

Automated Counting of Microbial Colonies¹

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The time required for direct counting of colonies on agar plates for estimating population density of viable microorganisms has precluded studies requiring measurement of such a parameter. Bowman, Blume, and Vurek have described a photo-electrical capillary tube scanner which automates the counting. Results obtained with an instrument similar to that of Bowman et al. have been intensively analyzed with respect to precision and accuracy. The sources of "errors" have been ascertained, and the instrument's potentialities and limitations are discussed.

The direct or visual counting of colonies on agar plates has long been a bottleneck in studies involving estimation of the density of viable microorganisms. Accordingly, optical density and electronic counting methods have been used. These are, of course, indirect methods that do not give estimates of viable but rather of total counts (2). Various methods for automation of colony counts have involved a scanning process in which light reflected from colonies is sensed by a phototube and processed electronically (3). A bulky, expensive, and complex system of logic circuitry and information storage is required to suppress counts registered from colonies counted in previous scans.

Bowman, Blume, and Vurek (1) have achieved a major simplification in instrumentation for the estimation of certain viable microorganisms with their capillary tube scanner system. The colonies develop in capillary tubes with nutrient agars, and the light scattered from a colony registers as a single electrical pulse as the tube is translated mechanically relative to, or scanned by, the phototube. This approach reduces considerably the required electronics.

This paper is an assessment of the results obtained with an instrument similar to that of Bowman et al. (1). Our instrument does not give a linear, one colony-one count relationship. Therefore, a number of factors must be considered in converting the raw to meaningful data. Counts of almost 200,000 capillary tube readings were made during the course of this study.

MATERIALS AND METHODS

Instrument and accessories. The extent of variation from a nonlinear relationship (or "errors") is depend-

ent, in part, upon the choice of design specifications of a system.

The capillary tubes used are disposable 100- μ liter pipettes (Corning, catalogue no. 7099-S). They are placed, after filling with agar-cell mixture, on a supporting table that moves them along their length for scanning action.

The optical system is shown schematically in Fig. 1. The illumination and optical system of lamp A (General Electric, no. 1133), slits B and D, and lens C (10-mm focal length) produces a plane of light about 300- μ m in thickness over the entire cross sectional area of the tube. Slit B restricts the source to a small fraction of the lamp filament width. Lens F projects an image of the illuminated plane onto stop G. The central portion of the image, 70% of the cross sectional area of the capillary (1.15 mm of a capillary tube 1.38 mm in inner diameter), falls onto the surface of a photomultiplier tube (RCA 913A). An electrical signal proportional to the scattered light intensity is produced.

Bowman et al. (1) used a minimum-size threshold, so as to detect colonies with diameters of 3 to 8 μ m. Their objective was to obtain early estimates of viable populations; they made their counts within several hours of preparation of tubes. A low-threshold setting involves determination of an initial background count and a second count after incubation. The second count must be made before errors due to coincidence and overgrowth become appreciable.

Early estimate is not critical in our studies of the dynamics of microbial populations. Accordingly, the minimum-size threshold is set so that colonies with minimum diameters of 50 to 100 μ m and larger are detected. This obviates the need for a background count which is on the order of 0.3 count per tube. Some latitude for time of incubation prior to scanning is also afforded at our higher threshold. On the other hand, colonies with diameters of less than 50 μ m are not counted.

Therefore, the selection of a minimum-size threshold is dependent upon the purpose for which the counter is used.

As each capillary tube is scanned, the electrical

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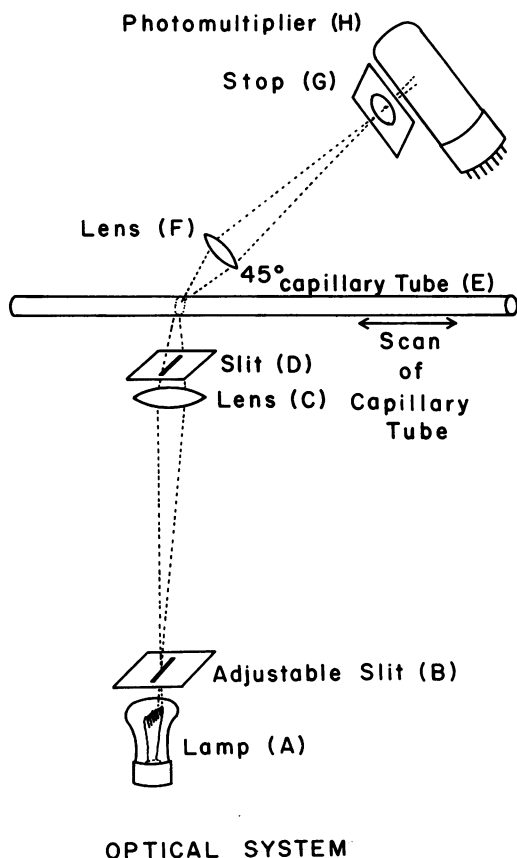


FIG. 1. Optical system.

pulse produced by the photomultiplier is shaped by a pulse height analyzer circuit with variable threshold. A given capillary is scanned in both directions, and the accumulated count was displayed on glow discharge counting tubes, or printed out on a typewriter, or coded and punched on paper tape.

The capillaries are transferred from notched racks for incubation to racks on the instrument. While the accumulated count is being recorded, a second pair of racks on the instrument picks up and positions each of the tubes on the movable table one position forward. The time required to count a tube, record its count, and position the next tube is 3.2 sec. This permits counting of enough tubes to give a statistically sound value with minimal, manual handling in a reasonable time.

Preparation of the capillary tubes. Diluted cell suspension is first mixed with 1% agar containing nutrients at 45 C. A previously sterilized manifold which holds 10 tubes (Fig. 2) is attached to a threaded cylinder, suction is applied, and the molten cell-agar mixture is drawn into the tubes. The capillaries are then rinsed under distilled water to set the agar and minimize contamination of the exterior of the tubes with agar. Later, at a convenient time, the capillaries are

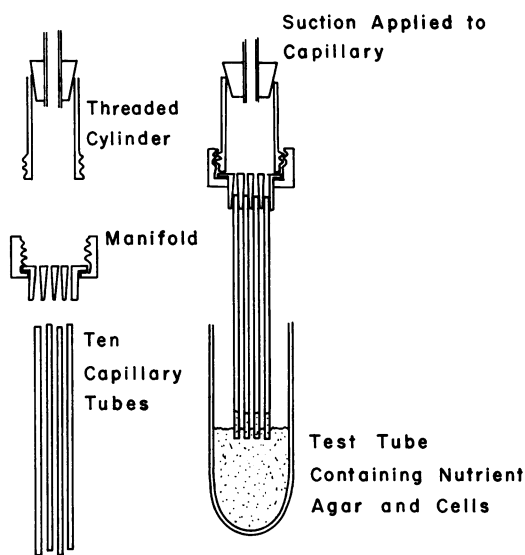


FIG. 2. Preparation of capillary tubes.

removed from the manifold, wiped clean with tissue paper, and placed on notched racks (for incubation); these racks permit up to 21 tubes to be placed on, or removed from, the counter as a group. About 10 different samples with 10 tubes each can be prepared by one operator in less than 45 min. This includes time for sampling, preparation of two dilution bottles, transfer to molten agar, stirring, filling the capillaries, washing, and placing them into racks.

Care is necessary in the preparation of these tubes. When the cells are mixed with agar, precaution must be taken to assure thorough mixing but not excessive agitation which creates foam. Foam bubbles refract light and may be counted as colonies by the counter. Capillaries which have not been washed and have an exterior deposition of agar may give blank "counts" of 10 to 15 per tube; hence the necessity of the rinsing and wiping operation mentioned above.

Organisms and media. Obligate aerobes such as *Bacillus subtilis* and *Azotobacter vinelandii* were unable to grow in a limited number of media tested in the capillary tubes.

The organisms used for our data included *Escherichia coli* B/r, *B. licheniformis* A-5, *Lactobacillus casei* NRRL B-1445, and *Saccharomyces cerevisiae* NRRL Y-567.

The medium for *E. coli* was 0.5% tryptone (Difco), 0.01% glucose, 0.25% yeast extract (Difco), and 0.1% KNO₃; pH 7.0. The medium for *B. licheniformis* and *L. casei* was 0.5% tryptone, 0.1% glucose, and 0.25% yeast extract; pH 7.0. The medium for *S. cerevisiae* was 0.5% tryptone, 0.01% glucose, and 0.25% yeast extract; pH 5.5. All media contained agar at the level of 1.0%. Note that the sugar concentration was low and that KNO₃ was incorporated in the medium for *E. coli*; these procedures were followed to avoid formation of gas (hydrogen) bubbles.

Direct count. Direct counts were made under 20X

magnification. All incubations were carried out at 33 C.

EXPERIMENTAL

Threshold setting. Comment has been made above concerning the choice of threshold settings. The instrument count versus threshold setting with *L. casei* in our instrument is shown in Fig. 3. The left hand portion of the uppermost curve shows the effect of errors due to coincidence of colonies with high number densities. Also minute-sized colonies are not counted. Accordingly, we have used a threshold setting of 1.0 for most of the work in which only mean values are desired. This setting will detect colonies in the range of diameters of 50 to 100 μm or larger. A categorical statement cannot be made regarding colony "size" because the type of growth of one organism gives a different pulse height as compared to the characteristic growth of a second organism.

Instrument response. The instrument counts versus direct counts for *E. coli*, *L. casei*, *S. cerevisiae* are shown in Fig. 4. Counts ranging from about 10 up to 400 show reasonably good response. However, random error is large on the left hand side of the curve.

A better method for calibration with the same data as in Fig. 8 is shown in Fig. 5. Here the plot

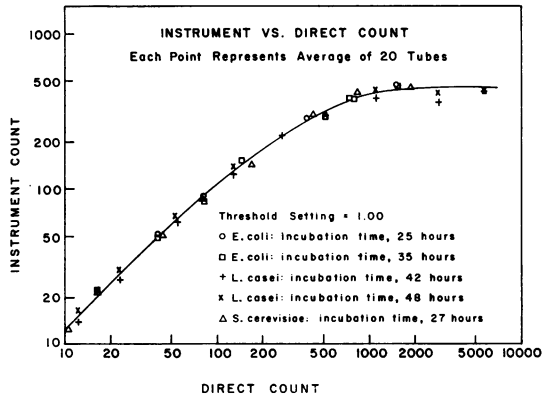


FIG. 4. Instrument versus direct count.

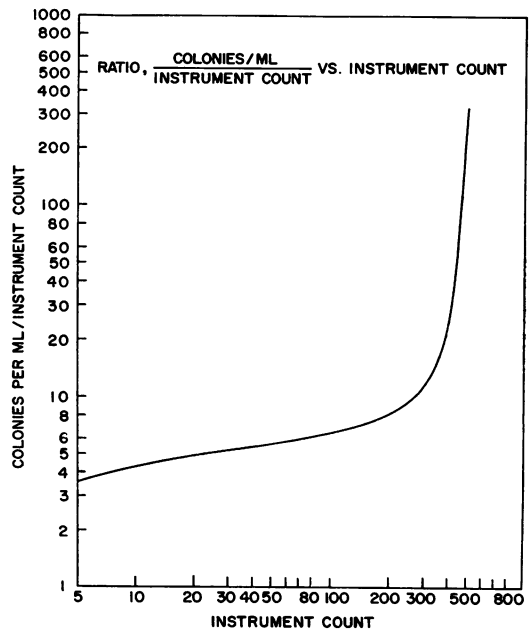


FIG. 5. Ratio of (colonies per milliliter/instrument count) versus instrument count.

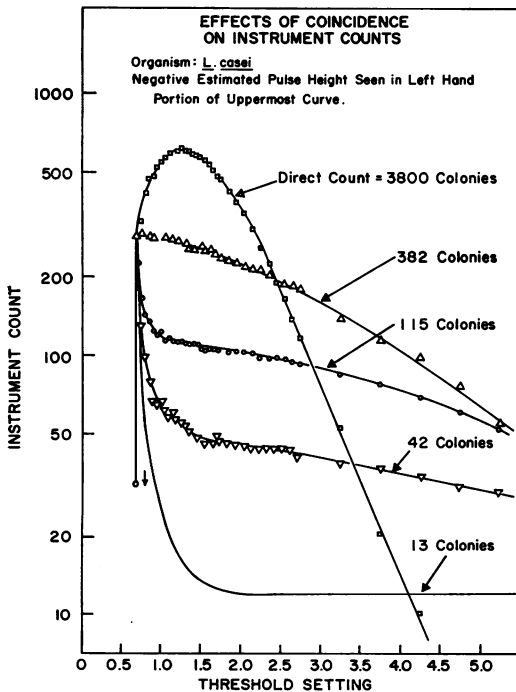


FIG. 3. Effects of coincidence on instrument counts.

of the ratio of colonies per milliliter of cell-agar mixture versus instrument count is presented. This calibration plot is used to convert the raw instrument count into "actual" count. For example, an instrument count of 100 on the x axis indicates 6.6 colonies per ml of agar per instrument count on the y axis. This represents a count of 660 visible organisms per milliliter of cell-agar mixture. From the dilution factor for the agar and dilution bottles, one can readily compute the number density in the original sample.

Variations from linear response. The extent of deviation from a linear response depends upon the

TABLE 1. Maximum overall coefficients of variation^a

Determination	Total no. of tubes					
	1	2	5	10	20	∞
Range: 40-400 colonies/tube (no separate correlation used)						
One dilution	25 ^b	19	15	12.6	11.5	10.6
Two dilutions	[25]	18	13.2	10.9	9.6	8.6
Three dilutions	[24]	[18]	12.7	10.3	8.9	7.2
Range: 9-900 colonies/tube (no separate correlation used)						
One dilution	53	39	28	23	20	17
Two dilutions	[52]	38	27	22	19	15
Three dilutions	[51]	[38]	26	22	19	15

^a Figures represent worst case within specified range.

^b Values are expressed as percentages.

number of colonies, their diameters, and their opacity.

The "errors" may be categorized into two major groups (i) preparation-associated errors and (ii) instrument-associated errors.

The former may result from agglomeration of bacterial cells, from clumps or chains not breaking, etc. The effect of such errors is such that it is not profitable, beyond a certain point, to prepare more tubes unless an entirely separate series of dilutions is made. Table 1 shows the coefficients of variation (COV) for 1 to 20 capillaries, 1 to 3 dilution series, and over 2 density ranges.

The instrument-associated errors are those errors which would not be present if the tubes were counted directly. These may be divided into two components: (i) correlation-induced errors or errors due to uncertainty in colony size, density, shape, etc.; and (ii) instrument-induced errors or errors due to the slightly random nature of the instrument's response to fixed inputs.

Two types of correlation-induced errors have been encountered. They are due to coincidence and overgrowth.

Coincidence results from more than one colony lying within the field or view at a given time. When the number density is high, two or more colonies may be counted as a single colony, or, if the colony diameter is larger, coincidence error becomes greater. Both lead to "low" counts. This is seen in the uppermost curve of Fig. 3.

Overgrowth is the error due to a colony having its center outside the actual scanned area but growing into the scanned area with incubation time. This leads to "high" counts in that the

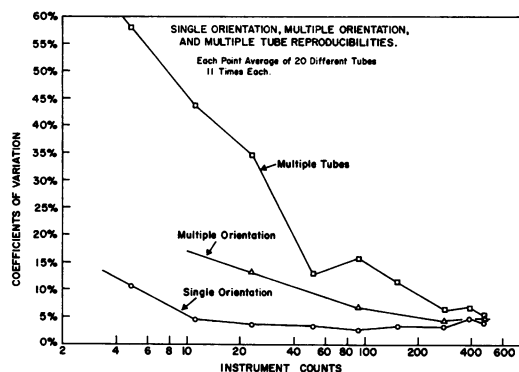


FIG. 6. Single orientation, multiple orientation, and multiple-tube reproducibilities.

"effective" scanned area is larger. Although coincidence becomes more probable as the product of the colony number density and the mean diameter of the colonies, overgrowth depends only on the diameter of the colonies.

The correlation-induced error is a consequence of the major simplifying assumption that the size and nature of the colonies need not be considered in the correlation between the instrument and actual counts. The correlation-induced errors are determinate errors and may be reduced by employing individual correlations for a given organism, its physiological state, incubation time (in the capillaries), etc.

The instrument-induced error is a random non-determinate noiselike variability in the counter's response to random variations, or drifts, in the electrical, optical, and mechanical variables. One such source of error is shown by the data in Fig. 6 on a plot of COV versus colony number for: (i) a single tube oriented in a fixed position, (ii) multiple orientation for a single tube (the capillaries are not absolutely straight and hence tend to rotate to their stable positions during repeated scan movement; hence the photomultiplier sees a different section on each scan), and (iii) multiple tube, multiple orientation in which random stochastic error between tubes is superimposed on error due to (ii). The error due to multiple orientation (ii) was obtained by manually turning each tube end for end between counts.

From the data in Fig. 7, it can be seen that virtually all of the instrument-induced errors are inherent given the microbiological, statistical, and geometrical constraints on the system; they are not due to significant variations or instabilities within the counter itself.

The overall uncertainty in the estimates of the instrument made from a single count on one capillary tube is the vector, not the arithmetic sum

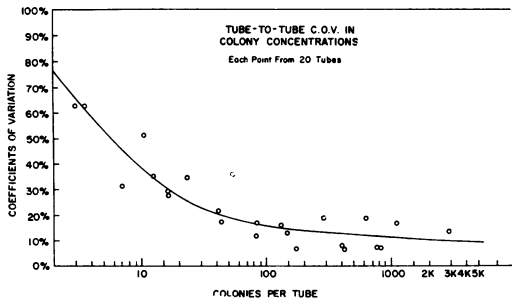


FIG. 7. Tube-to-tube coefficients of variation (COV) in colony concentrations.

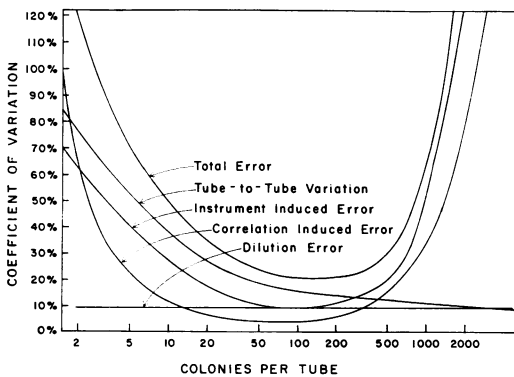


FIG. 8. Errors in predicted concentrations.

of the dilution error, the tube-to-tube variation, the correlation-induced error, and the instrument-induced error. Their COV are plotted against colonies per tube in Fig. 8.

Measurement of colony size distributions. We have hitherto been concerned with the determination of the colony number density in a given capillary tube. A logical extension of the capabilities of the counter lies in the assessment of sizes of those colonies. For example, such a measurement may indicate the physiological state of the cells in the population. Colony sizes as a function of tube incubation time and extrapolation should, in principle, yield a distribution of lag-phase times. It may also find application in determining the suitability of a given medium for supporting [or inhibiting, as has been suggested for antibiotic testing by Bowman et al. (1)] the growth of an organism.

The basic principle is that the larger a given colony is, the more light it will scatter. The greater the amount of scattered light, the higher will be the peak of the voltage spike at the amplified photomultiplier output. The distribution of these pulse heights can be measured by substituting a multichannel pulse height analyzer or a single

TABLE 2. Approximate diameters corresponding to preset thresholds

Threshold setting	Equivalent diameters (μm)			
	<i>B. licheniformis</i>	<i>E. coli</i>	<i>S. cerevisiae</i>	<i>L. casei</i>
0.84	116	111	86	54
1.28	168	159	123	77
1.71	198	188	146	92
2.15	227	210	163	103
2.96	255	242	288	118
3.78	282	267	208	131
4.55	303	288	223	141
5.80	332	316	245	154
6.90	354	336	261	164
8.11	375	356	277	174
10.73	415	394	306	192

channel analyzer. We used the latter less expensive alternative, namely, to count each tube repeatedly at different threshold settings. Eleven threshold values were used; the entire operation of advancing the thresholds and moving a subsequent tube into the field of view and starting over was automated so that no attention by the operator was required.

Although the light scattered by a colony is approximately proportional to the biomass of the colony, this relationship breaks down when the size or opacity of the colony becomes such that significant proportions of the incident light are absorbed within the colony. The density of the colony depends upon the organism and upon the conditions of growth. This is seen in Table 2, which gives the approximate equivalent diameters of *E. coli*, *B. licheniformis*, *S. cerevisiae*, and *L. casei* for various threshold values. The equivalent diameters were obtained by direct measurement of the size of colonies.

From direct measurements one cannot make firm statements concerning colony size as measured by the instrument; however, the automatic counter clearly shows the distinction between the more or less opaque colonies. The mean pulse height is an indicator of colony biomass under carefully controlled conditions. For unusually opaque colonies, mean diameter of colonies must be restricted to 250 μm and the number density must be restricted to less than 100 per tube so that coincidence is not a significant factor. Indeed large errors, i.e., regions of negative measured pulse heights, result under conditions other than these.

Effect of gas bubbles. Difficulties are encountered with organisms in media which result in formation of large amounts of hydrogen gas; the

TABLE 3. "Total" and "spore" counts (organism: *B. licheniformis*)

Determination	Spread plate count ^a (per ml)	Tube count ^a (per ml)
Control (20 C)		
<i>t</i> = 0	1.3 × 10 ⁸	1.3 × 10 ⁸
<i>t</i> = 30	1.3 × 10 ⁸	1.3 × 10 ⁸
Heat-treated (80 C)		
<i>t</i> = 5	7.7 × 10 ⁴	12.5 × 10 ⁴
<i>t</i> = 15	6.9 × 10 ⁴	7.3 × 10 ⁴
<i>t</i> = 30	7.0 × 10 ⁴	8.6 × 10 ⁴

^a Average of 10 plates.

^b Average of 20 capillary tubes.

TABLE 4. Effect of gas bubbles^a

Threshold	Gas present	Gas absent
0.84	176.0	231.0
1.28	76.0	99.0
1.71	30.0	35.0
2.15	11.0	9.9
2.96	3.8	0.1
3.78	3.0	0
4.55	3.0	0
5.80	2.6	0
6.90	2.6	0
8.11	2.5	0
10.73	2.3	0

^a Organism: *E. coli*. Average count of 30 tubes each.

instrument cannot discriminate between the bubbles and the colonies. *E. coli* was grown in medium which would not allow gas formation (the standard *E. coli* medium) and in the *L. casei* medium in which gas is produced. The count was substantially lower in the medium with gas bubbles at low-threshold values but higher at high settings (Table 3).

Use in determination of spores. The scanner can be used to estimate the number of spores in a mixed population of vegetative cells and spores. A non-heat-treated sample can be taken as the total count; a heat-treated sample can be taken as the spore count. Since there is a spectrum of heat-resistant states from the true vegetative cells to the heat-resistant spores, the investigator must operationally define a spore. In our case, we used

B. licheniformis as the test organism. A 1-ml amount of a mixture of cells was suspended in 3 ml of 0.066 M KH₂PO₄ and 0.066 M K₂HPO₄ (pH 7.0) buffer. The 4 ml was transferred to a test tube (16 by 150 mm) and placed in a water bath at 80 C. The temperature rose to essentially 80 C in just under 3 min. Those cells that survived the heat treatment were called spores (Table 4).

Comparison with other methods. The methods of determining viable count of *E. coli* were compared: the pour plate, the spread plate (4), and the capillary tube scanner. Twenty plates were made for both plate count procedures, and 20 capillaries were made from the same culture. All three gave essentially the same results. The pour plate gave a mean of 1.55 ± 0.11 × 10⁹/ml; the spread plate, 1.48 ± 0.55 × 10⁹/ml; and the scanner, 1.46 ± 0.046 × 10⁹/ml. The ranges were computed for 95% confidence limit.

In general, the capillary tubes gave a lower count than the Coulter counter as was to be expected. A correlation could be made between the two counts only when samples were taken from chemostats at steady state. However, fairly wide discrepancies between the two counts were found under certain conditions.

The capillary tube scanner system of Bowman, Blume, and Vurek (1) or one similar to it represents a major breakthrough in the determination of the number density of certain microorganisms. Its use enables one to conduct studies which could not hitherto be carried out because of the excessive manual work involved in estimation of viable microorganisms. It should be of particular interest to those interested in the physiology of bacterial spores.

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