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Cytometric approaches to red blood cells

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Abstract - Recently developed methods for making quantitative measurements of red cell hemoglobin and morphology are described. High-speed image processing is used to make six simultaneous red cell measurements relating to size, hemoglobin content, central pallor, and shape. Sampled population distributions are obtained for each parameter. The blood sample is characterized by the principal distributional parameters. Also, an objective assessment of the blood sample is made relative to the diagnosis of anemia. Results from typically encountered blood conditions are presented and analyzed in detail to illustrate the various distributional parameters involved and to illustrate the potential of these new measurements to extract relevant diagnostic information.

INTRODUCTION

This paper presents new, recently developed methods for making quantitative measurements of red cell morphology on individual cells. These measurements are combined objectively to characterize the populations of cells in a blood sample, e.g. illustrated in Figure 1, combining the hemoglobin content of cells with distribution parameters and morphology, and relating this information to the diagnosis of anemia.

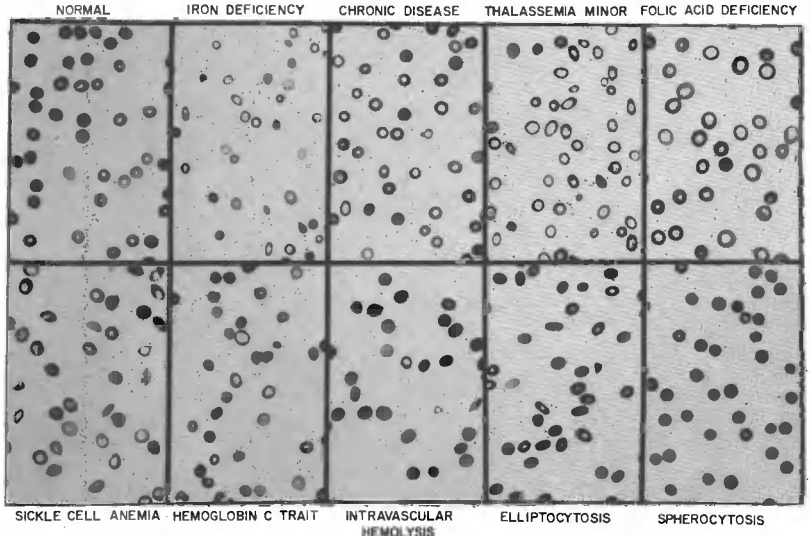


Figure 1

Red cells from nine types of anemias and red cell disorders, compared to normal red cells.

MATERIALS AND METHODS

A monolayer blood film, preparation is made [1] and the unstained cells are analyzed at high resolution microdensitometrically. Microscope stage motors move the slide and focus a microscope objective. Conventional microscope optics project cell images upon a television scanning imaging sensor. As the microscope stage is moved in a scanning pattern, different red blood cells are located for analysis. Each time the microscope stage stops all of the cells in a given field are measured. This continues until a large number of cells have been analyzed, typically 1,000 cells. Two microprocessors are used. One of them controls stage movement and focusing and keeps track of subpopulation parameters that are transferred to it as a result of cell identification and measurement. The second microprocessor acquires digitized images, performs the cell measurements, classifies each cell, and sends the results to accumulate parameters for each subpopulation.

These techniques have made possible, precise, detailed measurements of individual cells, measurement of size, hemoglobin, pallor and shape [2, 3, 4]. Accumulation of sample cell population distributions for each measurement are also obtained.

Each digitized image field may be described as a function (x,y), where each value is a measured point of absorbance obeying the Beer-Lambert absorption law;

$$\eta\,(x,y) = \log \frac{I_o}{I_t} = \frac{\varepsilon_{\lambda b} m}{a} \qquad (1)$$

where I_o = incident light, I_t = transmitted light, λ = specific extinction coefficient of hemoglobin (μm^2/pg) at a chosen wavelength (λ) and with a specific microspectrophotometer bandpass (b), m = mass of hemoglobin in picograms (pg) for each x,y image field measurement point inside a red cell, and a = the measurement spot size in (μm^2). Assuming the boundary points of a cell have been properly determined, and for a single red cell in the field of view;

$$H = \frac{\alpha}{\varepsilon_{\lambda,b}} \sum_x \sum_y \eta(x,y) \qquad (2)$$

Equation 2 defines the measurement of the mass of hemoglobin (H) for that cell. Hemoglobin spectral measurements on dried cells are rather distinctively characteristic in the sense that the peak optical density is in the region of 412 nanometers, and not in the region of one of the predominately stable forms from hemoglobin solutions, of methemoglobin (405nm), oxyhemoglobin (415nm), or deoxyhemoglobin (426nm) as might be expected [5].

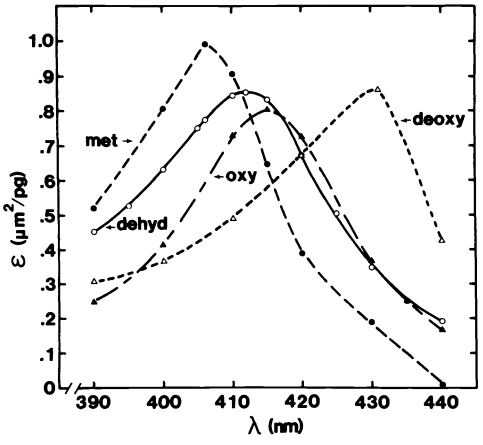


Figure 2

Extinction values for methemoglobin (met), oxyhemoglobin (oxy), dehydrated hemoglobin (dehyd) and deoxyhemoglobin (deoxy).

Figure 2 illustrates the spectral curves of the extinction coefficients of methemoglobin, dehydrated hemoglobin, oxyhemoglobin and deoxyhemoglobin. The extinction values for met, deoxy, and oxyhemoglobin were calculated from the literature and converted to the units of m moles/liter to units of um^2/pg to correspond with the scanning microdensitometry form of the Beer-Lambert absorption law for mass determination given in equations shown above. We have only recently determined the extinction spectrum of dehydrated hemoglobin, illustrated here. This was measured at 7.7 nm on a previously described microspectrophotometer [6, 7]. The results were corrected to .31nm to compare with the other spectra [5]. For precise and accurate determinations of hemoglobin, in addition to the optimum measurement wavelength (λ), it is also necessary to consider the bandpass (b) of the densitometer used in the measuring instrument in order to obtain the proper value of the extinction coefficient ($\varepsilon_{\lambda,b}$).

Volume Individual cell volume (v) is calculated as;

$$V = K_1\Psi + K_2H + K_3\Phi + K_4 \qquad (3)$$

where Ψ = cell area (μm^2), H = cell hemoglobin content (pg), Φ = cell pallor (%), and k_1 thru k_4 are specified constants. Mean cell volume (MCV) calculated through the use of equation 3 compares well with MCV determinations using impedence flow techniques [4].

RESULTS

As described high speed image processing is used to make simultaneous measurements on each cell of size and hemoglobin content. Central pallor, and shape are also measured and have been described previously [2, 3]. These measurements are combined in three different ways and compiled together to provide the analysis results for a blood sample. This analysis is called the QRCM (Quantitative Red Cell Morphology) test. Figures 4, 5, 6 are typical of results with this method. The analysis report consists of 3 parts: 1) the Red Cell Differential, 2) plotted Population Distributions of measurements on the sampled population of red cells, and 3) a Diagnostic Profile.

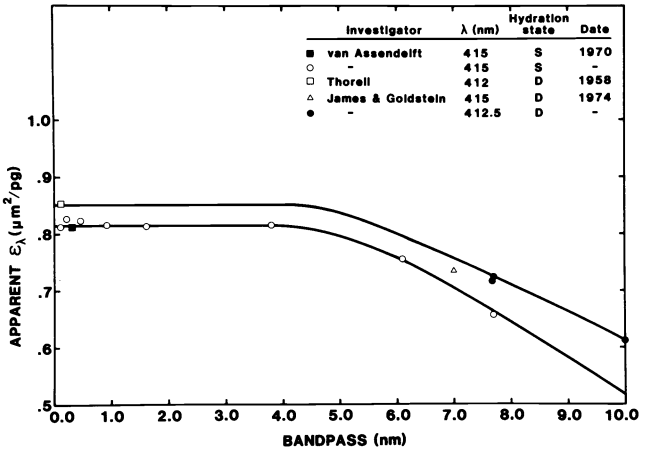
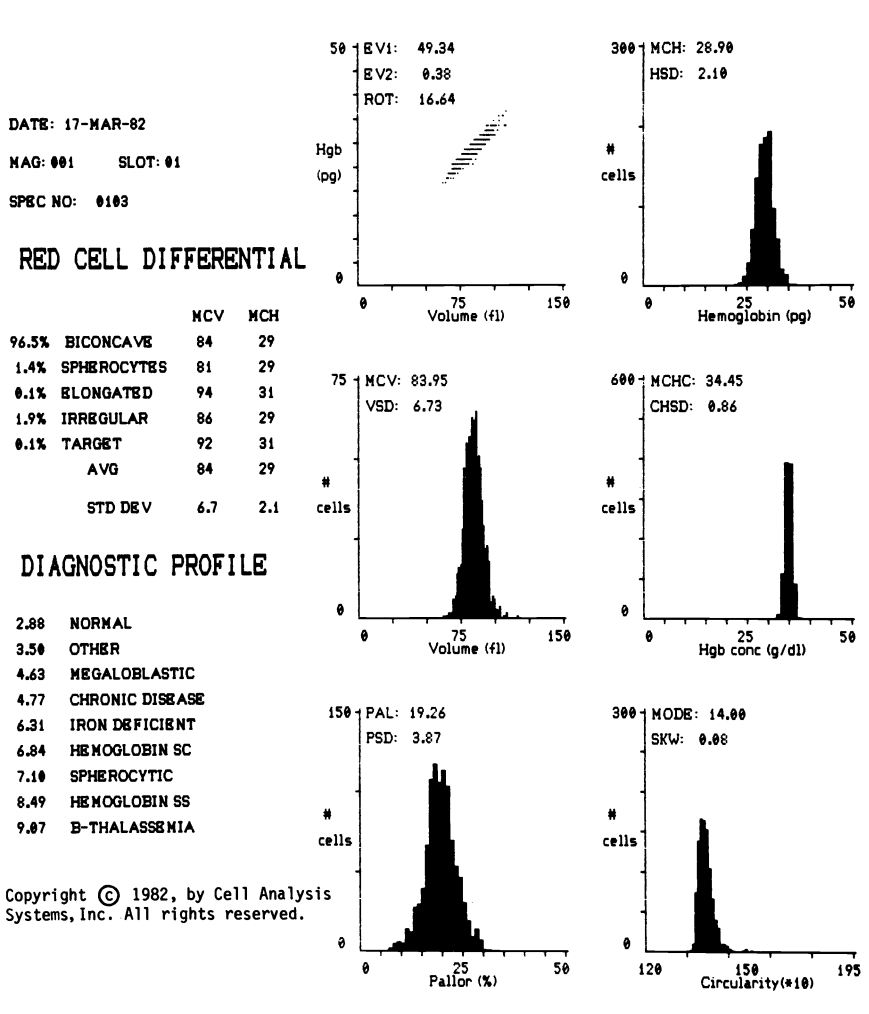


Figure 3

Apparent extinction coefficient as a function of bandpass. The plotted data ○ and ● are from our measurements of hemoglobin in solution and in dried cells, respectively. The other data are from the literature. The hydration state is indicated as in solution (S) or dried (D).

Figure 4

Typical QRCM report from the blood of a hematologically normal person.



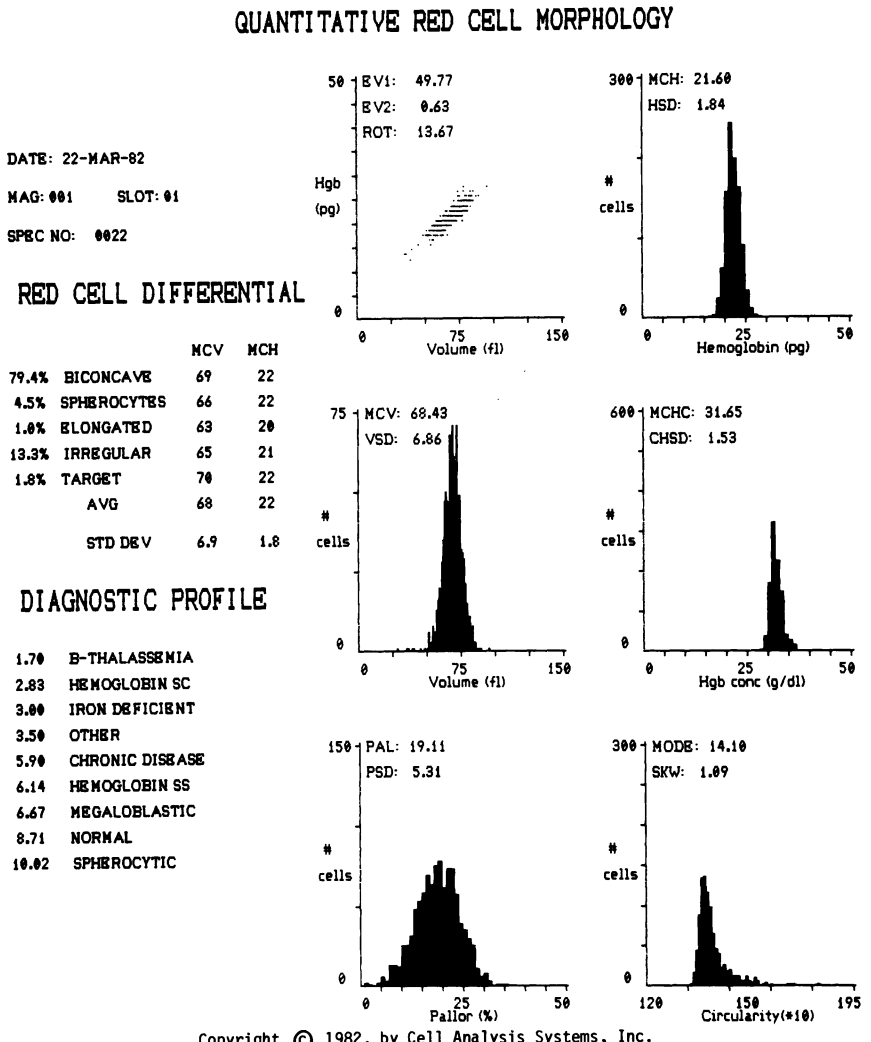


Figure 5
Typical QRCM report from a person with β-thalassemia.

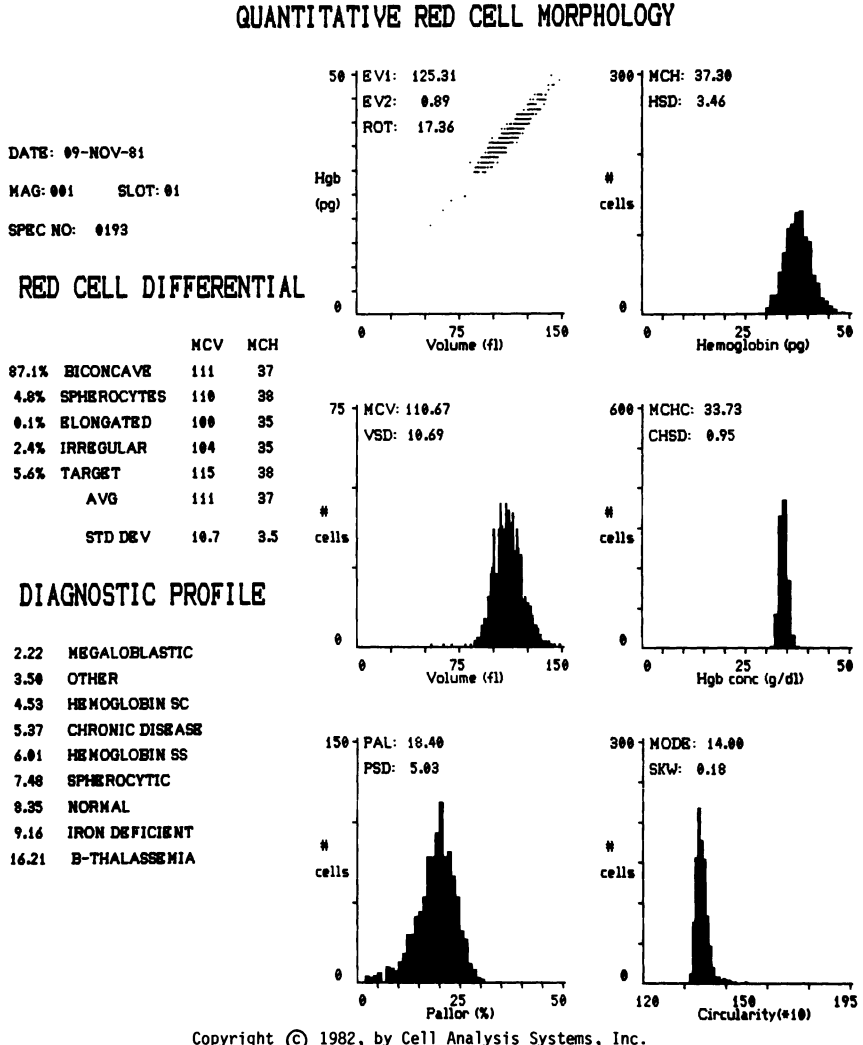


Figure 6
Typical QRCM report from a person with megaloblastic anemia.

The Red Cell Differential

This part of the analysis results provides a partitioning of the sampled population of red cells into five subpopulations, providing the % of cells in each subpopulation along with the corresponding subpopulation mean cell volume, calibrated in femtoliters (fl), and the subpopulation mean cell hemoglobin content, calibrated in picograms (pg). The average (AVG) and the standard deviation (STD DEV) of cell size (MCV) and the hemoglobin content (MCH) are also provided for the entire population of red cells. The decision procedures applied to each individual red cell during analysis and compilation of this report have been previously described [2]. Target cells are detected by the presence of a 3 peaked absorbance profile, rather than the characteristic 2 peaked profile shown before.

Population Distributions.

The red blood cell measurement distributions provide a graphic or visual assessment of important cell measurements, as well as parameters of the various distributions. Distributions of individual cell size, hemoglobin, central pallor and shape are presented. For the univariate size, hemoglobin content, hemoglobin concentration, and pallor distributions, the descriptive statistics are the mean and standard deviation, provided as labeled values which are different in each plot, see Table I. For the bivariate distribution of hemoglobin and size, the eigenvalues (EV1 and EV2) and rotation (ROT) of the distribution in degrees around its centroid are provided, the centroid of this distribution is given by the MCV and MCH of the corresponding univariate distributions. The hemoglobin concentration distribution is a projection of the bivariate distribution of hemoglobin and size, along lines of constant slope, passing through the origin of the bivariate plot. Each line of constant slope, e.g. .32 or .34, represents a cell hemoglobin concentration, and the cells falling on that line in the bivariate space determine the height of the corresponding univariate hemoglobin concentration at that cell hemoglobin concentration level. For the shape distribution (circularity) the mode (MODE) and the skewness (SKW) are provided as descriptive parameters.

TABLE 1. Descriptors for sample distribution of cell measurements

MCH	Mean cell hemoglobin
HSD	Standard deviation of cell hemoglobin
MCV	Mean cell volume
VSD	Standard deviation of cell volume
MCHC	Mean cell hemoglobin concentration
CHSD	Standard deviation of cell hemoglobin concentration
PAL	Mean of cell pallor
PSD	Standard deviation of cell pallor

The Diagnostic Profile.

The Red Cell Differential and the statistics from Population Distributions provide highly descriptive information not previously available from routine red cell analyses. This information has significant diagnostic implications. The Diagnostic Profile condenses this information through the presentation of a ranked list containing some of the more commonly occurring blood conditions related to red cell disorders. The number in front of each diagnostic category indicates the closeness, or similarity, for the analyzed blood to the prototype for that category.

Presently, the similarity is computed from a subset of 6 measurement values obtained from the Population Distributions. These 6 measurements were chosen after performing a detailed study of patient blood samples of confirmed diagnosis.

The subset of 6 measurements, after transformation, define a measurement (or feature) vector;

$$X = [\ MCH,\ \ln(CHSD^2),\ \ln(EV2),\ PAL,\ ROT,\ \ln(SKW)\ ]$$

and essentially summarize the blood analysis information in the QRCM report.

These measurements are used to compute the Mahalobinis distance in 6 space;

$$d_j = [(X-U_j)^t \sum^{-1}_j (X-U_i)]^{1/2} \quad j = 1, 8$$

and thus provide a numerical assessment of the similarity of the blood analyzed to the prototypic categories. The numbers listed in front of each category indicate the similarity, d_j. A small number indicates a close similarity for the blood analyzed to the blood condition listed. Additionally, as indicated above, the list is also sorted so that the most probable category is at the top and the least probable is at the bottom. The OTHER category in the list has a preset distance value of 3.50. This implies that a distance greater than 3.50 in this 6-space is considered non-similar to the standard categories listed, and automatically forces the OTHER category to the top of the list.

DISCUSSION

Most of the routine measurement values available today in hematology for red cells are mean values, assuming the blood specimen represents one population of similar cells. Although, historically it has been recognized that there is significant diagnostic information in the morphology of red blood cells, these characteristics were only subjectively evaluated during hematological examination. With the exception of red cell size, there are no methods to rapidly obtain dispersion parameters. Subpopulation estimates are not available. In general, our ability to describe red blood cells has depended upon the sophistication of our observational and measurement apparatus. This new measurement system uses high-speed image processing and computer analysis to make multiple measurements on individual cells and identify and subcategorize cells on the basis of the measurements. This leads to more discriminating analytical parameters of population distributions, including dispersion parameters, to help in diagnosis. Also, these techniques have allowed for the first time, the measurement of sampled population distributions of cell hemoglobin concentration, the dispersion of which, as a new measurement value, i.e. CHSD (and also EV2), seems to be diagnostically significant.

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