to the object to serve for the background determination, and—on the other hand—should not exceed the memory capacity of the computer (4096 points in the present configuration).

The microscope stage is then directed with the joystick to center the cell to be measured within the chosen frame, after which the operator starts the scanning procedure; measurements being taken only while the stage is moving from left to right (Fig. 1A).

In this program, computation starts after completion of the scan. To obtain the correct integrated absorbance of the cell, determination of the background value and exclusion of all points not belonging to the object are both of importance.

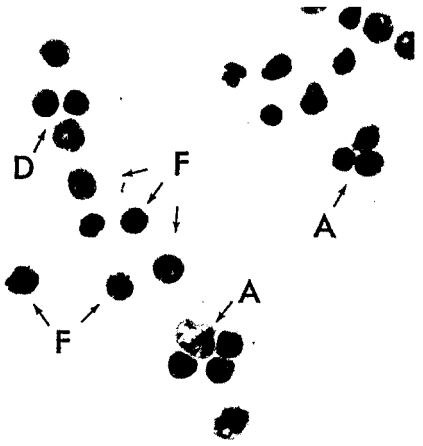

Fig. 2. Feulgen-stained human lymph node imprint preparation showing isolated cells (*F*) that can be measured with the FASTSCAN program, cells (*D*) for which DIODA is more appropriate, and cells (*A*) requiring the ARRAYSCAN program, including SELECT, to obtain integrated absorbance values

The separation between points belonging to the object and those belonging to the background, is initiated with the aid of an intra-histogram of measured light intensity values. This histogram is first averaged over 6 values at a time to obtain a well-defined singular background peak (Fig. 3). On the slopes of this peak, the intensity values I_l and I_r lying closest to values representing half the frequency of the background peak are determined. The threshold (I_t) is then set at a distance ($I_r - I_l$) from I_l in the direction of the object's intensity values. The operator can either accept this threshold or choose another by moving a vertical cursor visible on the display to the desired position. From now on the computer performs the isolation of the object after conversion of intensity values into absorbance values. Starting from the position determined by the center of the cross hairs, the program will search for a pair of adjacent points (A_1, A_2) satisfying the conditions $A_1 \geqq A_t > A_2 (A_t = -\log I_t)$. Starting from the first A_1, the program determines the contour by searching the neighbouring points counterclockwise until the next A_1 is found. From this point the procedure will be repeated, continuing until the first A_1 is reached and the boundary closed.

Meanwhile, a corresponding figure is visualized on the display to enable the operator to judge whether the object is isolated correctly from other light-absorbing points within the scanned area. If the contour is about to touch the border of the scanned rectangle, a warning message is displayed and computation stops. The scanning procedure can then be repeated after readjustment of the object or another object can be chosen and positioned.

While the contour is being drawn, the number of contour points is integrated; if this number exceeds the available memory buffer capacity (here 200 points) the message: CELL TOO LARGE TO STORE will appear. This can also occur when two objects cannot be separated by DIODA. The processing must then be stopped, and the ARRAYSCAN program followed by ERASE or SELECT, as described below, should be applied. When the number lies within the limit of the buffer capacity, processing is continued by filling the boundary (Fig. 4A). All the points indicated by 1 in Figure 4B are then counted to obtain the number of object points.

After the object points have been separated from all the other points, the absorbing part of the object has to be extended and the background defined. To this end, dilatation is performed by stepping outwards from the boundary already isolated, and assigning to the extended object those points that have a monotomically lower absorbance value. Computational considerations led us to arrange for these additional points to be displayed in rings aroung the original object image (see Figs. 4B and 4C).

The dilatation process stops when there are no more points to be assigned, or due to lack of symmetry in the set of assigned points. The outermost points of the extended object are called set *A*. They undoubtedly belong to the background, but an average background absorbance value computed solely from these points, would be systematically too low, because the absorbance value of points of set *A* is systematically lower than that of the neighbouring points, both inside and

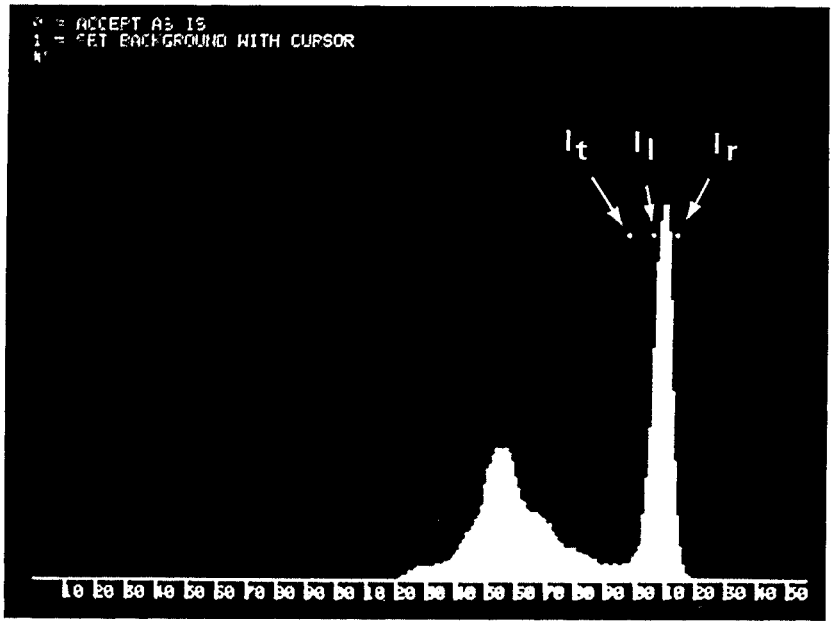

Fig. 3. Display on Tektronix scope of intra-histogram of measured light intensities. I_l and I_r = intensity values at half of the frequency of the background peak, I_t = threshold intensity value calculated by the computer program (for details see text). If desired, another threshold can be chosen by the operator

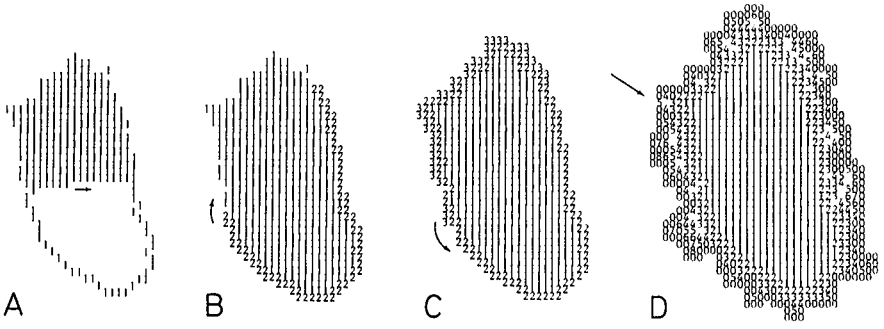

Fig. 4. **A** Scope image of boundary of *object points* processed with the DIODA program, being filled with 1s to show the *measured area*. **B** and **C** Extension of *area of object points* with rings indicated by 2, 3, 4 ... to obtain the complete *object area* together with background set A (for details see text). **D** Fully extended object and background image. At →, extension had to be stopped prematurely to prevent addition of points belonging to the neighbouring object

outside the extended object. Therefore, another set of points is added, set *B*, which comprises the outside points i.e., those belonging to the innermost ring around the extended cell (indicated in Fig. 4D by 0). Taking care not too introduce a false background value, those points of set B are excluded which apparently belong to a neighbouring object, i.e., having a too high intensity value. In Figure 4D this has for instance occurred at the position indicated by the arrow where the 0s are missing. In this way, the background is obtained as the weighted sum of the averages of sets *A* and *B*.

The background thus defined, is the closest possible one around the cell or nucleus; influences of disturbances (noise or gradient) throughout the measuring rectangle being minimized. The number of background points obtained amounts to 50 and 100% of that of the measured object, which is quite close to the optimal situation. At the end of the procedure the total absorbance value, the number of measuring points, and the mean local absorbance value are displayed.

Because measurements are only taken while the stage is moving from left to right, the scan time is about twice that required for the FASTSCAN procedure; computing time is about 10 s, independent of the size if the scanned rectangle, because the dilatation is the most time-consuming part of the procedure. The gain in the integrated absorbance value is relatively small ($<5\%$) and in fact introduces more uncertainty with respect to the total DNA content, but is required to obtain a properly defined background value. To speed up the procedure, the scanning stage can be re-positioned with the joystick to center another cell during the computation and display procedures.

With this program 50 cells per hour can be measured.

ARRAYSCAN

This is the program of choice for preparations in which the cells touch or lie very close to each other. After arrays are scanned (sampling from left to right only), an intra-histogram of light intensity values is displayed on the Tektronix scope, showing the background threshold computed by the program. The determination of this threshold is performed as described for the DIODA procedure (Fig. 3). After the threshold is set, the operator can ask for a display of the remaining image in 10, 17 or 26 ($=n^2+1$) intensity levels (Fig. 5A) or one in which 20 intensity levels are represented by an alphanumeric character. It is then possible to ERASE or SELECT parts of the image by the use of a rectangle marked by two cursors which are movable in the way described above (Figs. 5B and 5C), or by means of a point (generated on the display) that can be moved with the joystick (Fig. 5D). After this procedure, the integrated absorbance of the remaining points is computed and shown together with the remaining image. It is also possible at this time, to ask for a histogram of the points in the remaining area.

Results

To check the reproducibility of the individual measuring procedures and the reliability of the results obtained by any of them, isolated cells in well-spread areas with a clean background were measured successively with the ARRAY-SCAN, FASTSCAN and DIODA programs. These series of measurements were repeated 5 times for each of the cells (Tables 1–5). In all cases the dimensions of the measuring area were chosen just large enough to allow easy adjustment of the objects. In preparations where the background is not homogeneous the use of relatively large areas can affect the values obtained with the FASTSCAN program (Table 6). As could be expected, in such cases, the closest approximation of the values resulting from the other programs was obtained when small measuring areas were used. As already mentioned under *Material and Methods*, however, for the DIODA program the measuring area must be large enough to provide measuring points for the background determination. When the mea-

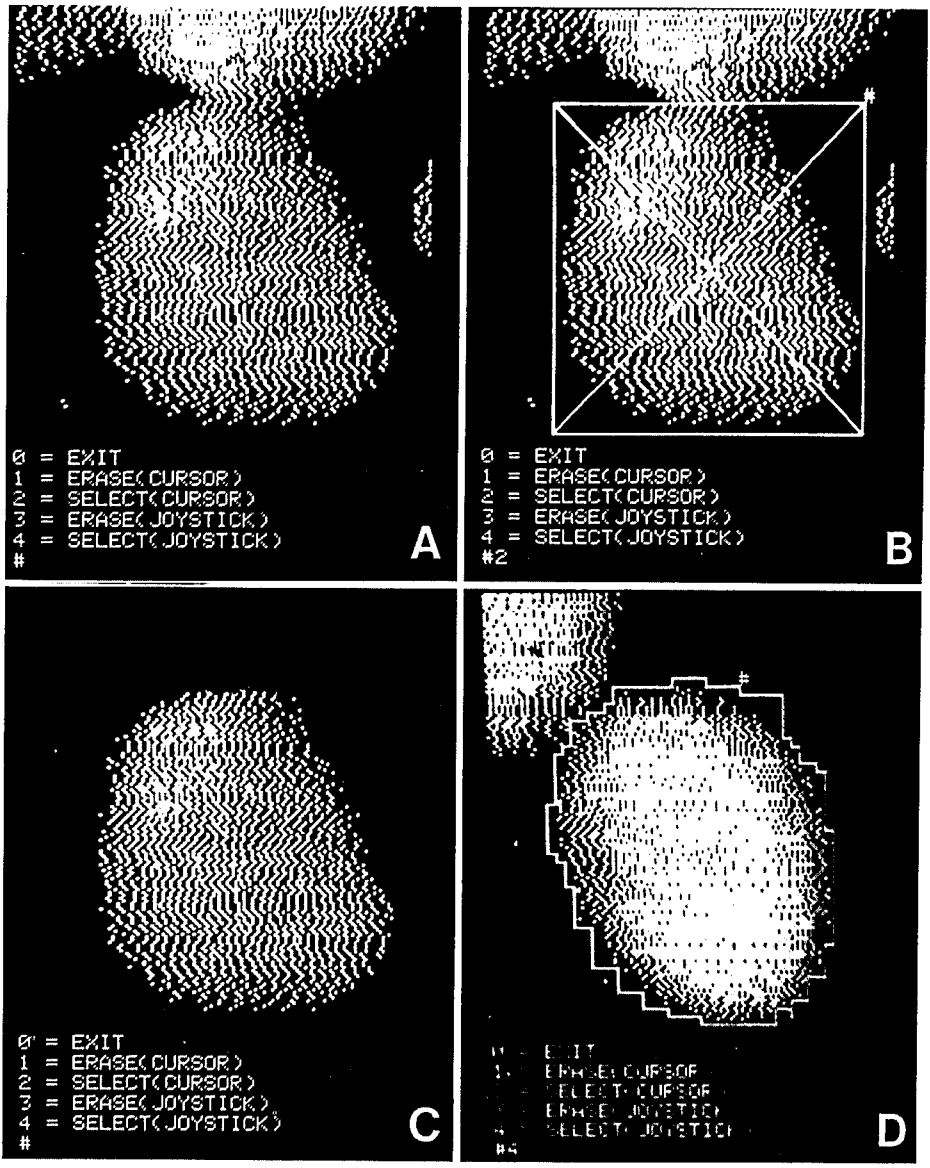

Fig. 5. **A** Display in 10 intensity levels of points with an absorbance greater than the threshold value. **B** Display after choice of a rectangle enclosing the part of the image that is to be SELECT-ed. **C** Display of remaining image for which the integrated absorbance value has to be calculated. **D** Display after SELECT procedure performed with a point moved by means of joystick

Table 1. Integrated absorbance values of Feulgen-stained chicken erythrocyte nuclei

Cell	Program		
	ARRAYSCAN	FASTSCAN	DIODA
1	2610 (1.2) 1.00	2584 (0.6) 0.99	2620 (1.1) 1.00
2	2565 (1.7) 1.00	2592 (2.4) 1.00	2602 (1.0) 1.01
3	2695 (1.5) 1.00	2629 (1.4) 0.98	2661 (1.6) 0.99
4	2656 (2.3) 1.00	2625 (1.8) 0.99	2665 (0.5) 1.00
5	2671 (2.4) 1.00	2682 (2.2) 1.00	2675 (2.2) 1.00

Measurements were taken at 0.5 μm intervals. Each cell was measured 5 times in each program ( )=C.V.

Table 2. Integrated absorbance values of chicken erythrocytes after gallocyanin chrome alum staining

Cell	Program		
	ARRAYSCAN	FASTSCAN	DIODA
1	4466 (1.6) 1.00	4489 (3.5) 1.00	4423 (1.7) 0.99
2	4788 (1.6) 1.00	4797 (2.3) 1.00	4728 (0.9) 0.99
3	4450 (4.0) 1.00	4516 (1.1) 1.01	4652 (1.8) 1.04
4	4448 (1.5) 1.00	4450 (1.5) 1.00	4500 (2.2) 1.01
5	4727 (2.0) 1.00	4661 (1.0) 0.99	4677 (1.8) 0.99

Table 3. Integrated absorbance values of guinea pig exudate granulocytes stained for alkaline phosphatase activity ($\times 10^{-1}$)

Cell	Program		
	ARRAYSCAN	FASTSCAN	DIODA
1	2029 (0.3) 1.00	2014 (0.9) 0.99	2019 (0.7) 0.99
2	1644 (0.3) 1.00	1662 (0.8) 1.01	1658 (0.8) 1.01
3	2017 (0.5) 1.00	2023 (0.5) 1.00	2024 (0.5) 1.00
4	1116 (0.3) 1.00	1128 (0.6) 1.01	1102 (0.6) 0.99
5	1247 (0.7) 1.00	1237 (0.6) 0.99	1252 (0.6) 1.00

surement of relatively large objects threatened to exhaust the limited memory core capacity available for this program, the number of data was reduced by scanning at sampling intervals of 1.0 μm. In such cases the integrated absorbance value was multiplied by 4 for comparability with the other data.

As an additional check, we compared the data obtained when either the SELECT OR ERASE procedure was performed to obtain the individual integrated absorbance values from an ARRAYSCAN of two touching objects (Ta-

Table 4. Integrated absorbance values of Feulgen-stained human white blood cells from valve centrifuge preparations

Cell	Program		
	ARRAYSCAN	FASTSCAN	DIODA
1	7158 (0.3) 1.00	7458 (0.7) 1.04	7211 (0.8) 1.01
2	7184 (0.8) 1.00	7419 (1.8) 1.03	7162 (1.0) 1.00
3	6985 (0.6) 1.00	7140 (1.8) 1.02	7091 (0.2) 1.01
4	7118 (0.6) 1.00	– –[a]	7069 (0.3) 0.99
5	6939 (1.0) 1.00	6782 (2.4) 0.98	6908 (1.0) 1.00

[a] Too close to measure with FASTSCAN

Table 5. Integrated absorbance values of Feulgen-stained human fibroblasts cultured on glass slides

Cell	Program		
	ARRAYSCAN	FASTSCAN	DIODA
1	7121 (1.1) 1.00	7108 (0.9) 1.00	7098 (0.9) 1.00
2	7424 (1.2) 1.00	7516 (0.4) 1.01	7487 (1.4) 1.01
3	6936 (1.1) 1.00	7017 (0.5) 1.01	6982 (0.4) 1.01
4	7015 (1.1) 1.00	7005 (0.7) 0.99	7083 (0.8) 1.00
5	7726 (1.5) 1.00	7767 (1.0) 1.00	7694 (1.1) 1.00

Table 6. Integrated absorbance values of Feulgen-stained chicken erythrocyte nuclei measured with the FASTSCAN program using different measuring areas

Cell	Width of Area in µm		Cell	Width of Area in µm	
1	8	2691	2	8	2651
	16	2722		14	2870
	24	2821		20	3001
	32	2979		36	3117
	40	3005		80	3050

ble 7). According to expectation, due to the absorbance in certain background points there is a slight difference (less than 1% in the preparations tested) between the sum of the two values obtained by the SELECT procedure for each of the objects and the total integrated value calculated over the whole array.

Table 7. Integrated absorbance values of nuclei of touching Feulgen-stained cells in lymph node imprint preparations, measured with the ARRAYSCAN-SELECT procedure

Cells	Total	Sum of select values	Difference in %
1–2	14460	14308	1.1
3–4	13878	13878	0
5–6	13543	13542	0
7–8	13935	13916	0.1
9–10	14129	14085	0.3

Discussion

Although the need for quantitation of cytochemical staining results is neither doubted nor disputed by the majority of cytochemists, it must be concluded that only a relatively small percentage of these investigators are actively engaged in the application of microscopical measuring methods (Rosenquist, 1975). In general cytophotometry and cytofluorometry are still considered to be laborious, time-consuming (and expensive) and also beset with many pitfalls.

In our opinion, this is—at least partially—due to the fact that in general too little is known about the rapid progress which has been achieved in the field of microscopic-cytochemical methods, and the measuring equipment now available, which allow reliable application of these tools in biomedical and clinical research (see e.g. Melamed and Kamentsky, 1975; Pearse, 1972).

Several types of reliable cytophotometers that are reasonably simple to operate, are commercially available at present. Instruments that utilize one or another scanning principle have so far found the widest application (Altman, 1975). For the relative advantages and disadvantages of image plane, flying spot object-plane. TV, or stage scanners, see e.g. Bedi and Goldstein (1976), Wied (1966) and Wied and Bahr (1970).

The present paper describes a versatile combination of measuring programs (HIDACSYS) developed to enable determination of the integrated absorbance or fluorescence values of individual cells, nuclei, or chromosomes, properly corrected for background, as quickly as the local conditions in the microscopical preparation permit.

Routine microscopical preparations usually contain not only areas with optimally separated cells but also areas with closely packed cells. These areas—and even those containing cell clusters—cannot be excluded from measurement because some of the cells in them may contain essential information.

Improved preparatory techniques can reduce the number of artificial cell clumps, but it seems unlikely that it will become possible to eliminate clumping completely. This means that the measuring program must also allow measurements in areas where objects lie very close or even touch each other. In such densely packed areas it may be difficult to find sites to determine the background

correction properly in the immediate vicinity of the object under study. It is the special advantage of the ARRAYSCAN and DIODA programs that they allow determination of the correct background directly around the measured object.

The definition of the correct background may still seem somewhat ambiguous. Measurements taken too close around say a nucleus can be influenced by stray-light errors resulting from the refraction of light at the margin, while – as already indicated – background sampling further away is sensitive to potential gradients (see e.g. Mayall and Mendelsohn, 1970).

Although the present investigation was not primarily designed to solve this problem, the results of the measurements described here show that for the material under study, the three HIDACSYS programs – each of which having a slightly different method of background correction – at least lead to integrated absorbance values that do not differ significantly. Further testing of the programs, during measurements on larger numbers of cells showed that the values obtained are quite accurate provided staining and embedding have been performed under optimally standardized conditions (Van Duijn et al., 1977; Duijndam et al., 1977). This was also demonstrated by investigations performed by Van Vloten et al. (1974), Van der Want and Sprey (1976), Goldstein et al. (1977), and Chatterjee et al. (1977).

The investigation of Duijndam et al. also showed that – due to the scanning stage principle applied and the quality of the optics of our cytophotometers – optical errors, and especially glare, can be kept so low that correction for these effects, as proposed by Bedi and Goldstein (1976), is not necessary.

DIODA has its own ability to delineate the objects' boundary and to exclude close-lying parts of other objects present in the scanned area. To the best of our knowledge, such programs have so far only been realized on large computers (see e.g. Mendelsohn et al., 1966; Groen, 1977) and not with a mini computer like a PDP 11/10.

ARRAYSCAN, in combination with the ERASE or SELECT options, allows isolation of the chosen object from other light-absorbing particles lying extremely close to it, and is preferentially applied when the interfering material touches the measured object. Comparison in this respect with the APAMOS program provided by Zeiss and the MIDAS program of Leitz, shows that the latter are relatively time-consuming, because deletion of such particles requires moving of a rectangle over the display of the scanned area where – as a result of the chosen threshold – both the object and adjacent particles are visible. The data within the rectangle can be deleted, but this demands painstaking positioning of the rectangle in cases of touching objects.

At the moment, the HIDACSYS programs are regularly used in investigations on the Feulgen-DNA content of individual nuclei in somatic cell hybrids, nuclei of snail neuron imprint preparations, cell nuclei in cases requiring diagnosis of cervical, urinary, or mamma tumors, and the Sézary syndrome (Dons et al., 1974; Bosman and Van Vloten, 1976). They are applied to measure dye deposits in cells stained for alkaline or acid phosphatase activity, and also to study immunocytochemically labelled cells or models consisting of agarose microbeads (Streefkerk et al., 1974).

In our configuration HIDACSYS runs under the (time-sharing) MINIBOSS operating system, which can supply full control to up to five scanning cytophotometers simultaneously. In a single-users version under DEC's RT-11 operating system the same facilities need 6K memory less.

The main advantage of the HIDACSYS system is its versatility: it allows rapid interchange of programs when the local situation in the microscopical cell preparation requires so.

FASTSCAN is the fastest program, and allows the measurement of 60 to 70 cells per hour in preparations of good quality. However, in preparations with a gradient in the background absorbance the results tend to be susceptible to the size of the measuring rectangle.

DIODA, with its ability to delineate the object under investigation from its surroundings, is slightly more time-consuming, partly because signal sampling is done in one direction only, and partly because data processing is not started until scanning is completed. On this basis its performance amounts to 40 to 50 cells per hour.

When the ARRAYSCAN program is used without the SELECT or ERASE procedure, it is somewhat faster than DIODA the maximum being 55 cells per hour. With the inclusion of SELECT and/or ERASE, about 20 to 25 cells can be measured per hour. With frequent switching from one program to the other, this number is of the order of 30 to 40. One of the main limitations on performance, is of course the fact that the motor-driven stage has a maximum speed of 200 steps a second.

The ARRAYSCAN program can also be used to produce a data file as a basis for pattern analysis, e.g. for chromatin analysis (with or without prior data reduction). A modification of the FASTSCAN program called PROFILSCAN is used to make measurements in photographic images of individual chromosomes and to determine the DNA content and DNA distribution along the chromosome (Van der Ploeg et al., 1974a, 1974b; Geraedts et al., 1975; Bosman et al., 1977) as well as the Quinacrine or Giemsa banding patterns (Van der Ploeg et al., 1977).

From the foregoing it may be concluded that the HIDACSYS program system is accurate and versatile and that it permits effective measurement of different types of cell preparation at a reasonable speed.

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