

Comparison of methods of counting skin window coverslips

A statistical analysis

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Using the skin window technique, many investigators have expressed their results in terms of an increase or decrease of one cell type versus another on the skin window coverslips. These percentages of cell types are often then discussed as to how they represent the cellular aspect of inflammation and/or hypersensitivity. The usual method of arriving at these percentages of cell types is to count approximately 200 cells representative of the relative proportion of cell types seen on scanning a skin window coverslip. This method of counting (referred to as the "representative" method) was thought to be too subjective, and a new method of counting was elaborated (referred to as the "fixed" method). Using the "fixed" method, one counts all the cells which fall under a given unit (e.g., a pointer tip) as the entire coverslip is viewed in a fixed pattern. To compare the two methods, the scatter of the samples obtained by the two methods was calculated by finding their standard deviations about the true percentages of cell types on each coverslip sampled. The true percentages of cell types were obtained by counting all the cells on the coverslips. Eight to 10 samples were taken from each of 7 coverslips by both methods (126 samples from a total cell population of 58,617). Samples obtained by the "representative" method had standard deviations twice those of the "fixed" method. The "fixed" method was found to come closer to the binomial standard deviations of the true percentages of cell types on the coverslips than the "representative" method. Hence the "fixed" method of counting is proposed as a new means of sampling skin window coverslips.

The skin window technique was originally described by Rebuck and Crowley.¹ First, the volar surface of the forearm is cleansed with alcohol, then a one square centimeter area of skin is abraded with a scalpel until fine bleeding points are seen. A drop of antigen is applied and covered with a cardboard-backed sterile glass coverslip. The coverslips are removed at timed intervals, air dried, and stained by the May-Grünwald-Giemsa method. An arbitrary number of cells is counted, and the percentages of each cell type are calculated.

This technique has been used by many investigators¹⁻¹² to study the cellular aspect of inflammation and hypersensitivity. Most of their results are expressed

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as percentages of cell types counted on the skin window coverslips. For example, atopic subjects who had positive scratch tests to ragweed developed high skin window eosinophil percentages to ragweed but not to tetanus toxoid (control antigen). Thus eosinophils were postulated to represent a cellular aspect of immediate-type hypersensitivity.²

The usual method of arriving at these percentages of cell types (referred to as the "representative" method of counting^{3, 11, 13, 14}) seemed too subjective to us, so a search for a more systematic method was begun.

This investigation compares the result of the aforementioned search (referred to as the "fixed" method of counting) to the usual "representative" method of counting skin window coverslips.

MATERIALS AND METHODS

Definitions of the cell types viewed on the skin window coverslips are elaborated because the cells on the coverslips are more variable in their form than are cells seen on a peripheral blood smear. Consequently, different investigators may or may not count the same cell as belonging to a particular cell type. For example, it makes a difference in the percentage of eosinophils reported if one counts eosinophils which have discharged their granules or only eosinophils with intact cytoplasmic membranes.

The following definitions were used in this study: An eosinophil had a two-lobed nucleus and a cytoplasm containing red granules, surrounded by an intact cell membrane. A polymorphonuclear cell had a nucleus containing two or more lobes and a finely granular cytoplasm. A mononuclear cell had a single-lobed nucleus having more finely dispersed chromatin than did the polymorphonuclear cell, and the nucleus was surrounded by a pale blue cytoplasm. A basophil had an obscured nuclear pattern, because the nucleus was overlaid by purplish granules surrounded by an intact cytoplasmic membrane.

Certain cells were not counted because they could not be clearly described. These were cells that were caught in a blue film and too dark to be clearly distinguished, cells obscured by overlying each other, or cells without distinct cytoplasmic boundaries. Cells around the outer edge of the coverslip (a margin of two 250 power microscopic fields) often were indistinct and therefore not counted.

Two methods were used in sampling the coverslips. In the fixed (F) method, all cells that fell under a given unit (Fig. 1) were counted continuously as the entire coverslip was passed over in a fixed pattern. Since the total number of cells varied from one coverslip to another, several procedures were used.

The initial procedure for every coverslip was to count continuously the cells passed over by the tip of a pointer (Fig. 1) as successive power fields were viewed following the pattern indicated in Fig. 2.

If there were less than 200 cells initially counted, the cells were recounted using the pattern indicated in Fig. 2; but instead of counting only cells continuously passed over by the pointer tip, cells passed over by a square or column of the grid or the entire grid (Fig. 1) were also counted. The portion of the grid used depended on how far below 200 cells the initial count fell, e.g., if only 30 cells were counted by the initial procedure, then one could use the entire grid as the unit for counting in an attempt to reach a cell count of 200. Some coverslips have less than 200 cells totally; if this occurs, a total cell count would be done.

If 200 cells were counted before the entire coverslip could be viewed, then the technique was altered in the following order for increasing numbers of cells on the coverslip: (1) one counted more than 200 cells by the initial procedure, i.e., counting more cells (greater than 200) in order to pass over the entire coverslip with the tip of the pointer; (2) one repeated the count (e.g., with the pointer tip again) but used the pattern indicated in Fig. 3; (3) one counted only cells located under two boxes of the grid (Fig. 4) for each field width in a 250 power field, following the pattern indicated in Fig. 2 or 3. Occasionally more than one

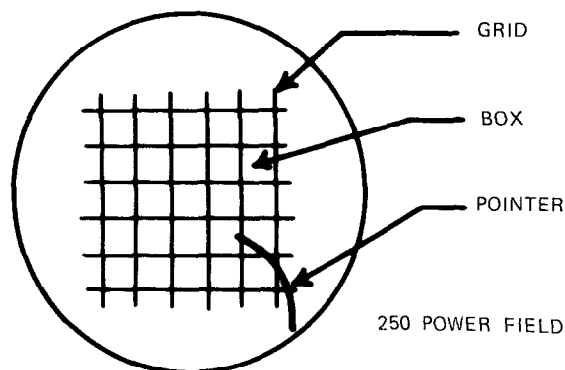


FIG. 1. A 250 power microscopic field is illustrated. In this field are the **units** used in counting cells, i.e., the tip of the pointer, a box, column, or entire grid. Figs. 1 to 4 are diagrams of the fixed method of counting.

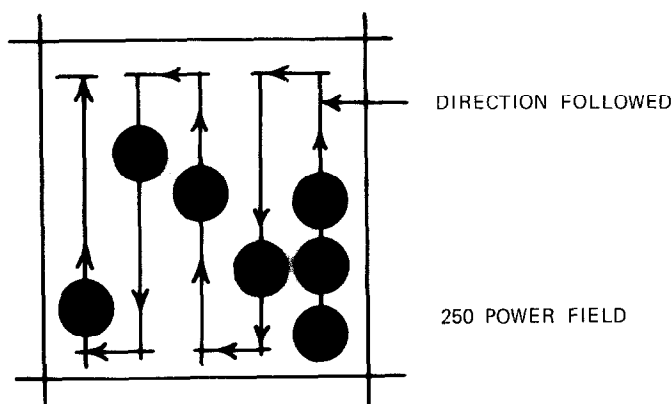


FIG. 2. The initial pattern followed by a unit used for counting cells is shown. The boxed area is the coverslip. Each **black circle** represents one 250 power field. At the end of each vertical passage of the field across the coverslip, one shifts the field laterally exactly one field width so that in due course the entire coverslip is counted. The number of 250 power fields to cover a coverslip varies with the dimensions of the coverslip used.

procedure was used in obtaining samples by the fixed method from the same coverslip. The procedure which brought the count close to 200 cells was the one used most often.

With the usual, or representative, method, 200 cells were counted. After the coverslip was scanned, cells were counted from one or more small cell clusters that were representative of the proportion of cell types seen on scanning the coverslip,^{3, 11, 13, 14} and/or large representative groups of cells were counted by passing one field over the cell aggregates in a line from outer edge to outer edge (Fig. 5).¹¹

Equal numbers of samples (8 to 10) were taken with each method from 7 coverslips. One hundred twenty-six samples were examined from a total cell population of 58,617 cells (total of cells on 7 coverslips).

Standard deviations (S.D.) were calculated in order to compare the accuracy of the two methods of counting. The formula used was: $S.D.^2 = \frac{1}{N} \sum_{i=1}^N (T-x)^2$, where T was the true percentage of the particular cell type sampled (computed by counting all the cells on the

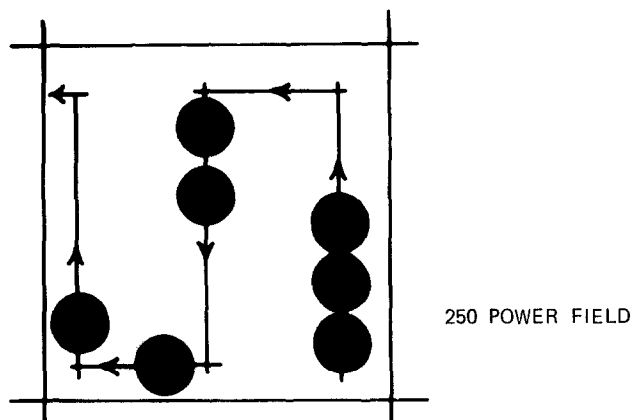


FIG. 3. When the coverslip has a large number of cells, one shifts laterally exactly two field widths and resumes counting.

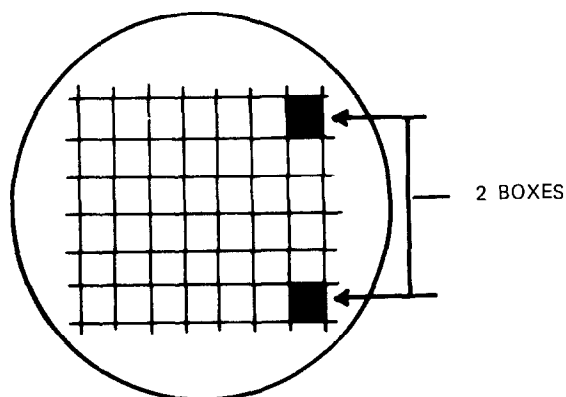


FIG. 4. Instead of counting all cells **passed over** by a unit through a field's width, only the cells falling under two boxes **at each end** of a field's width are counted; then the coverslip is advanced to the next field. One can follow the pattern in Fig. 2 or 3. This procedure is used when very large numbers of cells are on the coverslip.

coverslip), x was the sample's percentage of the particular cell type, and N was the number of samples taken.¹⁵

If one assumes that the methods of counting gave a random sampling of the total population of cells, the standard deviation of the percentages of cell types counted by the two methods should approach the "ideal" standard deviation, binomial standard deviation. The ideal standard deviation (I.S.D.) is the standard deviation one would expect if the samples' percentages of cell types in a certain method were obtained in a random manner from a large cell population after a number of samplings. Thus the formula, $I.S.D.^2 = \frac{T(1-T)}{C}$, where T is defined as above and C was the number of cells in a sample, allowed us to compare the two methods as to their randomness.¹⁵ For example, for the coverslip with 1,487 cells in Table I, one can see that $T = 0.37$ for the polymorphonuclear cells; therefore, the 2I.S.D. by the representative method equals $2\sqrt{\frac{(0.37)(0.63)}{200}} = 6.6$ per cent as is given in Table I. C always equaled 200 cells for the representative method. With the fixed method, numbers of cells counted in each sampling of a single coverslip varied slightly, so an

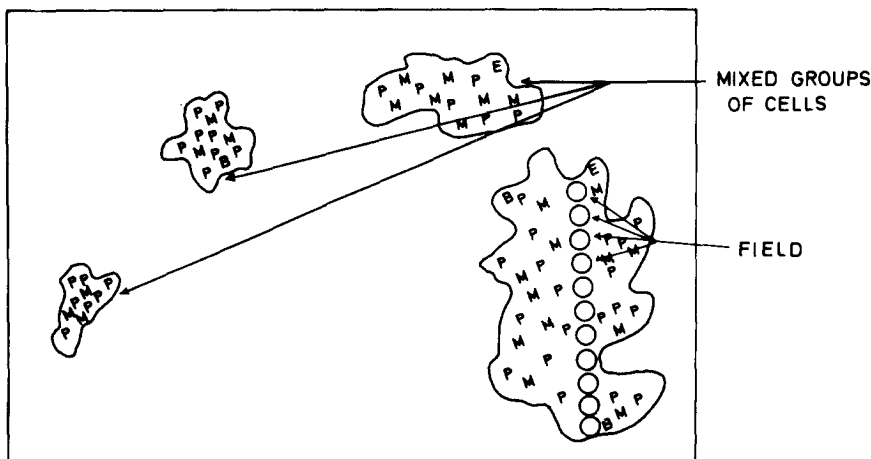


FIG. 5. Cell aggregates are totally counted using the representative method. Also diagrammed is how a large cell aggregate is counted by the representative method (all cells counted that fall under the circles).

arithmetic mean of the samples' counted cells was used for C. Usually this mean was close to 200.

RESULTS

It took between 5 and 10 minutes to count a coverslip by the representative method and between 10 and 15 minutes by the fixed method. But most importantly for the conclusions of studies using the skin window technique, the fixed method of counting gave percentages of cell types closer to the actual or true percentages of cell types on the coverslips than did the representative method (Table I). For example, using values from Table I, one can see that from a total cell count of 1,487 cells, 37 per cent of the cells were polymorphonuclear cells. For samplings by the fixed method, the samples' percentages varied from the true per cent by 7.4 per cent (2 S.D.). If, in the fixed method, samples were collected in a random manner, the 2 S.D. should approach 5.4 per cent (2 I.S.D.), which it did (7.4 per cent). For samplings by the representative method, the samples' percentages varied from the true per cent by 33.4 per cent (2 S.D.). If, in the representative method, samples were collected in a random manner, the 2 S.D. should approach 6.6 per cent (2 I.S.D.); however, it did not (33.4 per cent).

It can be seen from Table I that the standard deviation by the representative method of counting was usually more than double the standard deviation by the fixed method. Also, the fixed method of counting can be seen to approach the binomial expression of S.D., whereas the representative method rarely gave standard deviations close to the I.S.D.

DISCUSSION

The problem of obtaining samples that show the true percentages of cell types on skin window coverslips has been mentioned by other investigators.

TABLE I

Total No. of cells	Method of count	P			M			E		
		T	2S.D.	2I.S.D.	T	2S.D.	2I.S.D.	T	2S.D.	2I.S.D.
1,487	F	37.0	7.4	5.4	62.5	7.8	5.6	0.5	0.8	
	R		33.4	6.6		33.0	6.8		0.8	
4,271	F	26.0	11.6	5.0	74.0	11.6	5.0			
	R		33.6	6.2		33.6	6.2			
4,540	F	49.8	18.0	6.6	48.2	17.0	6.6	2.0	3.4	2.2
	R		36.8	6.6		36.0	6.6		2.4	2.2
6,999	F	11.2	5.2	4.4	88.8	4.6	4.4			
	R		10.7	4.4		10.7	4.4			
10,099	F	23.1	3.6	6.0	76.9	3.6	6.0			
	R		40.6	6.0		54.4	6.0			
11,042	F	67.3	8.4	6.6	26.4	10.4	6.2	6.3	2.6	3.0
	R		24.0	6.6		27.0	6.2		4.2	2.6
20,179	F	83.8	7.4	5.0	11.7	5.0	4.4	4.5	3.0	2.8
	R		13.0	4.8		11.6	4.4		3.8	2.8

P = polymorphonuclear cells; M = mononuclear cells; E = eosinophils; T = true per cent of cell type (derived from total cell count); 2S.D. = 2 standard deviations from the T; 2I.S.D. = 2 ideal standard deviations from the T; F = fixed method of counting; R = representative method of counting.

Riis¹¹ noted that the cells were not in a homogeneous suspension on the coverslips and counted 200 cells from "evenly distributed" clusters of between 50 and 100 cells. Feinberg and associates¹³ counted 1,000 cells from "representative fields." Boggs and associates¹⁴ scanned the coverslips for areas in which the cells appeared "evenly distributed" and counted 100 cells. Seinfeld and McCombs³ scanned the coverslips and counted 500 cells from aggregates in which eosinophils predominated. In these studies and others,^{5, 16, 17} statistical evidence that the fields chosen for counting approached the true proportion of cell types on the coverslips was lacking. The representative method, which was used by most of the above-mentioned investigators, is not supported by the findings of this study. As to why the representative method gave such variation, one could speculate that scanning the skin window coverslip gives only a guess as to what the percentages of cell types actually are on the coverslip—and cell types vary greatly in their proportions from one cell aggregate to another, even though they are often referred to as "evenly distributed."^{11, 14}

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