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In-Use Evaluation of the Omnicon Automated Tumor Colony Counter[1]

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The reproducibility and accuracy of the Omnicon (Bausch and Lomb Inc., Rochester, NY) automated tumor colony counter for counting tumor colonies growing in double layer soft agar is evaluated and the reproducibility is compared with manual tumor colony counting. Replicate within day run-to-run colony counts of the Omnicon show a median correlation coefficient (r) of >0.985, and day-to-day median r of >0.980. In contrast, for manual colony counting, the best intra-observer reproducibility achieved is a r of 0.943 and the best inter-observer reproducibility is a r of 0.831. Analysis of results from individual culture plates counted by the Omnicon on 5 separate days shows a median coefficient of variation of 10% with 77% of the culture dishes showing coefficients of variation of colony counts over 5 days of less than 20%. Counting of culture plates during incubation shows that the Omnicon is counting tumor colonies developing after plating of a single cell suspension.

Key terms: Clonogenic culture, tumor colonies, tumor cloning, soft agar culture

The recent development of short-term culture of human primary tumors has offered the clinical laboratory the possibility of monitoring dynamic rather than static properties of human tumor cells. Specifically, the human tumor cloning system (HTCS) applied to solid tumors by Salmon *et al.*, allows the growth in a two-layer soft agar system of tumor colonies from clonogenic, presumptive "stem" cells (5,6). The first proposed application of this system to clinical oncology was the *in vitro* determination of chemotherapeutic sensitivity and resistance of tumors to allow individualizing of chemotherapeutic regimens (5). Further clinical applications have been proposed, including posttherapy evaluation of persistent or recurrent tumor (1) and evaluation of grade and stage of tumor based on *in vitro* growth characteristics (2).

Soon after the potential clinical usefulness of the HTCS was recognized, an automated tumor colony counter was developed (3) to speed up the process of counting colonies and determining chemotherapeutic drug resistance or sensitivity by computation of the inhibition of colony growth produced by preincubation of cell samples with cytostatic agents. Since the automated colony counter also gives information on the size distribution of tumor colonies, additional data bearing on tumor colony growth parameters *in vitro* have become available and are potentially useful new data which may contribute to all of the proposed clinical applications of HTCS cited here. However, before the full potential of the automated colony counter can be realized, the reproducibility and accuracy of its basic function of counting tumor colonies in soft agar culture must be established. The present report deals with initial in-use experience with the automated colony counter.

Materials and Methods

In the Pathology Department of Radboud Hospital, all urologic and gynecologic tumors received are cultured by the two layer soft agar method. Routine surgical specimens of solid tumors are brought sterile and unfixed to the Department of Pathology. A pathologist using sterile technique examines the material and chooses tissue for soft agar culture (approximately 1–2 g) and routine histopathology. Tissue selected for soft agar culture is further processed according to the detailed description recently published (6).

Briefly, the tissue is minced with scissors for 5 min through a metal sieve of 60 holes/inch into McCoy's wash medium. The suspension of cells is then treated with collagenase and DNase for 2 hr (7), syringed manually once through a 25 g 1 inch needle and washed twice in McCoy's medium. Trypan blue-excluding cells are determined using 0.1 ml of the cell suspension, and the concentration of nucleated cells

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is determined in a Burker-Turk haemocytometer. The concentration of cells is then adjusted to 3×10^6 nucleated cells/ml McCoy's wash. Culture dishes (35 mm) are seeded with 5×10^5 nucleated cells per dish in double layer soft agar exactly as described elsewhere (5) except that conditioned medium is not used. Culture dishes are examined within 24 hr after plating to evaluate presence of clumps seeded into culture and to rule out infection of the culture. Colonies of tumor cells are examined beginning 1 week after plating. A colony of tumor cells is defined as a spherical three-dimensional cellular aggregate with smooth borders containing 30 or more cells (1).

Air-dried Papanicolaou stained smears of the cell suspension used for plating the cultures are made from all specimens. These smears allow visual assessment of the cell composition and morphology of the cell types brought into culture. Following the growth of colonies, detailed morphologic assessment of the colonies as well as other cells and particles in the agar is carried out on agar layers removed from the culture dishes, mounted on standard glass slides, and stained with the periodic acid Schiff (PAS) reaction. From selected cultures, formalin-fixed paraffin blocks are made and 4–6 μm sections are prepared, similar to routine histologic preparations (4). These sections are stained with haematoxylin and eosin as well as other routine histologic stains as appropriate for the type of tumor.

Automated colony counting: The Omnicon (Bausch and Lomb Inc., Rochester, NY) was used for the present evaluation of automated colony counting. A detailed description of the theory and operation of this instrument has been published (3). The instrument used in the present work was similar to that previously described except that it was fitted with an inverted microscope and a fully automatic 36-dish microscope stage. This allowed walk-away operation with counting of 36 culture dishes taking an average of 40–45 min.

For the present study, culture dishes from the routine workload of the laboratory including a spectrum of both gynecologic and urologic tumors were fixed in 4% glutaraldehyde. These cultures were unselected except that an attempt was made to include cultures showing the full range of colony counts: <30 to >1500 colonies/plate. For automated analysis, 108 culture dishes were counted by the Omnicon as three 36-dish sets. For determination of within day run-to-run reproducibility, the sets were counted twice in succession with no changes in position of the dishes, settings of the instrument controls, etc. A total of 13 different within day comparisons were made on different days. For determination of day-to-day reproducibility, the same 108 culture dishes as 36-dish sets were counted five times on 5 or 6 different days over a period of 8 days with demounting and remounting of the cultures as well as readjustment of the illuminator source and scanner sensitivity each day. This provided a total of 35 different day-to-day comparisons. Care was taken however, to orient the culture dishes identically each day using an indelible ink mark on the side of the culture dish. Because of drying and cracking of the agar layers with ageing, (in spite of glutaraldehyde fixation, hydration and refrigeration of the culture dishes) 29 of the culture dishes became unusable during the course of the study leaving a total of 79 culture dishes from which evaluable data were obtained.

Manual colony counting: For comparison with automated colony counting, 155 culture dishes were counted by three different observers working in the culture laboratory and responsible for routine counting of colonies. For technical reasons, primarily deterioration and drying of the agar, these culture dishes were not the same dishes used for Omnicon counting but were from the same types of material with a similar distribution of numbers of tumor cell colonies. All colony counting was done with the culture plates coded so that the observer did not know whether or not he had seen the culture plate previously, nor did the observer know the results of previous counts, if any.

Results

Omnicon reproducibility: Figure 1*A* shows the distribution of r values of duplicate Omnicon counts on the same day, each point representing one 36-dish set. The modal r value is >0.990 and median r value is >0.985.

The day-to-day r values of automated colony counting are presented in Figure 1*B*. Each point represents the r value of

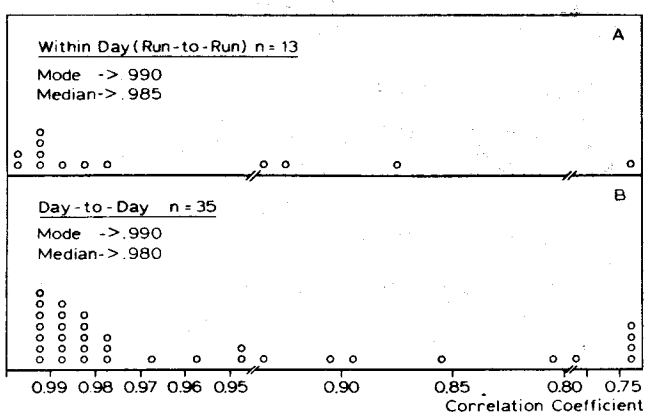

FIG. 1. Distribution of correlation coefficients of within-day run-to-run (*A*) and day-to-day (*B*) replicate Omnicon tumor colony counts. Each point represents one 36-plate set counted twice on the same day (*A*) or on two different days (*B*). *Ordinate:* number of observations; *abscissa:* correlation coefficient.

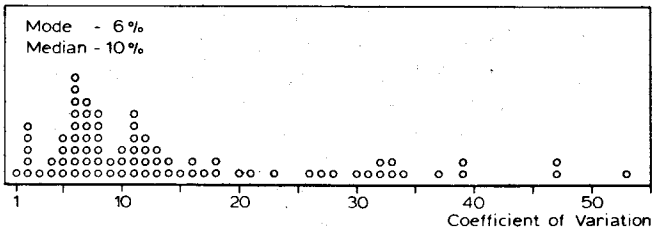

FIG. 2. Distribution of coefficients of variation of tumor colony counts for 79 evaluable culture dishes. Each point represents one culture dish counted with the Omnicon automated tumor colony counter on 5 different days.

colony counts from one of the three 36-dish sets on 1 day with counts obtained from the same set on 1 of the other days. Thus, each day's colony count is compared with every other day's count for each of the sets of 36 dishes. The modal day-to-day r value is >0.990 and the median >0.980.

Since the distribution of both run-to-run and day-to-day r values showed considerable skewness with several day-to-day r values less than 0.750, the variability of Omnicon counting was evaluated individually for each culture plate. Figure 2 presents the distribution of coefficients of variation (CV) for each culture dish computed from the colony counts on 5 different days. The modal CV is 6%, the median CV 10%, and 61 of the 79 evaluable culture dishes show a CV of automated counting less than 20%. When the mean number of colonies per dish is compared with the CV, a tendency to lower CV with higher colony counts is seen in all three test sets ($r = -0.115$, -0.217, and -0.380).

Reproducibility of manual counting: For comparison with automated colony counting, 155 culture dishes were counted by at least two observers and/or by the same observer twice to assess intra- and inter-observer variability. Table I presents the results of these studies. The best reproducibility is achieved by observer A counting the same plates twice. Observer C also shows good reproducibility with duplicate counting of the same culture dishes (consistency), but clearly produces very different results from observers A and B. Ob-

server A had, at the time of the present study, 1.5 yr experience with the soft agar culture technique after training in one of the most active and experienced HTCS laboratories. Observers B and C were trained by observer A and all three observers count culture plates routinely in our laboratory. It is clear that the best reproducibility achieved by manual counting falls short of that achieved by automated counting. In addition, the inter-observer variability is such as to call into question the use of data in a laboratory where more than one observer counts culture dishes.

Comparison of automated and manual counting: All culture plates counted by the Omnicon were also counted by observer A. The r value for the three sets of culture dishes comparing automated and manual counting were 0.513, 0.630 and 0.891, respectively, similar to the inter-observer reproducibility shown by manual counting (Table 1). In general, the manual counts were lower by factors of 2 to 5 than the Omnicon counts ("Discussion").

Accuracy of automated colony counts: Because of the poor correlation of manual colony counts by different human observers, the accuracy of the Omnicon was assessed independently of manual counting. The essential question to determine the accuracy of automated colony counting is whether the automated counts reflect development of tumor colonies in culture over time, dependent only on the presence of living cells in the original cell suspension. Table 2 shows an example of such a study. MatLyLu rat prostate carcinoma cells, maintained in our laboratory, were planted in soft agar culture. Because of the high plating efficiency and rapid growth of this undifferentiated tumor, 5×10^3 cells were used per culture plate in place of the 5×10^5 cells used for human primary tumors. As controls, MatLyLu tumor cells were prefixed in neutral buffered formalin for 2 hr, washed twice with PBS and planted in culture. During incubation, pairs of culture plates were removed from culture and counted (Table 2). Because of the high likelihood of infection of the culture plates during the time they were being counted by the Omnicon in an open laboratory and the subsequent risk of infection of other cultures in the incubator, the plates were not reincubated after counting. Thus, the data points in Table 2 represent different pairs of culture plates from the same tumor cell suspensions, processed and set in culture at the same time.

As shown in Table 2, the Omnicon counted no colony development in the culture plates planted with formalin-fixed cells, while the unfixed cells formed microscopically verified colonies over 9 days in culture. The development during 3 weeks incubation of Omnicon-counted tumor colonies from a human breast carcinoma is also shown in Table 2. Colony growth is slower and the plating efficiency is lower in primary human tumor cultures.

Table 1
Inter- and Intra-observer Reproducibility of Manual Tumor Colony Counts[a]

	Observer		
	A	B	C
A	0.943 (25)[b]	0.831 (150)	0.478 (114)
B		0.733 (18)[b]	0.643 (110)
C			0.929 (15)[b]

[a] Correlation coefficient (number of culture plates).
[b] Pooled data for 3 observers, each observer counting the same culture plate twice: total $N = 58$; $r = 0.877$.

Table 2
Development of Tumor Colonies in Soft-Agar Culture[a]

Days in culture	MatLyLu Rat Carcinoma[b]		Human Breast Carcinoma Pleural Fluid[c]
	Formalin fixed	Unfixed	
1			4 ± 1
2	3 ± 0	0 ± 0	
3			12 ± 3
6	2 ± 1	419 ± 40	22 ± 2
9	2 ± 2	521 ± 136	
10			45 ± 10
13			45 ± 3
16			43 ± 1
19			60 ± 7
21			70 ± 35

[a] Mean ± SE; two culture plates per data point. Omnicon counts
[b] 5×10^3 Cells plated per culture dish.
[c] 5×10^5 Cells plated per culture dish.

Discussion

The present study indicates that the Omnicon automated tumor colony counter functions with a high reproducibility. The median within day run-to-run r of >0.985 and median day-to-day r of >0.980 are significantly better than the reproducibility achievable by manual colony counting (Table 1).

In order to achieve this reproducibility, instrumental variables must be carefully controlled. The most sensitive hardware component of the Omnicon is the light source. It is important that the light source be adjusted each day such that maximum illumination, judged by level of TV-scanner saturation, is as high as possible and the same from day-to-day. It was noted in the present study that increased variability in colony counts was recorded just before the light bulb burned out. The addition of a feedback circuit for monitoring light intensity at the field of view might obviate this problem.

The cultures which were subject to high day-to-day CV were examined manually and compared with cultures producing low variability. The single most important cause of lack of day-to-day reproducibility of colony counts of individual culture dishes is the presence of tumor colonies at different levels in the agar layers. The most common levels of colony growth seem to be the upper surface of the upper agar layer and the interface between the upper and lower agar layers. Two or more layers of tumor cell colonies in a single culture dish seem to cause difficulties with the automatic focussing of the Omnicon such that different planes of focus are chosen from run to run and day to day, with inevitable variability in colony counts resulting. Preliminary manual inspection of culture dishes to ascertain that the level of colony growth within the culture dish is reasonably uniform should eliminate many of the culture dishes which give high CV with automated counting.

The observation that day-to-day CV tend to be inversely related to colony count in individual culture dishes may also be related to problems with the automated focussing of the Omnicon. If the number of tumor cell colonies in a culture

dish is so low that the instrument does not have a tumor cell colony to use for focussing in the first field examined, variable planes of focus will be used from run-to-run. A minimum threshold number of colonies per culture dish as a prerequisite for accepting Omnicon counts may help in eliminating from automated counting those culture dishes which show higher variability.

The Omnicon rejects fields of view from counting if the total optical density of the field is too high. In the present study, data from a particular culture dish were not included in the statistics if 10 or more fields (of a total of 35 scanned per culture plate) were rejected because of high optical density. This was an arbitrary choice based on the counting statistics; further refinement of this threshold for rejecting Omnicon counts may be possible.

The most difficult problem in evaluating Omnicon results and comparing them with manual counting of tumor cell colonies is the question of accuracy. The most widely accepted present definition of a tumor cell colony in the double-layer soft-agar system is a reasonably regular round structure composed of 30 to 50 or more cells. Since identification by inverted phase microscopy of individual cells in colonies is difficult, it seems that in practice the most important criterion used by laboratory personnel is colony size. Since the size of a tumor colony composed of 30 cells can vary significantly depending on the tumor type, the number of cells actually required to meet the definition of tumor cell colony may vary both among human observers and for the Omnicon. This may explain in large part the poor inter- and intra-observer reproducibility of manual counting as well as the poor correlation between the Omnicon and manual counting (3). During the initial phases of the present study, comparison of Omnicon and manual counts and further examination of fixed, PAS stained agar layers demonstrated that our laboratory's criteria for tumor colonies had become too stringent, requiring in practice 100 or more cells for a structure to be accepted as a tumor colony.

The tumors used in the present study to evaluate Omnicon performance included a range of gynecologic and urologic tumors. Since the individual tumor cells of different tumor types vary widely in size, a tumor colony in agar composed of 30 cells will also vary in size. Thus, for optimal tumor colony counting, the size threshold accepted by the Omnicon may need to be varied depending on the specific tumor type.

The fact that growth of tumor colonies in agar during the period of incubation is clearly reflected in automated counts from a rapidly growing animal tumor and from a more slowly growing primary human tumor (Table 2) supports the interpretation that the Omnicon is counting tumor colonies from two very different tumor types. Since colonies are not recognized by the Omnicon during incubation of formalin-fixed cells in the soft agar system, artefacts such as drying or cracking of the agar do not seem to contribute to the colony count produced by the Omnicon.

In general the reproducibility of the Omnicon is adequate for routine use in counting of tumor colonies in soft agar, and is clearly better than the reproducibility achieved by manual counting. Further evaluation of this instrument will allow criteria to be formulated to identify the types of cultures and properties of specific culture dishes which cause poor reproducibility of automated counting of specific cultures. The present study suggests that these may constitute approximately 20% of the total culture dishes in a routine tumor cloning laboratory. The remaining 80% of cultures, evaluable by automated colony counting, will also provide additional data of potential value such as colony size distribution during growth in culture and changes in size distribution caused by chemotherapeutic agents. This additional data is unavailable from manual counting techniques.

Acknowledgments

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