PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

The Rapid Analysis of Single Marine Cells by Flow Cytometry [and Discussion]

P. H. Burkill, R. F. C. Mantoura and M. Cresser

Phil. Trans. R. Soc. Lond. A 1990 **333**, 99-112
doi: 10.1098/rsta.1990.0141

The rapid analysis of single marine cells by flow cytometry

By P. H. Burkill and R. F. C. Mantoura

Plymouth Marine Laboratory, Prospect Place, Plymouth PL1 3DH, U.K.

Analytical flow cytometry (AFC) is a novel technique for the rapid (more than 10^3 s^{-1}) analysis and sorting of single cells based upon simultaneous, multiple measurements of laser-induced particle fluorescence, light scatter and impedance. Originally developed for biomedical use, AFC is now being adapted to analyse single-celled organisms such as phytoplankton and bacteria which are present as trace but functionally important components in seawater. Marine AFC has been used to analytically differentiate and sort these organisms from the heterogeneous assemblage of particles present in seawater. Chlorophyll autofluorescence is an unique biomarker for photosynthetic organisms and has been used to analyse phytoplankton cytometrically both in the laboratory and at sea. A theoretical and practical framework for the cytometric quantitation of cellular chlorophyll in phytoplankton based on autofluorescence is presented. Other subcellular constituents such as enzymes, lipids, nucleic acids and toxins in phytoplankton have recently been analysed by AFC using immuno-, induced or applied fluorescent labelling techniques. Examples are presented together with novel developments in fringe areas of cytometry that are likely to influence AFC of single marine cells in the near future.

1. Introduction

The most striking and distinctive feature of our planet, when viewed from space, is the amount of water on its surface. Most of this water is contained within the oceans which collectively cover 3.61×10^8 km^2, or 71%, of the Earth's surface. The clear blue water of the oceans may appear lifeless from space, but floating within them, at all depths from the surface to the deep ocean floor are complex communities of tiny plants and animals on whose activities the life of all larger oceanic animals depend: these plants and animals form the plankton, a community of organisms which drift with the movements of water.

The ecosystems of the open ocean are fuelled by the energy fixed from sunlight by the phytoplankton, tiny single-celled plants, which are fed upon by a host of herbivorous zooplankton that in turn form the food of fish. The wastes and other excreted matter from phytoplankton and zooplankton are utilized by bacteria that remineralize organic material thereby replenishing nutrients required for phytoplankton growth.

In addition to sustaining oceanic life, phytoplankton play an important role in the global carbon cycle, through taking up inorganic carbon in surface waters. A proportion of the organic carbon formed by photosynthesis eventually sinks to the seabed, thereby removing carbon from the upper waters. This mechanism creates a

Phil. Trans. R. Soc. Lond. A (1990) **333**, 99–112
Printed in Great Britain

deficit in the surface water carbon content which is replaced by carbon dioxide from the atmosphere (Lewis 1989). Understanding the magnitude of this process is critical to further understanding of the global carbon cycle and perhaps ameliorating the 'greenhouse effect'.

In spite of the fundamental importance of phytoplankton to marine food chains and to global biogeochemical cycles, these organisms form a trace constituent of seawater. Since phytoplankton depend on light to sustain their growth, their concentration is highest in surface waters. The average phytoplankton content in the top 100 m of the open ocean can be estimated, based upon data in Whittaker & Likens (1975), to be around 300 mg wet mass per 1000 kg of seawater or 0.00003% w/w. The analysis of phytoplankton in seawater clearly requires sensitive analytical techniques. One such technique is analytical flow cytometry (AFC) which is now beginning to be used to analyse phytoplankton in seawater and to determine some of their subcellular constituents.

2. Analytical flow cytometry

AFC is a novel biomedical technique that is being increasingly applied in many other biological fields due to its quantitative capability, versatility, sensitivity, speed, statistical precision, and ability to identify cell subsets in, and sort them from, heterogeneous populations. Quantitation in AFC relies upon fluorescence and light scatter measurements from individual cells, and the power of the technique derives from the ability to make these multiple measurements of each cell simultaneously, at very rapid rates. Typically, up to 5000 cells can be analysed per second and sorting rates of 3000 per second with greater than 90% purity can be achieved (Watson 1987). As the technique has been reviewed recently (Van Dilla *et al.* 1985; Shapiro 1988), only an outline is given here.

The principles of AFC, as illustrated in figure 1, depend upon an hydrodynamically focused, single-cell suspension streamed coaxially through a flow chamber so that individual cells pass singly through the focus of a high intensity excitation light source. Coherent wave lasers are frequently used because their light beam is very stable and easily focused to small volumes, and the high light flux excites sufficient fluorescence from individual cells to be measured in the few microseconds taken to traverse the light beam. Each flash of scattered light emitted from a cell, or fluorescence from autofluorescent cells or those containing suitably stained molecules, is picked up by a light collection system and spectrally filtered in sequence by dichroic mirrors. These reflect specific wavelengths into photomultiplier tubes (PMT) which are optically screened by band-pass filters. The quantity of light incident upon each PMT, responding within its given colour band, is then proportionally converted to an electrical signal. This is amplified, digitized, stored transiently in computer memory and thence stored on disk for subsequent display and analysis.

Commercial cytometers are usually equipped with two light-scatter detectors (narrow forward and orthogonal angle) as well as two or three fluorescence PMTs. Although a single laser is normal, many instruments can be equipped with two or more lasers to correspondingly increase the number of fluorochrome excitation wavelengths. Less complex cytometers may use arc-lamp excitation. Flow chambers may vary in their hydrodynamic, optical, mechanical and electrical characteristics to achieve high sensitivity and good stream stability. Flow sorting instruments use 'jet-in-air' chambers, while highest sensitivity is achieved with quartz cuvette

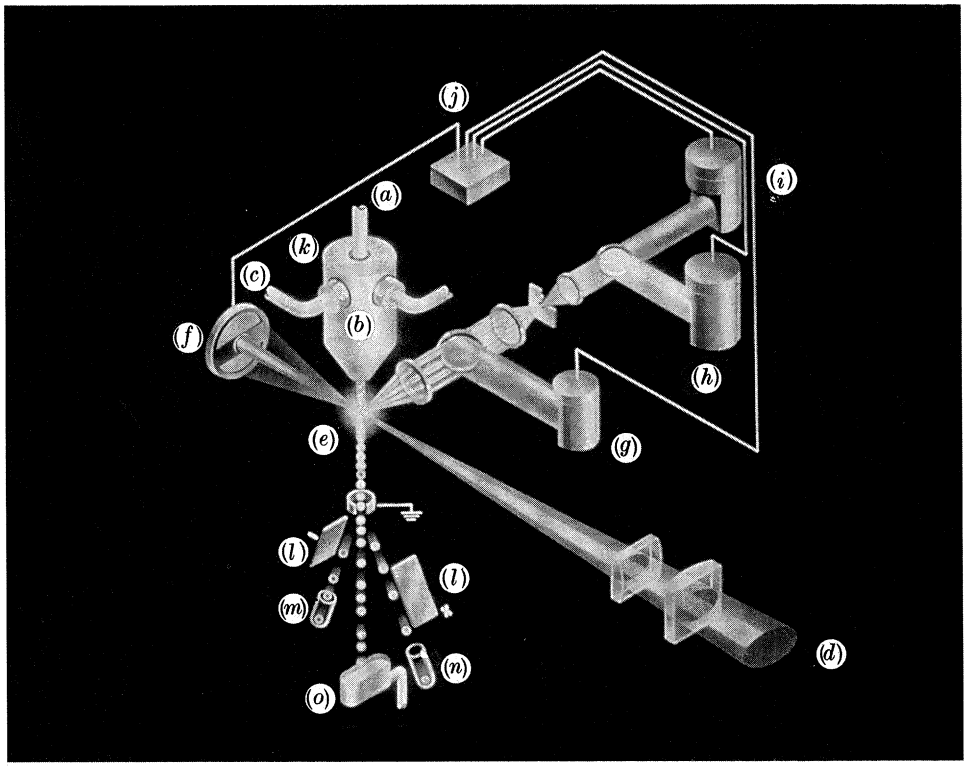


Figure 1. A schematic diagram of single cell analysis and sorting by AFC. Cells are introduced (*a*) to a flow chamber (*b*) where they are hydrodynamically focused by sheath fluid (*c*). Monochromatic light from an argon-ion laser (*d*) irradiates each particle as it passes singly through the beam. Particles scatter, and may fluoresce, light from the analysis point (*e*). A photodiode (*f*) quantifies the forward light scatter to measure particle size, while photomultiplier tubes (*g*, *h*, *i*) pick up the 90 light scatter for cellular refractive index, and two-colour, wavelength-selective fluorescence to measure biochemical properties of the particle. Signals from these sensors are amplified, digitized and stored transiently in computer memory before stored on disk for subsequent analysis (*j*). The sample stream is vibrated by a piezoelectric transducer (*k*), producing uniform liquid droplets at 35000 s^{-1} travelling at 10 m s^{-1}. Droplets containing cells exhibiting the pre-set scatter and fluorescence characteristics are electrostatically charged at the droplet formation break-off point. As these droplets pass charged plates (*l*), they are deflected into sort containers (*m*, *n*). Particles not meeting the required sort criteria pass undeflected into waste (*o*). Particles may be analysed and sorted at up to 5000 s^{-1}, with collection purities of 98%. (From Burkill 1987*a*.)

sensing. Specialized instruments also measure cell volumes from alteration in electrical impedance as the cell flows through an orifice. Date processing and display procedures have been developed which handle up to eight-dimensional fully cross-correlated data in a single pass through the data-set, using microcomputers (Watson 1987).

Cell sorting is based upon developments in ink jet printing (Sweet 1964). Two populations may be sorted from the sample stream which undergoes oscillation, driven by a piezo-electric crystal mechanically coupled to the flow chamber. The crystal, driven at 30–40 kHz, produces uniform liquid droplets in which a few percent contain one or more cells. The 'sort logic' circuitry compares processed signals from the sensors with preset ranges. When the amplitude falls within the

preset range, an electronic time delay of a few microseconds is activated. This triggers an electrical droplet-charging pulse at the moment the cell arrives at the droplet formation break-off point. The droplet-charging pulse causes a group of droplets to be charged, and, subsequently, deflected by a static electric field into a collection vessel. Cells failing the preset sort criteria do not trigger a droplet-charging pulse, and so pass undetected into the waste collector. Sorted cells may be used for identification, manipulation or further analysis.

AFC applications are diverse and are increasing as the technology and new fluorochromes develop. Detection limits are typically equivalent to 10^3 (but may be exceptionally as low as 150) molecules of free fluorescein per cell (Watson 1987). With innovations under development this could be improved further.

Cellular fluorescence may be derived from four categories:

(1) *autofluorescence* in which the fluorescent molecule of interest occurs naturally in the cell;

(2) *immunofluorescence* due to fluorochrome coupled antibodies;

(3) *induced fluorescence* in which the ligand interacts with a cellular constituent to release the fluorophore;

(4) *applied fluorescence* in which the fluorescent ligand is applied and is accumulated within the cell.

The analysis of single marine cells will be considered under these headings in the section below.

3. Cytometric analysis of single marine cells

The first flow cytometer dedicated to marine science was installed in an U.S. oceanographic laboratory in 1983 (Yentsch *et al.* 1983). Since then 15 other marine laboratories in seven countries have acquired flow cytometers (Chisholm *et al.* 1988*a*) and cytometric applications in marine sciences is now expanding fast. Marine flow cytometry has been reviewed recently (Burkill 1987*a*), and only the more recent developments in this field together with studies particularly pertinent to the topic of trace analysis will be presented here.

Autofluorescence

As an example of the utility of autofluorescence in AFC, we discuss the quantitation of cellular chlorophyll in phytoplankton. Photosynthetic pigments particularly the chlorophylls and carotenoids are perhaps the most frequently measured variables in biological oceanography. Pigment distributions in oceanic particles provide not only a quantitative distinction of the contribution of phytoplankton to the heterogeneous microbial biomass, but also information on its photosynthetic capacity, chemotaxonomy and its control of ocean colour. Pigments and their breakdown products have also been used as quantitative source markers in trophic and geochemical fate studies of algal carbon in the marine environment.

Over the past 20 years, the analysis of chlorophyll has evolved from simple spectrophotometry (Jeffrey & Humphrey 1975) and fluorometry (Holm-Hansen *et al.* 1965) of pigment extracts to shipboard advanced chromatographic techniques using HPLC (Mantoura & Llewellyn 1983), which can separate and identify the component pigments and their detrital breakdown products.

Several workers have now demonstrated that the characteristic red autofluorescence of chlorophyll found in phytoplankton populations can also be very

sensitively detected in individual cells using AFC (Yentsch *et al.* 1983). The coexistence of other cells or particles with different or additional fluorescence signatures (e.g. orange phycobiliprotein in crytophyceae and cyanobacteria, Hoechst-stained bacterial DNA, non-fluorescent detrital particles) has also been optically resolved, enumerated and sorted from the chlorophyll containing cells (reviewed by Burkill 1987*a*).

To fully exploit the analytical potential of AFC to determine the cellular chlorophyll concentration in phytoplankton, it is necessary to overcome three fundamentally separate problems.

1. The wide dynamic range of cellular chlorophyll encountered in marine phytoplankton (from 5 to 5×10^4 fg chlorophyll-a per cell) and the resultant need to vary laser irradiation power.

2. The fact that the *in vivo* fluorescence (F) in phytoplankton originates mainly from a chlorophyll-protein complex (photosystem II, PS-II) whose fluorescence yield (F_Y) varies with its composition of accessory pigments (carotenoids, phycobiliproteins) and with the photophysiological history and status of the phytoplankton cell (Falkowski *et al.* 1986; Neal *et al.* 1989; Sosik *et al.* 1989).

3. Standardization in the absence of stable chlorophyll beads.

Our studies on the above trace analytical problem (Mantoura & Burkill 1990) are summarized here to illustrate the analytical systematics needed to quantify cellular chlorophyll in phytoplankton cells. AFC was accomplished using an EPICS 751 Analytical Flow Cytometer (Coulter Electronics) fitted with a 5 W argon laser. Laser power was varied between 5 and 1000 mW at 488 nm line selected because of the spectral match between Ar lasing lines available and the excitation spectra of algal pigments. Fluorescence emissions from phytoplankton cells and from Fluorescein isothiocyanate (FITC) labelled beads were split via a 590 nm dichroic mirror into wavebands of 560–590 nm and greater than 650 nm for the detection of phycoerythrin (PE) and chlorophyll respectively. FITC beads (Coulter Fluorospheres Types 1058, 3570 and 4160) served as inanimate model particles to investigate the effect of laser power (I) on F_Y and to standardize the AFC calibration between experiments. The chlorophyll and carotenoid contents of phytoplankton species were determined by gradient elution reverse-phase HPLC (Mantoura & Llewellyn 1983) in 90% acetone extracts of cells trapped on glass fibre filters before and after AFC. The cell biovolumes and densities were enumerated using the Coulter Multisizer (TA-II). Phytoplankton species investigated were the diatom *Phaeodactylum tricornutum* Bohlin, the green alga *Dunaliella tertiolecta*, the dinoflagellate *Amphidinium carterae* Hulbert from the Plymouth Collection, and the cyanobacteria *Synechococcus sp* clone DC-2 and *Agmenellum quadruplicatum* PR-6.

Figure 2*a* shows the effect of increasing laser power (I) on the intensity of red fluorescence (F) of the FITC-beads, the diatom *P. tricornutum* and the small cyanobacteria DC-2. Under low I (less than 25 mW) typical of low power AFCs and of benchtop fluorometers, fluorescence (F) is linearly related with I indicating constant fluorescence yield (F_Y). However, at high I, both beads and autofluorescing cells exhibit a pronounced nonlinear decrease in fluorescence. This is due to photosaturation effects (Shapiro 1988) associated with a finite population of excited-state chlorophyll molecules in the PS-II system of the algae (Pellegrino & Alfano 1982). Although reversible quenching effects were evident from the reanalyses of beads and cells, photodegradation of chlorophyll and carotenoid pigments was also evident from HPLC analyses of phytoplankton cell collected after AFC > 250 mW.

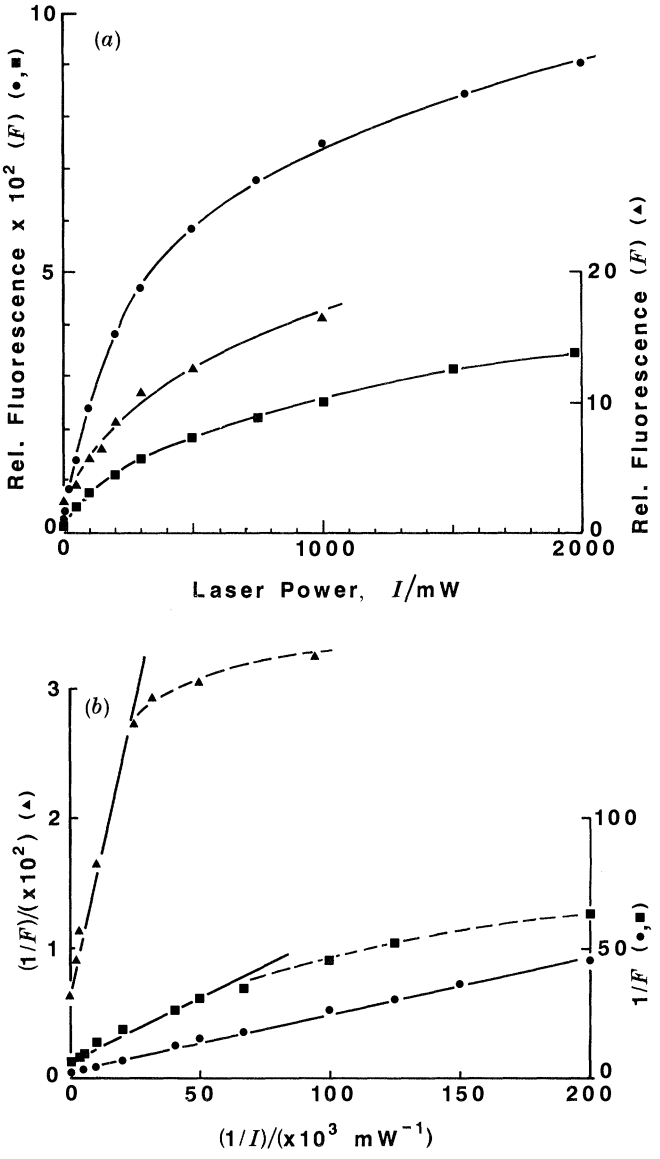


Figure 2*a*. The effect of increasing Ar laser power (I) at 488 nm on the integrated red (greater than 650 nm) fluorescence (F) if FITC labelled latex beads (●) and of chlorophyll in the diatom *Phaeodactylum tricornutum* (■) and the cyanobacterium *Synechococcus sp* DC-2. (▲). (*b*) Fluorescence photosaturation modelling of F against I from (*a*) by reciprocal plotting of $1/F$ against $1/I$.

We have developed a simple AFC fluorescence photosaturation model for phytoplankton (Mantoura & Burkill 1990) which allows the estimation of the total (analytical) cellular chlorophyll content $[A_t]$ from the F against I relationship. If $[A^*]$ is the steady-state concentration of excited state molecules of chlorophyll then neglecting any photodegradation, it can be shown that the fluorescence F is related to I by

$$F = k_f\,[A^*] = k_a\,k_f\,[A_t]\,I/(\Sigma + k_a\,I),$$

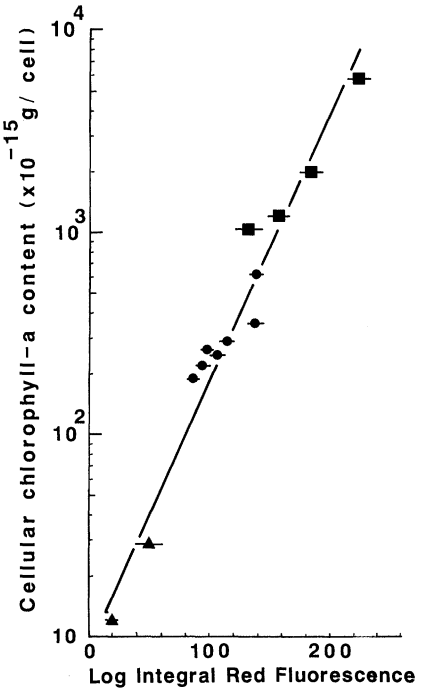


Figure 3. Plot showing the calibration of LIRFL against cellular chlorophyll-a content of sorted subsets of three species of phytoplankton (▲, *Agmanellum quadruplicatum*, a cyanobacterium; ●, *Phaeodactylum tricornutum*, a diatom; ■, *Amphidinium carterae*, a dinoflagellate). Irradiation was with the 488 nm laser line of an argon-ion laser set at 200 mW: fluorescence was measured at more than 630 nm. Chlorophyll-a was determined by high-performance liquid chromatographic analysis of a known number of sorted cells.

where k_a is the photon excitation rate of chlorophyll, k_f is the fluorescence decay rate of A*, and Σ is sum of rate constants for various radiationless transitions of A* (e.g. quenching, thermal losses, annihilation (Geacintov & Breton 1982)) back to ground state.

By rearrangement of this inverse hyperbolic equation, the above equation can be expressed as a reciprocal plot of $1/F$ against $1/I$ (figure 2 *b*) from which it is possible to estimate $[A_t]$, the cellular concentration of chlorophyll. The deviations from linearity at high values of $1/I$ in the case of the algal cells may indicate a higher light capture efficiency at low I linked with the presence of additional light harvesting pigments (e.g. fucoxanthin in diatoms and phycoerythrin in cyanobacteria).

Figure 3 shows that it is possible under fluorescence photosaturation conditions, to determine cellular chlorophyll-a concentrations over three orders of magnitude by AFC. More recently, we have extended these studies to over 26 algal isolates spanning four orders of magnitude (Mantoura & Burkill 1990).

The ideal AFC standard would be photoinert chlorophyll-conjugated beads, but since these are likely to be unstable (unpublished results), the use of 'proxy' beads which are spectrally overlapping with chlorophyll, can be used after intercalibration with HPLC or with F, provided the optical configuration of the AFC is not altered. We conclude, that for quantitative AFC of any induced or autofluorescent constitution of particles, it is always necessary to verify fluorescence linearity under low laser power and to parametrize fluorescence quenching under high laser power.

Immunofluorescence

Chlorophyll and other photosynthetic pigments are involved in light capture in photosynthesis. The so-called 'dark phase' of photosynthesis involving the Calvin cycle follows light capture and involves inorganic carbon uptake mediated by enzymes. Immunofluorescent probes have recently been used with AFC to characterize two proteins involved in photosynthesis in phytoplankton. Orellana *et al.* (1989) have reported assays for ribulose,1-5,bisphosphatecarboxylase (RuBPCase) and peridinin/chlorophyll-a binding protein (PCP) in six species of diatom and dinoflagellate phytoplankton. Since PCP is found only in autotrophic dinoflagellates where it acts as the light harvesting antenna complex, it can therefore be used to taxonomically differentiate this group from other phytoplankton taxa. RuBPCase is universally found in all phytoplankton, where it catalyses inorganic carbon fixation in photosynthesis. This enzyme comprises an octometric aggregation of eight large and eight small subunits. The large subunit of RuBPCase which is evolutionarily highly conservative, is antigenically similar in chlorophyll-a containing cells, thereby allowing it to be a marker for all phytoplankton cells (Orellana *et al.* 1988).

Induced fluorescence

Paralytic shellfish poisoning (PSP) is caused by saxitoxin, a neurotoxin produced by particular strains of dinoflagellates such as *Protogonyaulax tamarensis* under bloom conditions. Saxitoxin acts as a potent blocking agent of voltage-sensitive sodium ion channels in neuromuscular membranes. Disruption of ion passage prevents signal transmission in the neuron, resulting in paralysis and death (Guire *et al.* 1985). As the lethal dose in humans is extremely low (less than 1 mg), there is a strong need for sensitive analytical methods for evaluating PSP. A flow cytometric assay for saxitoxin has been developed based upon hydrogen peroxide oxidation to a fluorescent product in different species of *Protogonyaulax* (Yentsch 1981).

An assay for phytoplankton viability and metabolic activity based upon esterase activity has been developed by Dorsey *et al.* (1989). This assay is based upon techniques for the measurement of esterase activity in mammalian cells. These assays use fluorescein diacetate (FDA) as a fluorogenic compound which, since it is hydrophobic, readily penetrates cells. Within the cell, non-specific esterase activity hydrolyses FDA into fluorescein which is highly fluorescent. As fluorescein is hydrophilic, it does not readily leak out of the cell, thereby leaving metabolically active cells fluorescent and dead cells non-fluorescent.

Applied fluorescence

One of the major biosynthetic products of phytoplankton photosynthesis is lipid. Phytoplankton lipids, particularly non-polar lipids, may have commercial potential as a fuel fraction. Cytometric analysis of phytoplankton lipid content and cytometric sorting of live phytoplankton based upon their lipid content has been carried out using Nile Red (Cooksey *et al.* 1987; Mann & Yentsch 1990). Nile Red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one) fluoresces yellow–gold when dissolved in neutral lipids and red when in more polar lipids such as phospholipids. It can be readily excited at 488 nm, while the fluorescence of the neutral lipid bound strain is typically measured at 550 or 575 nm (Shapiro 1988; Cooksey *et al.* 1987).

Nucleic acid stains have been used extensively in the analysis of mammalian cell cycling and this has facilitated transfer of cell cycle techniques into the marine field. Fluorescence by the supravital fluorochromes, Hoechst 33342 and 4,-6-diaminido-2-phenylindole (DAPI) have been used to differentiate proportion of cells in G1, G2 + M

or S phase. Cell cycle analysis has been carried out on eukaryotic autotrophs, but has recently been investigated in marine prokaryotes (Chisholm *et al.* 1986; Robertson & Button 1989). Spermatogenesis in diatoms has also been analysed using DNA-specific fluorochromes (Vaulot & Chisholm 1987).

Although bacteria have been analysed by flow cytometry in clinical microbiology (Van Dilla *et al.* 1983) and freshwater ecology (Hutter & Eipel 1978), it is only very recently that marine bacteria have been analysed (Robertson & Button 1989). Bacteria are ecologically and biogeochemically important in marine systems where they may form around half the plankton biomass and more than 99% of its active surface area (Button & Robertson 1990). Although marine bacteria are numerous in seawater with concentrations of 10^8 to 10^9 l^{-1} in surface waters, they are extremely small. Whereas a starved *E. coli* cell may be only 0.6 μm^3, marine bacteria as small as 0.006 μm^3 have been detected cytometrically using DAPI-DNA staining techniques (Robertson & Button 1989). Even at these low (*ca.* 5 fg DNA $cell^{-1}$) levels, the cytometric analysis allowed differentiation of the bacterial cell cycle. The DNA contents of individual marine bacteria have, so far, been shown to be rather constant. It is not yet known whether the activity of individual bacteria is constant, or whether DNA is an insensitive assay of cellular activity in these organisms.

4. Analysis of single cells at sea

The most exciting frontier in marine flow cytometry involves the analysis of natural populations at sea (Chisholm *et al.* 1988 *a*). However, shipboard flow cytometry presents many analytical problems. To understand the heterogeneous composition of natural communities, discrete populations must be identified by microscopy. Once defined cytometrically, as shown in figure 4, populations are sorted by sorting cytometers which are themselves large and complex. At the Plymouth Marine laboratory, we use a 6 m container as a mobile cytometry laboratory in conjunction with a mobile, 500 kg chiller to cool the laser, for shipboard operation (Burkill 1987 *b*). Shipboard analysis therefore involves moving *ca.* 4 t of delicate cytometric equipment. As accurate laser alignment is critical for high analytical sensitivity, a complete realignment is required whenever the cytometer is moved. The ship itself is a highly mobile platform which vibrates at a range of frequencies. Minimizing vibration transmission requires careful fixing of the laser optical bench. Fortunately these problems can be overcome and several marine groups are now proving that shipboard flow cytometry is a powerful tool for analysing plankton communities at sea.

Two conceptual schemes have been used for analysing natural populations; in one of these, particles could be consecutively characterized as organic or inorganic, living or dead, autotrophic or heterotrophic and finally separated into different pigment groups (Yentsch *et al.* 1983); in the second, different phytoplankton taxa (Dinoflagellates, Cryptophytes, Rhodophytes, Coccolithophores, pennate Diatoms, Chlorophytes) have been successfully separated on the basis of Coulter volume, polarization properties of forward light scatter, orthogonal light scatter, autofluorescence emission and excitation (Olson *et al.* 1989). The focus for shipboard work has been on elucidating the vertical depth distributions of phytoplankton in different ocean regions with cytometric discrimination between cell types with different light scatter and autofluorescent properties.

Dual laser irradiation at 488 and 514 nm has been used to demonstrate that tiny

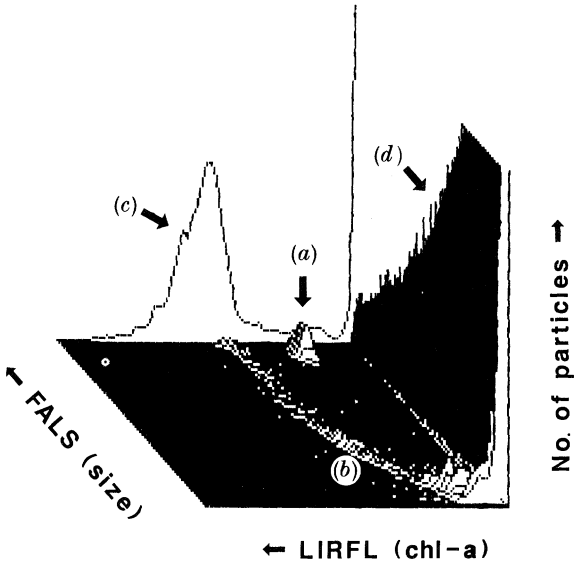


Figure 4. Orthographic plot of cellular chlorophyll content (LIRFL) and size (FALS) of phytoplankton and other particles present in summer plankton community at 41 m depth in the Celtic Sea (50° 30′ N, 07° 00′ W). Population (*a*) comprises 2.2 μm beads added as an internal cytometric standard. Other populations identified by microscopic analysis of material sorted at sea were prokaryotic and eukaryotic photosynthetic picoplankton (*b*), nanophytoplankton (*c*) and a large but cytometrically undefined population of detritus and heterotrophic cells (*d*).

0.8 μm *Synechococcus* cells are both abundant in the open ocean and contain a different pattern of pigmentation from both coastal water populations, and from most cultured strains (Olsen *et al.* 1988). This discovery may resolve an enigma in oceanography: Lewis *et al.* (1986) had previously concluded that *Synechococcus* was insignificant in the open ocean, from differences between remotely-sensed photosynthetic action spectra (PAS) of the open ocean and PAS derived from laboratory *Synechococcus* cultures. The discovery that open ocean prokaryotes have absorption properties similar to those of eukaryotic phytoplankton and different from most cultured prokaryote strains, demonstrates that we can get a distorted perspective of oceanic life from studying only those organisms that can be cultured (Olson *et al.* 1988).

A novel prokaryote group has been recently discovered by flow cytometry within the oceanic phytoplankton: the prochlorophytes (Chisholm *et al.* 1988*b*). These minute (less than 0.8 μm) phytoplankters are abundant (50000–120000 cells ml^{-1}) deep in the euphotic zone. The cells are so sensitive to prolonged high light that they lyse or fade when subjected to observation by epifluorescence microscopy. Fortunately the laser illumination period in flow cytometry is so short (10^{-5} s) that they can be detected (Legendre & Yentsch 1989).

Analysis of natural populations has been based upon fresh material because traditional phytoplankton preservation techniques has drastically affected pigment autofluorescence. A method of preservation of phytoplankton for cytometric analysis, based on sample fixation in 1% glutaraldehyde followed by storage in liquid nitrogen, has been described (Vaulot *et al.* 1989). This technique performs best for small-sized phytoplankton while some large (more than 3 μm) cells preserve poorly.

5. Future developments

Future developments in this field depend on a number of factors. These include adaptation of instrumentation to meet marine criteria, provision of relevant instrument standards and controls, application of immunofluorescent probes and appropriate computer software to analyse heterogeneous natural populations. Commercial cytometers developed for biomedicine have limitations for the analysis of marine populations which exhibit larger ranges of cell sizes, shapes and concentrations than is typically found in biomedical populations. Modifications for specific marine analysis have been addressed, for instance, increasing sampling rate (Olson 1989), increased sensitivity (Vaulot & Ning 1989). Dedicated cytometers for phytoplankton analysis are now being developed (Peeters *et al.* 1989; Cunningham 1990). As these technical modifications are taken up by the marine community, the improved instrument design will increase availability of flow cytometers and further expand the use of cytometric instrumentation in the study of single marine cells.

Cytometric analysis depends on good instrument fluorescence standards, which are often lacking in marine cytometry. For instance, quantitatively labelled chlorophyll-containing beads are not yet available, although these are under development (Flow Cytometry Standards Corp, Research Triangle, North Carolina, U.S.A.). When available, these beads will improve cytometric chlorophyll measurements.

The application of immunological tagging techniques based on mono or polyclonal antibodies will open up new fields of marine cytometry. Many marine antibodies have been developed for phytoplankton species (Shapiro 1989), cyanobacteria (Campbell 1988), toxins (Baden *et al.* 1988; Guire *et al.* 1988; Hokama *et al.* 1988); ammonium oxidizing bacteria (Ward & Carlucci 1985), nitrate reductase (Balch *et al.* 1988) and oligonucleotide probes (Binder 1990).

Much of the painstaking work on cell cycle analysis in cultured phytoplankton and bacteria described above will hopefully allow estimates of the *in situ* growth rates, and hence productivity, of natural populations (Chisholm *et al.* 1986). When this occurs, quantifying marine productivity will be greatly simplified.

Sophisticated computing techniques such as neural network analysis (Frankel *et al.* 1989) will be increasingly used to handle data management and analysis and to make the fullest use of multi-parametric data.

Marine scientists eagerly await the coupling of analytical techniques of sufficient sensitivity to quantitate carbon, nitrogen and stable isotope ratios in single cells with flow cytometric sorting of cells from heterogeneous natural populations to further understand the dynamics of single cells in the ocean.

This paper was sponsored in part by funds from the Natural Environmental Research Council to the Plymouth Marine Laboratory's Research Project 2, Individual Research Project 3 and the Community Research Project on Biogeochemical Ocean Flux Study.

References

Baden, D. G., Mende, T. J. & Szmant, A. M. 1988 Brevetoxins and binding: sodium channels versus antibodies. In *Immunochemical approaches to coastal, estuarine and oceanographic questions* (ed. C. M. Yentsch, F. C. Mague & P. K. Horan), pp. 134–144. New York: Springer-Verlag.

Balch, W. M., Yentsch, C. M., Reguera, B. & Campbell, W. 1988 Examining nitrate reduction by phytoplankton with an immunoassay. In *Immunochemical approaches to coastal, estuarine and oceanographic questions* (ed. C. M. Yentsch, F. C. Mague & P. K. Horan), pp. 263–276. New York: Springer-Verlag.

Binder, B. 1990 Oligonucleotide probes in flow cytometry. *Signal Noise* **3**, 1.

Burkill, P. H. 1987*a* Analytical flow cytometry and its application to marine microbial ecology. In *Microbes in the sea* (ed. M. A. Sleigh), pp. 139–166. Chichester: Ellis Horwood.

Burkill, P. H. 1987*b* Shipboard flow cytometry: sea-trials and analysis with sorting of photosynthetic picoplankton in the Celtic Sea. *Cytometry* S**1**, 94.

Button, D. K. & Robertson, B. R. 1990 The status and promise of flow cytometry as a tool in aquatic bacteriology. *Signal Noise* **3**, 3–4.

Campbell, L. 1988 Identification of marine chroococcoid cyanobacteria by immunofluorescence. In *Immunochemical approaches to coastal, estuarine and oceanographic questions* (ed. C. M. Yentsch, F. C. Mague & P. K. Horan), pp. 208–229. New York: Springer-Verlag.

Chisholm, S. W., Armbrust, E. V. & Olson, R. J. 1986 The individual cell in phytoplankton ecology: cell cycles and applications of flow cytometry. *Can. Bull. Fish. Aquat. Sci.* **214**, 343–369.

Chisholm, S. W., Olson, R. J. & Yentsch, C. M. 1988*a* Flow cytometry in oceanography. *Eos, Wash.* **69**, 562.

Chisholm, S. W., Olson, R. J., Zettler, E. R., Goericke, R., Waterbury, J. B. & Welschmeyer, N. A. 1988*b* A novel free-living prochlorophyte abundant in the euphotic zone. *Nature, Lond.* **334**, 340–343.

Cooksey, K. E., Guckert, J. B., Williams, S. A. & Callis, P. R. 1987 Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. *J. microbiol. Methods* **6**, 333–345.

Cunningham, A. 1990 A low-cost, portable flow cytometer specifically designed for phytoplankton analysis. *J. Plankton Res.* **12**, 140–160.

Dorsey, J., Yentsch, C. M., Mayo, S. & McKenna, C. 1989 Rapid analytical technique for the assessment of cell metabolic activity in marine microalgae. *Cytometry* **10**, 622–628.

Falkowski, P. G., Wyman, K., Ley, A. C. & Mauzarall, D. M. 1986 Relationship of steady state photosynthesis to fluorescence in eukaryotic algae. *Biochim. biophys. Acta* **849**, 183–193.

Frankel, D. S., Olson, R. J., Frankel, S. L. & Chisholm, S. W. 1989 Use of a neural net computer system for analysis of flow cytometric data of phytoplankton populations. *Cytometry* **10**, 540–550.

Geacintov, N. E. & Breton, J. 1982 Exciton annihilation and other nonlinear high intensity excitation effects. In *Biological events probed by ultrafast laser spectroscopy* (ed. R. R. Alfano), pp. 158–189. New York: Academic Press.

Guire, P. E., Duquette, P. H., Amos, R. A., Behrens, J. C., Josephson, M. W. & Chambers, R. P. 1985 Preparation of model haptens to express common epitopes of PSP toxins. In *Immunochemical approaches to coastal, estuarine and oceanographic questions* (ed. C. M. Yentsch, F. C. Mague & P. K. Horan), pp. 145–154. New York: Springer-Verlag.

Hokama, Y., Honda, S. A. A., Kobayashi, M. N., Nakagawa, L. K., Shirai, L. K. & Miyahara, J. T. 1988 Monoclonal antibodies (monabs) to ciguatoxon and related polyethers. In *Immunochemical approaches to coastal, estuarine and oceanographic questions* (ed. C. M. Yentsch, F. C. Mague & P. K. Horan), pp. 155–166. New York: Springer-Verlag.

Holm-Hansen, O., Lorenzen, C. J., Holmes, R. W. & Strickland, J. D. H. 1965 Fluorometric determination of chlorophyll. *J. Conseil* **30**, 3–15.

Hutter, K.-J. & Eipel, H. E. 1978 Flow cytometric determinations of cellular substances in algae, bacteria, moulds and yeasts. *Antonie van Leewenhoek* **44**, 269–282.

Jeffrey, S. W. & Humphrey, G. F. 1975 New spectrophotometric equations for the determination of chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanz* **167**, 191–194.

Legendre, L. & Yentsch, C. M. 1989 Overview of flow cytometry and image analysis in biological oceanography. *Cytometry* **10**, 501–510.

Lewis, M. R. 1989 The variegated ocean: a view from space. *New Scient.* **124**, 37–40.

Lewis, M. R., Warnock, R. E. & Platt, T. 1986 Photosynthetic response of marine picoplankton at low photon flux. *Can. Bull. Fish. Aquat. Sci.* **214**, 235–250.

Mann, E. & Yentsch, C. M. 1990 Neutral lipids in individual cells. *Signal Noise* **3**, 4.

Mantoura, R. F. C. & Burkill, P. H. 1990 (In preparation.)

Mantoura, R. F. C. & Llewellyn, C. A. 1983 The rapid determination of algal chlorophyll and

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

carotenoid pigments and their breakdown products in natural waters by reverse phase high performance liquid chromatography. *Anal Chim Acta*, **151**, 297–314.

Neal, P. J., Cullen, J. J. & Yentsch, C. M. 1989 Bio-optical inferences from chlorophyll-*a* fluorescence: what kind of fluorescence is measured in flow cytometry? *Limnol. Oceanogr.* **34**, 1739–1748.

Olson, R. J. 1989 Increase sampling rate on an EPICS flow cytometer. *Signal Noise* **2**, 5.

Olson, R. J., Chisholm, S. W., Zettler, E. R. & Armbrust, E. V. 1988 Analysis of *Synechococcus* pigment types in the sea using single and dual beam flow cytometry. *Deep-Sea Res.* **35**, 425–440.

Olson, R. J., Zettler, E. R. & Anderson, O. K. 1989 Discrimination of eukaryotic phytoplankton cell types from light scatter and autofluorescence properties measured by flow cytometry. *Cytometry* **10**, 636–643.

Orellana, M. V., Perry, M. J. & Watson, B. A. 1988 Probes for assessing single-cell primary production: antibodies against Ribulose-1,5-bisphosphate carboxylase (RuBPCASE) and peridinin/chlorophyll-a protein (PCP). In *Immunochemical approaches to coastal, estuarine and oceanographic questions* (ed. C. M. Yentsch, F. C. Mague & P. K. Horan), pp. 243–262. New York: Springer-Verlag.

Peeters, J. C. H., Dubelaar, G. B. J., Ringelberg, J. & Visser, J. W. M. 1989 Optical plankton analyser: a flow cytometer for plankton analysis. *Cytometry* **10**, 522–528.

Pellegrino, F. & Alfano, R. R. 1982 Time-resolved fluorescence spectroscopy. In *Biological events probed by ultrafast laser spectroscopy* (ed. R. R. Alfano), pp. 27–50. New York: Academic Press.

Robertson, B. R. & Button, D. K. 1989 Characterizing aquatic bacteria according to population, cell size and apparent DNA content by flow cytometry. *Cytometry* **10**, 70–76.

Shapiro, H. M. 1988 *Practical flow cytometry*. New York: Alan R. Liss.

Shapiro, L. 1989 Immunofluorescent characterisation of the eukaryotic ultraplankton. In *Immunochemical approaches to coastal, estuarine and oceanographic questions* (ed. C. M. Yentsch, F. C. Mague & P. K. Horan), pp. 242. New York: Springer-Verlag.

Sosik, H. M., Chisholm, S. W. & Olson, R. J. 1989 Chlorophyll fluorescence from single cells: Interpretation of flow cytometric signals. *Limnol. Oceanogr.* **34**, 1749–1761.

Sweet, R. G. 1964 High frequency oscillography with electrostatically deflected ink jets. *Tech. Rep.* 1722–1. Stanford Electron. Lab., Stanford University.

Van Dilla, M. A., Dean, P. N., Laerum, O. D. & Melamed, M. R. (eds) 1985 *Flow cytometry: instrumentation and data analysis*. London: Academic Press.

Van Dilla, M. A., Langlois, R. G., Pinkel, D., Yajko, D. & Hadley, W. K. 1983 Bacterial characterisation by flow cytometry. *Science, Wash.* 220; 620–622.

Vaulot, D. & Chisholm, S. W. 1987 Flow cytometric analysis of spermatogenesis in the diatom, *Thalassiosira weissflogii* (Bacillariophyceae). *J. Phycology* **23**, 132–137.

Vaulot, D., Courties, C. & Partenski, F. 1989 A simple method to preserve oceanic phytoplankton for flow cytometric analysis. *Cytometry* **10**, 629–635.

Vaulot, D. & Ning, X. 1989 Abundance and cellular characterization of marine *Synechococcus* spp. in the dilution zone of the Changjiang (Yangtze River, China). *Cont. Shelf Res.* **8**, 1171–1186.

Ward, B. B. & Carlucci, A. F. 1983 Marine ammonia- and nitrite oxidising bacteria: serological diversity determined by immunofluorescence in culture and in the environment. *Appl. env. Microbiol.* **50**, 194–201.

Watson, J. V. 1987 Flow cytometry in biomedical science. *Nature, Lond.* **325**, 741–742.

Whittaker, R. H. & Likens, G. E. 1975 In *Primary productivity of the biosphere* (ed. H. Leith & R. H. Whittaker), pp. 305–328. Berlin: Springer Verlag.

Yentsch, C. M. 1981 Flow cytometric analyses of cellular saxitoxin in the dinoflagellate *Gonyaulax tamarensis* var *excavata*. *Toxicon* **19**, 611–621.

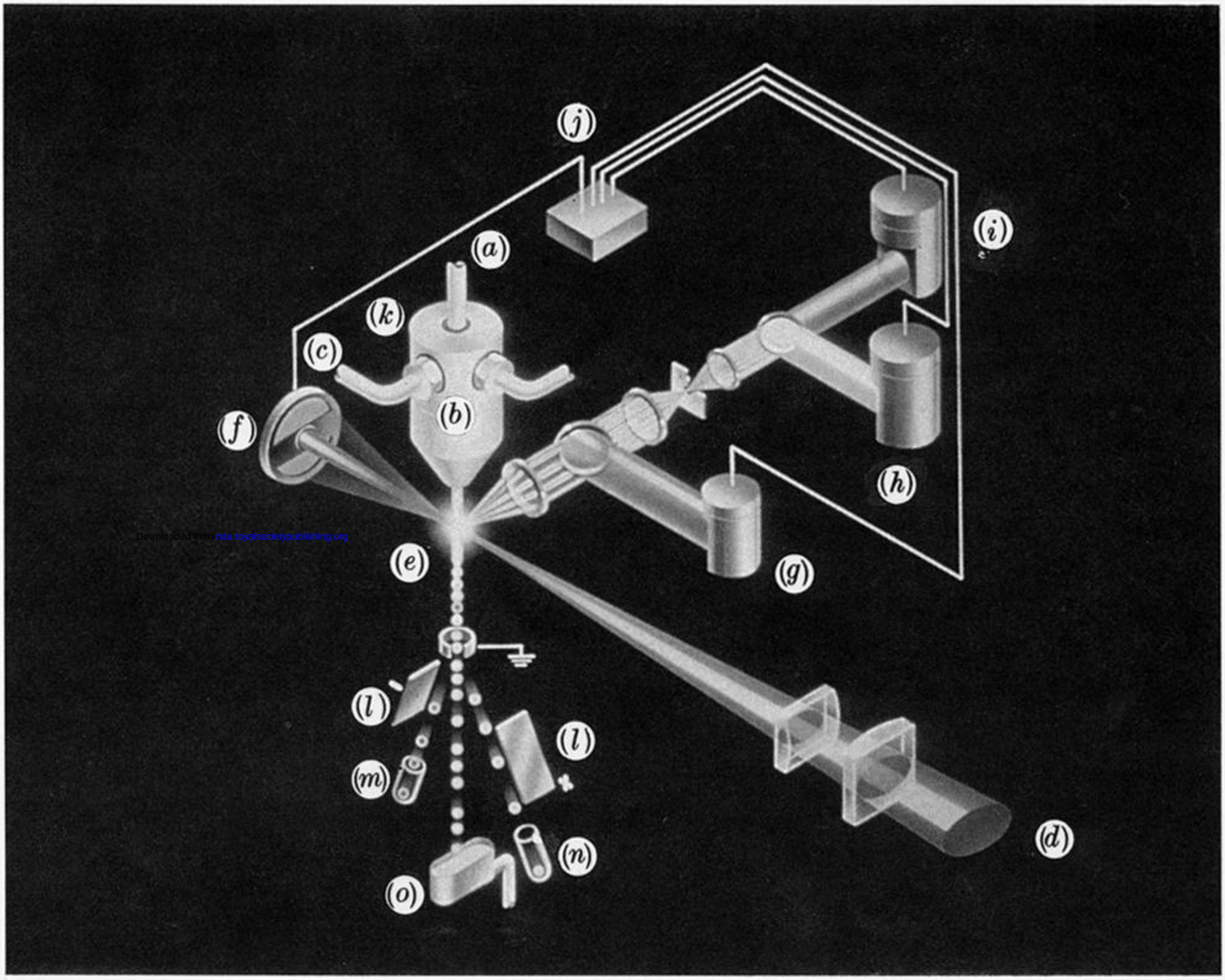
Yentsch, C. M., Horan, P. K., Muirhead, K., Dortch, Q., Haugen, E., Legendre, L., Murphy, L. S., Perry, M. J., Phinney, D. A., Pomponi, S. A., Spinrad, R. W., Wood, M., Yentsch, C. S. & Zahuranec, B. J. 1983 Flow cytometry and cell sorting: a technique for analysis and sorting of aquatic particles. *Limnol. Oceanogr.* **28**, 1275–1280.

Discussion

M. Cresser (*Aberdeen University, U.K.*). Dr Burkill mentioned briefly the possible distribution of radiant energy between fluorescence and photosynthesis when measuring chlorophyll in single-cell organisms. I have had problems with this when looking at photoacoustic spectra of pigments in intact leaves. To what extent is control of the organism environment a problem in this context?

P. H. Burkill. The photophysiological history of algae controls the proportion of the total chlorophyll in the ground state and which is therefore available for excitation by laser light (see Pellegrino & Alfano 1983). I believe Guillard and co-workers at Woods Hole Oceanographic Institute, U.S.A., attempted photoacoustic spectroscopy on filtered phytoplankton cells but obtained only limited success in determining pigment content; it was, I believe, not sensitive enough to be useful.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES



igure 1. A schematic diagram of single cell analysis and sorting by AFC. Cells are introduced (*a*) a flow chamber (*b*) where they are hydrodynamically focused by sheath fluid (*c*). Monochromatic ght from an argon-ion laser (*d*) irradiates each particle as it passes singly through the beam. articles scatter, and may fluoresce, light from the analysis point (*e*). A photodiode (*f*) quantifies ie forward light scatter to measure particle size, while photomultiplier tubes (*g*, *h*, *i*) pick up the) light scatter for cellular refractive index, and two-colour, wavelength-selective fluorescence to easure biochemical properties of the particle. Signals from these sensors are amplified, digitized id stored transiently in computer memory before stored on disk for subsequent analysis (*j*). The mple stream is vibrated by a piezoelectric transducer (*k*), producing uniform liquid droplets at 5000 s^{-1} travelling at 10 m s^{-1}. Droplets containing cells exhibiting the pre-set scatter and uorescence characteristics are electrostatically charged at the droplet formation break-off point. s these droplets pass charged plates (*l*), they are deflected into sort containers (*m*, *n*). Particles not eeting the required sort criteria pass undeflected into waste (*o*). Particles may be analysed and rted at up to 5000 s^{-1}, with collection purities of 98%. (From Burkill 1987*a*.)