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The use of 5-cyano-2,3-ditolyl tetrazolium chloride and flow cytometry for the visualisation of respiratory activity in individual cells of *Micrococcus luteus*

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Summary

5-cyano-2,3-ditolyl tetrazolium chloride is a redox dye which may be reduced to a fluorescent formazan derivative. We describe its use, together with flow cytometry, for the visualisation of respiratory activity in individual cells of *Micrococcus luteus*, and in combination with exogenous NADH for the distinction in a frozen/thawed population of 'injured' cells which have an impaired permeability barrier to the pyridine nucleotide.

Key words: Tetrazolium salt; Respiratory chain; Fluorescent formazan derivative; Flow cytometry

Introduction

Bacterial cultures, especially in nature and under conditions of stress (such as freezing, drying and starvation), display significant heterogeneity in terms of the percentage of viable cells, and with respect to their gross cellular metabolic activities (for review see [1,2]). An important task is therefore to find reliable and rapid methods for estimating the number of cells with different characteristics in the whole bacterial population. To this end, the application of flow cytometric methods seems very promising, since they make it possible to distinguish the properties of individual cells in the population.

Recently, using the lipophilic cation rhodamine 123 and flow cytometry, we were able to discriminate live, dead and dormant cells in a culture of *Micrococcus luteus*

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grown in a lactate-limited chemostat at a very low dilution rate. This discrimination was based on the different extent of membrane energisation exhibited by cells in the different subpopulations in the culture [3].

However, it is well known that membrane energisation in respiring mitochondrial and bacterial systems can be maintained at an apparently high level (as judged by the extent of lipophilic ion uptake) even if respiration is decreased to less than 80% of its initial level (e.g. [4,5]). Thus, it is plausible that monitoring of the respiratory activity of individual cells may be much more sensitive to their metabolic state than is the apparent extent of membrane energization. 5-cyano-2,3-ditoly tetrazolium chloride (CTC) is a recently developed tetrazolium salt [6] which has the important (and presently unique) characteristic that it may be converted by the respiratory chain to an insoluble but fluorescent formazan. It has been applied to the characterization of animal cells both histochemically [6,7] and by flow cytometry [8,9]. Very recently, Rodriguez et al. [10] used CTC for staining *Pseudomonas putida* and for the microscopic assay of active planktonic and immobilised bacteria in environmental samples. In the present work we have developed the use of CTC and flow cytometry for the characterization of the respiratory activity of bacterial cultures in both normal conditions and after freezing.

Materials and Methods

Organism and media

Micrococcus luteus, Fleming strain 2665, was maintained on Nutrient Agar (Difco). *Escherichia coli* K12 strain C6 from the laboratory collection was also used [11]. Bacteria were grown aerobically at 30°C in shake flasks in a rich medium consisting of 1.3% (w/v) Nutrient Broth E (Lab M).

CTC reduction

Flow cytometric experiments were carried out essentially as described by Davey et al. [11] and Kaprelyants and Kell [3] using a Skatron Argus 100 instrument (Skatron Ltd, PO Box 34, Newmarket, Suffolk) with a sample flow rate of 0.5–1 $\mu\text{l}/\text{min}$ and a sheath fluid pressure of 1.5 kPa/cm^2 . This instrument [12,13] contains low-angle ($<15^\circ$) and large-angle light-scattering detectors, plus a variety of fluorescence detectors selectable using appropriate filters. In the present experiments, we illustrate data obtained solely from low-angle light-scattering (LS1) and a fluorescence detector with the following optical characteristic: excitation 530–550 nm, band stop 560 nm, emission 580 nm and beyond. Cells (ca. $10^8 \cdot \text{ml}^{-1}$) were normally incubated in 50 mM potassium phosphate, pH 7.4 with 4 mM CTC for various times described in the legends to the figures at room temperature prior to flow cytometric analysis. Measurements were carried out according to the manufacturer's instructions, except that an additional bacteriological filter (Anotop 25, pore size 0.1 μm ; Anotec Separations Ltd, Banbury, Oxon) was placed in the sheath-fluid line. The instrument was run under the control of a Viglen (London) IIHDE microcomputer (IBM-PC-AT-compatible, 80286 processor, EGA screen), with software supplied by the manufacturer. The photomultiplier voltages were normally set at 400 V and 650 V for the light-scattering and fluorescence channels, respectively, and all measurements

were gated by the light-scattering channel. Cells were carefully dispersed before measurement by repeated passage through a 0.4 mm needle to avoid clumping artefacts.

Cell viability measurements

Standard pour plates consisting of 1.3% Nutrient Broth E (Lab M) solidified with 1.5% purified agar (Lab M) were used. Cell dilutions were made at room temperature in quadruplicate using the lactate minimal medium described above. Cells were carefully dispersed by repeated passage through a 0.4 mm needle to avoid clumping artefacts. Plates were incubated at 30°C for 3 days and counted manually.

Total cell counts

Unstained cells were counted using a phase-contrast microscope and an improved Neubauer counting chamber.

Freezing conditions

Late-exponential phase cells were kept in culture broth, stored in 1 ml aliquots at minus 20°C overnight, and thawed at room temperature prior to analysis.

Chemicals and biochemicals

Chemicals used were of analytical grade and were obtained from Sigma or BDH, save that CTC was obtained from Polysciences, Eppelheim, Germany.

Results and Discussion

The first task was to establish the best conditions with which to study the staining of cells with CTC using flow cytometry and the appropriate filter block (see Materials and Methods). Fig 1 demonstrates the time dependence of CTC-formazan accumulation by endogenously respiring *M. luteus* cells as judged by the distribution of

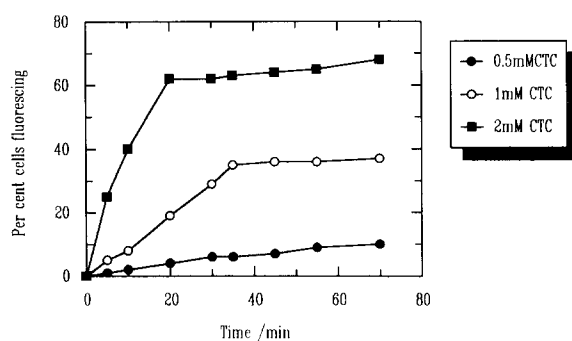


Fig. 1. Time dependence of the appearance of fluorescent cells during the reduction of CTC by a suspension of *M. luteus*. Cells were taken from late-logarithmic-phase culture and diluted 10-fold in 50 mM phosphate buffer. The experiment was carried out as described in Materials and Methods. The percentage of cells fluorescing are scored as the percentage of cells whose fluorescence was sufficient to cause them to appear in a flow cytometric channel number above 20.

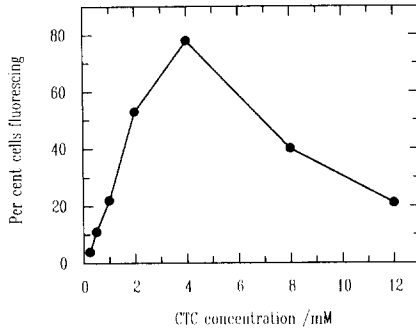


Fig. 2. Effect of CTC concentration on its reduction to a fluorescent formazan derivative by cells of *M. luteus*. The experiment was performed as described in the legend to Fig. 1, and scored in the same way, save that cells were incubated for 50 minutes.

fluorescent cells in the flow cytometer. Evidently 40–45 min of incubation of the cells with CTC is sufficient to reach the maximum accumulation of formazan in the cells. The addition of the respiratory chain inhibitor 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) had little effect on the kinetics of CTC reduction, suggesting that CTC interacts rather directly with the respiratory chain dehydrogenases (and see [9]). The optimal initial concentration of CTC was 4–5 mM (Fig. 2) close to that found in the case of *P. putida* [10]. The presence of a maximum on the curve of Fig. 2 may be explained in terms of an inhibition of respiration by CTC (or formazan) at high concentrations, as was also suggested in [10]. Although this point was not studied in detail, the kinetics of CTC reduction (Fig. 1) suggest that formazan is the toxic moiety. Fig. 3 demonstrates typical flow cytometric behavior of freshly harvested *M. luteus* cells stained with CTC under optimal conditions. In these experiments, the PMT voltage for fluorescence (650 V) was chosen such that control (unstained) cells

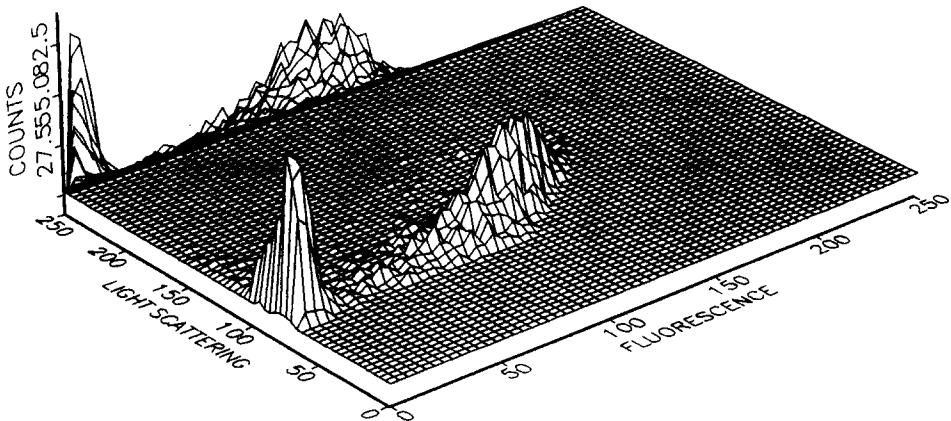


Fig. 3. Flow cytometric light-scattering and fluorimetric behaviour of a late-logarithmic-phase culture of *M. luteus* diluted 10-fold in 50 mM phosphate buffer and incubated with 4 mM CTC for 50 min. The experiment was otherwise carried out as described in the legend to Fig. 1.

had no fluorescence above channel 20. It is clear that even a freshly prepared late-logarithmic cell culture is significantly heterogeneous with respect to its ability to reduce CTC, and 20% of the cells revealed close to zero respiratory activity (staining with rhodamine 123 showed that these were not non-cellular debris; data not shown). Similar results were obtained for the Gram-negative bacterium *E. coli* (data not shown); it is particularly noteworthy that in contrast to the situation with rhodamine 123 [3], no EDTA pretreatment of the cells was necessary to effect uptake of the CTC. In other studies we also found that the percentage of cells fluorescing above channel number 20 when stained in this way was well correlated with the appearance of living cells (as judged by plating) after resuscitation of starved population of *M. luteus* [Kaprelyants and Kell, in preparation].

The ability to monitor the respiratory activity of individual cells thus gives us the

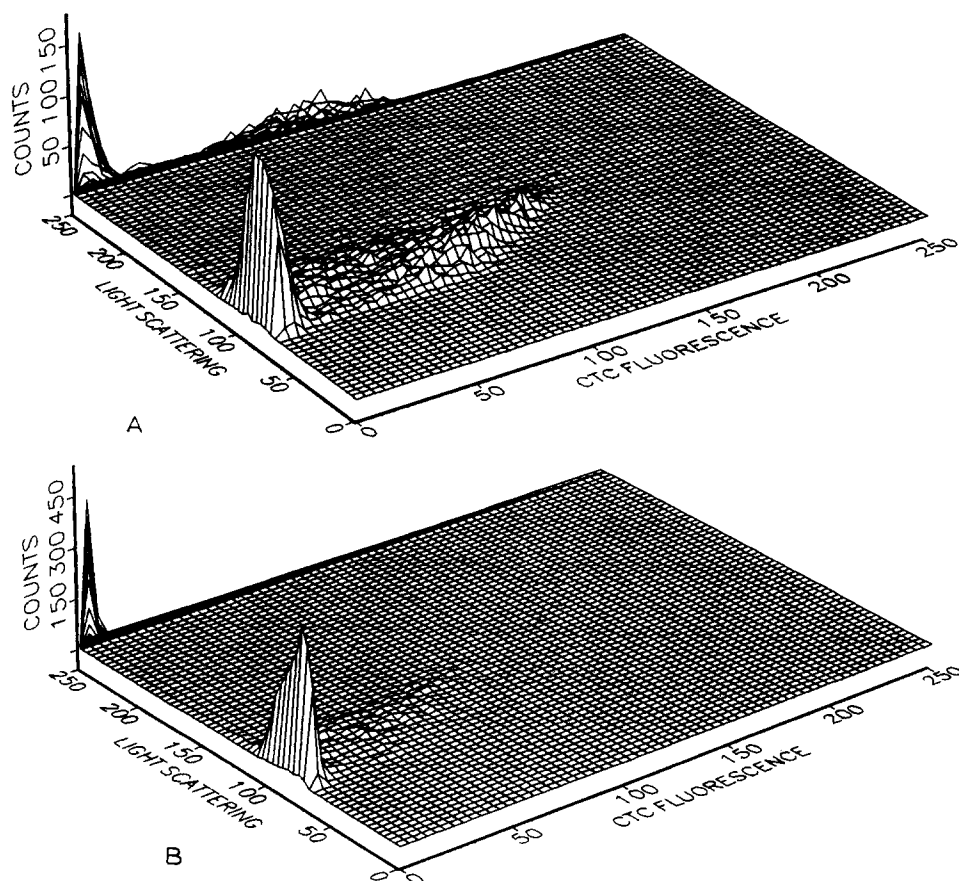


Fig. 4. Flow cytometric light-scattering and fluorimetric behaviour of a sample of *M. luteus* that had been frozen and thawed. Cells were prepared as described in Materials and Methods, diluted 10-fold in 50 mM phosphate buffer and incubated with 4 mM CTC for 5 min (A) with or (B) without 1 mM NADH. The experiment was otherwise carried out as described in the legend to Fig. 1.

possibility of designing experiments for the quantitative determination of injured cells in a population deliberately subjected to freezing. It is well known that some bacteria in stressed populations become injured, as reflected in their elevated sensitivity to surface-active agents [14]. This effect has been used for enumerating injured bacteria by plating them on selective media consisting of detergents [14]. However, this approach can reflect only injuries connected with damage to the outer portion of the cell envelope of Gram-negative bacteria [14,15], whilst it is damage to the cytoplasmic membrane that is most important in determining the further viability of bacteria after freezing [15]. Thus, it has been shown that an increase in the permeability of the cytoplasmic membrane to NADH after freezing (which in contrast to normal cells resulted in the stimulation of endogenous respiration by NADH), was well correlated with a decrease in the viability of *E. coli* [15]. The flow cytometric behaviour of frozen/thawed *M. luteus* cells after the addition of CTC and in the presence or absence of exogenous NADH is shown in Figs. 4 and 5. After the

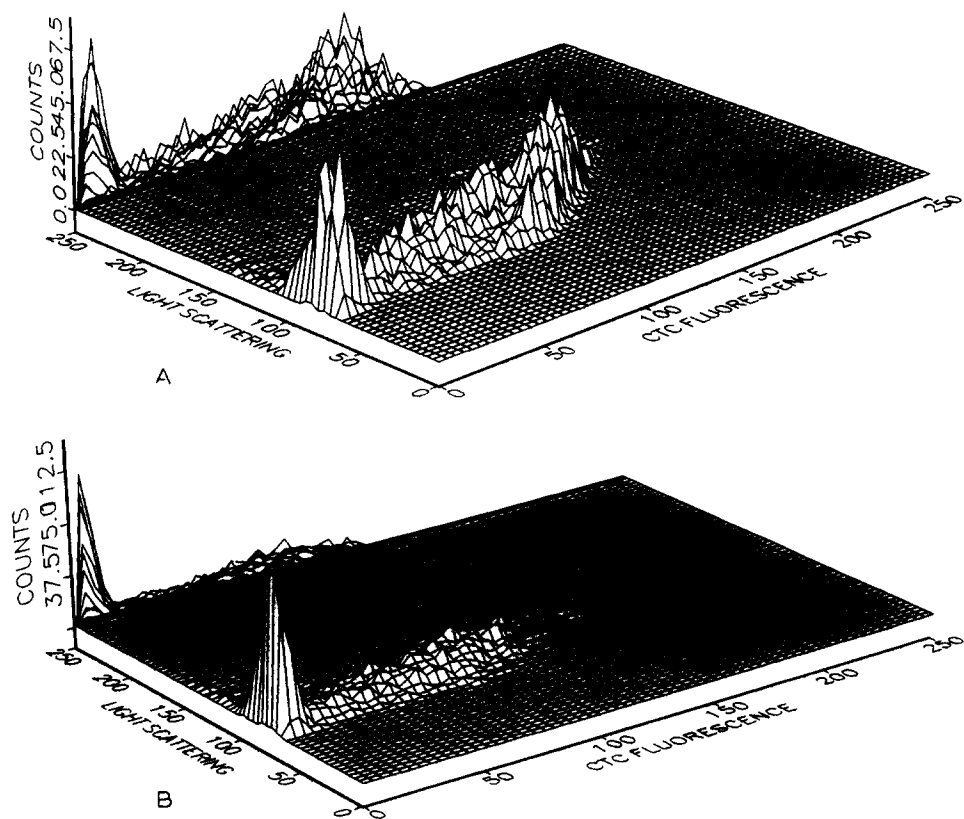


Fig. 5. Flow cytometric light-scattering and fluorimetric behaviour of a sample of *M. luteus* that had been frozen and thawed. Cells were prepared as described in the Materials and Methods section, diluted 10-fold in 50 mM phosphate buffer and incubated with 4 mM CTC for 17 min (A) with or (B) without 1 mM NADH. The experiment was otherwise carried out as described in the legend to Fig. 4.

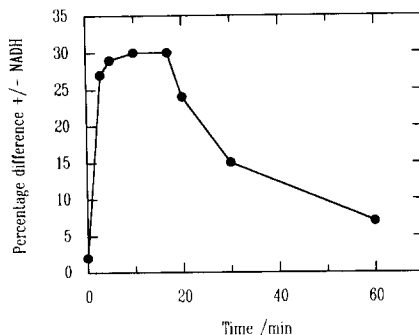


Fig. 6. Flow cytometric fluorescence behaviour of a sample of *M. luteus* that had been frozen and thawed. Cells were prepared as described in Materials and Methods, diluted 10-fold in 50 mM phosphate buffer and incubated with 4 mM CTC for the times indicated, either with or without 1 mM NADH. The experiment was otherwise carried out as described in the legend to Fig. 4. The ordinate gives the difference between the samples with or without added NADH in the percentage of cells whose fluorescence was in a channel number greater than 20.

first 5 min of incubation in the presence of CTC (Fig. 4) approximately 25% of the population gave a significant fluorescence when NADH was also present (Fig. 4a) but only 1% when it was not (Fig. 4b). After 17 min of incubation with CTC (Fig. 5), the percentage of cells fluorescing above a channel number of 20 had increased in both cases (\pm NADH), whilst their difference had not. Further incubation of the cells resulted in a decreasing difference in the distribution pattern for the two types of sample, and after 60 min incubation both distributions were similar to that of Fig. 3 (intact cells). The kinetics of CTC reduction in the two samples are summarised in Fig. 6. It is suggested that the fast reduction of CTC in the first few minutes of incubation in the samples with NADH reflects the existence in the population of cells with an injured permeability barrier but with an intact respiratory chain [15,16]. These cells very rapidly reduce CTC to formazan, to a concentration comparable with that in intact cells (as judged by the channel number of the fluorescence), whilst some endogenous substrates left in the cells after freezing and thawing permit a slower reduction of CTC in the samples without NADH. We can conclude that at least 25–30% of the cells in this frozen population of *M. luteus* are injured (although the final viability of this sample, as judged by plating on a rich medium which permitted repair processes to take place [16,17], was 90–95%). CTC seems a promising new weapon in the armoury of the microbial flow cytometrist.

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