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Accuracy of optical density measurement of cells.

1: Low resolution

A. W. M. Smeulders and T. K. ten Kate

In this paper we analyze factors influencing the accuracy and precision of optical density measurement of light microscopical objects. The study is applied to the DNA content of Feulgen-stained cells using a low-resolution, TV-equipped microscope connected to a digital image processing system. Factors influencing the accuracy of density measurement include staining (not considered here), image formation (glare, focus, diffraction), image sampling (distribution, sampling density, noise), and computational accuracy. These factors are reviewed with respect to potential remedies. Evaluation of the measurement error contribution is performed on the level of the individual cell and on the specimen level. It is concluded that a coefficient of variation of 5% (in contrast to 3.5% using flow cytometry of the same specimen) in the measured values of normal cells of one specimen is attainable using adequate shading correction. With relatively simple computational methods, image cytometry may be well suited for pathology practice.

I. Introduction

A recurrent technique in the field of cytometry is to determine the amount of cellular substrate quantitatively via measurement of the optical mass. A prerequisite for quantitative measurement is that the visualizing dye be bound specifically and proportionally to the amount of cellular substrate. For DNA, the most prominent cellular component, the Feulgen procedure satisfies this demand.¹ In cytometrical practice, two ways of cell measurement are often encountered. Flow cytometry involves the analysis of single cells in a suspension flowing past a sensor. Here, usually fluorescence imaging is used. The alternative is image cytometry in which a microscope, equipped with an image sensor, extracts measurements from the cells or tissue on a glass slide. Although the advantages of fluorescence imaging have been argued for image cytometry as well [see, for example, Ref. 2], for practical reasons absorbance microscopy is usually preferred.

From a clinical point of view, measuring the optical mass, particularly when representing DNA, is important for diagnosis, prognosis, and therapy of many

tumors and tumorlike diseases.³ It is not so much the absorbance value of an individual cell that counts, but rather the DNA profile of a specimen acquired by accumulating the individual DNA values of many cells in a histogram. The presence of uncommon peaks in the profile reveals (small) cell populations in the specimen with abnormal DNA values.

In this context it is our aim to combine the practical advantages of image cytometry in measuring absorbance of cells dissociated from their connecting tissue^{4,5} with the advantages of flow cytometry. They are accuracy and precision of measurement and speed of analysis. The accuracy of the DNA measurement usually is expressed as the coefficient of variation of a homogeneous cell population (e.g., of normal cells in a resting phase). For flow cytometers (using fluorescence imaging) the accuracy can be as high as 1–4%.^{6,7} This figure includes the biological variation in the DNA content of the cells in one sample (under normal conditions ~0.5%). These figures, in principle, permit the detection of tumor lines with even small deviations from the normal DNA profile. They do not, however, tell the whole story. In common flow cytometers no image of the object is formed. In these flow cytometers, making a distinction among measured values of single cells and the values due to cell clumps and artifacts is, therefore, difficult, if not impossible. This is a drawback of flow cytometry as some tumors with only small proportions of abnormally valued cells may become indistinguishable from the portion of artifacts or cell clumps in the sample. The second advantage of flow cytometers, their speed, permits the construction of DNA profiles of 10,000–100,000 cells/specimen in ~15 min. From a practical point of view, however,

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Table I. Summary of Aspects of Densitometric Accuracy

Part	Aspect	Main operand	Effect on value of Z
Microscope	Glare	Intensity	Systematically too low
	Shading	Intensity	Systematic error
	Diffraction	Intensity	Systematically too low
	Focus	Intensity	Systematically too low
Digitizer		Area object	
	Resolution	Intensity	Larger variation
	Noise	Intensity	Larger variation
	Offset	Proportionality	Systematically too low
	Gain	Proportionality	Systematic error
Computation	Distribution	Intensity	Systematically too low
	Overlap	Area object	Systematically too high
	Segmentation	Area object	Systematic error
	Backgr. region	Backgr. int.	Larger variation
			Systematically too low
	Backgr. value	Backgr. int.	Systematic error

flow cytometers require a complex procedure to operate them, limiting the average throughput of specimens on a flow cytometer to about one per hour. Although the actual analysis is very fast, the preparation of apparatus and specimen stands in the way of improving efficiency.

Image cytometry is a potential alternative for routine cell specimen analysis as it resolves some of the drawbacks of flow cytometry. In image cytometry artifacts and cell clumps can be excluded from the DNA profile on the basis of shape or texture analysis. This makes image cytometry better suited to detection of low-portion tumors. Second, image analysis systems are in principle capable of analyzing cells in their original context, providing additional insight into the architecture of the tumor. In practice, image cytometry has the considerable advantage that specimens can be handled and stored dry. The machine is also simpler to operate, making the total time for one specimen almost equal to the time the analysis takes. Before image cytometry is used in routine practice, two of the present disadvantages should be resolved. First, the processing speed should be increased to offer a practical alternative to flow cytometry. Employing state-of-the-art technology,^{8,9} this goal can be achieved. Second, the accuracy of image cytometers for DNA measurement should be improved considerably to become comparable with flow cytometry.

Therefore, in this paper we analyze aspects of the accuracy and precision of densitometry. The overall accuracy of the DNA specimen profile is determined by the accuracy and precision (varying the location in the image) in the measurement of each cell. We consider a video microscope connected to a digital image

processor. We do so for low-resolution systems to guarantee a reasonable processing speed per slide.

The theory of densitometry has been dealt with in great detail by Goldstein¹⁰⁻¹² and Mendelsohn and Mayall^{13,14} for stage-scanning systems. A similar analysis of video-based systems has not been given yet. The practice as follows from descriptions of densitometry algorithms is not always as precise as to reconstruct the method in detail. Sometimes the size of the image is reduced to considering only the cell in the middle.¹⁵ Mostly, some sort of shading correction is applied, sometimes unspecified,¹⁶ sometimes additive,¹⁵ and sometimes of the multiplicative type.¹⁷ Many programs have interactive selection of segmentation parameters which may give an undesired bias in the densitometric result. The background intensity is measured either from selection of the background peak modus or by computation of the background intensity from a local region.¹⁵ These aspects will be reviewed to arrive at a reliable and fast densitometric algorithm.

II. Analysis of Optical Density Measurement

The basic densitometric formula is given by the Lambert-Beer law:

$$Z = \sum_N f \left(\frac{I(x,y)}{I_0(x,y)} \right),$$

where Z is the total absorbance of the object, N is the area of the object, I is the intensity at location (x,y) , and I_0 is the intensity at (x,y) if the object were not there. Function f gives the relationship between the intensity and the absorbance, including the molar absorptivity and chromophore concentration. In case of absorbance microscopy, f ideally is minus the 10-base logarithm function. The formula indicates where errors in the value of Z may originate. They are either in the area N , in the individual intensity values, in the background intensity estimation, or in the deviation of f from the logarithm. As summarized in Table I, each error may occur in each of the three parts of the measuring system: the microscope, the digitizer, and during the processing of the image, each of which are discussed.

The influence of glare on the densitometric result of stage-scanning systems is studied in Ref. 10, where it adds some 1% to the signal due to the restricted illumination. A glare of 1% limits the measurable density values in practice to 2 ($= -\log 0.01$). For total field illumination, as is the case here, higher glare values are found, ranging to 5%, limiting density to 1.70. The glare results in a relative error in Z , like Goldstein in Ref. 10:

$$\left(\frac{\Delta Z}{Z} \right)_{\text{glare}} = -\log \left(\frac{(1-G) \cdot I(x,y) + G \cdot I_0}{I(x,y)} \right) / \log \left(\frac{I_0}{I(x,y)} \right),$$

where G is the glare present in the system expressed as a fraction of I_0 . In practice with 5% glare and a homogeneous absorbance of 0.25, the error amounts to -6%.

The electronic offset in the TV signal is an example of a completely additive effect.

The effect of locally reduced light flux (shading) is a local diminution of the signal by a factor $\Delta g_s(x,y)$. A similar effect as the optical shading effect is the locally unequal sensitivity of the camera tube, the gain. Apart from gradual changes $[\Delta g_g(x,y)]$ of the gain over the entire tube, there may also be present small local spots with a strongly decreased gain. The effect on Z for $\Delta g_s(x,y) + \Delta g_g(x,y) = \Delta g_c$ using a global value for I_0 is a reduction by a factor Δg_c , which may go up to 15% in the corners of the image. Using a local value for $I_0(x,y)$, there is no net effect on the value of Z .

The four optoelectronic aspects were divided into (partially) additive aberrations (glare and electronic offset) and multiplicative effects (shading and gain) on certain model assumptions. Whether this division of the effects over additive and multiplicative is completely valid is not relevant. It suffices to conclude that all four are aspects of optoelectronic origin that can be approximated as a linear description distortion in the intensity value. In commercially available image processing systems, a choice is offered between multiplicative (shading) correction alone (at reduced resolution¹⁸), or the user is given the choice between either additive or multiplicative correction (also at reduced resolution¹⁷). Of course, with either method a blank field may be perfectly reconstructed, but this background correction is not sufficient for densitometry: gray-scale correction is needed. Next we compare the performance of the additive method with the performance of the multiplicative one. We also compare linear correction (i.e., multiplicative and additive correction). In this case two correction images are needed. These two images are I_{E1} , which is a full-resolution empty image at 100% intensity averaged 64 times to be certain of the absence of noise, and I_{E2} , which is similarly recorded now using a 0.5 absorption filter. The linear correction is computed by

$$I_C = \frac{M \cdot (I_R - I_{E2})}{2 \cdot (I_{E1} - I_{E2})} + 1,$$

where I_R is the recorded image, and I_C is the corrected one. $M = \text{median}(I_{E1} - I_{E2})$ is a scaling factor to avoid in the corrected pixel values errors in rounding off to integer. It is anticipated at this stage that linear correction will outperform the other two correction methods as it contains the virtues of both.

The distributional error is inherent to absorbance densitometry. It originates from the fact that taking the logarithm of the intensity values averaged over the spot area is not the same as taking the logarithm of every pinpoint in the spot area and averaging these logarithms afterwards. By fitting a plane through the gray values in the neighborhood of (x,y) , Groen¹⁹ derives the approximate error in Z :

$$\left(\frac{\Delta Z}{Z}\right)_{\text{distr}} = - \frac{h^2 \sum \sum D(x,y)}{24 \sum \sum D(x,y)},$$

where $D(x,y) = \log I_0(x,y) - \log I(x,y)$, h is the grid constant, and

$$D(x,y) = \left(\frac{\partial D(x,y)}{\partial x}\right)^2 + \left(\frac{\partial D(x,y)}{\partial y}\right)^2.$$

For an object with $N = 200$, $D = 0.2$, and $\partial D/\partial x = \partial D/\partial y = 0.1/h$, the formula gives $\sim 0.4\%$. For double contrast ($N = 200$, $D = 0.4$, and $\partial D/\partial x = \partial D/\partial y = 0.2/h$), the outcome doubles (0.8%). From the formula it can be seen that the distributional error leads to a decrease in the observed value of Z and that the effect of the distributional error depends on the overall and on the internal contrast of the cell. The effect is also related to the sampling resolution as $D(x,y)$ will decrease with an increasing sampling density. Since the value of the distributional error is limited even for considerable values of internal contrast, the effect has not been considered in the low-resolution experiments conducted below.

The diffraction effect in scanning microdensitometry plays a role at distances up to $2 \times [\lambda/(2\pi \text{N.A.})]$ (Refs. 12 and 20). The diffraction error using $\text{N.A.} = 0.65$ and $\lambda = 550 \text{ nm}$ plays a role up to $0.25 \mu\text{m}$. For low-resolution systems, therefore, it does not play a significant role and has not been studied any further (see also Ref. 26).

The effect of defocusing on the image formation is theoretically well known²¹ to approximate a low-pass filtering of the image. Hence, although defocusing leads to a decrease of the local intensity values, it will not greatly influence the measured value of Z when the decrease in intensity values is compensated for by an increase in the apparent size of the object. In this way, in the increased mask of the object all optimal mass may be captured, leaving only a second-order effect. For a $100\times$ oil objective ($\text{N.A.} = 1.3$), Goldstein¹¹ finds a decrease of 4% in the value of Z/λ out of focus when computed by summing all density values above a fixed density threshold >0.05 . Had he used a threshold (and image size) varying with the overall contrast, the result would have been even less by the above argument. For our circumstances, working at low resolution, we have found that the effect is very small, especially when the focus is done automatically. Therefore, we have not studied it any further here, but we will return to this subject in Ref. 26.

As concerns the influence of noise, a typical TV camera has a SNR of 40 dB or 6 (useful) bits/pixel only. A rule of thumb²² is to take the quantizing step size three times the rms of the noise. Per pixel averaging sixteen successive image recordings is then necessary to obtain eight reliable bits or 58 dB. When the aim is density measurement alone, a noise-reducing effect comes from Z , resulting from a sum over N

pixels. This restricts the error in Z to $\sigma/\sqrt{N}$ and hence reduces the noise in the intensities (σ) by a factor of 0.1 to 0.03. It should also be noted that this averaging is on density values, but the noise is in the intensity values. Hence, computing Z from noisy intensity values leads to a bias, albeit a very small bias (see Appendix). For densitometry it may seem sufficient to accept the reduction of $\sigma/\sqrt{N}$ in the error of Z due to averaging over the N object pixels and leave the time-consuming averaging over image recordings out. The noise, however, will also indirectly influence the value of Z through variations in the segmentation of the object, to be discussed below.

Another aspect relevant for the accuracy of density measurement is the spatial resolution. Usually here the Nyquist theorem is quoted stating that all information of a frequency-limited continuous image (λ_N) can be reconstructed by sampling the image with $\lambda_S < \frac{1}{2}\lambda_N$. For a microscope the λ_N is given by $0.61\lambda/\text{N.A.}$, where λ is the wavelength of the light source. However, the Nyquist criterion is meant for image reconstruction with a sinc-function. For image reconstruction with a block function, effectively employed using a data array in the computer, much higher sampling rates are needed²³ (see also Refs. 24 and 25). But a more fundamental objection to the application of the Nyquist criterion here is that we are engaged with measurement rather than image reconstruction requiring other criteria. These criteria may be found in the number of object points needed to make a reliable estimate of Z , as was discussed above under noise considerations, or the ability to get a proper segmentation of the object. Resolution-dependent factors are considered in a sequel paper.²⁶

With respect to the computational aspects, the error in Z due to segmentation obviously will lead to underestimation of Z when the object is delineated too small. On the other hand, the area should not be extended over the outskirts of the object where no density is present, as regions with an average zero density in effect only contribute to the noise in the value of Z .¹⁹ A well-known compromise between these two extremes is to use a segmentation algorithm based on thresholding the image with a dissection level I_t . All intensity values with $I < I_t$ belong to the object. Here a variable dissection level is set a few standard deviation units away from the background peak by subtracting the width at 5% of the peak height from the left intensity value where the 5% occurs.²⁷ In this way no background pixels are included in the object. Some of the density at the borders of the object remains uncounted, however. The magnitude of the error may be derived by assuming that the cell border rises in a straight plane from the surrounding background. For a threshold located somewhere on the slope of this profile, an increase in the threshold value I_t will result in an increase in the value of Z by

$$\left(\frac{\Delta Z}{Z}\right)_{\text{segm}} = -\frac{4\pi \cdot \log(T)}{m \cdot \bar{Z} \cdot S},$$

where $-\log(T)$ is the average of the densities over the

increased area. For $-\log(T) = 0.02$, an average density of $\bar{Z} = 0.2$, a perimeter of $S = 100h$, and an intensity slope of $m = 10/h$, the error is 0.13%. The segmentation introduces a systematic underestimation of the value of Z which is approximately linear with the reciprocal of the perimeter. Therefore, it has an influence on Z depending on the type of cell.

In a strict sense there is a difficulty estimating the background value from the digitized image as it is the local value of the intensity when the object would not have been there. To overcome the impossibility of removing the cell, $I_0(x,y)$ is replaced by one value I_0 for the entire object derived from a substitute region in the image, the background region. The estimation of I_0 may thus be split into two parts: determination of the background region and determination of the value I_0 from this region. At this point one of the reasons to do accurate shading correction becomes clear, namely, to make the background region representative for the location of the cell. Usually, the background region is defined as the region where no cell pixels lie, giving one value of I_0 for all objects in the image. Care should be taken to exclude those parts of the image in the background region that have absorption, excluding a bias of I_0 . The influence of a bias on the value of Z is in first approximation given by

$$\left(\frac{\Delta Z}{Z}\right)_{\text{bias } I_0} = -\frac{\log(e)}{\bar{Z}} \cdot \frac{\Delta I_0}{I_0}.$$

To prevent the bias we exclude pixels with an intensity value deviating more than one standard deviation from the mode of the background intensity peak and all their eight connected neighbors. In the literature, sometimes, additional care in the computation of I_0 is proposed by defining a background region locally, one to each object.¹⁵ As shading correction is satisfactory in our case, we have not employed a local background region.

Given the background region, various ways are available to derive the background value from it. First, consider the case where the background intensity is defined as the modal value of the background peak. This is an integer value introducing an error in the density values $D(x,y)$ of

$$\Delta D = \frac{\Delta D}{\Delta I} \cdot \Delta I = \frac{\log(e) \cdot \Delta I}{I}.$$

The related error in Z is

$$(\Delta Z)_{\text{mode}} = \sum_N D(x,y) - N \cdot \Delta D_0.$$

The error $D(x,y)$ is likely to average out leaving the error D_0 as the main component. The relative error in the density of the cell will be of the order of

$$\left(\frac{\Delta Z}{Z}\right)_{\text{mode}} = \frac{\log(e) \cdot \Delta I_0}{I_0 \cdot \bar{Z}},$$

where $\bar{Z}$ is the average density of the object. When $I_0 = 200$ with a truncation error $\Delta I_0 = 0.5$ and $\bar{Z} = 0.2$, the error is 0.5% due to selecting an integer value for I_0

Fig. 1. The set of eight cells used in the experiments. Top left is CELL 1.

alone. Alternatively, if the background intensity is estimated by averaging sufficient background pixels with floating point precision, Groen¹⁹ derives the following equation for the quantization error:

$$\left(\frac{\Delta Z}{Z}\right)_{\text{quant}} = \sqrt{\frac{1}{N} + \frac{1}{N_b}} \cdot \frac{\log(e)}{\bar{Z} \cdot \sqrt{12} \cdot I} \cdot \left(1 - \frac{1}{I_{\max}}\right).$$

In this formula N_b is the number of pixels of the background region, and $I_{\max}$ is the maximal intensity value. The equation demonstrates that, to minimize the error in Z , the number of background points has to be at least equal to the number of object pixels. In a situation where $N = 500$, $N_b \gg N$, $I_{\max} = 255$, $\bar{Z} = 0.2$, and $I_0 = 200$, the relative error in Z due to background quantization is only 0.01%. The last three expressions show the importance of estimating I_0 in floating point precision from a background region cleaned from pixels having absorption.

Finally, in the analysis of a cell specimen, overlapping cells influence the shape of the DNA profile. The effect can be reduced largely by detecting them on basis of their shape.^{28,29} We will use a method based on the bending energy of the object contour³⁰ looking for sharp kinks in the contour.³¹ The method has a success rate of ~90%, only leading to failures when cells lie on top of each other.

III. Experiments

The above issues have been verified in experiments with the aim of quantifying the influence of each error compared to the effectivity and processing efficiency of a correction. A microscope-TV system was used, with a UEM frame and a dry-lens plan 40× objective (N.A. = 0.65). The overall sample spacing is 0.31 μm . For this resolution the nuclei of normal cells have an area ranging between 190 and 700 h^2 , where h is the grid

constant (0.31 μm). The halogen light source was tuned at the beginning of each day to give an intensity value of ~200 (with $I_{\max} = 256$) for an empty field. The light was filtered at the wavelength of maximum absorption of the Feulgen stain ($\lambda = 550 \text{ nm}$, $\Delta\lambda = 10 \text{ nm}$). The TV camera contains a chalnicon tube with a specified linearity of 95% and a SNR better than 55 dB. For the SNR of the system we measured a value of 40 dB. To move the stage in x , y , and z directions the system is equipped with a 10-kHz stepping table with step size of 0.25 μm and an autofocus device operating on the TV signal. The signal was also fed into an image processor: the Kontron IPS. The IPS supports a wide variety of image processing options, a small part is used here. Images are processed in arrays of 512×512 picture elements (pixels) stored in 8 bits.

Three types of experiment were performed, each time using a different set of cells. In all instances an experiment began by reading a freshly recorded image into the computer. For the first type of experiment a set of eight different cells was selected—varying in size, internal contrast, and density—from a non-Hodgkin lymphoma (see Fig. 1). They were selected to cover the entire spectrum, rather than being a representative sample for a specimen. The internal variation of a specimen is normally less. The cells were positioned at 3×3 equally spaced locations covering the field of view. They were measured one time at each of the nine locations.

In the base-line experiment the following program was used:

- Digitize one image;
- Threshold the image at intensity value I_t derived from the histogram²⁷;
- Concatenate the resulting object pixels when they are neighbors into objects;

Table II. Effect of Z of Different Cells (CELL) Given by the Mean (and c.v.) Values Derived Without Shading Correction (NULL), with Additive (ADD), Multiplicative (MULT), or Linear (LIN) Shading Correction^a

CELL #	NULL	ADD	MULT	LIN
1	47.67 (19.0)	46.17 (5.0)	47.35 (1.5)	47.06 (3.5)
2	43.01 (24.8)	40.52 (3.9)	40.91 (2.0)	40.66 (3.2)
3	—	44.07 (8.3)	43.63 (3.5)	41.42 (3.3)
4	138.52 (24.0)	114.33 (4.8)	116.96 (1.3)	114.70 (2.6)
5	115.99 (23.4)	109.60 (3.9)	106.21 (1.9)	109.44 (2.5)
6	52.69 (46.4)	51.67 (5.4)	52.41 (1.8)	52.02 (2.8)
7	93.86 (35.7)	90.58 (5.8)	94.04 (1.4)	88.86 (2.9)
8	61.14 (30.2)	59.45 (5.5)	61.65 (2.2)	58.28 (2.2)

^a Each cell measured at nine different positions covering the field. One measurement of the NULL experiment is missing due to the impossibility of segmenting the cell automatically.

Table III. Correlation Coefficients Computed from Two Measurements on a Vickers M85 Densitometer (VICK) Correlated with the Measurement on a TV System Applying No Shading Correction (NULL), or Additive (ADD), Multiplicative (MULT) or Linear (LIN) Shading Correction

VICK	NULL	ADD	MULT	LIN
1	0.84	0.97	0.98	0.97
2	0.83	0.99	1.00	0.99

Remove object touching the image edge;
Sum the log-intensity values of the N object pixels
(= Y);
Define $Z = Y - N \cdot I_0$, where I_0 is the modulus of the
intensity histogram (background peak);

For the set of eight cells, the mean and coefficient of variation values (c.v.) resulting from one measurement at the nine different locations in the image are given in Table II (first column).

Incorporating additive, multiplicative or linear shading correction as described above and repeating the same experiment reduces the c.v. values as may be seen from Table II. The correction is particularly powerful for the multiplicative and linear correction.

To get a standard for the densitometric value for the same set of cells, a measurement was also performed on an M85 stage-scanning microdensitometer. Linear correlation of the results obtained there with the results from the TV-based system gives Table III. From the table it can be seen that, for this set of cells, a good correlation is found only for multiplicative or linear shading correction. Multiplicative and linear correction leads to considerable, almost equal, improvements.

In the next type of experiment, the reproducibility was tested by measuring nine times the first cell from the set of eight, now located in the middle of the field only. The reproducibility expressed in the c.v. value is given in Table IV.

By including linear shading correction in the basic algorithm, the reproducibility may be further improved by reducing the noise in the image by averaging M image recordings. For two different background estimation methods (selection of the background mode $\text{mod}(I_0)$ and the average over the background region $\bar{I}_0$), the previous experiment with one cell was

Table IV. Reproducibility of the Measurement, Expressed in c.v., Based on Nine Measurements of a Sample Cell Located in the Middle of the Field with Various Types of Shading Correction

CELL	NULL	ADD	MULT	LIN
1	0.51%	0.83%	0.88%	0.86%

Table V. Reproducibility Over Nine Measurements of CELL 1 Expressed in c.v. (%) with Various Numbers of Averaging Successive Image Recordings^a

	Number of image recordings used in averaging			
	1X	4X	16X	64X
$\text{mod}(I_0)$	0.86	0.59	0.50	0.48
$\bar{I}_0$	0.51	0.43	0.26	0.17
N with $\bar{I}_0$	0.49	0.25	0.08	0.08

^a Measurements were carried out with selection for the background value of the modal value, $\text{mod}(I_0)$, of the background peak, i.e., an integer value for I , and, with the average value over the background region, $\bar{I}_0$. The last row indicates results of $\bar{I}_0$ for one fixed segmentation in all nine measurements.

Table VI. Optical Density, with the c.v. Between Brackets, Averaged Over Nine Measurements on CELL 1 for Various Segmentation Mask Extensions

	Number of dilations of the segmentation mask			
	0X	2X	4X	6X
	41.85 (0.26%)	42.18 (0.78%)	40.89 (1.12%)	40.33 (3.18%)

repeated. The results for increasing values of M are found in Table V. The table shows that background estimation by averaging is superior to mode selection as expected. Also, as anticipated from the measured SNR of 40 dB, no considerable improvement is found between $M = 16$ and $M = 64$.

To understand the remaining c.v. values, the experiment was repeated with one fixed-object segmentation mask for all nine measurements. The results for the background-averaging method are in the third row of Table V. They show the c.v. values resulting from the noise in the intensity values are negligible in comparison with the influence the noise has on the segmentation result. The variation in segmentation of the object due to noise thus adds most of the variation in the outcome of Z .

The next experiment was on the influence of the segmentation on the value of Z . From now on, adding the averaging of sixteen image recordings to the analysis program, four settings of the segmentation algorithm were compared. The initial segmentation result was expanded by laying rims of pixels (called dilations) around the object. Null, two, four, and six dilations were tested; the first of which corresponds to the segmentation algorithm used thus far. For CELL 1, located in the center of the field, nine repeated measurements were performed displaying mean and c.v. in Table VI. The table shows a slight increase in Z when the object is expanded slightly (two dilations) as explained above and thereafter, surprisingly, decreases

Fig. 2. The DNA profile of the breast specimen obtained from flow cytometric analysis of 59,123 cells.

slightly. The c.v. values increase with the size of the object as expected. Dilation of the object does not lead to improvement of the reproducibility and is, therefore, not included in the following steps.

To verify the effect of the threshold value on the value of Z , the density was calculated for nine different recordings for seven different values of the threshold at intervals of Z on the intensity scale around I_t . The

increase appeared to be 0.11% per intensity increase of I_t , in good accordance with the theory derived in the previous section.

The methods were also evaluated on the specimen level. For that purpose one breast specimen was analyzed on a PARTEC flow cytometer. The DNA profile resulting from analyzing 59,123 cells can be seen in Fig. 2. The c.v. of this population amounts to 3.5% measured by the width at 61% of the height of the normal cell. Material of the suspension of the same specimen was analyzed with the image analysis a few times, each time using a fixed part of the slide consisting of twenty-five microscope fields containing ~ 1100 cells (see Fig. 3).

IV. Discussion

In the previous sections we summarized factors influencing the accuracy and precision of densitometry of cells by a video microscope system and studied them in practice for a low-resolution system. For these systems, shading correction appears to dominate all other factors. Measurements of a variety of cells at different places in the field of view show that multiplicative correction of shading performs considerably better than additive correction (see Table II). The result of the multiplicative correction is hardly improved by linear correction, if at all. This is a surprising result as linear shading correction encompasses the virtues of multiplicative correction. It may be concluded that the major component of shading has a multiplicative effect. This may be due to the use of a chalnicon tube and may be reduced further when using a CCD camera. In our experiments we used shading correction images at full resolution, made free of noise by averaging sixty-four recordings. We have found it important not to process the images otherwise by re-

Fig. 3. The DNA-profile of the breast specimen of Fig. 2, obtained with image processing subsequently adding more sophistication: (a) basic program (BP); (b) BP plus additive shading correction; (c) BP plus multiplicative shading correction; (d) BP plus linear shading correction (LP); (e) LP plus background value based on averaging (ALP); (f) ALP plus averaging sixteen image recordings (16 ALP); (g) 16 ALP plus automated removal of overlapping cells.

ducing their resolution¹⁷ or fitting a polynomial plane through them.¹⁸ With our implementation almost perfectly flat images can be obtained, making segmentation, background estimation, and high-measurement accuracy much simpler. For multiplicative (or linear) shading correction we arrive at a reproducibility of measurement of 1.8% (or 2.5%) when the cell is positioned arbitrarily in the field. One half of the remaining variability can be explained as the remainder of shading correction, as for a cell positioned at the center of the field the reproducibility is 0.9% (or 0.9%). Half of this remaining error is due to the influence of the noise on the segmentation (see Table V, row 3). All figures demonstrate that a satisfactory accuracy is attainable at low resolution, still using the full width of the microscopic field. The central field reproducibility of 0.9% compared favorably with the reproducibility obtained by others.¹⁵

In the experiments on one and the same part of a specimen, containing ~1100 cells, the most important improvement in the measurement accuracy again comes from the shading correction. Thereafter, the peak width is 5.3% compared with 14.7%, when no shading correction is applied. The other corrections avoiding round off in intensity measurement (I) or noise reduction by averaging the image sixteen times, bring only little improvement in the width of the profile. Overlap rejection techniques, finally, have a considerable influence on the shape of the profile showing one of the virtues of image cytometry over flow cytometry.

The remaining 5.0% width of the normal cell peak is to be compared with the 3.7% from the flow cytometer on the same specimen. The figure of 3.7% includes an unknown specimen-dependent factor for the biologic variation. The analysis of 1100 cells by an image cytometer in absorbance mode thus leads to 1.3 times broader peak than the analysis of 59,123 cells by a flow cytometer in fluorescence mode. Part of the difference between 5.0% and 3.7% is due to imperfections left behind after shading correction, as follows from the c.v. values in Table II. Nevertheless, the factor 1.3 is a satisfactory result, as the image cytometer is working in low resolution. At present, without any optimization as to the speed, a fully automated analysis requires 10 min. Optimization of the program or the processor could lead to an improvement of speed by a factor 2–10. But even the present algorithm may compete with the performance of a flow cytometer on the basis of throughput per day.

When a higher accuracy is demanded, a higher resolution and a higher N.A. will be needed, implying that the distributional and diffractive errors need to be corrected as well. Resolution and related factors are the subjects of study in a sequel paper.²⁶

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Appendix

For a homogeneously stained object (value I), consider the case of uniformly distributed noise added to the intensity values with average zero and amplitude a . In the continuous case the density is given by

$$\bar{Z} = \int_{I-a}^{I+a} \log\left(\frac{i}{I_0}\right) \cdot \frac{1}{2a} \cdot di$$

leading to

$$\bar{Z} = \frac{I-a}{2a} \log\left(\frac{I-a}{I_0}\right) - \frac{I+a}{2a} \log\left(\frac{I+a}{I_0}\right) + \frac{1}{\ln(10)}.$$

Third-order Taylor approximation gives

$$Z = -\log\left(\frac{I}{I_0}\right) + \frac{a^2}{6 \cdot I^2 \cdot \ln(10)} + R(a),$$

where $R(a) < a^4/[4 \cdot I^4 \cdot \ln(10)]$. The relative error in the value of Z is

$$\frac{\Delta Z}{Z} = -\frac{a^2}{\bar{Z} \cdot I^2 \cdot \ln(10)}.$$

For $a/I = 0.01$ and $\bar{Z} = 0.2$ this gives $\Delta Z/Z = -0.02\%$. Intensity noise leads thus to a bias in the value of Z , albeit the bias is very small.

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