Microbial Determinations by Flow Cytometry

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Recent improvements in the optics and electronics of flow cytometry systems, as well as in staining techniques, permit the assay of such minute cellular constituents as the DNA and protein contents of micro-organisms. To assess the usefulness of this technique, DNA and protein content distributions were determined in *Escherichia coli*, *Lactobacillus brevis*, *Lactobacillus casei*, *Chlorella kessleri* 8k, *Saccharomyces cerevisiae*, *Candida utilis*, *Schizosaccharomyces pombe* and *Euglena gracilis*. Investigations of the DNA content distributions of polyploid strains of *Saccharomyces cerevisiae* indicated that the method can be used to determine ploidy. The rapidity of flow cytometry measurements allows accurate determinations in large populations.

INTRODUCTION

Pulse cytophotometry was first used for the determination of intracellular substances in micro-organisms in 1974. Using appropriate fluorescence stains and staining techniques, the RNA and protein contents of intact respiratory and respiratory-deficient yeasts of the genus *Saccharomyces* could be measured during vegetative growth. In these experiments, flow cytometry was used for the first time to determine the ploidy of yeasts. Initially the fluorescent light signals of the yeast DNA were too faint to be recorded by flow cytometry. Thus, the cytometrical measurements of RNA and protein in yeast populations were restricted according to ploidy (Hutter, 1974). Since that time, flow cytometrical evidence of microbial DNA has been extensive (Falchuk *et al.*, 1975; Skogen Hagenson, 1976; Paau *et al.*, 1977a, b; Bailey *et al.*, 1977; Slater *et al.*, 1977; Hutter & Eipel, 1978a).

The aim of this investigation was to test the usefulness of flow cytometric methods in microbiological assays.

METHODS

Micro-organisms. These are listed in Table 1.

Preparation of cultures. Organisms were incubated in 100 ml medium in 300 ml Erlenmeyer flasks unless indicated otherwise. *Chlorella kessleri* 8k was incubated for 7 d at 25°C and 6 klux in Kessler & Czygan (1970) medium. *Escherichia coli* was incubated with shaking for 24 h at 37°C in Merck Standard I nutrient broth. Lactobacilli were grown for 48 h at 37°C in medium containing (per litre) 5 g sodium acetate, 2 g diammonium citrate, 0.2 g MgSO₄·7H₂O and 0.05 g MnSO₄·H₂O (adjusted to pH 6.2 to 6.5). Yeasts were grown with shaking for 3 d at 28°C in yeast extract peptone medium containing 1% (w/v) Difco yeast extract, 2% (w/v) Difco Bacto-peptone and 2% (w/v) glucose. *Nectria coccinea* Pers. ex Fr. was incubated at 25°C for 5 d in medium containing (per litre) 30 g Merck malt extract and 2 g Difco yeast extract. Due to the filamentous growth of these organisms, the cell suspension was filtered through a polyamide sieve with a mesh size of 65 µm (Hutter *et al.*, 1978a); the filtered suspension consisted of spores, microconidia and macroconidia. *Euglena gracilis* was incubated for 8 d at 20°C in daylight. The medium, prepared in 100 ml Erlenmeyer flasks, consisted of garden soil (1 cm deep), 0.5 g hard cheese and 50 ml tap water; it was shaken continuously and heated in a boiling water bath for 1 h. After cooling, flasks were inoculated with 5 ml from an old *E. gracilis* culture (Streble & Krauter, 1974).

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Table 1. *Micro-organisms used*

Organism	Source
<i>Chlorella kessleri</i> 8k	Prof. Dr Kessler, Botanisches Institut, Universität Erlangen, F.R.G.
<i>Escherichia coli</i>	Prof. Dr Emeis, Institut für Angewandte Biologie, RWTH Aachen, F.R.G.
<i>Saccharomyces pastorianus</i>	
<i>Schizosaccharomyces pombe</i>	
<i>Candida utilis</i>	
<i>Saccharomyces cerevisiae</i> (Baker's yeast)	Monheimer Presshefe- und Spritwerke GmbH, 4019 Monheim, F.R.G.
<i>Nectria coccinea</i> Pers. ex Fr.	Dr Klein, Forstbotanisches Institut, München, F.R.G.
<i>Saccharomyces cerevisiae</i> (211, 300, 301, 312, 400, 401, 415, 500)	Prof. Dr Pohlitz, Institut für Strahlenbiologie, Frankfurt, F.R.G.
<i>Euglena gracilis</i>	Prof. Mestre, Laboratoire de Biologie Cellulaire, Université de Paris-Sud, France
<i>Lactobacillus casei</i> 20021	German Collection of Microorganisms, GSF, München, F.R.G.
<i>Lactobacillus brevis</i> 20054	

Fluorometry of microbial cells. Cells fixed in 70% (v/v) ethanol were washed and resuspended in 1 ml 0.2 M-Tris buffer; 0.5 ml portions of these suspensions were used for DNA or protein staining.

Protein staining. Cells were treated overnight in fluorescein isothiocyanate (FITC) solution (0.03 mg ml⁻¹ in 0.2 M-Tris buffer, pH 7.5). The staining solution was removed by centrifugation at 3000 g and the cells were washed three times in 0.2 M-Tris buffer. Immediately before flow cytometry, the cells were resuspended in fresh Tris buffer (Hutter & Eipel, 1978b).

DNA staining. Cells were treated in 5 ml RNAase solution (Serva; 0.1% in 0.2 M-Tris buffer, pH 7.5) for 1 h at 37°C and then for 5 min at 20°C in 2 ml pepsin solution (Serva; 0.5% in 0.2 M-Tris buffer, pH 7.5). They were stained for 15 min in 5 ml propidium iodide solution (Calbiochem; 0.05 mg ml⁻¹ in 0.2 M-Tris buffer, pH 7.5). The staining solution was removed by centrifugation and the cells were resuspended in 2 ml 0.2 M-Tris buffer.

Apparatus. The flow cytometer used was a Cytofluorograph FC-200-50 (Ortho Instruments, Westwood, Mass., U.S.A.), equipped with a 50 mW argon laser. The excitation wavelengths were 514.5 nm for propidium iodide (DNA staining) and 488 nm for FITC (protein staining). A dichroic mirror with a dividing edge at 570 nm was inserted into the fluorescence analysis path to separate red and green fluorescence. For propidium iodide measurements, a 590 nm barrier filter was used in front of the red channel photomultiplier. A special interference filter with a band pass from 515 to 545 nm was used for FITC. Forward light scatter was measured at angles between 1.5° and 19° to the illuminating beam. Two light scatter sensors were mounted on each side of the beam. The light scatter correlated approximately with cell volume.

RESULTS AND DISCUSSION

Since Strugger's (1949) pioneering work, fluorescence microscopy has become an important tool for microbiological analysis. The information that could be gleaned from measurements with fluorochromed organisms was, however, poor due to the lack of instruments for quantitative determinations. This limitation is overcome by quantitative cytophotometry which avoids time-consuming single cell measurements by using rapid flow-through systems allowing the analysis of more than 1000 cells s⁻¹. The time needed to measure the fluorescence of single cell components depends on their specific 'stainability', but is of the order of 5 µs (Kamentsky *et al.*, 1965; Dittrich & Göhde, 1969; Kamentsky, 1970; Göhde, 1972).

Flow cytometric measurements can only be correctly interpreted where microbial populations consist of single cells. Micro-organisms which grow in chains, pairs, clusters, tetrads or in filamentous forms or clumps, and those which produce mycelium or pseudo-mycelium, cannot be measured without pre-treatment. For this reason, *Nectria coccinea* suspensions were filtered before use (see Methods).

The DNA histograms of *Lactobacillus brevis* (Fig. 1c), *Lactobacillus casei* (Fig. 1d), *Escherichia coli* (Fig. 1f), *Chlorella kessleri* 8k (Fig. 1g) and *Nectria coccinea* Pers. ex Fr.

Fig. 1. Flow cytometric measurements of the DNA content of (a) *Schizosaccharomyces pombe*, (b) *Candida utilis*, (c) *Lactobacillus brevis* 20054, (d) *Lactobacillus casei* 20021, (e) *Euglena gracilis*, (f) *Escherichia coli*, (g) *Chlorella kessleri* 8k, (h) *Nectria coccinea* Pers. ex Fr., (i) *Saccharomyces cerevisiae* and (j) baker's yeast.

(Fig. 1h) each showed a single peak whereas the histograms of *Schizosaccharomyces pombe* (Fig. 1a), *Candida utilis* (Fig. 1b), *Euglena gracilis* (Fig. 1e), *Saccharomyces cerevisiae* (Fig. 1i) and baker's yeast (Fig. 1j) consisted of two or more peaks, representing two-, three- or four-fold higher DNA contents. Alternatively, aggregated cells may have been responsible for the extra peaks or, in the case of yeasts, the initiation of a new round of bud formation may have been measured. Figure 1(i) shows a semi-continuous culture of proliferating yeast cells, most of which are in the G2 phase. The end of this period is marked by cell

Fig. 2. Protein distributions of (a) *Escherichia coli*, (b) *Chlorella kessleri* 8k, (c) *Saccharomyces pastorianus* and (d) baker's yeast.

membrane separation (Hutter *et al.*, 1978*b*). The DNA distributions of the first two peaks in Fig. 1(a, b, e, i and j) are consistent with the separation of the cell cycle into G1, S, G2 and M phases (Howard & Pelc, 1953). DNA replication occurs in the S phase, mitosis in the M phase, while the G1 and G2 phases are 'gap' periods. Discontinuous DNA synthesis was not observed with the bacterial, algal or mould cells. Due to the low signal to noise ratio, the fluorescence light signals for *Lactobacillus brevis* and *Lactobacillus casei* were too faint to permit interpretation.

Euglena gracilis gave the most significant results. The first peak was situated by channel 111 and had a coefficient of variation of 3.23% [determined, assuming a Gaussian distribution for the peak, from $(FWHM/PCH) \times 0.425$, where FWHM is the full peak width at half maximum (in channels) and PCH is the position of the peak (channel number)]. The second peak was situated by channel 222 and the third by channel 334. This type of fluorescence distribution has not previously been used to measure microbial DNA.

The protein contents of the different organisms increase and protein is synthesized during the cell cycle (Hutter & Eipel, 1978*b*). Protein content distributions in *Escherichia coli*, *Chlorella kessleri* 8k, *Saccharomyces pastorianus* and baker's yeast are shown in Fig. 2. The yeast cells used in these experiments were actively growing (see Methods). Biochemical studies have indicated that cellular components may be synthesized either discontinuously or continuously (Williamson & Scopes, 1961; Williamson, 1965; Duffus, 1971; Halvorson *et al.*, 1971; Mitchison, 1971; Hartwell, 1974; Smith & Berry, 1974). The protein distributions in *Saccharomyces* yeasts (Fig. 2c, d) did not show the separate peaks of the corresponding DNA distributions; thus, while DNA synthesis is discontinuous in these yeasts, protein is synthesized continuously throughout the cell cycle.

A number of methods have been developed to determine the ploidy of yeast cells, using cytological, biochemical and genetic criteria (Emeis, 1962; Esser & Kuenen, 1965; Laskowski *et al.*, 1960; Laskowski, 1962; Hutter, 1975). Some of these methods were difficult and time-consuming. Using flow cytometry, we have developed a method of ploidy determination which is both rapid and precise. Since DNA plays an important role in the building

Fig. 3. DNA distributions of *Saccharomyces cerevisiae* strains with different ploidy: diploid (211), triploid (300, 301, 312), tetraploid (400, 401, 415) and pentaploid (500).

up of chromosomes, the DNA content of the nucleus must increase in proportion to ploidy. In previous biochemical methods, ploidy was determined by measuring the DNA content of the cell mass and then counting the number of individual cell nuclei. Using flow cytometry, the DNA content of individual cells is measured and used to determine ploidy.

The DNA distributions in exponentially growing populations of eight *Saccharomyces cerevisiae* strains with different ploidies were examined (Fig. 3). Clear shifts in the frequency distribution were observed for different strains, for example Fig. 3(a, b). Consistent with its ploidy, the tetraplont 401 (Fig. 3f) had more DNA than the diplont and triplonts (Fig. 3a to d). However, the supposed tetraplonts 400 and 415 (Fig. 3e, g), as well as the pentaplont 500 (Fig. 3h), had different ploidy levels. This could only be explained by a modification

of the chromosomes in the course of establishment of the yeast genus, leading to aneuploidy or even polyploidy. The presumed tetraplonts 400 and 415 seemed to be more like diplonts, while the presumed pentaplont 500 was more like a tetraplont. While the DNA distributions of the diplont and triplonts consisted of only two peaks, the tetraplonts and the pentaplont showed additional small peaks in their DNA histograms which were not consistent with the results of Howard & Pelc (1953). The first small peaks of these histograms are probably caused by damaged or degenerated cells with a weak fluorescence intensity. We conclude that flow cytometry can be used to determine the degree of ploidy of yeasts.

Our results show that flow cytometry can be of value in determining cellular components of different micro-organisms. Its main advantages are the speed at which measurements can be made, its sensitivity to more than 10^{-14} g DNA per cell (Kornberg, 1974; Laskowski *et al.*, 1960) and the possibility of screening large populations.

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