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Ploidy Determination of *Candida albicans*

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The dimorphic yeast *Candida albicans*, as a member of the fungi imperfecti, has been assumed to be in the haploid, or imperfect, state. The deoxyribonucleic acid content of this species has been measured by flow microfluorometry, a technique capable of analyzing single cells. These results were compared with flow microfluorometric deoxyribonucleic acid determinations on a series of strains of *Saccharomyces cerevisiae* of known ploidy (haploid, diploid, triploid, and tetraploid). These ploidy levels were readily distinguished by the flow microfluorometry procedure. By this criterion, *C. albicans* was found to contain a diploid amount of deoxyribonucleic acid. Ultraviolet radiation survival and chemical mutagenesis experiments support the conclusion that both clinically isolated and laboratory strains of *C. albicans* are diploid.

The genus *Candida* is a heterogeneous collection of asporogenous yeast species which do not seem to lend themselves to a stringent scheme of classification (13). This entire collection is held to be comprised of imperfect, or haploid, yeasts (5). The dimorphic yeast of medical importance, *Candida albicans*, as a member of this genus, has also been considered to be in the imperfect, or haploid, state. One of our primary interests in this species has been to generate mutant strains which are impaired in the process of transformation from the yeast (blastospore) form of growth to the pseudomycelial (germ tube) phase of growth often found in infected tissue. Since the species can be grown in a simple medium (9), it seemed puzzling that even the most elementary form of mutants, namely, auxotrophs, were largely unavailable, although requirements for adenine (2) and smooth and rough colonial variants (7) had been reported. Our own attempts at auxotroph isolation were largely unsuccessful; therefore, we performed a series of experiments which were designed to investigate the underlying mechanism which prevented the routine isolation of auxotrophic mutants from *C. albicans*.

One obvious explanation for this lack of auxotrophic strains could be that *C. albicans* was of a higher ploidy than haploid. Diploid strains would necessarily yield auxotrophic mutants at a far lower rate, since either mutations would have to be introduced in each of the two alleles present for a given locus by a mutagen or a dominant mutation would be required in order that the mutation be expressed. Both of these conditions occur at a relatively low rate as compared with the isolation of recessive auxotrophic mutations from comparable haploid cell lines. Indeed, Van der Walt and Pitout (12) reported the isolation of cell lines from *C. albicans* differing in cell volume as well as DNA content. Their conclusion that there were ploidy differences for *C. albicans* was based solely on a chemical DNA determination. We report here on a sensitive flow technique designed to precisely measure the relative DNA contents of yeast strains. We have used this method to determine the ploidy of *C. albicans* strains for both laboratory and clinically isolated cell lines. This technique may have general application for making ploidy determinations as well as DNA content measurements for yeast.

MATERIALS AND METHODS

Strains. *C. albicans* strain 3153A and clinical isolates C136, C139, and C670 were obtained from Helen R. Buckley of the Temple University School of Medicine, Philadelphia, Pa. *Saccharomyces cerevisiae* strains α_6-131-20 (haploid) and A364**a** (haploid) were obtained from Anita K. Hopper of the University of Massachusetts School of Medicine, Worcester, Mass. Also obtained from Hopper were the following: AP3**a**α (diploid), generated by crossing α_6-131-20 with A364**a**; AP3**aa** (diploid) and AP3$\alpha\alpha$ (diploid), both formed by mitotic recombination induced by UV mutagenesis of AP3**a**α; a tetraploid of the **a**/**a**/α/α mating type formed by mating AP3$\alpha\alpha$ with AP3**aa**; a triploid (**aa**α) strain formed by mating AP3**aa** with α_6-131-20. For the results reported here, the haploid strain was A364**a**, and the diploid strain was AP3**a**α.

Media and culture conditions. All strains were grown to stationary phase in YEPD medium (1% yeast extract, 2% peptone, and 2% glucose) at 25°C on a New Brunswick rotary shaker at 200 rpm. The stock cultures were maintained on Sabouraud agar at 4°C. For experiments demanding cells in the exponential

phase of growth, the cells were followed turbidimetrically and harvested at approximately 10^7/ml. These cells were at least four to five generations from the cell levels obtained at the stationary phase of growth.

The *C. albicans* strains were routinely tested for chlamydospore production on corn meal agar and for germ tube formation with the defined medium of Lee et al. (9).

Preparation of cells for FMF analysis. For flow microfluorimeter (FMF) analysis, cells were harvested from the desired phase of growth, washed with 0.9% saline twice, and fixed in 70% ethanol for 10 h at 4°C. The fixed cells were washed once with 0.9% saline and suspended in an aqueous solution of 0.1% bovine pancreatic RNase (Calbiochem) for 1 h at 37°C. Pepsin (Sigma) was then added to 1,000 U/ml, and the cells were incubated for an additional 5 min at 25°C. The cells were then collected by centrifugation and were suspended in an aqueous solution of 5 mg of propidium iodide per ml for 1 h at 25°C (6).

After staining, the cells were rinsed and suspended in normal (0.9%) saline. The setup, principle, mechanics, and operation of the FMF were similar to those described by Steinkamp et al. (11). The wavelength of the exciting laser beam was 488 nm. Yeast counts were automated by a Coulter Counter built into the FMF.

The intensity of the fluorescence (which has been found to be proportional to DNA content [3]) emitted from each of the propidium iodide-stained samples was recorded electronically. The final results were obtained as pulse amplitude frequency distributions, using a 256-channel pulse height analyzer. The results were generated in such a fashion that increased channel number is reflective of increased DNA content. The displayed histograms were photographed, and the data are reported in this communication as graphic reproductions of the distributions of these histograms. In certain instances, for comparative purposes, two distributions obtained in separate determinations were superimposed on each other. These superimpositions were directly generated by the FMF machine, which has a computer-assisted memory so that a previous distribution can be recalled and directly superimposed on a current result. The relative intensities of the peak channel values were recorded in volts. These values were then used to provide the calibration procedure described in the text.

UV radiation survival. Cells for UV radiation survival experiments were derived from late-log cells ($\sim 8 \times 10^7$ cells per ml) grown in YEPD medium at 25°C as described above. The cells were washed in sterile distilled water, and serial dilutions in sterile distilled water were performed. Aliquots were plated onto YEPD plates. The YEPD plates were exposed to UV light (254 nm at 520 ergs/mm^2 per s) for increasing periods of time. The survival curves were generated by counting the resulting colonies.

Chemical mutagen survival (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and ethyl methane sulfonate). Cells were harvested from the mid-log phase of growth in YEPD. The cells were washed twice in sterile distilled water and serially diluted to 5×10^6 to 6×10^6 cells per ml. Ethyl methane sulfonate or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was added to a final concentration of 1 mg/ml. Aliquots were removed as a function of time and diluted 1,000-fold. Survival curves were generated by removing 0.1 ml of these dilutions to YEPD plates and counting the resulting colonies.

RESULTS

Although the technique of FMF has been of substantial importance in the monitoring of mammalian cells in tissue culture (see reference 4 for review), the extension of this work to microbial systems has been limited.

Development in FMF instrumentation now permits rapid measurements (3,000 cells per s [6]) of DNA contents of single yeast cells. Chemical analyses of DNA content on whole cells provide an average nucleic acid content. The FMF technique allows individual cells which have been stained with a fluorescent stain to pass through a laser beam. The resulting fluorescence is measured by a photomultiplier tube. The intensity of this fluorescence is then measured by a pulse height analyzer which assigns each individual measurement to one of a predetermined number of channels. The number of channels is arbitrary and is determined in advance of the measurement. The intensity of the fluorescent signal is proportional to cellular content of the component (in this case, DNA) being measured. In other words, the higher the individual cellular content of the component one is determining, the higher the fluorescent intensity and therefore the higher the channel number that this particular measurement is assigned to. One advantage of the FMF machine is that it provides a distributional analysis of the DNA content in addition to the mean DNA content per cell. Two recent reports have used this technique to monitor yeast DNA synthesis patterns during various fermentations and also DNA synthesis in rhizobia in alfalfa nodules (6, 10). These reports indicated that relatively low DNA content in these cells was capable of being detected by the FMF procedure.

Since the data that one obtains from measurements of this type are of a relative nature, it was necessary to determine if this system was capable of resolving nearly isogenic cell lines of different ploidies. The question under consideration was, Can the FMF machine distinguish isogenic cell lines differing in average DNA content by a factor of 2?. Further, it was also necessary to assign a quantitative measure to the DNA content being analyzed through the use of some internal standard. Both of these requirements could be met through the use of nearly isogenic strains of known ploidy derived from stocks of *S. cerevisiae*.

For this purpose, isogenic haploid, diploid, triploid, and tetraploid strains were obtained from Hopper. These cell lines were grown to stationary phase and prepared for FMF analysis as indicated in Materials and Methods.

The DNA contents for the four *S. cerevisiae* strains analyzed were well separated and easily resolvable (Fig. 1). These data are summarized in Table 1; as can be seen, each of the ploidies measured were separated by ~1 unit (±10%). These data were in close agreement with the values predicted from the ploidy expected. Therefore, it was a simple procedure to ask the question, Which of the distributions represented in Fig. 1 most closely described the distribution found for stationary-phase cells of *C. albicans*? This distribution closely resembled that of the diploid *S. cerevisiae* (Fig. 1; Table 1).

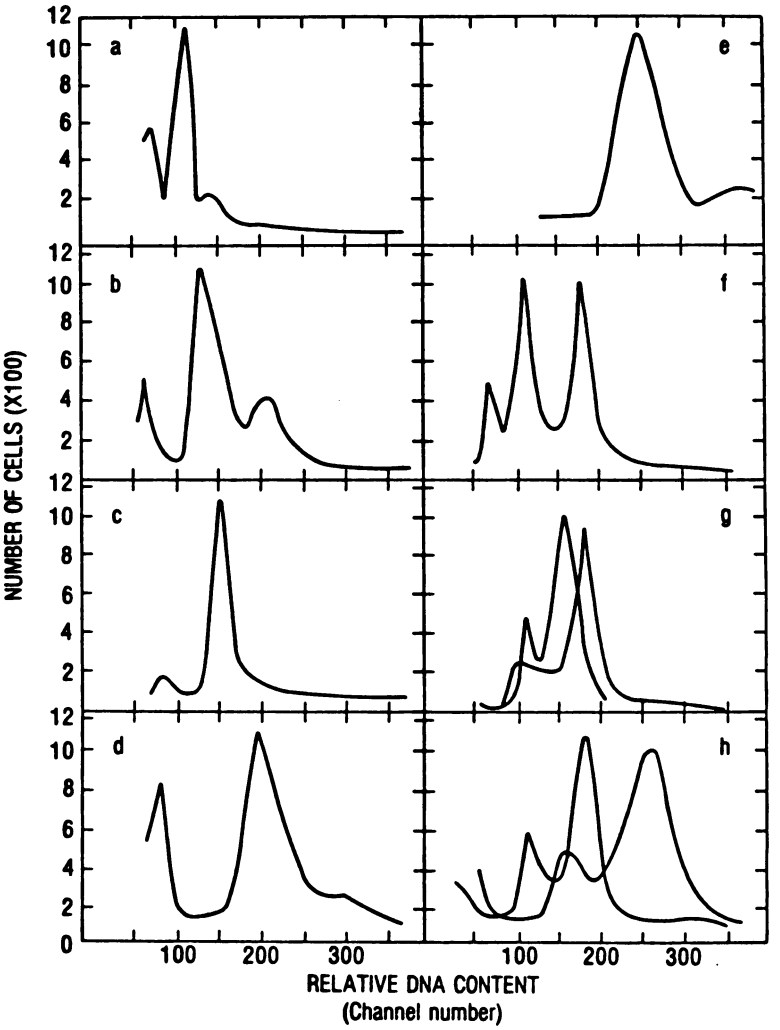

FIG. 1. *Results of FMF analysis of strains of S. cerevisiae differing in ploidy as compared with C. albicans strain 3153A. FMF patterns were derived from individually analyzed stationary-phase cells stained with propidium iodide and subjected to FMF analysis as described in the text. The data were taken from tracings of photographs taken of the distribution recorded by the pulse height analyzer. The numbers on the abscissa represent channel numbers. The greater the channel number, the greater the intensity of the fluorescent output signal and thus the greater the amount of fluorescent dye contained by an individual cell. Therefore, for the purposes of this analysis, the higher the channel number, the greater the DNA content. (a) Haploid S. cerevisiae; (b) diploid S. cerevisiae; (c) C. albicans; (d) triploid S. cerevisiae; (e) tetraploid S. cerevisiae; (f) haploid S. cerevisiae and C. albicans run simultaneously; (g) diploid S. cerevisiae result electronically superimposed on the C. albicans result; (h) tetraploid S. cerevisiae result electronically superimposed on the C. albicans result.*

TABLE 1. *Relative ploidy after normalization to the peak channel value of the fluorescent output pulse obtained from the diploid S. cerevisiae*[a]

Strain	Ploidy			
	Expected	Determined by FMF		
		Determination 1	Determination 2	Avg
S. cerevisiae A364a	1	1.09	1.07	1.08 ± 0.04
S. cerevisiae AP3aα	2	2	2	—
S. cerevisiae (aaα)	3	2.98	2.99	2.99 ± 0.01
S. cerevisiae (aaαα)	4	4.10	4.48	4.29 ± 0.27
C. albicans 3153A		2.04	2.0	2.02 ± 0.03
C. albicans C136			2.14	
C. albicans C139			1.80	
C. albicans C670			1.98	

[a] The mean value for all *C. albicans* determinations was 1.99 ± 0.12. The data displayed in Fig. 1 came from determination 1.

Since there was some concern that this result might be attributed to a differential staining capacity of *C. albicans* as compared with *S. cerevisiae*, we repeated these measurements with ethidium bromide as a stain (propidium iodide was used to generate the results displayed in Fig. 1). The results were identical (data not presented). In addition, we examined a series of three clinical isolates supplied to us by Buckley. All of these strains were found to contain a diploid amount of DNA (Table 1).

It should be emphasized at this point that although we have shown that a number of strains of *C. albicans* contained a diploid amount of DNA as compared with *S. cerevisiae*, there are two ways of interpreting these results. The first of these is that this strain of *C. albicans* is diploid and has a DNA complexity approximately equivalent to the DNA complexity of *S. cerevisiae*. The second interpretation is that *C. albicans* is haploid and that the DNA complexity is twofold that of *S. cerevisiae*.

One obvious approach to resolve these two possibilities is to determine the DNA complexity by determining the reassociation value ($C_0t_{1/2}$) of *C. albicans* DNA. We made this determination (data to be presented elsewhere), and we found a $C_0t_{1/2}$ value of 14, as opposed to a $C_0t_{1/2}$ value of 13 for *S. cerevisiae* (1). However, the DNA complexity for *S. cerevisiae* has been reported by different groups to vary at least over a twofold range (1, 8). Therefore, this determination of $C_0t_{1/2}$ may not be definitive, since it is a factor of 2 with which we are concerned.

An alternative approach remained to resolve the two possible explanations which the FMF data allowed. The two explanations would make radically different predictions in terms of survival of *C. albicans* to mutagenic agents. If *C. albicans* were indeed haploid but had a genome size twice that of *S. cerevisiae*, this species should be far more susceptible to mutagenic agents, such as UV light and chemical mutagens. The reason for this prediction lies in target theory. This theory makes the following assumptions. First, the probability of a lethal mutation is constant per unit length of DNA (and, thus, genome size). Second, the larger the number of copies of a given gene necessary for a given function, the larger the amount of time required by a given dosage of a mutagenic agent to inactivate all copies of this gene and thereby cause a lethal event. With only one copy of a given gene and twice the number of potential sites at which a lethal event might occur, *C. albicans* should therefore be even more susceptible to mutagenic agents than are the haploid strains of *S. cerevisiae*.

Survival to UV light was proportional to ploidy for the standard strains of *S. cerevisiae* (these were the same strains used for the FMF procedure) (Fig. 2). The greater the ploidy number, the more resistant these strains were to UV light.

C. albicans demonstrated inactivation kinetics nearly identical to those of the diploid *S. cerevisiae* (Fig. 2). This result again indicated that *C. albicans* is diploid. It could not be ruled out, however, that *C. albicans* was naturally more resistant to UV light for reasons which

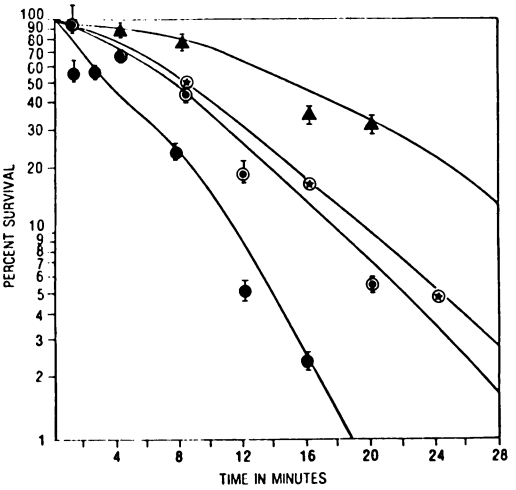

FIG. 2. *Survival of S. cerevisiae strains of different ploidies as compared with C. albicans strain 3153A after exposure to UV light. The cells were exposed to UV light as indicated in the text. The points plotted are averages with the diagram at each point indicating the range of the measurements. Symbols: ▲, tetraploid S. cerevisiae; ⊙, diploid S. cerevisiae; ●, haploid S. cerevisiae; ⊛, C. albicans.*

were not related to genome size or DNA content. Therefore, this result was further investigated by using the chemical mutagens nitrosoguanidine and ethyl methane sulfonate, using a similar protocol in which the mutagen concentration was held constant over time. For both of these mutagens the results were the same. *C. albicans* displayed inactivation kinetics nearly identical to those of the diploid strain (Fig. 3 and 4).

Perhaps a more striking comparison of *C. albicans* with the haploid *S. cerevisiae* is presented in Fig. 5. In this instance mid-log-phase cells were prepared for FMF analysis as described in Materials and Methods. When using log-phase cells, both the G1- and the G2-phase cells can be easily distinguished. The DNA content of these cells in G1 differs from that of cells in G2 by a factor of 2 by definition. The G2 distribution of the haploid *S. cerevisiae* precisely overlapped the G-1 distribution of *C. albicans* (Fig. 5). Further, if one assumes that the distance between the G1 and G2 peaks of haploid *S. cerevisiae* correspond to an arbitrary 1 unit of DNA, then one predicts that the distance between the G1 and G2 peaks of *C. albicans* should correspond to 2 units of DNA on this same scale, if *C. albicans* contains twice the amount of DNA that haploid *S. cerevisiae* contains. As also can be seen in Fig. 5, this was indeed the case.

DISCUSSION

We have presented several different lines of evidence which indicate that *C. albicans* is a diploid species and has a genome size equivalent to that of *S. cerevisiae*. None of these lines is self-sufficient, but if they are taken in aggregate, the conclusion seems likely. We cannot rule out in absolute terms the alternative explanation, that *C. albicans* is haploid but contains DNA with twice the complexity of *S. cerevisiae* DNA. This explanation would also demand that *C. albicans* be twice as resistant to mutagenic agents which are quite different with regard to their mode of action. For example, ethyl methane sulfonate is an alkylating agent, UV light affects DNA repair, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenizes at the growing point of the replicating DNA molecule. Therefore, we feel that this alternative conclusion is far less tenable.

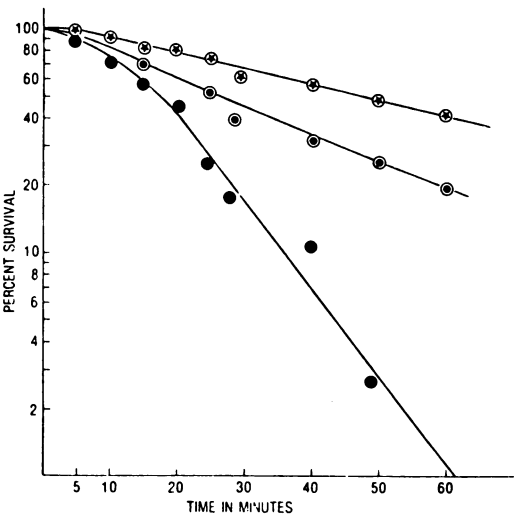

FIG. 3. *Survival of S. cerevisiae strains of different ploidies as compared with C. albicans strain 3153A after exposure to ethyl methane sulfonate. Cells were exposed to ethyl methane sulfonate as indicated in the text, and survival curves were generated. Symbols: ⊛, C. albicans; ⊙, diploid S. cerevisiae; ●, haploid S. cerevisiae.*

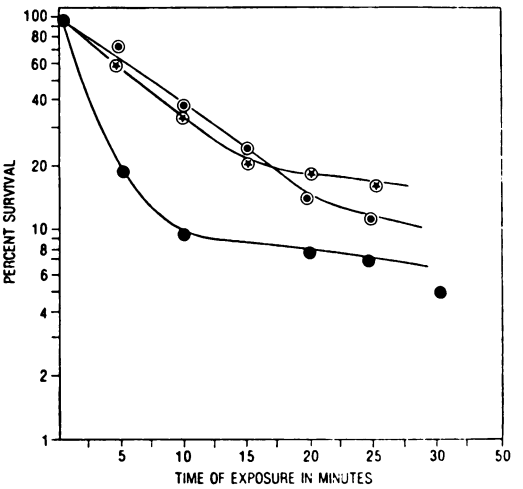

FIG. 4. *Survival of S. cerevisiae strains of different ploidies as compared with C. albicans strain 3153A after exposure to N-methyl-N'-nitro-N-nitrosoguanidine. Cells were exposed to N-methyl-N'-nitro-N-nitrosoguanidine as indicated in the text, and survival curves were generated. Symbols: ●, haploid S. cerevisiae; ⊙, diploid S. cerevisiae; ⊛, C. albicans.*

C. albicans as found both in the laboratory and in the clinic is likely to be a diploid yeast. This means that this species is in the perfect stage. With this concept in mind it should shift the emphasis for those investigators who have been trying to induce mating between putative haploid cell lines to generate a perfect stage for this species with the hope of establishing a genetic system. The emphasis probably should be placed on finding a haploid cell line with the goal of mating these haploid, or imperfect, yeasts.

Further, the finding that *C. albicans* is likely diploid explains the difficulty referred to in the introduction of obtaining auxotrophically mu-

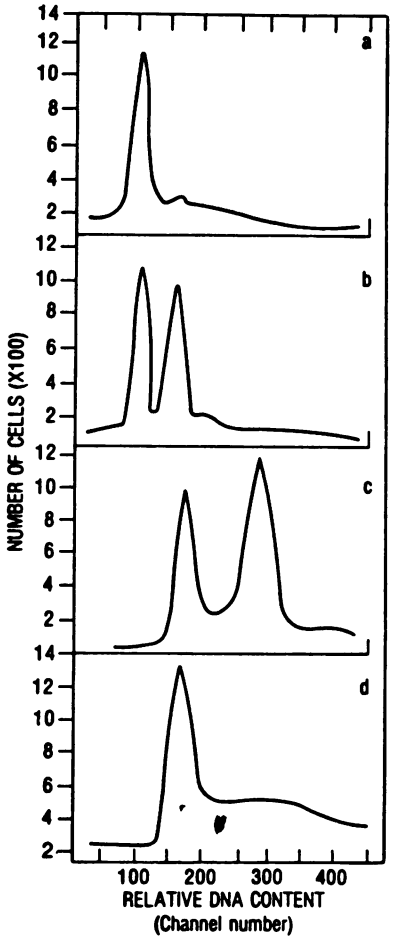

FIG. 5. *FMF results from haploid S. cerevisiae in stationary and exponential phases of growth as compared with C. albicans strain 3153A in the stationary and exponential phases of growth. The conditions and means of displaying these data were the same as those described in the legend to Fig. 1. (a) Haploid S. cerevisiae, stationary growth phase; (b) haploid S. cerevisiae, exponential growth phase; (c) C. albicans, exponential growth phase; (d) C. albicans, stationary growth phase.*

tant cell lines for this species. Mutants obtained from diploid cell lines usually express dominant mutations. This means that they will be obtained at a far lower frequency, since the majority of mutational events in a diploid organism are compensated for by the normal allelic gene. Dominant mutations, of course, are not compensated. The other possibility for mutational events in a diploid organism would be that both allelic genes for a given function would be simultaneously mutagenized. This is likely to occur at an even lower rate than that of dominant mutations.

The power of the FMF DNA determination method is that DNA content measurements can be made which do not depend upon the cells being in any particular growth phase. Chemical determinations for DNA content often assume the cells to be in stationary phase. The numbers generated are either averages per cell number or averages per dry weight. Both of these normalizing factors can be misleading. For example, if the cells in stationary phase were not all in the G1 phase when making a chemical determination of DNA content, then the DNA content would be overestimated. By being able to visualize the distributions of cellular DNA content with the FMF machine, problems of this nature can be eliminated, since one relies on peak distributions for making relative DNA content determinations.

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