11-1990

Color Image-Analyzed Fluorescence Microscopy: A New Tool For Marine Microbial Ecology

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COLOR IMAGE-ANALYZED FLUORESCENCE MICROSCOPY: A NEW TOOL FOR MARINE MICROBIAL ECOLOGY

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Epifluorescence microscopy is currently the method of choice for measuring the biomass of picoplankton and nanoplankton.

BACTERIA, cyanobacteria, protozoa and small eukaryotic phytoplankton are now known to be fundamental components of marine planktonic ecosystems (Azam *et al.*, 1983; Sieburth, 1984). These organisms have high potential growth rates, are efficient converters of material and energy, and usually constitute most of the plankton biomass (Davis *et al.*, 1985; Fuhrman *et al.*, 1989). Their different trophic levels are often closely coupled. These characteristics require that experimental and sampling designs for studying the microbial populations incorporate appropriate (i.e., small) temporal and spatial scales. The corollary to this is that large numbers of samples must be taken. If these can be processed rapidly, sampling designs can be modified to existing conditions. Quantifying the abundance and biovolume of these microbial cells is difficult because these organisms are extremely small and cell biovolume is a cubic function of linear dimensions. These difficulties are major obstacles to developing realistic trophodynamic models.

Epifluorescence microscopy is one of the major methods used to verify the recently accepted paradigm in marine ecology (Pomeroy, 1974). Fluorescence microscopy was developed in the first half of this century (Clark, 1961) but only became practical with the development of efficient optical filters and stable light sources in the 1950s and 1960s. It was first used in marine ecology by Francisco *et al.* (1973) to count bacteria in natural waters. The method has since been improved (Hobbie *et al.*, 1977) and diversified through the use of a variety of fluorochromes (Porter and Feig, 1980; Haas, 1982; Caron, 1985). These techniques can now be used to provide a variety of information about the microbial community at the level of individual cells. Although the main use of epifluorescence microscopy is to classify cell types and determine standing stocks, methods have been developed to measure bacterivory (McManus and Fuhrman, 1986; Bird and Kalff, 1986; Sieracki *et al.*, 1987) and uptake rates of dissolved organic molecules by protists (Sherr, 1988). Advances with fluorescent analogues and antibodies are leading to additional methods for characterizing cells by epifluorescence microscopy (Ward, 1990).

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Flow cytometry is a complementary technology for measuring fluorescence characteristics of individual cells. This technology was developed for biomedical use in the 1970s (Horan and Wheeless, 1977) and was first used for analysis of aquatic particles by Olson *et al.* (1983) and Yentsch *et al.* (1983). These instruments measure the fluorescence emission and light scatter of individual cells passing through a light (or laser) beam in a flow stream. The two methods are briefly compared at the end of this article.

Epifluorescence microscopy is currently the method of choice for measuring the biomass of picoplankton (0.2 to 2.0 μm) and nanoplankton (2.0 to 20 μm) cell populations, but it is tedious and requires an experienced microscopist. We have pursued one solution to these problems—the application of semi-automated image analysis techniques to microscope images. Advances in computer hardware and software technology are now making powerful image analysis systems widely available for scientific applications, including fluorescence microscopy (Arndt-Joven *et al.*, 1985). True-color microscope images can be detected and digitized using video technology and readily available image digitizers. These digitizers ("frame-grabbers") produce two-dimensional arrays of numbers (picture elements, or pixels) from video images that relate to image brightness and color. Both the spatial and color resolution of these digital images can exceed that of human vision.

Our lab has been developing the software and techniques for using image analysis as a tool to improve measurements of bacteria, cyanobacteria and phototrophic and heterotrophic protist populations in the plankton. Our goal is to use color image analysis of microscope images to rapidly identify, count and measure these cells. This paper is intended to provide a brief overview and status report on the use of color image-analyzed fluorescence microscopy in microbial plankton ecology. More detailed descriptions of image-analyzed fluorescence microscopy can be found elsewhere (Sieracki *et al.*, 1985; Inoue, 1986; Bjørnsen, 1986; Sieracki *et al.*, 1989a; 1989b; Sieracki and Webb, 1990).

Samples are prepared for color image analysis in basically the same way as for visual epifluorescence microscopy (Hobbie *et al.*, 1977; Haas, 1982). Typically, a small volume of a whole water sample (1-25 ml)

is fixed, stained with one or more fluorochromes (e.g., 4',6-diamidino-2-phenylindole (DAPI), acridine orange, proflavine), and filtered onto a black-stained, 0.2 μm pore-size polycarbonate filter. Counts of cells per microscope field are converted to abundance estimates, which are often used as simple biomass indicators alone. Since cell size distribution of these populations can vary considerably, it is preferable to measure individual cells to estimate mean cell biovolume. Typically cell length and width is measured on a subsample of cells visually, using a micrometer in the microscope ocular or from photographic prints or slides. These measurements are then converted to biovolume assuming simple cell shapes, such as spheres, prolate spheroids or cylinders with hemispherical ends. With image analysis it is possible to make faster, more precise size measurements on the two-dimensional cell images. We have developed a method that uses the complete cell perimeter to estimate cell biovolume from digital images (Sieracki *et al.*, 1989b). Once the cell abundance and biovolume is known, the biomass of the different cell populations can be calculated†.

Color Image Detection

The first and foremost requirement for image analysis is accurate detection of the image. This depends upon the quality of the microscope optics, camera and digitizing part of the system. Microscope optical quality is of paramount importance for delivering a clear, detailed image to the camera. Optics with high transmission efficiency are needed since fluorescing cells are generally dim. This is especially important for color image analysis since the sensitivity of color cameras is generally much lower than that of black and white cameras.

Color image-analyzed fluorescence microscopy was used successfully at sea for the first time on the recent U.S. Joint Global Ocean Flux Study (U.S. JGOFS) pilot study of the north Atlantic spring bloom (Sieracki *et al.*, 1990). Examples of digitized, true-color images of nanoplankton cells from this study are shown in Fig. 1 (p. 32). Fine structure can be seen including the unique appendage(s) and food vacuole contents of the cell in Figs. 1a, b and e and the distinctive sheath surrounding the cell in Figs. 1c and d. Zooming an image by pixel replication (Fig. 1e) does not add any useful magnification but demonstrates the digital nature of the image. The quality of these color images permits accurate cell measurement and classification (see below).

Automatic Threshold Selection for Cell Sizing

Since epifluorescent images are generally composed of bright fluorescing cells against a dark background, the computer can detect cells in the image using a brightness threshold. Pixels above the threshold are considered part of a cell and pixels below it are background. In two-color (red and green) images of fluorescing cells (Fig. 2a, p. 33), we average the two colors to measure cell size (Fig. 2b). Because image detectors cannot produce a perfect image, the edge of a cell appears as a gradient between the dark background and the bright cell, rather than a distinct edge. This is illustrated in a cross-section of brightness across a cell (Fig. 2d) where the edge is sloped, rather than a sharp vertical line. It can be seen that different threshold brightness levels will result in different apparent cell sizes (Fig. 2c). For this reason accurate threshold selection is necessary for accurate cell size measurement. An objective, automated method for finding a threshold that is based on the image characteristics is preferable to subjective, visual methods. We have developed such an automated method that finds the threshold as a minimum of the second derivative of the cell brightness profile (Sieracki *et al.*, 1989a). The method was developed by analyzing images of commercially available standard fluorescent microspheres of different sizes and brightnesses and was found to be accurate and reliable for measuring a variety of pico- and nanoplankton cells.

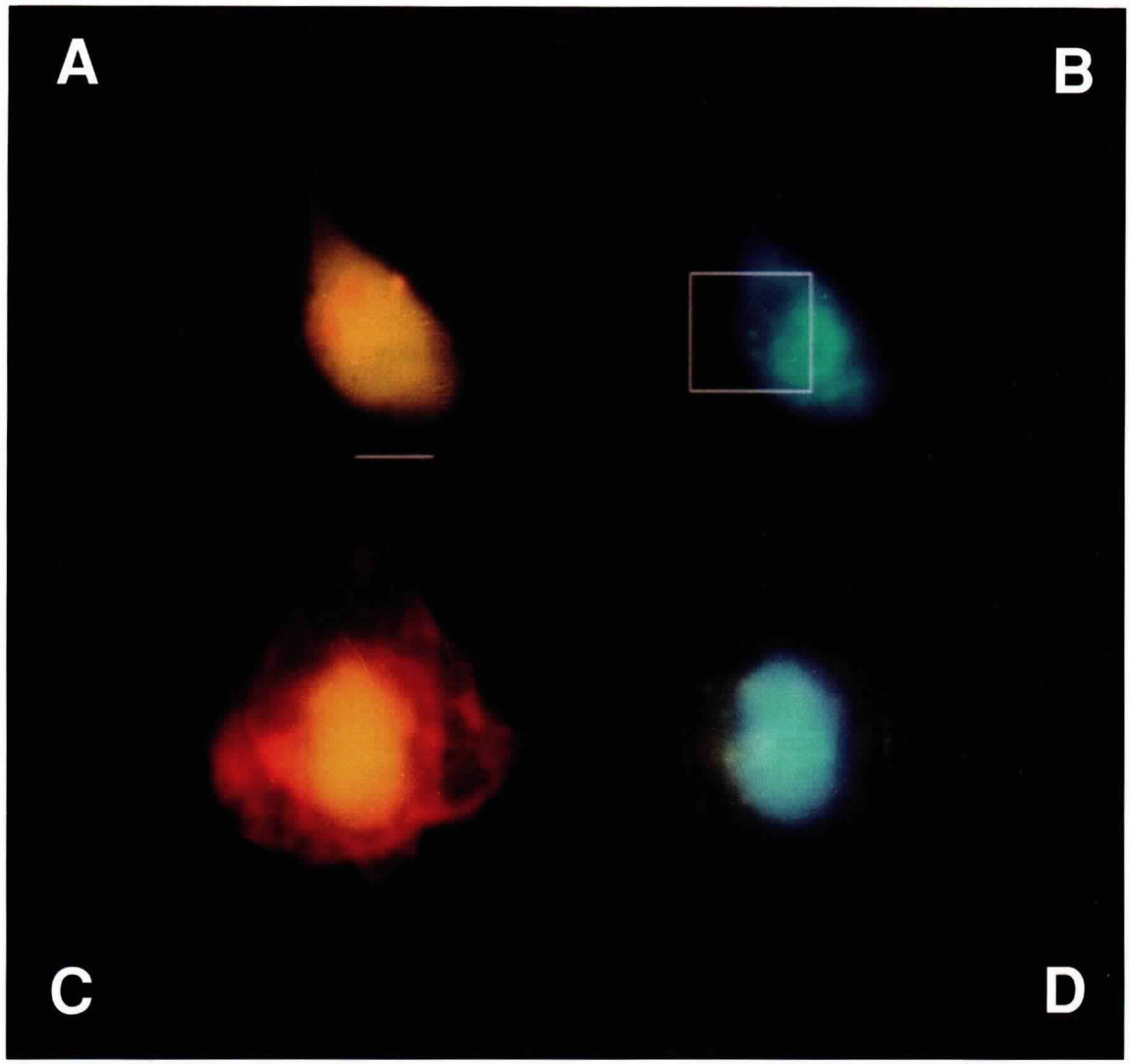
New Imaging Technology

Conventional video camera technology is limited by electronic signal standards created decades ago for commercial broadcast purposes. This is also true of cameras using new imaging technology but producing standard video signals. Recent advances in imaging instrumentation have led to the production of cameras with significantly improved quantitative characteristics, such as the cooled, charge-coupled device (CCD) camera. A CCD is a silicon-based photodetector that, when exposed to light, accumulates an electrical charge linearly proportional to the number of photons hitting it. A two-dimensional array of CCDs can be used to form an image. In addition to this extreme linear response, cooled CCD cameras have numerous important advantages over video cameras. Their dynamic range, the difference between the dimmest and brightest objects that can be detected in the same scene, is much greater than video. Little of the geometric distortion and instability resulting from the scanning electron beam of the video camera is present in a CCD. When cooled, a CCD has very low background noise. Finally, quantum efficiency, i.e., the percentage of photons striking the detector that are actually detected, is much higher (up to 75% in the visible spectrum) than either video (20%), the human eye (1%), or photographic film (1%).

Astronomers have developed CCD cameras to image the most distant galaxies ever visualized (Waldrop, 1986). Anyone who has looked at DAPI-stained bacteria through an epifluorescence microscope appreciates the similarity to a clear night sky. These cameras are now being used successfully with fluorescence microscopy in biomedical research (Hiraoka *et al.*, 1987), and we have begun using a cooled CCD camera (Photometrics Ltd., model 200) for imaging fluorescing plankton cells. The camera produces a direct digital image without the need for a frame-grabber,

The first and foremost requirement for image analysis is accurate detection of the image.

† Biomass of cell population p, B_p (μg C l^{-1}), is the product of cell concentration, C_p (cells l^{-1}), mean cell biovolume, V_p (μm^3 cell^{-1}), and a biovolume-to-carbon conversion factor, F_p (μg C μm^{-3}): $B_p = C_p \times V_p \times F_p$.



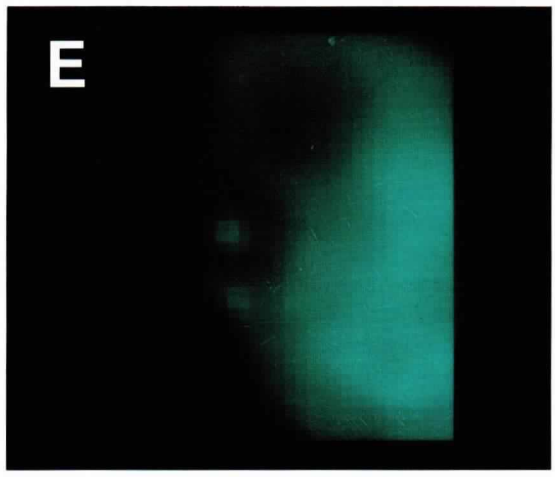
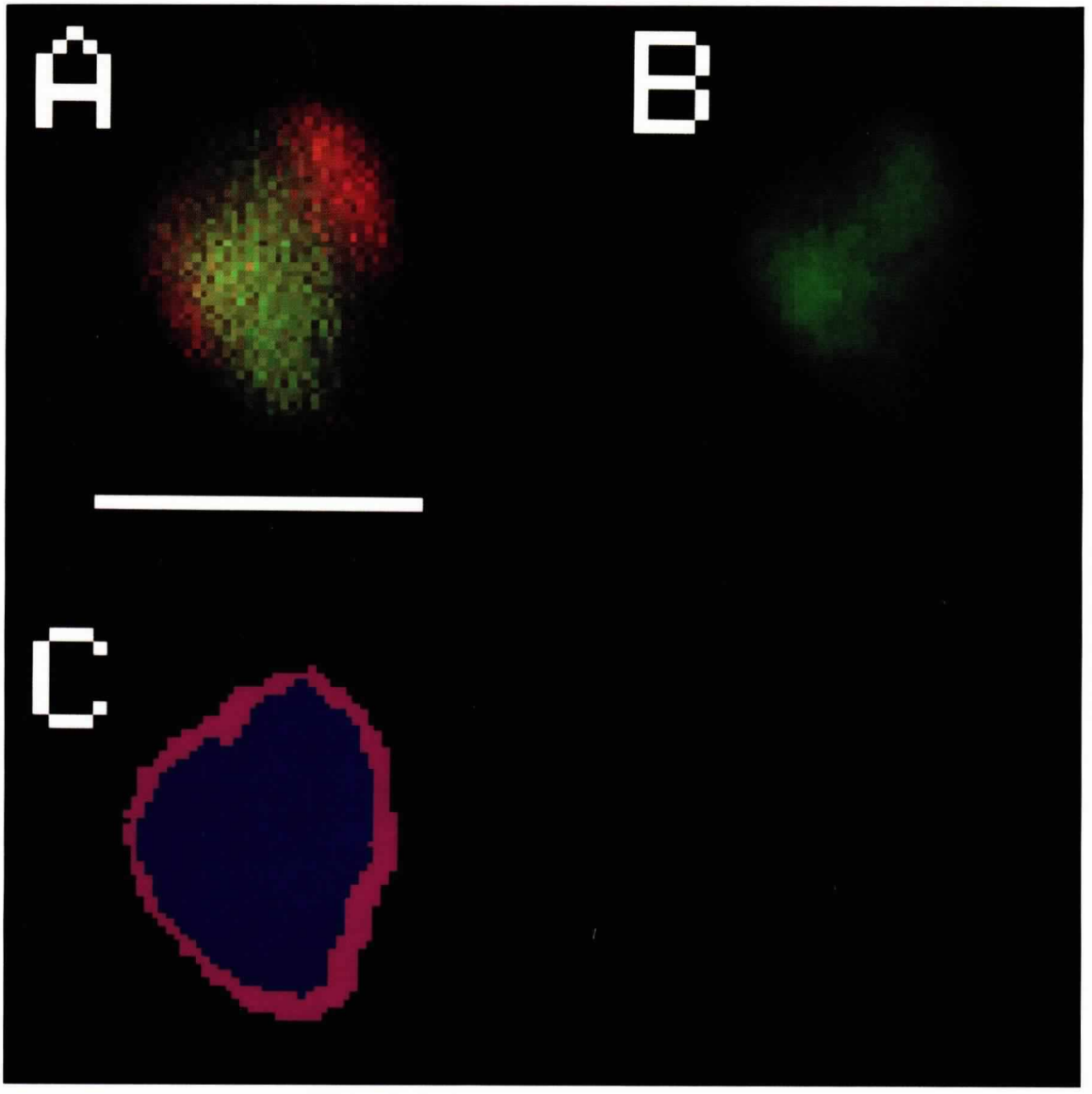


Fig. 1: Examples of true-color, digitized images of cells from the U.S. JGOFS North Atlantic spring bloom experiment. These two heterotrophic flagellates were collected from a depth of 13 m on May 19, 1989, near the 47°N, 20°W study site. Samples were dual-stained with DAPI and proflavine and excited with blue (a, c) and ultraviolet (b, d, e) wavelengths. The digital nature of the image is apparent (e) when a portion of the cell in (b), a food vacuole containing bacteria, is zoomed 8X by pixel replication. These images were produced using our color system consisting of a Zeiss Axioplan microscope with a 63X Neofluar objective, a Sony 3-chip color CCD camera (DXC-750), a Matrox video digitizer (MVP-VME/NP), and a Sun 3/160 host computer. Sixteen video frames were averaged to reduce noise, and the red, green and blue components were individually contrast enhanced. Scale bar is 5 μm.



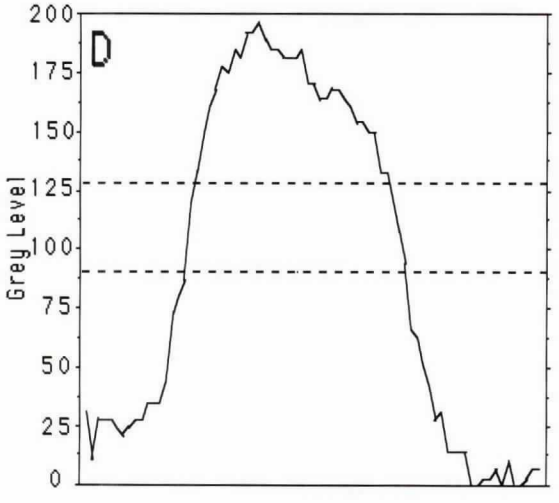


Fig. 2: Digital image of a phytoflagellate cell from Chesapeake Bay showing (a) the original 2-color (red and green) image and (b) the smoothed average of the red and green images, used for cell sizing. The false color silhouettes (c) show the effect of two thresholds: 90 (red outline), selected using an optimal, automated technique, and 128 (blue outline), arbitrarily chosen to exemplify a sub-optimal threshold. These thresholds are also shown as dashed lines on the brightness profile (d) taken from a transect of the cell in (b). Scale bar in (a) is 5 μm. Cell was stained with proflavine and viewed under blue excitation (450-490 nm).

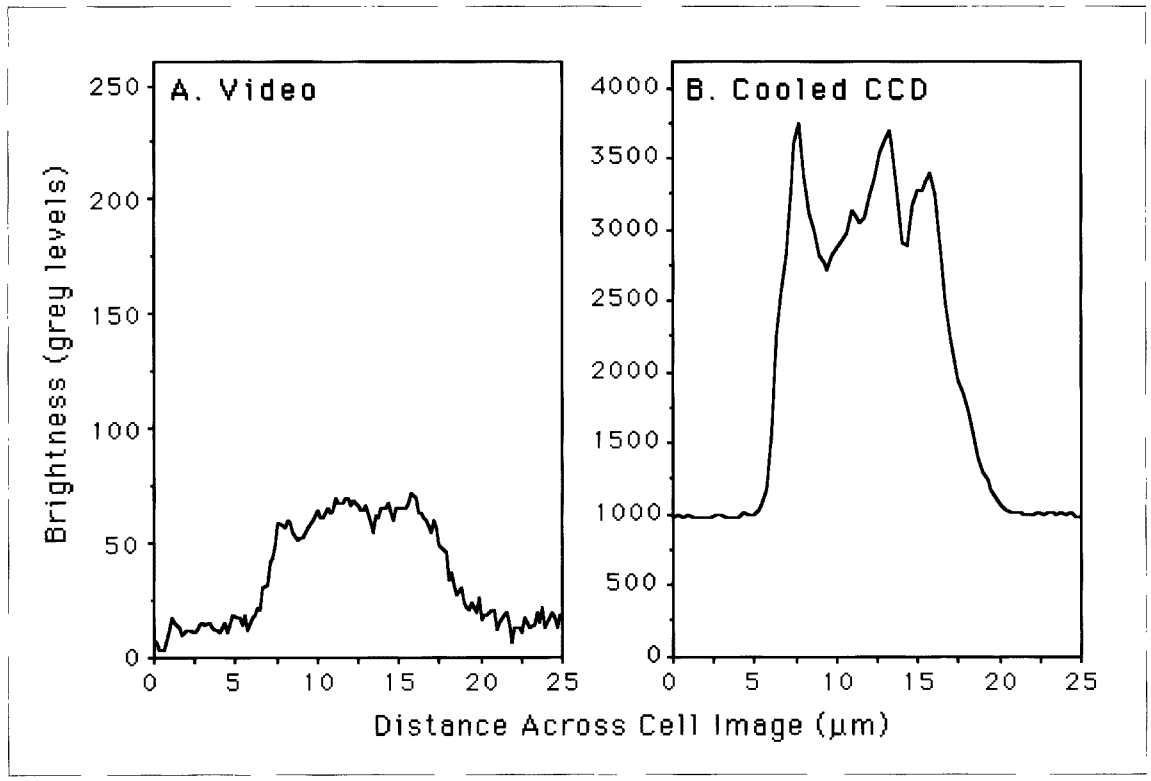


Fig. 3: Brightness cross-sections across an image of the same heterotrophic flagellate cell using (a) a standard video camera and (b) a cooled CCD (charge coupled device) camera. The cooled CCD shows greater brightness resolution and lower noise than the video camera. The sharp edges of the cell profile allows easier thresholding and should yield more precise measurements of cell size. Note that the video image is digitized into 256 brightness levels while the CCD provides 4096 real brightness levels.

and exposure times are controlled much like a photographic camera. The low noise, high efficiency and high brightness resolution of this camera are evident when compared to a standard video camera (Fig. 3). These characteristics result in a sharp, distinct cell edge in the image (Fig. 3b) that makes automatic thresholding, and thus cell size measurements, more precise and reliable. The camera produces grayscale images that are used to accurately measure bacteria and cyanobacteria. These cells are distinctive enough in abundance, size, and fluorescence that color is not necessary for their analysis.

Particle Classification by Color

In the protistan groups of interest, the presence or absence of chloroplasts within cells cannot be made on the basis of external morphology using conventional bright-field microscopy. The presence of photopigments, however, is easily detected by fluorescence microscopy. Color images are therefore necessary to classify particles in the nanoplankton size range using image analysis. Detrital particles are distinguished from living cells on the basis of their DAPI-staining characteristics. DAPI binds specifically to DNA, so the nucleii of eukaryotic cells fluoresce bluish-white under ultraviolet (UV) excitation (365 nm), while detrital particles, which are often the same size and shape as cells, fluoresce yellow. Cells are then measured and classified as phototrophic or heterotrophic on the basis of the presence or absence of photopigments, which can be observed under blue excitation (450-490 nm). These wavelengths cause chlorophyll and phycoerythrin, the two dominant fluorescing pigment types in marine plankton, to fluoresce red and orange, respectively. This simple classification scheme is essentially the same as that used in visual microscopy. Color video cameras separate the image (i.e., the microscope field) into red, green and blue images that can be independently analyzed. A study of visually classified particle types from Chesapeake Bay indicates that the classification scheme outlined above can be used to classify particles as detritus and phototrophic and heterotrophic cells automatically by color image analysis (Fig. 4). This is done by using simple ratios of the average particle emission brightnesses in red, green and blue under UV and blue excitation.

Conclusions

Color image analysis has considerable potential for aiding marine microbiologists in quantifying and characterizing the marine microbial plankton community. Sample analysis is currently an interactive, semi-automatic procedure with considerable operator intervention. It takes about two hours per sample to do a complete analysis of pico- and nanoplankton cell abun-

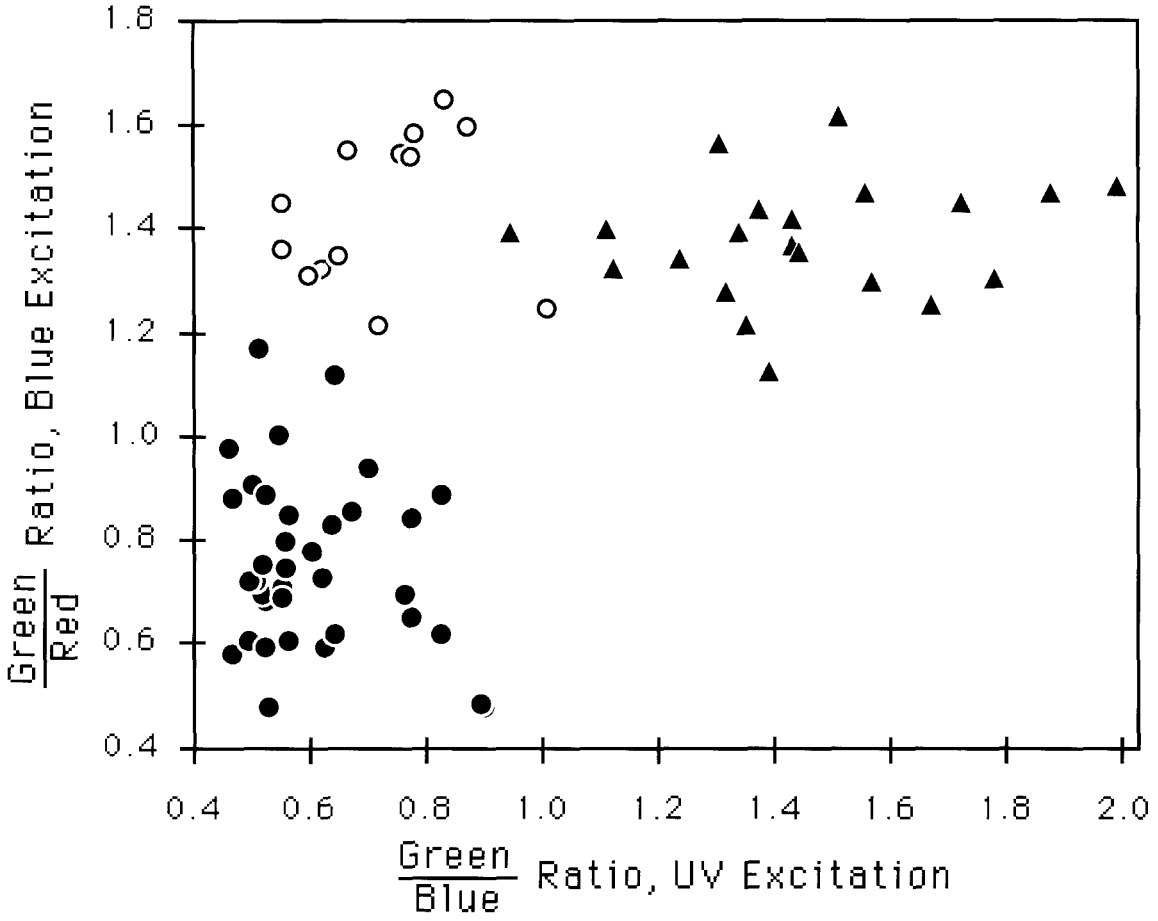


Fig. 4: Detrital particles (▲) and heterotrophic (○) and phototropic (●) nanoplankton cells can be distinguished on the basis of their different color emissions under blue and ultraviolet (UV) excitation in samples dual-stained with DAPI and proflavine. The ratios of the average brightness of pixels making up each cell in the red (R), green (G), and blue (B) color channels are used. Since DAPI-stained DNA in living cells appears bright blue under UV excitation, they have a lower G:B ratio than detritus. Although the nucleus and cytoplasm of both heterotrophs and phototrophs appear green under blue excitation, the presence of chlorophyll or phycoerythrin in phototrophs gives them a brighter red fluorescence. The G:R ratio of phototrophs, therefore, is generally lower than that of heterotrophs. This sample is from Chesapeake Bay.

dance and biomass. Much of this time is spent focusing the microscope and visually classifying cells. This time could probably be reduced by half with more automated classification, and the development of such algorithms is currently under way.

Color image analysis is comparable to flow cytometry in its ability to provide information about plankton populations at the single cell level. Color image analysis, while currently not as fast as flow cytometry, yields considerably more information per cell. Flow cytometry provides between two and five measurements per cell, depending on the number of lasers and photo-detectors used. A digital image can contain from ten to hundreds of pixels per cell (depending on cell size) in each of two or three colors detected. These pixels define the spectral emission of fluorescence and the two-dimensional size and shape of each cell. For this reason, cell sizing is probably more accurate with image analysis. Flow cytometry uses light scatter as an indicator of cell size but this is affected by cell shape and optical characteristics. Sizing by image analysis is more direct and can account for cell shape (Sieracki *et al.*, 1989b). The biovolume of cells with more complex morphologies, such as larger diatoms, dinoflagellates and ciliates, cannot be so easily measured from two-dimensional images, however. Image analysis also provides the operator the satisfaction of actually seeing the cells being measured as opposed to the several photo-detector signals per particle produced by flow cytometers. Flow cytometry has several advantages over semi-automated image analysis, including a much faster analysis rate and the ability to analyze and sort living cells. Direct comparisons of the capabilities of flow cytometry and image analysis have not yet been made, so much of the above is speculative at this point.

Color image analysis also facilitates the classification of particles and identification of cells into trophic groups and, in some cases, taxons. Cells are easily discriminated from non-living particles on the basis of the presence or absence of a DAPI-staining nucleus. In the north Atlantic spring bloom study we were able to identify and measure a significant population of heterotrophic dinoflagellates in the thermocline by epifluorescence microscopy and color image analysis (Stoecker *et al.*, 1990).

High-quality detection of fluorescent images is possible with current camera technology and can be used to gain considerable information on individual pico- and nanoplankton cells. This information allows cells to be automatically discriminated from detrital particles, accurately measured, and classified by trophic type. More precise measurements of picoplankton cell biovolume will be possible with the new CCD imaging technology.

Acknowledgements

We thank Sarah Rennie and Rajeeb Hazra for their creative software development. Larry Haas, Ken Webb and Carol Falkenhayn offered useful comments on the manuscript. Mary Anne Posenau of The Image Processing Laboratory, Flight Software and Graphics Branch of the NASA Langley Research Center, and Kris Mathews and Betsy Avis of Computer Sciences Corporation provided valuable assistance with the color figures. This work was supported by NSF grants OCE-8813356 and OCE-8817399.

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