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THE DEVELOPMENT OF A BIOLOGICAL TEST SYSTEM FOR CALIBRATING AND EVALUATING THE PERFORMANCE OF COMPUTER-CONTROLLED OPTICAL SCANNERS

NEW ENGLAND MEDICAL CENTER HOSPITALS
DEPARTMENTS OF PATHOLOGY & PHYSICS

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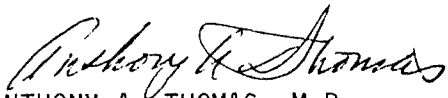

ANTHONY A. THOMAS, M.D.
Director, Toxic Hazards Division
6570th Aerospace Medical Research Laboratory

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A method was developed for processing human red blood cells (RBCs) for microstereology analysis of major cell dimensions. Using this preparative method, RBCs from five normal human donors were prepared for thin section electron microscopy. A test film containing 250 electron micrographs of human RBCs was prepared and analyzed by microstereology methods. Using these methods, the calculated RBC mean corpuscular volume (MCV) is 79.6 μm^3 and the mean RBC surface area is 130.1 μm^2. These results are consistent with results obtained by other quantitative microscopy techniques. The film is of high quality and preliminary

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testing indicates that the film is suitable for use in evaluations of computer soft ware that is designed to control computer-controlled optical scanners that are utilized for boundary tracing in biological materials.

INTRODUCTION

A technical goal of the toxicological pathologist is to automate quantitative diagnostic microscopy techniques through the use of computer-controlled optical scanner methods. In order to accomplish this goal, a number of technical problems must be solved. Computer software must be developed to: (1) enable the computer program to identify specific cells and organelles from digitized representations of electron optical images; (2) direct the computer-controlled optical scanner to trace cell and organelle boundaries; and (3) abstract microstereology data from encoded segment lists that represent the coordinates of the boundaries of specific classes of cells and organelles. Boundary tracing within biological systems is in of itself a complex and difficult problem because of the low contrast that is inherent in 60 nm thin sections of plastic embedded tissues, and variations and inconsistencies in gray levels at the perimeters of the structures of interest. This is in contradistinction to the phase boundaries within alloys and minerals which are generally quite easy to trace with computer-controlled optical scanners because the boundaries are well defined and of high contrast.

In order to develop computer soft-ware that will direct a computer-controlled optical scanner to trace biological boundaries it is necessary to have a suitable biological test system that can be used to evaluate the efficiency and accuracy of programs and to calibrate the computer-controlled optical scanner. The purpose of this report is to describe such a test system. Human red blood cells were selected as the test material because they are reasonably uniform in size and shape, the cells are readily obtained by venipuncture from normal donors, and the red cell is reasonably electron dense as viewed by thin section transmission electron microscopy. Further, since major red cell dimensions including diameter, surface area and volume have been carefully measured by several independent methods (Ponder, 1948; Canham and Burton, 1968; Evans and Fung, 1972) they are particularly useful as biological calibration standards against which new quantitative methods can be evaluated. This report also contains the first analysis of normal human red cell dimensions using microstereology methods.

MATERIALS AND METHODS

The RBC-Agar Embedding Technique

The following technique was devised to produce a biological standard consisting of randomly dispersed human red blood cells embedded in plastic and suitable for examination by thin section electron microscopy and analysis by microstereology techniques. Human RBC's were collected by venipuncture from five healthy male donors, heparinized, lightly centrifuged and the buffy coat was removed. Clinical data on the donors is listed in Table 1.

TABLE I

CLINICAL DATA ON HUMAN RBC DONORS

Donor	Age	Hct	RBC Count X10^{6}*	MCV (μm^3)*
J.W.	17	42	5.75	72.5
R.W.	34	46	5.60	82.1
R.S.	23	45	5.57	80.8
M.C.	21	48	5.99	80.1
J.F.	22	47	5.31	88.5
mean	23.6	45.6	5.65	80.8
± S.D.	±6.77	±2.30	±0.255	±5.78

* Determined with a Coulter Counter

The packed RBCs were washed twice in isotonic phosphate-buffered saline pH 7.4 and were fixed for 1 1/2 hours at 4°C in 1% glutaraldehyde containing 6.70 gm NaCl/L, 0.675 gm KH_2PO_4/L and 7.60 gm $Na_2HPO_4.7H_2O$/L. The cells were added dropwise to the fixative over a fifteen minute period until a ratio of fixative to cells of 10:1 was reached. After fixation, the cells were washed twice in isotonic phosphate buffer and resuspended in buffered saline to a final hematocrit of approximately 50%. Aliquots of cells from the suspensions were counted on a Coulter Counter. A freshly prepared 1% agar solution (prepared from "purified agar", DIFCO Corporation, Detroit, Michigan) was equilibrated at 48°C in a water bath. 0.5 ml of the agar was combined with 0.5 ml of the RBC suspension and they were thoroughly mixed on a Vortex mixer. The RBC-agar mixtures were allowed to cool to 22°C over a 20 minute period and then placed in a refrigerator at 4°C for 45 minutes. With cooling, the RBC-agar mixture solidifies. The resulting RBC-agar gel was diced into 1.5 mm^3 blocks. These blocks were post-fixed in 1% osmium tetroxide in phosphate buffer, pH 7.4, for one hour and dehydrated by serial passage through 70,80,95 and 100% ethanol solutions. The blocks were infiltrated with monomeric Epon 812 and the plastic was polymerized at 60°C. Thin sections were cut on diamond knives and the sections were stained with uranyl acetate and lead citrate.

Agar-Shrinkage Artifacts

A potential source of dimensional distortion with the RBC-agar technique is shrinkage of the specimen that might be introduced during

polymerization of agar. Shrinkage was determined experimentally to less than 0.6% which is regarded as trivial.

Electron Microscopy for Microstereology Analysis

Thin sections were mounted on 400-mesh copper grids and photographed in a Philips EM 300 electron microscope according to the systematic random sampling procedures as summarized by Weibel, 1969 . In brief, large sections of blocks were cut so that individual sections covered many grid squares. The upper left hand corner of each of 10-32 consecutive grid squares was photographed on Kodak SO 410 35 mm film at original magnification of 1800X. Electron microscope magnification was precisely calibrated before each set of electron micrographs with a 28,800 line/inch calibration grating (Ladd, Burlington, Vt.). 35 mm film strips containing the electron micrographs from individual donors were spliced together and coded for purposes of identification. Included on the final test film are 10 electron micrographs of thin sections of five RBC-agar blocks from each of five donors. Thus, the spliced test film contains a total of 250 electron micrographs of human red blood cells.

Microstereology Analysis of RBC's by Hand Counting

Microstereology analysis of red cells was carried out using a Weibel microprojection morphometrics table and a multipurpose test lattice system (Weibel, 1969). The multipurpose test lattice system consists of a triangular lattice of 168 test points with every other point connected to its following neighbor by a line. These lines, in aggregate, are defined as the "test line" (L_T). In order to perform microstereology analysis of red cells, electron micrographs are individually projected onto the screen of the Weibel microprojection table. The multipurpose test system of lines is printed on the table screen.

With the test system it is possible to determine the mean corpuscular volume and mean surface area for a population of RBC's using hand counting methods. Figure 1 illustrates how microstereology data are obtained with the Weibel multipurpose test lattice. The lattice is superimposed over an electron micrograph of a thin section of randomly oriented RBCs. The ends of each test line represent "test points" (e.g. 2 points per test line). The structure under each test point is identified and recorded on a differential counter. Next intercepts are tallied. An intercept is defined as a point where an element of the test line (L_T) crosses a cell boundary, as illustrated in Figure 1.

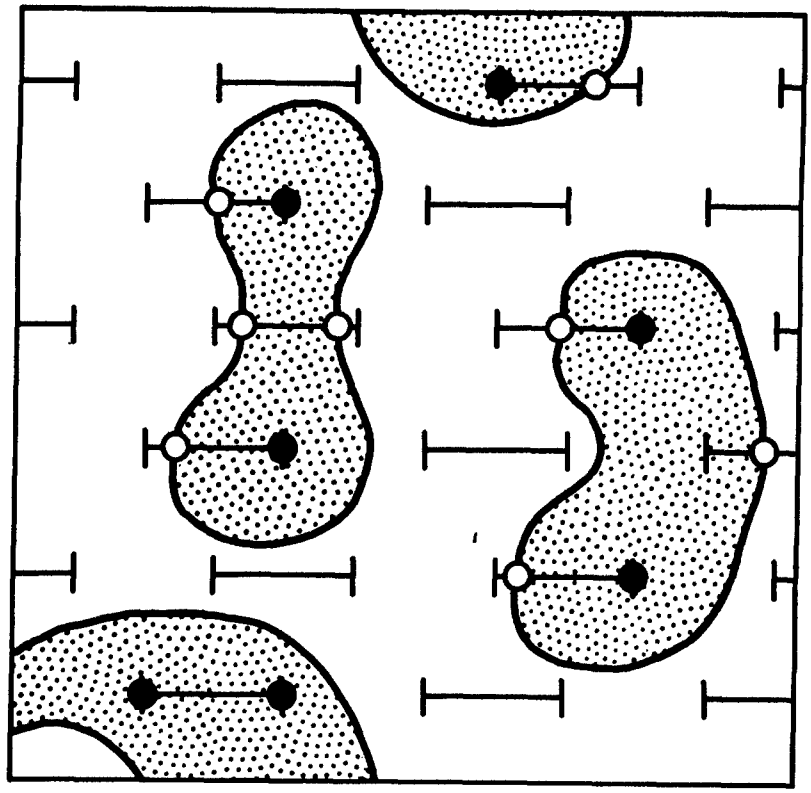

Figure 1. Diagramatic representation of part of the multipurpose test lattice system, shown superimposed over a drawing of sectioned RBCs (stippled profiles). Test points over RBCs (P_{RBC}) are represented as closed circles. Intercepts (I_{RBC}) are points at which the test line crosses the cell surface and these are shown as open circles.

Determination of Mean Cell Volume

For each donor, the mean RBC cell volumes and RBC surface areas were measured by microstereology methods. The number of RBCs per unit volume in the RBC-agar blocks were calculated. Partial volumes (e.g. Hematocrits) for RBC in the final plastic blocks were calculated according to the following formulae:

$$V_{V_{RBC}} \text{ (Hct)} = \frac{V_{RBC}}{V_T} = \frac{P_{RBC}}{P_T}$$

where $V_{V_{RBC}}$ is the volume fraction of the cell block that is occupied by RBCs, V_{RBC} is the volume of RBCs in the blocks, V_T is the total volume of the block, P_{RBC} is the number of test points over RBCs, P_T is the total number of test points (e.g. 168 per electron micrograph). The mean

corpuscular volume (MCV) of red blood cells is:

$$MCV = \frac{V_{V_{RBC}}\ mm^3}{N\ D} \qquad (2)$$

where N is the number of RBCs per mm^3 and D is a dilution factor representing the extent of dilution of the red cell solution in the final RBC-agar mixture ($D \simeq 0.5$).

RBC Surface Area Measurements

The total surface area of the RBCs is determined by the formulae:

$$SV = {}^{2\ I}/_{L_T} \qquad (3)$$

where I represents the number of intercepts at RBC surfaces and L_T is the total length of the lines in the multipurpose test line system (Weibel, 1969).

RESULTS

Electron Microscopy of Human Red Cells

Figure 2 shows prints of consecutive electron micrographs of human RBCs that were recorded on 35 mm film. The cells are of relatively high contrast and are suitable for microstereology analysis by a computer-controlled optical scanner.

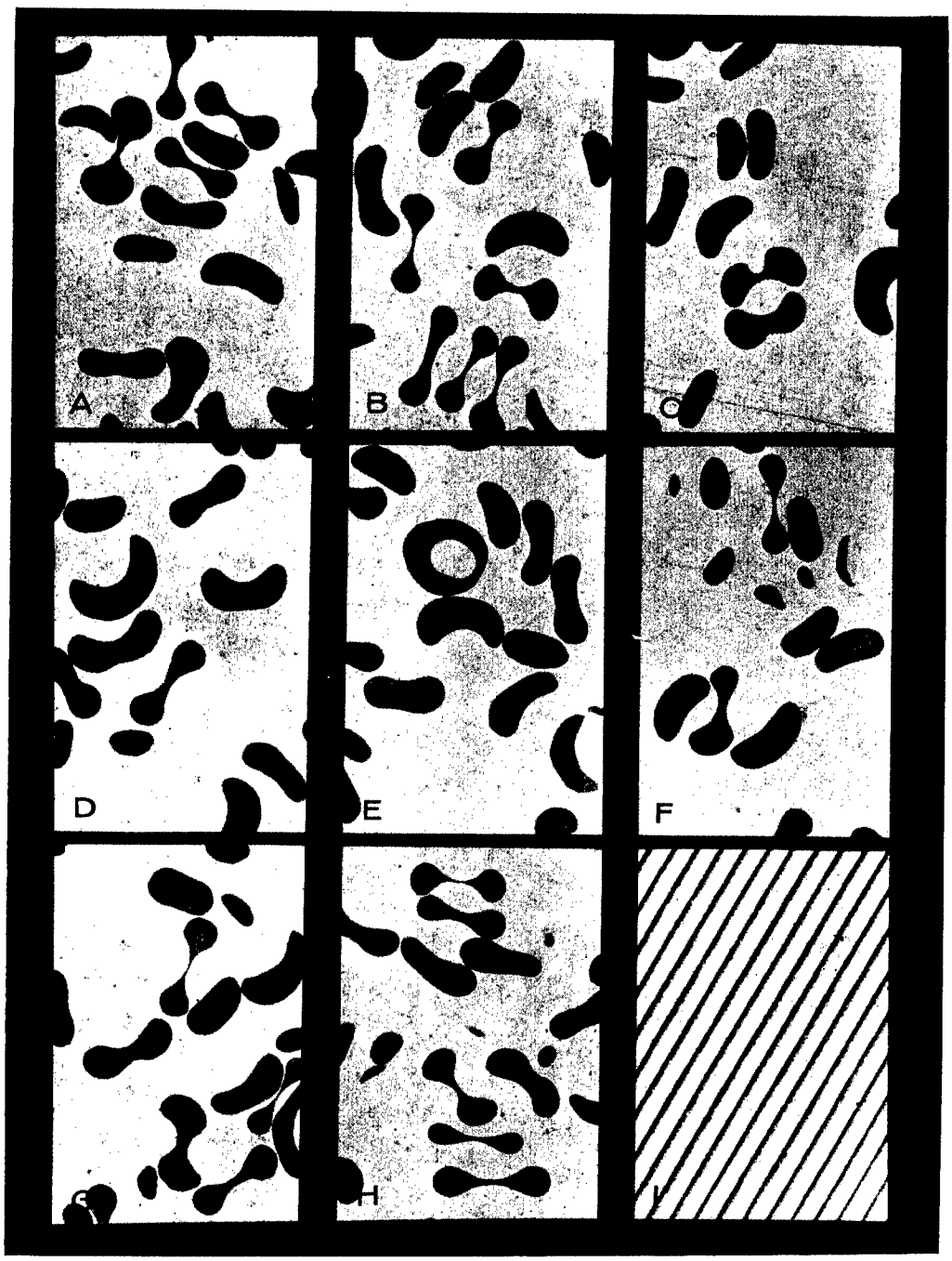

Figure 2. A-H: Consecutive electron micrographs from neighboring fields of a thin section of randomly oriented human RBCs in an RBC-agar gel. Magnification X2000. I: Electron micrograph of the germanium shadowed carbon replica that is used to calibrate the electron microscope magnification for each film strip. Calibration grating spacing is 28,800 lines per inch.

Determination of the Representative Sample

In applying microstereology methods to specific problems in biology, such as the determination of red cell dimensions, it must be determined how many frames (electron micrographs) should be pooled to achieve a representative sample. This is important because: (1) the specimens may be heterogeneous; and (2) a single electron micrograph represents a statistically inadequate sample. For the purposes of this study, the acceptable mean value for cell volume in each RBC-agar block was set at ± 10% of the value derived from pooled counts on large numbers of test frames (i.e. >25 frames). Figure 3 shows data obtained from counts on 32 consecutive frames from a single thin section of human RBCs. The curve shows that the cumulative means are within the ±10% limits when six frames are analyzed and they remain within the ±10% range with the addition of subsequent tallies.

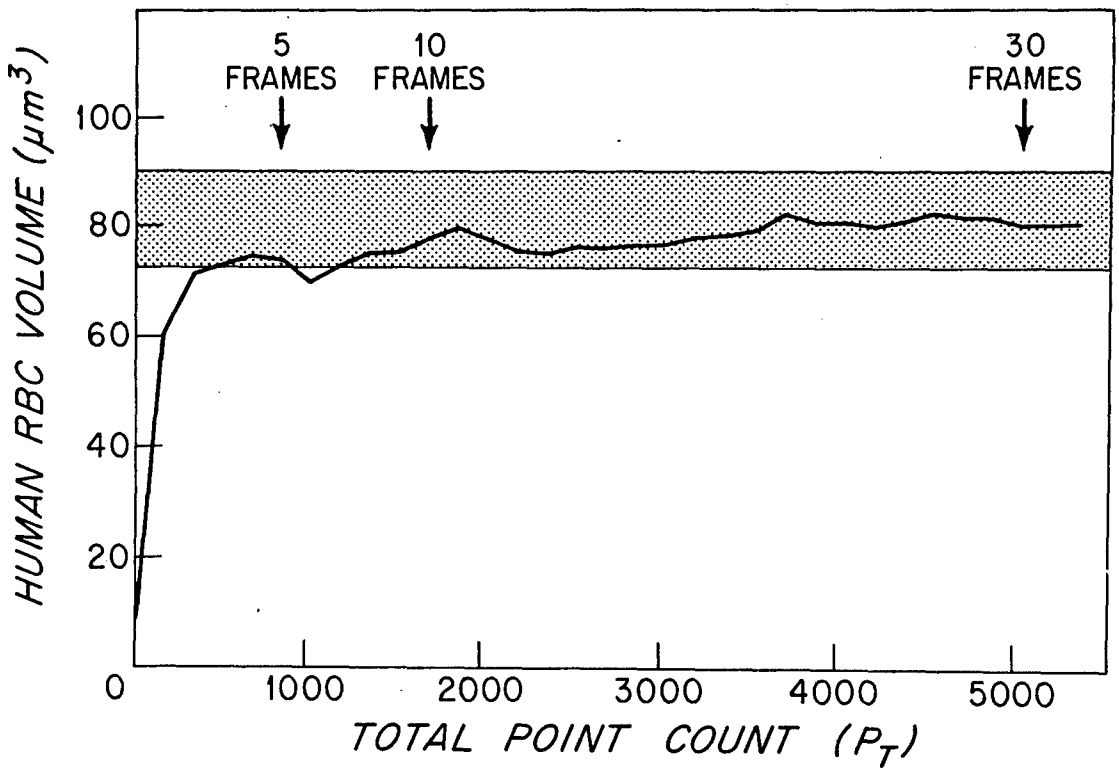

Figure 3. Cumulative mean values for the measurement of RBC mean corpuscular volume by microstereology hand-counting methods. 168 test points were tallied for each electron micrograph. The mean value obtained from counts on 10 micrographs (1680 test points) is essentially the same as the cumulative mean from 30 frames. The stippled zone represents ±10% of the final mean value.

Microstereology Data on RBC's

Table 2 shows the surface area and MCV values for five normal human donors, as determined by microstereology methods. The mean MCV value for the five donors was 79.6 μm^3. Mean cell surface areas ranged from 122.8 μm^2 to 133.9 μm^2 and the mean value for five normal human male donors was 130.1 μm^2. MCV values were compared with MCV values for the same donors as measured with the Coulter counter (Table 1). Values obtained by the two methods are not significantly different (students t-test, $P < .01$).

TABLE 2

MICROSTEREOLOGY DATA ON HUMAN RBC's

Donor	Hct	Surface Area (μm^2)	MCV (μm^3)	S/V ratio
J.W.	42	133.4	76.9	1.74
R.W.	43	133.7	75.0	1.78
R.S.	46	133.9	81.8	1.63
M.C.	47	122.8	76.8	1.41
J.F.	47	122.8	87.4	1.41
mean	45.00	130.14	79.58	1.628
± S.D	±2.345	±5.045	±5.049	±.1462

DISCUSSION

In this report, a method is described that can be used to prepare a biological test object, the human RBC, for thin section electron microscopy. Red cells are randomly and relatively uniformly distributed in the RBC-agar gels prepared according to this method. The cells are of reasonably high contrast and preliminary testing indicates that these film of RBC-agar blocks are suitable for use in a computer-controlled optical scanner, thus satisfying our technical objective.

The microstereology data on human RBCs that were developed in the course of this study are of considerable general interest. Estimates of RBC MCVs and surface areas are of considerable importance since these values are used in studies dealing with a broad spectrum of problems in cell biology, biophysics and biochemistry (Weinstein, 1974). Many investigators have attempted to determine these RBC dimensions by other methods and some of these results are summarized in Table 3.

TABLE 3

ESTIMATES OF HUMAN RED CELL MAJOR DIMENSIONS BY DIRECT MEASUREMENTS

Surface Area (μm^2 ± S.D.)	Volume (μm^3 ± S.D.)	Reference
138.1 ± 17.4	107.0 ± 16.8	Canham and Burton, 1968
134.0 ± 4.9	82.0 ± 4.9	Houchin et al., 1958
135.0 ± 16	94.0 ± 14	Evans and Fung, 1972

These previous studies have relied upon the light microscope for measurements on RBCs. In the current study, we have employed electron microscopy techniques to determine RBC dimensions. Although this approach has the advantage of the higher resolution of the electron microscope, there may be certain disadvantages. Specimens must be chemically fixed, dehydrated through graded ethanol solutions, embedded in plastic and thin sectioned. These procedures may introduce dimensional distortions (Weibel, 1969). Dimensional distortion from these potential sources of artifacts might account for our estimate of RBC surface area, 130.1 μm^2, which is 2.9 to 6.1% lower than the value estimated by other methods (Table 3). It is also entirely possible that our value is more accurate than that obtained by other methods. This question can only be resolved by a systematic study of the artifacts of thin section electron microscopy preparative techniques. It is noteworthy that the microstereology approach has certain advantages over conventional quantitative microscopy techniques. In particular, the method is shape independent thus making possible measurements on toxically injured cells of various shapes.

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