Technical methods

Enumeration of platelets using a model B counter

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The standard methods for counting blood platelets, using an engraved counting chamber and a light microscope, have many possibilities of error. The introduction of phase-contrast microscopy by Brecher, Schneiderman, and Cronkite (1953) has greatly facilitated the counting of platelets and improved the accuracy of the results. However, despite this improvement in accuracy the visual method is lacking in one basic essential, namely, speed in producing results. Because of this, a number of workers, in recent years, have sought for a more accurate and less laborious method for the enumeration of blood platelets. As the electronic counting of both erythrocytes and leucocytes has become established practice in many laboratories, similar methods have been investigated for counting blood platelets.

Using a Coulter counter, Eggleton and Sharp in 1963 described an accurate method for counting platelets in dilutions of platelet-rich plasma. Similarly Eastham (1963 and 1965), using the same instrument, devised a method for counting the platelets in whole blood. In this technique dilutions of whole blood were made in saline and an appropriate platelet suspension was obtained by differential centrifugation. Aliquots of the supernatant were then counted at two different threshold settings in order to distinguish between red cells and platelets. After a correction for coincidence had been made, the platelet count was obtained by subtraction.

In 1965, Bull, Schneiderman, and Brecher introduced a different method designed to eliminate the errors resulting from loss of platelets during centrifugation. The sample of whole blood was allowed to undergo sedimentation in a narrow-bore plastic tube for 10 to 15 minutes. An aliquot of the resulting supernatant platelet-rich plasma was then counted electronically. Because platelet-poor plasma was trapped within the red cell mass during sedimentation two corrections were made, one for the haematocrit and one for the excess platelets in the supernatant. This technique avoided some of the errors of the previous method but preparation was time consuming.

Williamson in 1966 further modified Eastham's original method by altering the dilutions and centrifugation speeds but retained the differential counting method for separation of red cells from platelets. However, it has been found that this technique could be adapted for use with a model B Coulter counter, by using instrument settings designed to count only particles of platelet size, thereby enabling whole blood platelet counts to be read directly off the instrument.

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METHOD USING WHOLE BLOOD

Five millilitres of blood, obtained by clean venepuncture, are taken into a specimen bottle containing 9 mg dipotassium EDTA. After careful mixing a 40 μl sample of this blood is placed in a wide-bore plastic tube containing 20 ml of particle-free saline (see appendix) to give an initial dilution of 1 in 500. In the case of a fingerprick specimen the 40μl of blood is placed directly into 20 ml of 10% EDTA-saline. This is to prevent platelets clumping. The contents of the tube are then mixed and centrifuged in a BTL bench centrifuge at 1,800 rpm for 40 seconds. This time does not include the rapid starting or the natural stopping of the centrifuge. A speed of 1,800 rpm or 560 *g* was selected because a slower rate, *eg*, 1,500 rpm, allowed an excess of red cells to remain in the supernatant. Similarly a faster speed, *eg*, 2,000 rpm, produced the same result, due to 'shuddering' of the centrifuge as it came to rest of its own volition. The selection of the centrifuge is critical as any degree of 'shuddering' will give inaccurate results.

After centrifugation, a further : 10 dilution of the resultant supernatant 'platelet-rich' suspension is made in the particle-free saline to give a final dilution of 1 : 5,000. The specimen must be adequately mixed before counting in order to avoid errors due to platelet sedimentation. An 0·05 ml and an 0·05 ml aliquot from the final dilution are then counted on the model B Coulter counter. These quantities enable the actual platelet count per cmm to be read directly from the counter. The final count is reported as the mean of the two results obtained from the different aliquots. In practice these figures show close approximation. The rate of counting was of the order of one platelet count every four minutes. It is important to ensure that the orifice tube does not become blocked during a count. The presence of blockage can readily be detected by miscroscopic observation of the orifice and by inspecting the manometer and the oscillograph pattern.

MODEL B COUNTER SETTINGS

Orifice tube	70μ
Aperture current	1/4
Amplification	1/4
Gain control	32
Aperture matching switch	64
Threshold	
Upper	50
Lower	5

Threshold module switch is put in 'separate' position.

ACCURACY OF THE PLATELET COUNT Fifty consecutive samples were counted from a master sample. The statistical analysis was as follows:

Range	130,300-136,000/cmm
Mean	133,900/cmm
Standard deviation	$\pm$1,650 platelets/cmm
Coefficient of variation	1·23%

TABLE I

VARIATION IN THE ACCURACY OF PLATELET COUNTS DEPENDING ON THE EXPERIENCE OF THE OPERATOR

	Length in Training			
	Eight Weeks	*Eighteen Months*	*Two and one-half Years*	*Authors' Results*
Number of counts	20	20	20	50
Range in number per cmm	164,000-199,000	222,000-260,000	258,000-283,000	133,000-136,000
Mean	186,300	238,000	272,000	133,900
Standard deviation	±10,600	±10,400	±7,790	±1,650
Coefficient of variation (%)	5·69	4·36	2·86	1·23

A coefficient of variation of 1·23% indicated a high degree of accuracy, but it was thought that the degree of accuracy might be variable, depending upon the expertise and training of the operator carrying out the tests. Therefore, three technicians at different stages in their training were asked to carry out a series of platelet counts. These results are shown in Table I. The mean coefficient of variation was 3·53%. In this laboratory the coefficient of variation, using phase contrast miscroscopy, is 6·9% and for other direct visual methods is 11·8%. Therefore, even in the hands of relatively inexperienced technicians a satisfactory degree of accuracy has been obtained.

COMPARISON OF VISUAL AND ELECTRONIC COUNTING OF THE SAME SAMPLES Blood samples were divided into two aliquots and 65 such paired samples were counted by visual and electronic methods. The visual counts were carried out under light microscopy and in the knowledge that the results were required for comparative purposes. The platelet counts included thrombocytopenic and thrombocythaemic samples. The range was from 31,000 to 1,460,000 platelets/cmm. The results are illustrated in Fig. 1 and recorded in Table II. The statistical analysis is as follows:

Number	65 paired samples
Range	32,000-1,460,000/cmm
Correlation coefficient	0·984
Degrees of freedom	64
P	0·00001

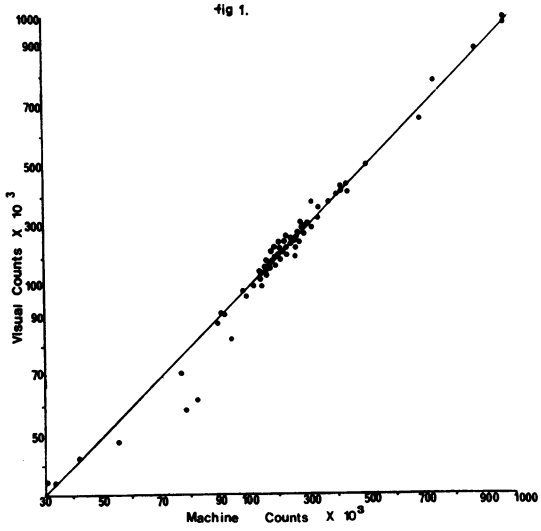

FIG. 1. *Graph illustrating comparative values for machine and visual counts.*

TABLE II

COMPARISON OF VISUAL AND ELECTRONIC PLATELET COUNTS ON THE SAME BLOOD SAMPLES

Coulter Counter	*Visual*	*Coulter Counter*	*Visual*
31	35	234	232
32	34	234	240
43	42	234	240
55	48	234	250
77	71	240	238
82	62	244	248
89	80	250	246
89	88	252	246
94	82	255	234
115	108	255	266
138	140	256	260
146	140	270	240
146	150	275	260
159	159	282	290
161	152	290	301
169	145	300	306
169	173	303	300
174	176	319	297
175	172	320	360
182	166	345	323
183	176	347	342
184	200	389	390
185	170	413	420
190	220	415	420
191	192	426	430
191	192	430	443
195	186	445	441
216	220	691	650
217	220	740	780
219	210	874	890
219	210	1,160	1,040
219	220	1,460	1,350
219	220		

[1]Platelet counts = × 10^3 cmm

STANDARDIZATION OF THE COULTER COUNTER A standard suspension, comparable to a platelet suspension, was prepared from a killed bacterial culture of *Micrococcus roseus*. This organism was selected due to its similarity in size compared with that of the platelet. The normal platelet diameter may vary from 0·5 μ to 3·5 μ. The Micrococcus has a narrower, yet similar diameter range, 1 μ to 1·5 μ. It is

a tetracoccus, but the majority grow in single form. The incorporation of glass beads into the nutrient broth culture medium enabled the bacterial clumps to be readily dispersed on shaking during the 48-hour growth period. The culture was then killed in 10% formol saline, washed in normal saline, and the final suspension standardized to give a concentration of 5×10^6 organisms per ml. The stability of the culture was found to be satisfactory. Dilutions prepared from the original master sample were counted daily in the first instance, and then at weekly intervals or longer over a period of nine months. These results are shown in Table III. Thereafter aliquots of the *Micrococcus roseus* bacterial suspension were counted regularly and used as a standard for the Coulter counter.

TABLE III

STABILITY OF THE *MICROCOCCUS ROSEUS* SUSPENSION

Day	*Count*	*Day*	*Count*
0	667·5	39	672·0
1	670·8	46	688·0
2	669·8	53	694·0
3	665·8	83	690·0
4	675·4	113	695·0
11	670·0	171	700·0
18	690·0	201	690·0
25	665·0	232	680·4
32	675·0	260	665·9

Counts are expressed as N $\times 10^3$ per cmm

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APPENDIX

The particle-free saline is prepared as follows:

A fresh 0·9 w/v sodium chloride solution is filtered initially through a glass fibre filter[1]. This is followed by pumping the roughly filtered solution through a 0·8 mμ millipore cellulose acetate filter. The resulting solution is a satisfactory diluting fluid for platelet counting, because the background count is invariably less than 100 counts per 0·5 ml aliquot.

[1]Glass fibre filter, type M. 2 in. diameter (Gelman Instrument Co. Michigan, USA)

Separation of haemoglobins on cellulose acetate

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Since cellulose acetate was first recommended for separation of abnormal haemoglobins (Kohn, 1958 and 1968) a number of papers have been published and various modifications recommended (*eg*, Friedman, 1962; Bartlett, 1963; Marengo-Rowe, 1965; Rosenbaum, 1966; Graham and Grunbaum, 1963). There seems to be little doubt that for routine purposes cellulose acetate is a very suitable medium for the separation of abnormal haemoglobins. The advantages over filter paper are speed and much neater and clearer separation patterns. With very few exceptions there is no need for the more sophisticated and complicated procedures such as agar gel, starch, or polyacrylamide. It was thought worthwhile to report a technique which is simple, reliable, and gave consistently satisfactory separations of abnormal haemoglobins as well as of Hb A_2 and Hb F (Figs. 1 and 2). A slight modification recently introduced proved to be particularly useful. By applying relatively high voltage across a narrow bridge gap and using standard equipment available in any laboratory, perfectly satisfactory separation of abnormal haemoglobins can be achieved within eight to 15 minutes (Fig. 1a). A discontinuous buffer system was found to be particularly useful.

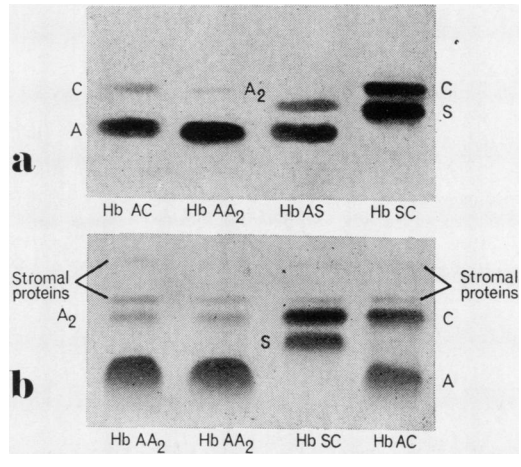

FIG. 1. *Haemoglobin separation:* a *300 V applied. 10 minutes' run. 3 cm wide bridge gap. Unstained strip. Note Hb A_2 migrating with Hb C mobility and absence of stromal protein bands.* b *200 V applied. 25 minutes' run. 5 cm bridge gap. Ponceau S. stained strip. Note the presence of two stromal bands, one of them nearly as strong as the A_2 band. This might lead to confusion.*