Concepts and Techniques for the Study of Algae

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1 Introduction

Algae can be studied in the field or in the laboratory (Fig. 1). A variety of conventional and modern techniques are available for isolation, characterization and identification of different species of algae. An alga is generally characterized on the basis of its size, colour, shape, form or growth habit. Some algae are visible to the naked eye whereas others can be seen with the help of a magnifying glass or a microscope. Algae are divided into two groups based on size: macroalgae and microalgae. Algae growing in the form of large, plant-like structures are called 'macroalgae' (e.g. Seaweeds and kelps). 'Microalgae' is a very broad term for all microscopic, photosynthetic protists. Microalgae that swim or drift within the well-lit regions of the water bodies are collectively termed "phytoplankton". Microalgae that inhabit the surfaces such as sea floor or river bottoms are called "benthic microalgae". The common unit for measuring microalgae is micrometer (μm). Phytoplankton can be differentiated on the basis of cell size into micro-phytoplankton (200–20 μm), nano-phytoplankton (20–2 μm) and pico-phytoplankton (2–0.2 μm). While the microphytoplankton and nanophytoplankton are easily identified with conventional techniques for instance light microscopy, the picophytoplankton can only be distinguished by fluorescence techniques.

Microscopes are essential tools for studying algae. Now, an incredible array of microscopes are available. Use of microscopes for the examination of algal cells requires the preparation of cells by appropriate methods for the particular type of

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Fig. 1 Studying algae in the field (**a**, **b**) Magnifying glass, (**c**) Portable spectrometer, (**d**) AlgaeTorch, a field instrument used to measure chlorophyll directly in the water body, from live algae (**e**) FluoroProbe, a multiwavelength field fluorometer (**f**) FlowCytobot, an in-situ automated submersible imaging flow cytometer (**g**) Images of algae obtained through FlowCam, an imaging cytometer (**h**) CytoBuoy, a real time monitoring flow cytometer

microscopy. Compound light microscopes provide enough magnification to see the cells of microalgae and macroalgae, but not the ultrastructure or many of the structures inside those cells. For that, we need an electron microscope. In the past few decades, transmission electron microscopy has added an entirely new aspect to our knowledge of the algae and has helped in understanding the structure and functions of cells and organelles. Analysis of a statistically significant quantity of cells requires some level of automation to be brought to the characterization. This job is fulfilled by flow cytometry. Innovation in fluorescent probe technology has led to diversification of the technique, allowing development of additional methods

for analysing labelled cells which incorporate the feature of automation. Various instruments have been devised for the automation of cell counting which go beyond the limitations presented by microscopy.

Algae contain different combination of pigments such as chlorophylls, carotenoids and phycobiliproteins which make them appear colourful. Pigment analysis offers deep insights into the taxonomic composition, distribution and abundance of natural algal populations in oceans. In addition, the signature pigments present in specific algae can be utilized as "fingerprints" in the study of harmful algal blooms (HAB). The spectrophotometric and fluorometric methods are routinely used to study the pigment composition in various algal species. In vivo fluorescence measurements allow real-time monitoring of phytoplankton distribution. HPLC provides detailed information about the composition of phytoplankton communities based on diagnostic signature or marker pigments which have been selected as taxonomical pigments for different algal groups such as zeaxanthin (for cyanobacteria) and prasinoxanthin (for Prasinophyceae), fucoxanthin (for diatoms), and chlorophyll *b* (for green algae) (Stauber and Jeffrey 1988; Millie et al. 1993; Jeffrey and Vest 1997). High precision liquid chromatography (HPLC) has been used to discriminate and quantify pigments directly from water samples.

2 Commonly Used Detection Tools for the Quantitative and Qualitative Analysis of Algae

Various tools provide distinctly different information and are chosen according to the application. Cells can be subjected to analysis of morphological changes and taxonomic diversity using light microscopy. For over a century light microscopy was the basic tool to observe phytoplankton. It is still invaluable to determine species composition. Since the late 1960s, both transmission and scanning electron microscopes have proved extremely useful in establishing accurate algal taxonomy using ultrastructural features such as ornamentation of body scales or architecture of flagellar roots. Later, fluorescence techniques such as epifluorescence microscopy and flow cytometry played a key role in the discovery of picoplankton. Flow cytometry, initially borrowed from the biomedical field, has become the method of choice to estimate cell abundance in the field, since it permits the counting and classification of several thousand cells per minute. Fluorescence microscopy, epifluorescence microscopy and flow cytometry are generally used for the detection of picophytoplankton and for the documentation of viable cells after staining. Quantification of cells can be easily achieved by flow cytometry, but ample species discrimination is difficult to obtain. Automated microscope based image cytometry can be used to analyse cells over time, a technique which is not possible using flow cytometry. Important criteria to consider for the selection of instrument include application, accuracy and speed of analysis, light sources etc.

2.1 Light Microscopy

Light microscopy is used to study most algae. It is the fundamental tool employed in an algae laboratory. In light microscope, visible light passes directly through the lenses and specimen. The simplest type of light microscope consists of only one glass lens, a magnifying glass (Fig. 1a, b). Microscope composed of more than one glass lens is called a compound microscope. Compound microscope includes condenser lens, objective lens and eye piece lens. Condenser lens focuses light from the light source onto the specimen. The objective lens is responsible for producing the magnifying image. Objective lens is commonly available in 4×, 10×, 20×, 40×, 60× and 100× magnifications. Light microscopes are available in two orientations: upright microscope and inverted microscope (Fig. 2a, b). Upright microscope is suitable for viewing microscope slides whereas inverted microscope is appropriate for examining culture vessels such as multi-well plates and petri dishes. Using an inverted microscope cells can be isolated straight from the well plates. It is also used in efficiently monitoring the enrichment cultures and single-cell isolates in the multi-well plates.

The type of microscope and observation method may differ depending on the specimen to be observed and the purpose of microscopic observations.

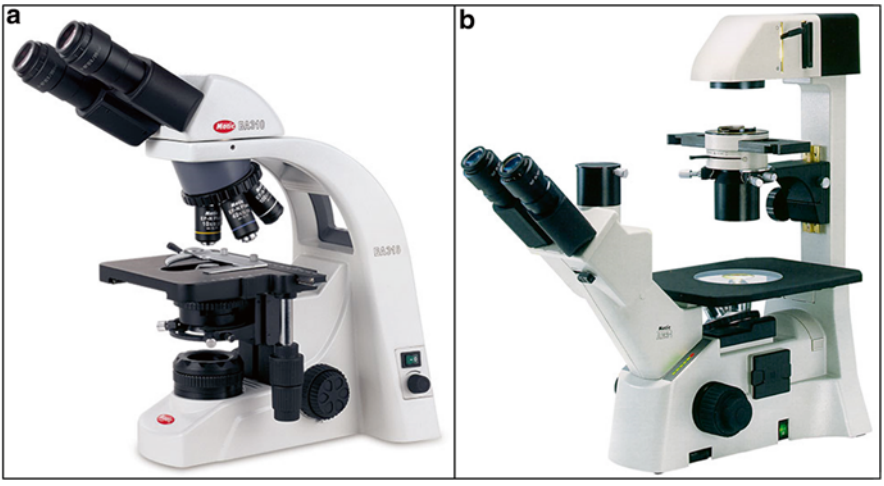

Fig. 2 Light microscope (**a**) Upright, (**b**) Inverted

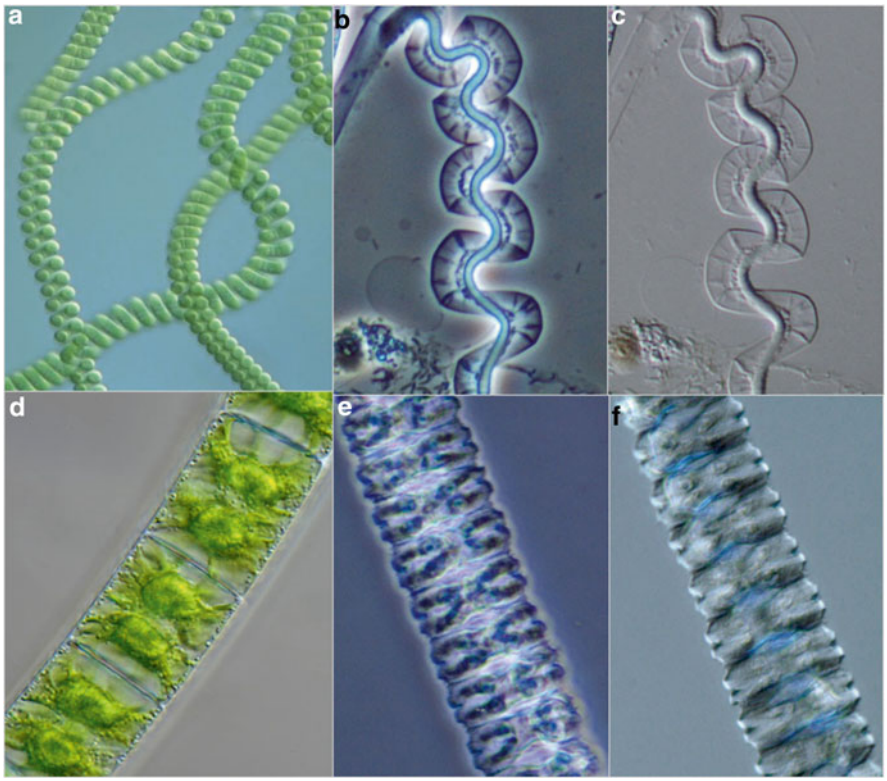

Fig. 3 (**a**–**c**) *Spirulina* and (**d**-**f**) *Zygnema* as observed by bright field, phase contrast and DIC microscopy (Image courtesy: Michael W. Davidson, The Florida State University)

2.1.1 Bright Field (BF) Microscopy

It is the most common observation method to observe an unstained or stained algal specimen. In a bright field microscope, the entire field of observation is illuminated and appears bright. The algal specimen is visible in the light path because of its natural pigmentation or stains that absorb light differentially (Fig. 3a, d).

2.1.2 Phase Contrast Microscopy

This method is appropriate for viewing live cells and colourless, transparent specimens. It exploits the difference between the phase of light passing through a relatively thinner region of the specimen and the phase of light passing through a denser or thicker region of the specimen. Hence the phase of light wave changes according to the refractive index of various regions of the specimen. The specimen appears as varying levels of brightness and contrast. The microscope is

built-in with a phase-contrast objective lens and a condenser lens. In positive phase contrast, the background light is phase shifted by +90° and hence specimens appear dark against a bright background. In negative phase contrast, the background light is phase shifted by −90° and hence specimens appear bright against a dark background. The contours of images are delimited by a distinctively bright diffraction halo (Fig. 3b, e).

2.1.3 Differential Interference Contrast (DIC) Microscopy

Differential interference contrast microscopy also known as Nomarski microscopy is used to enhance the contrast in transparent unstained specimens. For DIC observations, the microscope is equipped with a DIC prism or Nomarski prism and a polarizing plate. The image obtained using DIC is almost similar to that obtained through phase contrast microscopy but the edges of image appear shadowed, giving the image a three-dimensional appearance (Fig. 3c, f). DIC utilizes the phase difference in areas of the specimen when light passes through it to add contrast to images of transparent specimens.

Specimen preparation for light microscopy: Specimens to be observed with the light microscope are broadly divided into two categories: whole mounts and sections.

(i) Whole mount

A wealth of structural detail can be seen in wholemounts of living unicellular and filamentous algae (Fig. 4). Such preparations allow observations over time so that developmental phenomena can be followed. Phase contrast and DIC have been most useful for the observation of living cells. Sample preparation involve mounting the specimen in a mounting medium (water, tissue culture medium or glycerol) on a glass slide and covering it with a glass coverslip. The slide is then positioned on the specimen stage of the microscope and examined through the ocular lens, or camera. To take maximum advantage of the resolving power of the light microscope, specimens are usually prepared in a way designed to enhance contrast (difference in the darkness or colour of the structures being examined). A regular means of enhancing contrast is to apply particular dyes that colour or otherwise adjust the light transmitting properties of cell constituents.

(ii) Sections

Anatomical examination of macroalgal specimens are based on a very thin cross section either using a razor blade or a device called microtome.

Hand sections: Hand sections of macroalgae are useful in the study of cell walls, extracellular matrices and other anatomical details. Hand sectioning requires sharp and flexible razor blades. Sections are kept in sea water or freshwater for about a minute and then mounted in glycerine or examined directly. Sections can be stained with aniline blue or methylene blue to give good morphological description. Sections can also be stained histochemically using Toluidine blue, Alcian blue,

Fig. 4 Whole mounts of *Polysiphonia* (red algae) showing marine diatoms attached to it (Picture courtesy: Mr. Charles Krebs)

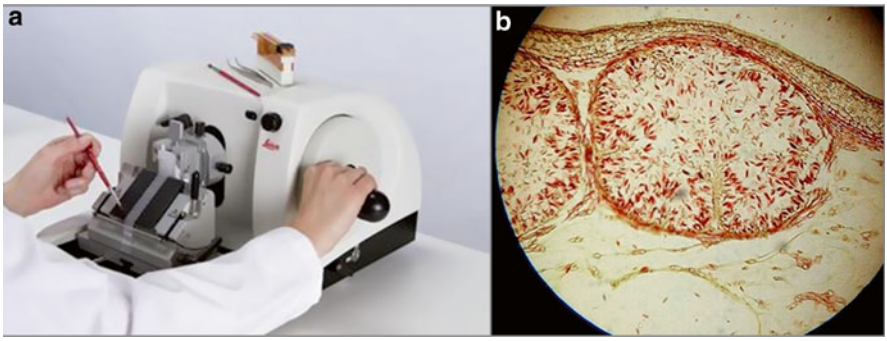

Fig. 5 (**a**) Microtome, (**b**) Section through a male conceptacle of *Fucus*, showing many antheridia borne on branched hairs (Picture courtesy: (**b**) Dr. Christopher Skilbeck)

Alcian yellow or PAS procedure (involving the use of Schiff's reagent) to identify the chemical components of cellular and subcellular structures. Algae are usually sensitive to dehydration hence a small drop of glycerine (mounting medium) is placed on the section. The sections are carefully covered with a cover glass. These glycerine mounts are sealed by applying clear nail polish to the edges of the cover glass.

Microtome sections: A microtome is a tool used to cut extremely thin sections (Fig. 5a, b). Specimen needs to be processed to avoid cell damage before sections

are prepared. The processing involves a sequence of steps: fixation, dehydration, embedding and the consequent sectioning using a microtome.

Fixation and dehydration: Fixation preserves the cells in their original state. Specimens are treated with a solution containing chemicals that penetrate the cell membrane and cross-link most nucleic acids, proteins and lipids. This cross-linking maintains the structural integrity of the cell. The most widely used fixatives are acids and aldehydes, for example, acetic acid, picric acid, formaldehyde and glutaraldehyde. Formaldehyde is the most commonly used primary fixative for light microscopy which cross-links amino groups on adjacent molecules, resulting in the formation of DNA- protein cross-links (DPCs). Other common fixative is glutaraldehyde which readily cross-links proteins. After fixation, the specimen is dehydrated through a series of alcohols or acetones.

Embedding: The specimen is then embedded in an embedding medium of liquefied epoxy resins, methacrylates or paraffin sand allowed to harden at appropriate temperature. Embedding media surround the specimen and infiltrates into it, harden upon cooling, hence provide mechanical support to the specimen both internally as well as externally during sectioning. The resulting blocks containing the specimen provide a stable base for fixing in the microtome and are ready for sectioning.

Sectioning: The block is mounted on the microtome holder. Microtome arm progresses the specimen towards the knife. The sections are cut with the knife and are picked up by a corner using fine forceps. The microtome knives are generally made up of polished steel or glass for light microscopy. The sections are then transferred to small drops of distilled water on a glass slide.

Before staining, sections are immersed in toluene or xylene, which removes the paraffin and wax, leaving the thin section attached to the slide. Sections embedded in epoxy resins and methacrylates (plastics) can be stained without the removal of the plastics.

Staining: Dewaxed sections are then stained with any of the morphological or histochemical stains, antibodies or enzymes showing affinity for a particular kind of cellular component. Most commonly used morphological stains are aniline blue or methylene blue. A few specific stains for histochemical studies are Bromophenol blue, Fast green, Acid fuchsin, Feulgen stain, Toluidine blue, Alcian blue, Alcian yellow or PAS procedure (involving the use of Schiff's reagent). A glass coverslip is mounted over the section using an appropriate mounting medium.

2.2 Fluorescence Microscopy

Fluorescence microscopy is a rapidly expanding technique in the field of phycology (Fig. 6). Fluorescent stains or fluorochromes absorb light at one particular wavelength and fluoresce (emit light) at a longer wavelength. In fluorescence microscopes, only fluorescent light emitted by the fluorochrome is utilized to form an image. Fluorescence microscopy techniques are most commonly used for seeing structures of cells, for observing physiological and biochemical events in the live algal cells, and for the enumeration of live/dead cells.

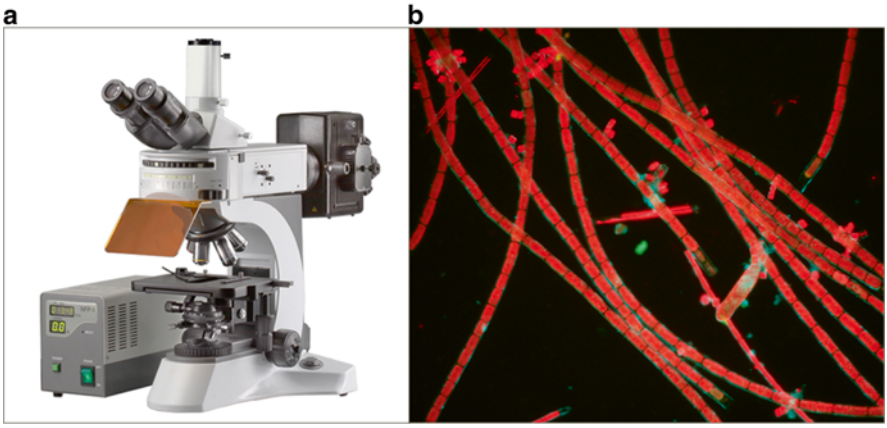

Fig. 6 (**a**) Fluorescence microscope, (**b**) Fluorescence micrograph of a filamentous alga (Picture Courtesy: (**b**) David W. Walker, Micscape)

2.2.1 Widefield Epifluorescence Microscopy

It is most commonly used to locate or detect specific proteins or other molecules in cells. In this technique the whole sample is simultaneously illuminated using a light source, usually a mercury lamp. Excitation filters are used to select the excitation wavelength. Excitation light is focused on the sample using an objective lens. The fluorescence emitted by the sample is directed to the same objective.

All algae contain chlorophyll *a* and exhibit fluorescence. Blue excitation causes chlorophyll *a* to emit or fluoresce red, green excitation causes phycoerythrin to fluoresce bright orange-red and phycocyanin to fluoresce orange yellow. An inverted microscope including epifluorescence illumination is a useful tool to detect and isolate cysts of many bloom forming algae (e.g., raphidophytes, diatoms, and dinoflagellates) from sediments. Cysts provide a fluorescent signal because of their cell wall and chlorophyll. Isolated cyst can be used for the cultivation of a particular alga.

Automated microscope based digital image cytometry, is where a fluorescence microscope is integrated with systems for image acquisition and analysis, and can be used to collect measurements of fluorescence per cell over time. In this type of microscopy, slow scan cameras are combined with a microscope interface and software for digital image acquisition and analysis. This type of microscopy is useful in single cell quantitative analysis. It is also useful in understanding the sources of variation in cell fluorescence which mainly arises due to differences in cell cycle position, variations in gene expression, the metabolic status of cells, and micro environmental differences.

2.2.2 Confocal Laser Scanning Microscopy (CLSM)

CLSM is a computerised microscope which involves the coupling of a laser light source to a light microscope and results in the generation of 3-D digital images of the microorganism (Fig. 7). Confocal means having the same focus. Confocal

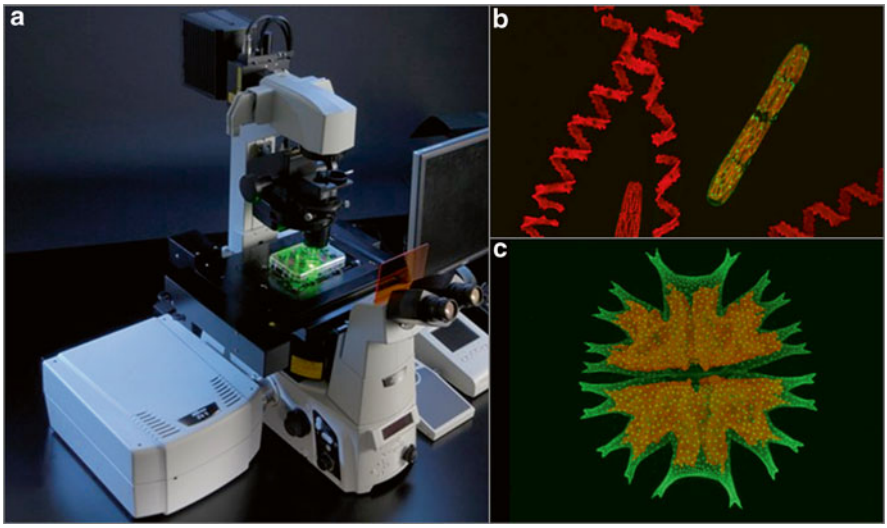

Fig. 7 (**a**) Confocal Microscope, (**b**) Confocal micrographs of *Penium margaritaceum* and *Spirogyra* algae. (**c**) *Micrasterias* (Image credits: (**b**) A. Andreas (**c**) J. Ochs'14)

microscopy solves one major problem, out-of-focus blur associated with regular fluorescence microscopy. Normally when a sample is illuminated by excitation light in the fluorescence microscope, full thickness of the specimen produces fluorescent signal at the same time. This adds to a background haze which does not permit most of the image to be in focus. Confocal microscopy is used to increase contrast and optical resolution of an image by filtering out the out-of-focus light from above and below the point of focus in the specimen. The confocal microscope filters out the out-of-focus light by means of a confocal pinhole situated in front of the image plane which acts as a spatial filter and allows only the in-focus portion of the light to be imaged. Light from above and below the plane of focus is eliminated from the final image. The ability of confocal microscope to produce sharp images of focal planes deep within a specimen by removing the involvement of out-of-focus light without any physical sectioning of the tissue, is termed optical sectioning. This method has revolutionized the ability to gather images from thick specimens. The confocal microscope uses a laser beam to illuminate a fluorescently stained or auto-fluorescent specimen.

Confocal microscopy combined with the use of fluorescent probes has been used in almost all cell based studies in phytoplankton. This technique has been used for assessing the ecotoxicity of chlorine and an oxidizing biocide on a marine diatom, *Cocconeis scutellum* Ehrenb (Nanchariah et al. 2007). It has also proven useful for the analysis of the three dimensional distribution of total and active cells in solid structures such as biofilms. In a confocal microscope, the illumination and light collection optics are configured to minimize the contributions from out-of-focus regions, providing a high resolution image from a very thin slice of specimen. This

resolution is further improved in multiphoton confocal microscopy, in which the fluorescence is excited by the nearly simultaneous observation of two or more photons of lower energy than would normally be needed for excitation (Shapiro 2003).

2.2.3 Inwater and Holographic Microscopy

In water microscopes are appropriate for real time imaging of microalgae in all aquatic environments (Fig. 8). These microscopes use a laser and camera to image, count, size and characterize objects in any body of water within the sample area. Resolution extends to objects as small as 0.5 μm and as large as 3 mm. Holography is a technique in which live three-dimensional and four dimensional holograms are

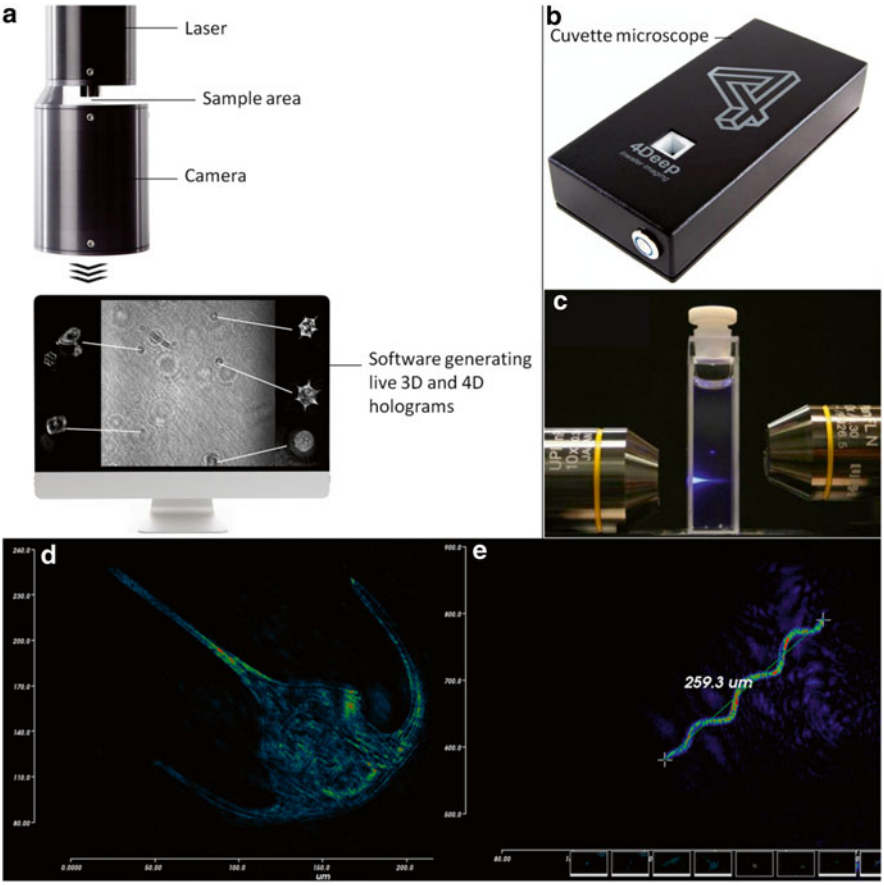

Fig. 8 In water microscopes: (**a**) Submersible microscope, (**b**, **c**) Cuvette microscope, (**d**, **e**) *Ceratium* (a diatom) and *Spirulina* as observed through submersible microscope (Image courtesy: 4Deep Inwater Imaging)

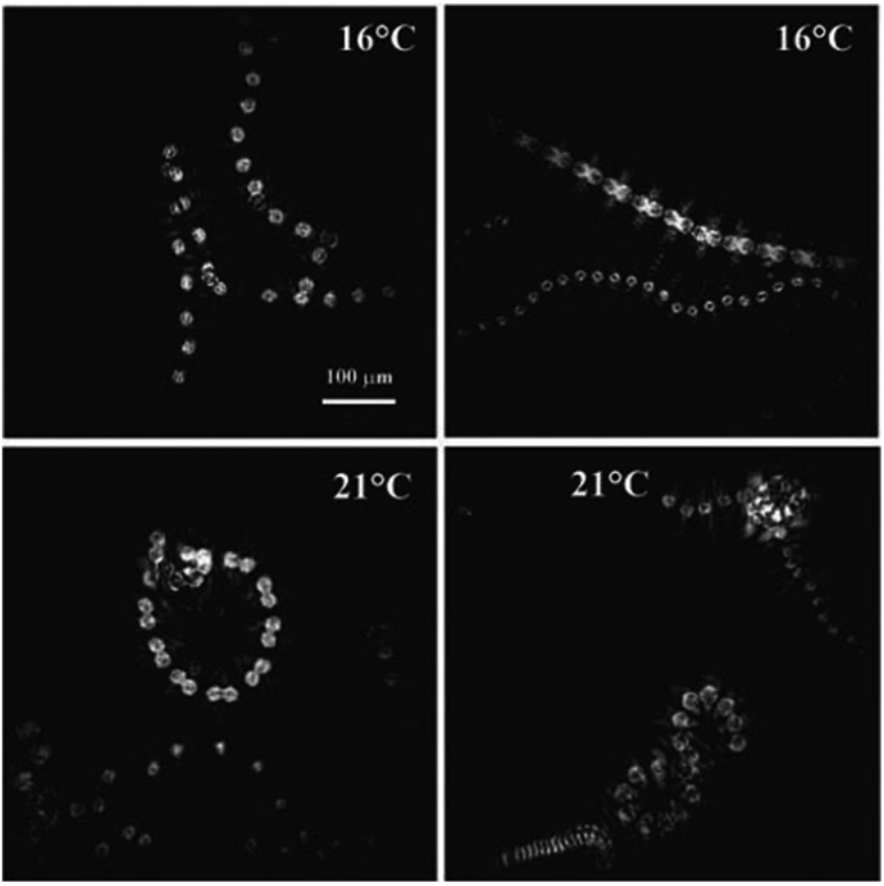

Fig. 9 Reconstructed images from holograms showing the effect of temperature on the motion of algae (*Alexandrium*). Circular motion at the elevated temperature (*bottom* pictures) is due to loss of rear flagella (Image courtesy: Prof. Hans Juergen Kreuzer, Department of Physics and Atmospheric Science, Dalhousie University, Halifax)

generated. It involves the use of a laser, interference, diffraction, light intensity recording and suitable illumination of the recording. The image changes as the position and orientation of the viewing system changes in exactly the same way as if the object were still present, thus making the image appear three-dimensional (Fig. 9).

2.3 Electron Microscopy

Electron microscopes use a focused stream of highly energetic electrons to see an object. This examination gives information regarding surface topography, morphology, ultrastructure and crystallographic structure (arrangement of atoms in an

object). The Electron microscopes have higher resolution and therefore can achieve higher magnification of up to two million times. This increase in resolution is possible since the electron beams have shorter wavelengths than that of the wavelengths of light. The drawback of the electron microscope is that the live cells cannot be observed.

Electron microscopes use electromagnetic lenses, instead of optical lenses to focus a beam of electrons instead of visible light to image the specimen. The fundamental steps implicated in all Electron microscopes are the following: A stream of electrons is produced by electron guns under high vacuum. This stream is focused into a monochromatic beam with the help of metal apertures and magnetic lenses. The accelerated electron beam impinges upon the sample and a variety of electron-specimen interactions take place. These interactions make electron microscopy possible. The products of electron-specimen interactions are detected and can be transformed into an image. There are different types of electron microscopy: Scanning electron microscopy, Transmission electron microscopy, Scanning tunneling microscopy, Atomic force electron microscopy, Immunoelectron microscopy.

2.3.1 Scanning Electron Microscopy

Scanning electron microscope (Fig. 10) permits the researcher to observe the external features of intact microorganisms. It gives information about the topography of a specimen surface. SEM can provide a three dimensional topographical information about the specimen surface (Fig. 11).

In scanning electron microscopy, primary electron beam moves over the surface of the specimen coated with a thin film of heavy metal such as gold or palladium.

Fig. 10 Scanning electron microscope

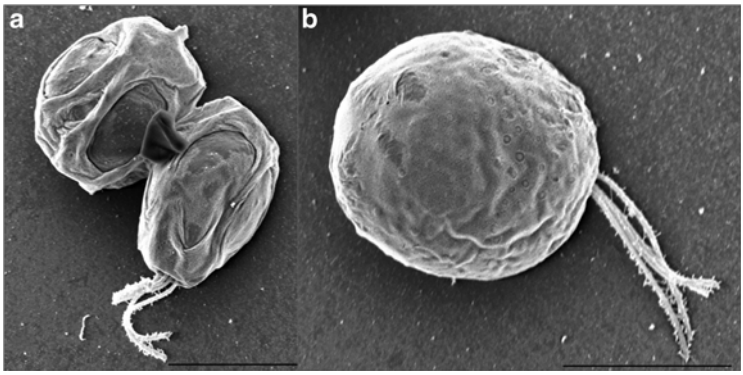

Fig. 11 Scanning electron micrograph of a green alga, *Tetraselmis indica*

This causes the emission of secondary electrons from the surface of the specimen. Detectors collect the secondary electrons and convert them into an electronic signal. These signals are then processed to generate an image on a cathode ray tube (CRT) screen.

Sample preparation for SEM

(i) Fixation and dehydration
Fixation preserves the algae in their original state by crosslinking nucleic acids, proteins and lipids. Specimens are treated with a chemical fixative such as glutaraldehyde and osmium tetroxide. The sample is usually immersed in 2–5 % glutaraldehyde in a buffer (phosphate buffer, cacodylate buffer or HEPES buffer) that maintains physiological pH. The sample is incubated overnight at 4 °C. The specimen is then rinsed a few times in their particular buffer. Following fixation, the specimen is dehydrated through a graded series of ethanol. The concentrations of ethanol usually start at 25 % and proceed at 25 %, 50–70 % followed by 85 %, 95–100 % ethanol. Usually three final changes in 100 % ethanol (EM grade) are used.

(ii) Specimen Drying
Specimens go through a procedure known as "critical point drying," in which all of the ethanol in the specimen is replaced with liquid carbon dioxide under pressure. This process removes moisture from the specimen without altering its features. A critical point drier (CPD) is used for this purpose. It is an automated process that takes around 40 min to complete. Once the dried specimen is taken out, it needs to be kept in a desiccated environment.

(iii) Specimen Mounting
Specimens are mounted on circular metallic stubs with the help of adhesives. An adhesive should be electrically conductive. One may use double-sided sticky carbon tape, copper tape, colloidal silver paste or colloidal graphite.

(iv) Sputter coating
In conventional Scanning electron microscopy specimen is sputter-coated with a metal such as gold or gold/palladium alloy prior to examination in the microscope. Sputter coating is a sputter deposition process which involves erosion of atoms from a target and their subsequent deposition onto the specimen. Sputter coating is performed using an automated sputter coater. Sputter coating is required to prevent the charging of specimen with electron beam and it also increases signal to noise ratio as heavy metals are good emitters of secondary electrons.

2.3.2 Transmission Electron Microscopy

Algal cells have membrane enclosed organelles and a rigid cell wall composed of polysaccharides. Cellular structures such as organelles, which allow the cell to function properly within its specified environment, can be examined at the ultrastructural level using Transmission electron microscope (TEM) (Fig. 12a). Ultrastructure has often been a reliable means of classifying microalgae. Transmission Electron microscopy provides detailed images of intracellular structures at a very high resolution and magnification (Fig. 13d).

Special techniques of thin sectioning are required to prepare a specimen for TEM, as electron beams have a poor penetrating power. In Transmission Electron Microscope (TEM), a focused beam of electrons is transmitted through a thin section of the specimen (around 60 nm). The electron beam then goes through the specimen. Depending on the specimen density, some of the electrons get scattered and disappear from the electron beam. When the electron beam emerges from the specimen, it carries information on the structure of the specimen. The unscattered electrons are magnified by a series of magnetic lenses and are recorded by hitting a

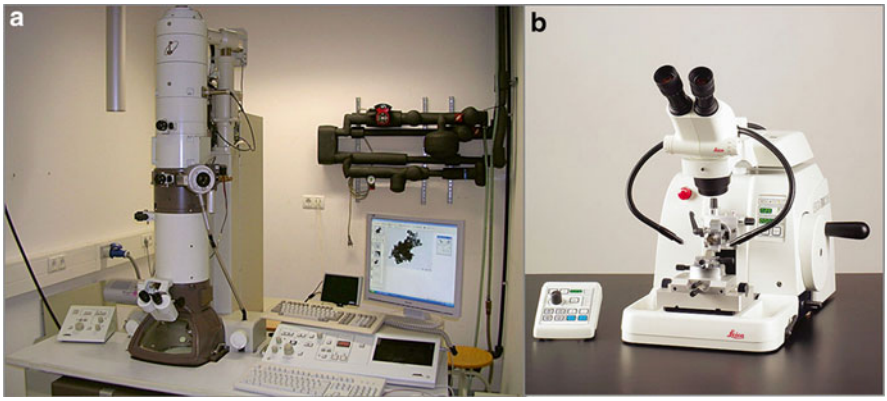

Fig. 12 (**a**) Transmission Electron Microscope, (**b**) An ultramicrotome

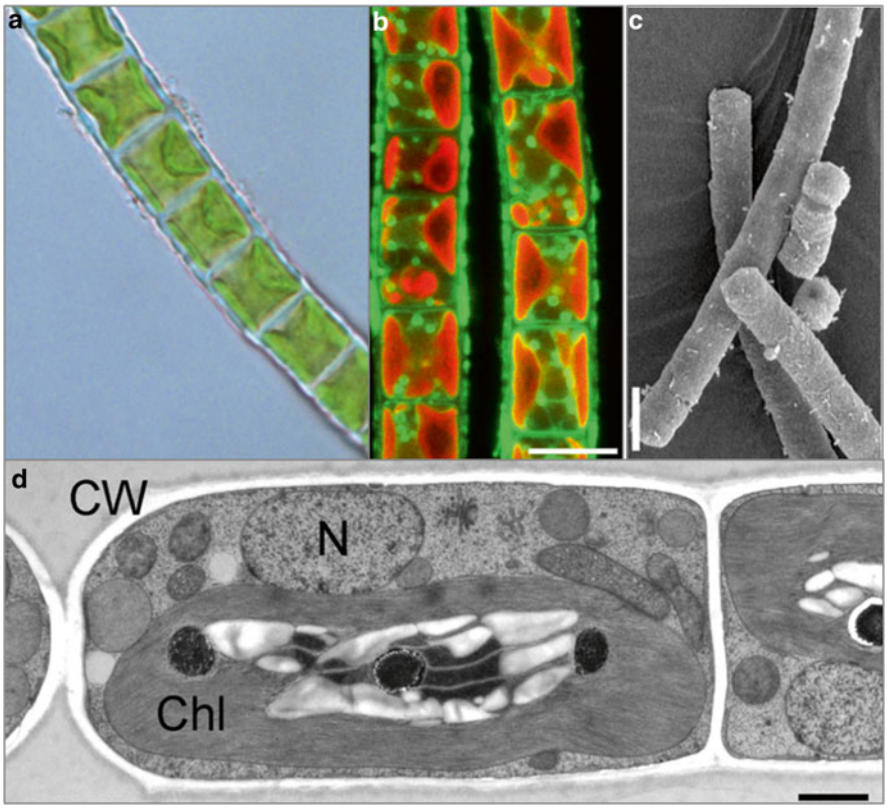

Fig. 13 Comparison of morphology and ultrastructure of an alga *Klebsormidium* as observed by (**a**) Light microscopy, (**b**) Confocal laser scanning microscopy, (**c**) Scanning electron microscopy, (**d**) Transmission electron microscopy (Image courtesy: Prof. Andreas Holzinger, University of Innsbruck, Austria)

CCD (charge-coupled device) camera or fluorescent screen, which produces an image of the specimen with varied regions of darkness depending on the density. To attain adequate contrast, the specimens are treated with compounds of heavy metals such as osmium, uranium, lanthanum, lead or gold (in immunogold labeling). These stains scatter electrons adequately and hence improve contrast.

Sample preparation in TEM

(i) Fixation and dehydration
Specimens are usually treated with 2–5 % glutaraldehyde in cacodylate buffer for primary fixation. The sample is incubated overnight at 4 °C. The specimen is then rinsed a few times in cacodylate buffer. Following primary fixation, the specimen is treated with 1 % osmium tetroxide in cacodylate buffer for secondary fixation. Glutaraldehyde cross links proteins during primary fixation, and lipids tend to be cross linked by osmium tetroxide during secondary

fixation. After fixation, samples are stained with 2 % aqueous uranyl acetate or phosphotungstic acid. The sample then undergoes dehydration through a graded series of ethanol, starting from 50 % followed by 50, 70, 85, 95 to 100 % ethanol. Usually three final changes in acetonitrile are used.

(ii) Infiltration
Infiltration is the replacement of the dehydrating fluid or transition solvent with plastic resin. In the case of Spurr's resin, changes of 1/2 resin (50/50 acetonitrile/spurr's resin), 1/3 resin, 2/3 resin and 100 % resin are used in progression. The purpose of infiltration is basically the complete penetration or infiltration of resin into the specimen. Slow rotation facilitates the penetration of the resin into the specimen.

(iii) Embedding
After infiltration, sample is embedded so that it can be sectioned. Embedding involves placing the sample in liquid plastic formulations or spurr's resin followed by its subsequent polymerization by heat or UV light. Special plastic BEEM capsules or gelatin capsules can be used as molds in embedding. After the polymerization (hardening) of plastic, the sample is sectioned into ultrathin sections and stained.

(iv) Sectioning
Sections are cut with a diamond knife mounted on an ultramicrotome (Fig. 12b). The sections are stretched with chloroform to eliminate compression, and mounted on pioloform filmed copper grids. Sections are stained with uranyl acetate and lead citrate. The grids are examined using a Transmission electron microscope (TEM) and digital images are collected using a CCD camera.

Advances in microscopes and microscopic techniques continue to be introduced to study cells, molecules, and even atoms. Among these are the scanning tunneling microscope, atomic force microscope and immunoelectron microscopy. These are particularly significant for studies of microorganisms at the molecular level.

2.3.3 Scanning Tunneling Microscope

The scanning tunneling microscope (STM) is used for studying the structure of an electrically conductive sample surface at atomic resolution (Fig. 14). STM can image individual atoms on a surface. STM integrates scanning capacity into vacuum tunneling capability. STM works by scanning a conducting tip over the surface to be examined, at a constant spacing. A voltage difference (bias) is applied between the tip and sample surface, which allows the electrons to tunnel through the vacuum between them. Information is obtained by monitoring the resulting tunneling current as the tip's position scan across the sample surface, and is displayed in the form of a STM image. Scanning tunneling microscopy has been used to acquire images at molecular resolution for the kappa- and iota-carrageenan algal polysaccharides, and to investigate the three-dimensional structure of C-phycocyanin (C-PC) isolated from blue-green alga *Spirulina platensis*.

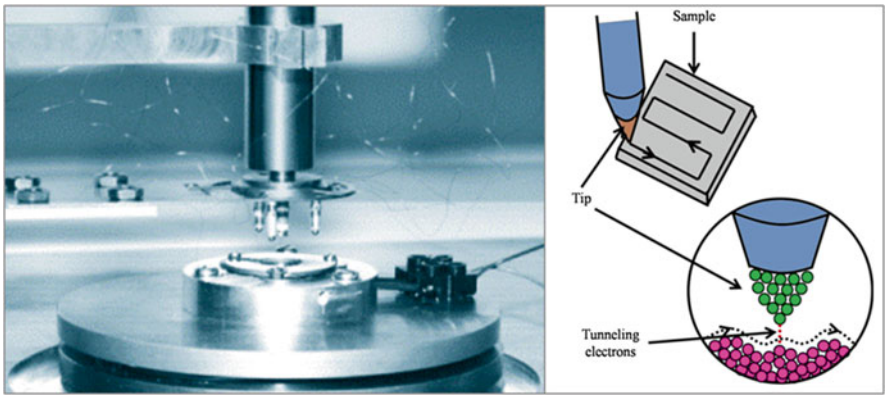

Fig. 14 Scanning tunneling microscope

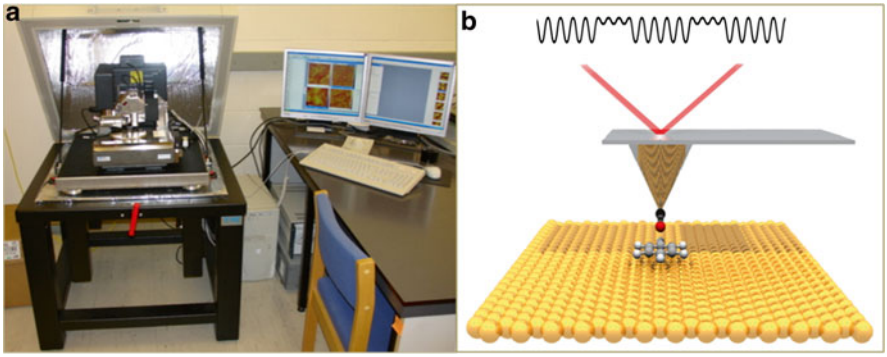

Fig. 15 (**a**) Atomic force microscope (**b**) The tip of atomic force microscope senses the changes in electronic forces as it moves across the sample surface at a constant height. Consequential movements of the AFM stylus are perceived by a laser beam to form images (Picture courtesy: (**a**) Stan Zurek, CC-BY-SA-3.0, Wikimedia Commons)

Atomic force microscopy (AFM) or scanning force microscopy (SFM) was developed as a variant of Scanning Tunneling Microscopy (STM), which makes it possible to study nonconductive or insulating samples (Fig. 15). Atomic force microscopy (AFM) allows the topographic structure of cells to be resolved under physiological conditions, without any fixation and dehydration artifacts. AFM is one of the leading tools for imaging sample at nanoscale (Fig. 16). Light and electron microscopes can produce two dimensional (X-Y) images of a sample surface, with a magnification ~1000× for an optical microscope and ~100,000× for an electron microscope. Nevertheless, these microscopes cannot determine the vertical dimension (z-direction) of the sample, e.g. the height (of particles) or depth (of holes, pits) of the surface topographic features. AFM, which exploits a sharp tip to

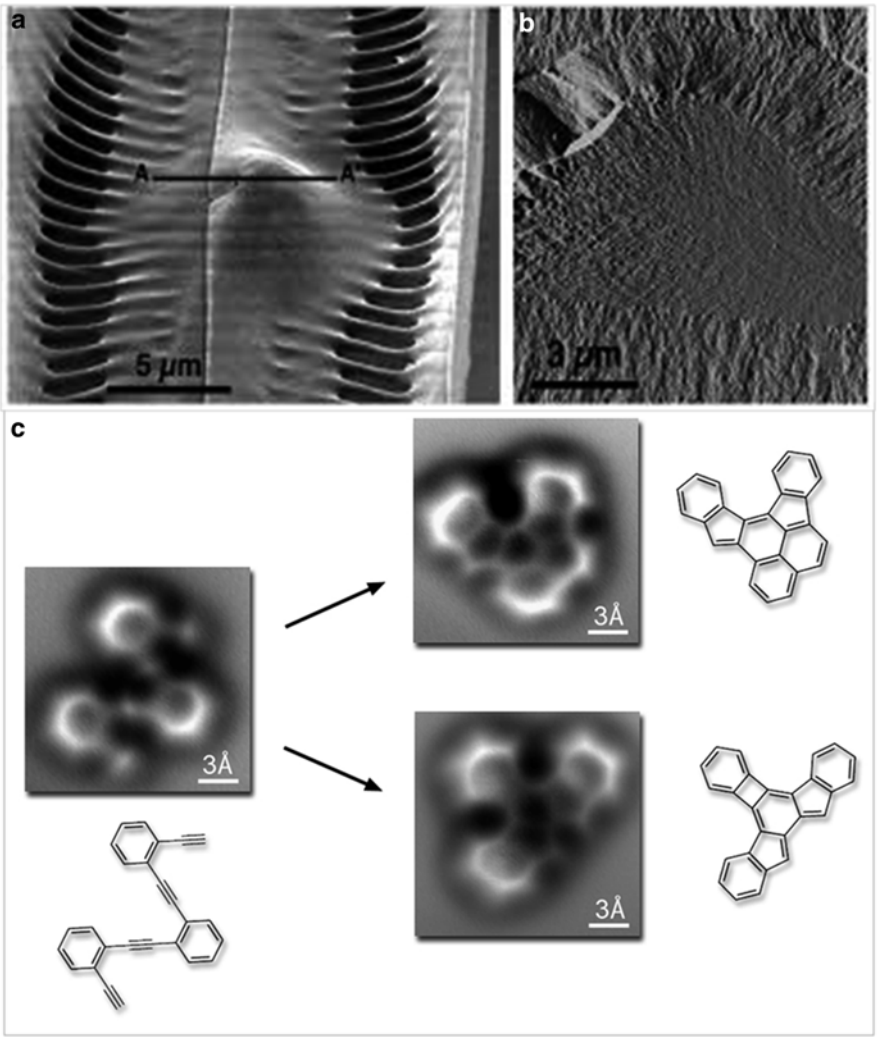

Fig. 16 (**a**, **b**) Comparison of a SEM micrograph and an atomic force microscope image of the diatom *Pinnularia viridis* valve, (**c**) The original reactant molecule imaged through AFM both before and after the reaction. The two most common final products of the reaction are shown (Image courtesy: (**a**, **b**) Dr. Simon A. Crawford, The University of Melbourne, Australia. (**c**) Lawrence Berkeley National Laboratory)

probe the surface features by raster scanning, can image the surface topography with extremely high magnifications, up to 1,000,000×. AFM measurements are made in three dimensions, X-Y, the horizontal plane and Z dimension, the vertical plane. Resolution at Z-direction is usually higher than X-Y. AFM has been used for the imaging of algal cells and for the characterization of the mechanical properties of glycoproteins that have potential utility as adhesives.

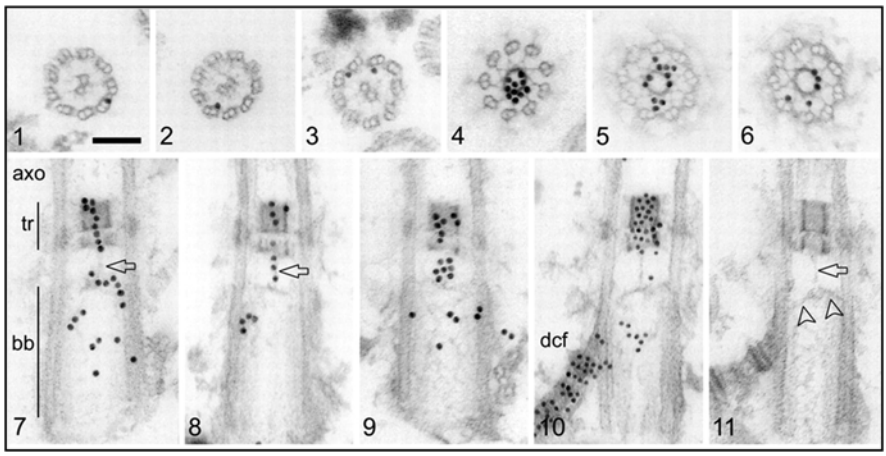

Fig. 17 Immunoelectron micrograph showing labeling of centrin using 15-nm gold particles in the cytoskeletons of *Chlamydomonas reinhardtii* (Image Courtesy: Dr. Stephan Geimer)

2.3.4 Immunoelectron Microscopy

This technique employs the use of antibodies to identify the intracellular position of particular proteins through electron microscopy (Fig. 17). Ultra thin sections of the specimen are labeled with antibodies (produced against the required antigen) conjugated with gold particles. The gold particles make the antibody markers electron-dense. Thus, immunoelectron microscopy appears as a technique that associates biochemistry and molecular biology with ultrastructural studies, by putting macromolecular functions into a cellular perspective. For immunoelectron microscopy cells are fixed and embedded in plastic. The plastic blocks are sectioned into ultrathin sections. The ultrathin serial (sequential) sections are labeled with a monospecific antibody raised against the protein of interest, and subsequently with protein-A gold, creating the antigen–antibody complex which is visible in the electron microscope. The sections are then imaged in the electron microscope.

2.4 Flow Cytometry

Cytometry is the measurement of physical and chemical characteristics of cells or other biological particles (e.g., nuclei or individual chromosomes). Flow cytometry involves the measurement of cells or particles in a flow system or fluid stream as they pass through a laser beam. Flow cytometry achieves multiparametric analysis of cells or other microscopic objects at high speed. Parameters are various physical or chemical characteristics of a cell (e.g. cytoplasmic granularity, cell size, nuclear DNA content, light scattering or fluorescence). Algal cells are inherently fluorescent

Fig. 18 Flow cytometer

but usually different fluorescent chemicals are used to label specific components of a cell which are subsequently excited by a laser to emit light at particular wavelengths. Flow cytometry integrates fluidics, optics, electronics and computational components (Fig. 18).

2.4.1 Basic components of a flow cytometer

(i) Fluidics System Fluidics aligns and carries the cells to laser interrogation point and takes away the waste. It utilizes hydrodynamic focusing to produce a stable particle stream within which cells or particles are aligned in a single file, to facilitate the proper analysis and sorting of cells and particles.

(ii) Optical System In flow cytometry, particles travel one at a time through a laser beam interrogation point. Optical components allow the microscopic particles to be illuminated by one or more lasers (Fig. 19a, b). As a particle passes through the focused laser beam it gives out optical signals or analogue signals (scattered light and fluorescence signals). Optical components resolve and route the optical signals to their individual detectors (photomultiplier tubes) after passing them through suitable filters.

(iii) Electronics Detectors or photomultiplier tubes (PMTs) convert optical signal into electronic signals (voltage pulses). These electronic signals are then converted to channel numbers (a digital value) by Analog-to-Digital Converter (ADC), so that they can be stored for display and analysis on a computer screen. Electronics are involved in signal acquisition, signal processing, computer display and analysis. For flow cytometers equipped with a sorting command (Fig. 20), electronics are involved in the formation, charging and deflection of individual droplets and to perform sorting of objects.

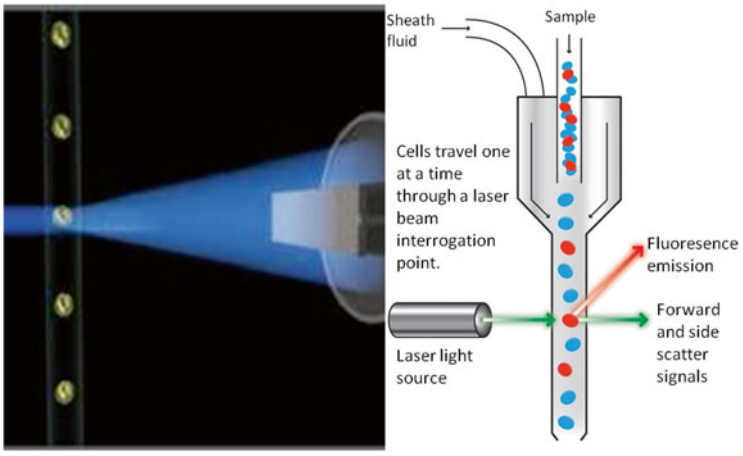

Fig. 19 The fluid stream is illuminated by a laser beam and the fluorescence and light signals scattered by each cell are detected by detectors after passing through filters

(iv) Computational Components Data collection process from samples using the FCM is termed 'acquisition'. Acquisition is performed through a computer coupled to the flow cytometer, and the software which regulates the digital interface with the flow cytometer. Acquired data comprises of individual measurements on thousands of signals, each corresponding to a particle flowing through the flow system. This data is stored according to the flow cytometry standard (FCS) format. Computational components take part in storage, display and analysis of data. A data storage file can be studied to obtain information on diverse cellular properties. These properties include the relative cell size, cell surface properties, granularity (internal complexity), autofluorescence intensity and relative fluorescence intensity of the object. Several commercial softwares for analysis of cell cycle and DNA content etc. are also available.

2.4.2 Flow Cytometry with Activated Cell Sorting (FACS)

Flow cytometry with activated cell sorting (FACS) is a method that allows separation or sorting of an object of interest from a heterogenous population (Fig. 20). Sorting of objects is achieved by electrostatic deflection. In this process a slight vibration is applied on the nozzle to produce small waves on the stream of cells, causing it to break into individual droplets after passing through the laser intersection. An electric charge is placed on the droplets having the object of interest. These droplets are then deflected or diverted by an electric field and collected into tubes based upon their charge, whereas the remaining uncharged droplets go into a waste stream.

Flow cytometry allows rapid automated characterization and classification of phytoplankton assemblages by measuring fluorescence characteristics (emission and light scatter) of individual cells at high speed (Phinney and Cucci 1989). The use of flow cytometry is very promising in the study of cell viability since it is a very

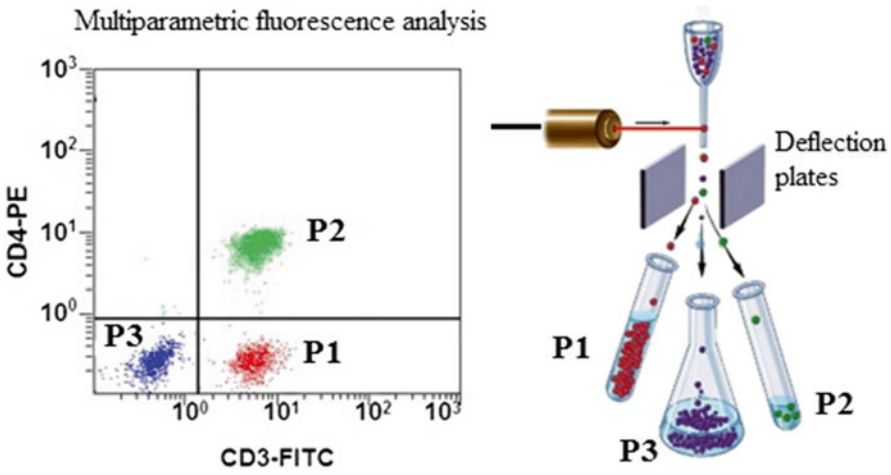

Fig. 20 Flow cytometry with activated cell sorting (FACS)

sensitive method for the quantitative examination of many cellular functions including membrane potential and enzyme activity. Using a flow cytometer it is possible to analyse and quantify a large sample of cells, thereby giving more statistically significant results. It also allows multi-colour fluorescence detection, increased sophistication and speedier data analysis. A flow cytometer can also evaluate the heterogeneity of a cell population due to the different levels of autofluorescence or stain intensity in single cells. Recently, flow cytometers have also been applied to determine dinoflagellate cyst viability in ballast water studies, which was traditionally assessed by microscopic examination of cyst germination over a period of at least 4 weeks (Binet and Stauber 2006). The results obtained using flow cytometry showed a high test precision and were in excellent agreement with the standard germination method.

Conventional flow cytometry analyzes single cells at a high rate but cannot analyse larger cells or chains, and to cover the full-size range of phytoplankton (~1 to several 100 μm) new instruments have been designed. These systems employ the use of an imaging flow cytometer to count, image and analyse the organisms as they pass through the instrument. This new generation of flow cytometers includes FlowCytoBot, which is coupled to an image in-flow system that covers the size range of 10 to ~100 μm (Fig. 1f). The cytometer, CytoBuoy, (Fig. 1h) has been used for the enumeration of cells per diatom chain and the results correlated well, and were much more precise and time efficient, than microscopic quantification (Takabayashi et al. 2006). Another instrument named FlowCAM (Fluid Imaging Technologies; Sieracki et al. 1998), is a portable plankton image analyser which combines the capabilities of both flow and a high resolution digital image cytometer (Fig. 1g). This instrument is of great interest since the analysis time when using FlowCAM is much less than that of microscopic enumeration and it does not require the sample to be fixed.

3 Isolation and Separation of Microalgae

Isolation is usually performed to obtain a pure strain of microalgae or to save a cross contaminated culture of algae. Algal cultures are usually unialgal (containing only one type of alga) clonal populations (a group of genetically identical cells). These cultures may be non axenic (have bacteria, fungi, or protozoa), or axenic (no bacteria, fungi or protozoa). There are five major techniques for achieving unialgal clonal isolates: streaking, spraying, serial dilution, single-cell isolations through capillary pipette and sorting with flow cytometry.

3.1 Streaking

This method is used for the isolation of small species of algae (<10 mm) that grow well on a substrate. Petriplates containing solidified agar growth medium are prepared, and the mixed phytoplankton sample is streaked with the help of a flame sterilized wire loop (Fig. 21). Plates are then covered, sealed and incubated under appropriate light and temperature conditions. Algal colonies are selected and removed from the plate with the help of a sterilized wire loop. These colonies are observed under the microscope to check that the colony is unialgal and the desired algal species has been isolated. Selected colonies are then transferred to liquid or agar growth medium.

3.2 Spraying

In this technique, a fine spray of cells is used for the inoculation of agar plates. Cells in a liquid suspension are sprayed using atomized sterile air so that they get dispersed onto the plate. These plates are incubated under appropriate conditions. Once the colonies have formed, cells are selected, removed and inoculated further.

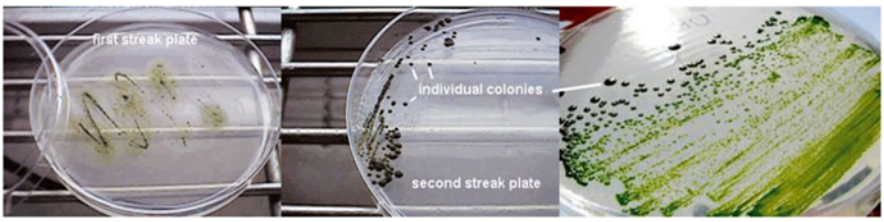

Fig. 21 Isolation of microalgae using streaking

Streaking and spraying techniques are useful for single-celled, colonial or filamentous algae that can easily grow on agar surface. Cultures of phytoflagellates such as *Cryptomonas* and *Chlamydomonas* can also be attained by these methods. Many algae are isolated by single-cell isolations or serial-dilution techniques. The most extensively used technique of single cell isolation is capillary pipette removal.

3.3 Micromanipulation or Capillary Pipette Removal

Capillary pipette removal or micropipette isolation is the most common method for single-cell isolation (Fig. 22). It is generally performed with a glass capillary or a Pasteur pipette. A capillary tube is heated in a fine flame, drawn out or extended, and then broken. The narrow end of the capillary should be approximately twofold the diameter of the algal cell to be isolated. Algal cell to be isolated is located in the drop of enrichment sample using an inverted microscope. While viewing, the cell is sucked up into the micropipette. The cell is then transferred to a drop of sterile medium on agar plate or glass slide.

3.4 Serial Dilution

A serial dilution is a step wise dilution technique which is useful for the organisms that are abundant in water sample. The dilution factor on each and every step is kept constant. This dilution technique is widely used to isolate random algal species

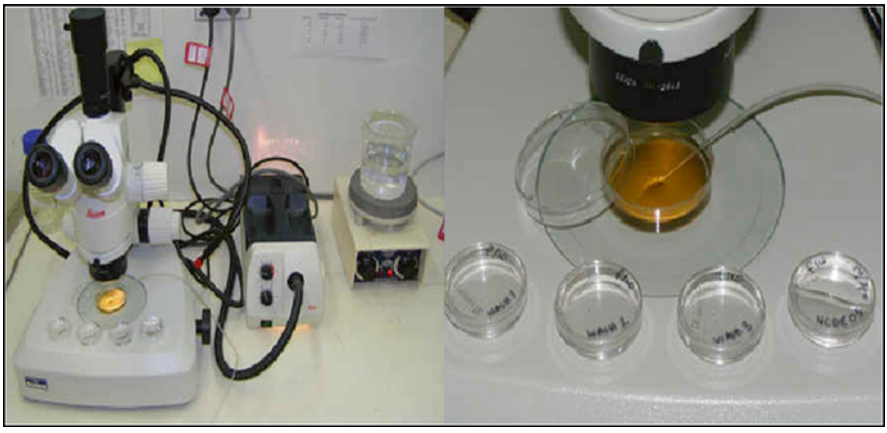

Fig. 22 Capillary pipette removal

present in field samples and consequently new algal species can be discovered. The dilution can be performed with distilled water, culture medium, seawater or filtered water from the field sample.

To perform a tenfold dilution (1 mL scale), 900 μL of diluting media is added into 100 μL of the algal sample by thorough mixing subsequent to each dilution step. Test tubes containing diluted samples are incubated under appropriate temperature and light conditions. Cultures are observed microscopically after 1–2 weeks. Concentrations having higher dilution e.g. 10^{-6} to 10^{-10} are expected to contain unialgal isolates. If the tubes have two or three different algal species, then capillary pipette removal may be used to achieve unialgal cultures.

3.5 Automated Single Cell Isolation

Flow cytometry with activated cell sorting (FACS) is an automated single cell isolation technique that is extensively used for cell sorting. This technique has been effectively used for sorting microalgae from water containing different strains of algae. Sorting is principally based on chlorophyll autofluorescence (CAF) and green autofluorescence (GAF) to discriminate algae such as phytoflagellates, dinoflagellates, diatoms or prokaryotic phytoplankton. FACS is a proficient and sensible tool for the isolation of microalgae from a field sample. Samples are acquired by FACS and several 2-D plots record the distribution of cells in forward scatter or FSC (measures the size of the cell), side scatter or SSC (measures the granularity of the cell) and in all fluorescence channels. Usually the sorting traits are achieved using a dot plot (Fig. 23) which combines inner cell complexity or granularity (SSC) and the chlorophyll autofluorescence of cells.

4 Growth Measurements of Microalgae

Under favorable conditions, microalgae grow constantly by cell division and yield an exponentially growing culture. Growth of microalgae slows down because of the depletion of nutrients and decreased light penetration as the culture becomes more crowded. After reaching their stationary phase for the existing conditions, the density of the cultures will not increase further.

Calculating the growth rates of cells are an important part of microalgal studies. Growth of microalgae can be measured in terms of cell counts in a known volume of water. Counting microalgae requires the most regular lab equipment-a microscope.

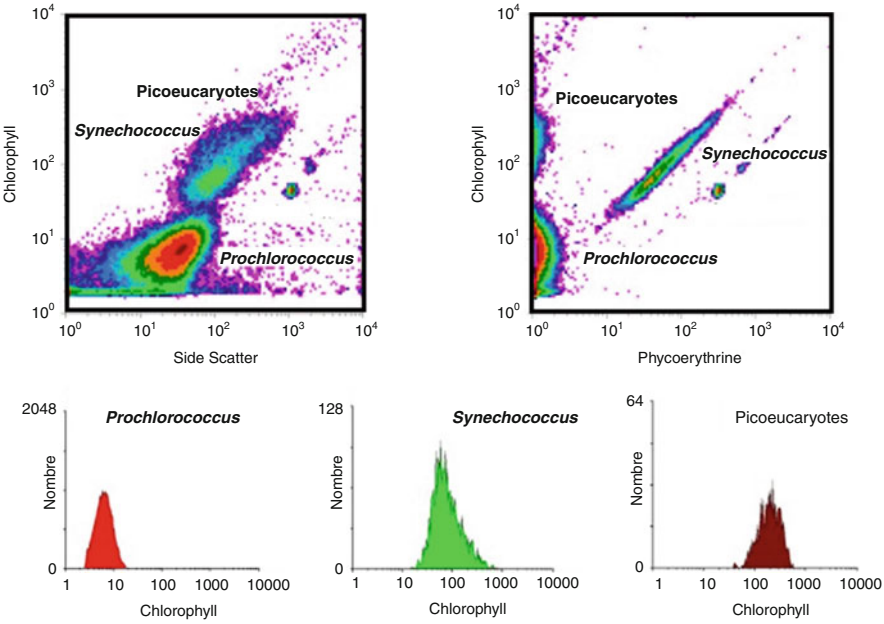

Fig. 23 Flow cytometry *dot plots* (from a sample of photosynthetic picoplankton) and activated cell sorting (FACS) (Image courtesy: Daniel Vaulot, CNRS, Station Biologique de Roscoff, CC-BY-SA 2.5, Wikimedia Commons)

It is performed with "counting chambers". Counting necessitates some knowledge of the algal taxonomy. Algal counts in mixed assemblages (i.e., in field samples or during competition experiments) are performed using Sedgewick-Rafter chamber, Palmer-Maloney Slide or inverted microscope method. Measuring cell density and counting algae in unialgal samples (i.e., during growth and bioassay experiments) requires the use of hemocytometer, coulter counter or flow cytometer. Microalgae contain pigments and increase in their number leads to an increase in the intensity of the colour of the culture which can be easily quantified using a spectrophotometer or fluorometer. Optical density and chlorophyll *a* measurement are generally used for quantification of algal biomass over a growth cycle.

Growth curves are prepared using data obtained from any of the above mentioned methods. Cultures are sampled at definite time intervals, depending on the algal growth rate. The growth curve is plotted using the number of cells or biomass against time (in days). From these curves, specific growth rate and division time can be calculated.

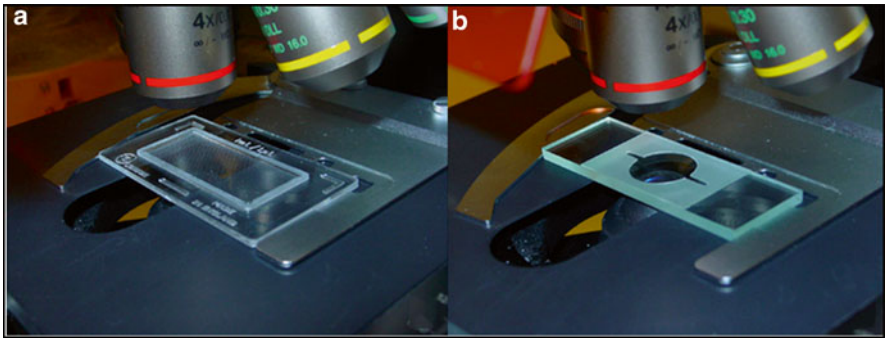

Fig. 24 (**a**) Sedgewick Rafter Chamber, (**b**) Palmer-Malony Cell (Picture courtesy: Agriculture, Fisheries and Conservation Department, HKSAR (Hong Kong Special Administrative Region))

4.1 Counting Cells in Mixed Assemblages

4.1.1 Sedgewick-Rafter Chamber Method

The Sedgewick Rafter counting Chamber is specifically used for the quantitative estimation of cells in a defined volume of fluid (Fig. 24a). The dimensions of this chamber are 50×20×1 mm. The chamber is made up of a grid of 100×1 mm squares. The chamber has an area of 1000 mm^2 and traps a volume of 1.0 ml when a cover glass is placed over it. The chamber is filled with the well mixed sample using a micropipette. A coverslip is properly positioned on the chamber. The sample is allowed to stand for at least 15 min to allow algae to settle to the bottom. Counts are performed using the lower power objectives (4× or 10×) of the compound microscope.

4.1.2 The Palmer-Maloney Slide

The Palmer-Maloney Slide comprises a glass slide onto which is epoxied a circular chamber, of volume 0.1 mL and has two narrow loading channels opposite to each other (Fig. 24b). This chamber is applicable for the enumeration of large algal cells (up to 150 mm) as well as very small nanoplanktons. However, for large algae present at low concentration, it may be appropriate to use a chamber that can hold a larger volume of sample (i.e., Sedgewick-Rafter Chamber).

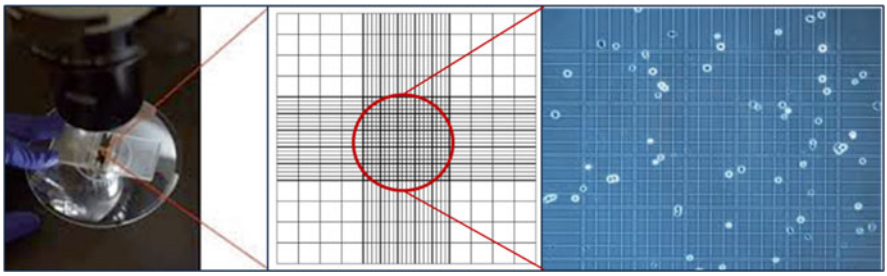

Fig. 25 A hemocytometer with its counting grids

4.1.3 Inverted Microscope Method

Inverted microscope is very advantageous for counting algae in mixed assemblages. In this microscope depth of the counting chamber does not prevent the usage of high power objective lenses. In the inverted microscope method, a known volume of algal sample is added to the chamber and allowed to stand for at least an hour to allow the algae to settle to the bottom. Utermőhl devised a standard settling and enumeration technique using inverted microscope and his own sedimentation chambers. The technique involves the gravitational sedimentation of a known volume of preserved algal samples into an Utermőhl chamber. Subsequently, counts are made on at least 20 random fields and a minimum of 200 cells are counted.

4.2 Counting Unialgal Samples

4.2.1 Hemocytometer Method

In case of unialgal samples, a chamber such as hemocytometer is commonly used to estimate the culture density. The modern-day hemocytometer has a double-chamber layout and counting grids developed by O. Neubauer (Fig. 25). This configuration permits the user to do two cell counts for every sample without cleaning the hemocytometer. The two counting areas or chambers are etched up with grids. Each chamber is composed of nine squares, each square 1×1 mm ($1\ mm^2$). The chamber is 0.1 mm deep. Hence the total area of each chamber is $9\ mm^2$ and the total volume is $0.9\ mm^3$. These nine squares are further subdivided into small areas. The squares at the four corners are subdivided into 16 intermediate squares, whereas the central square is subdivided into 25 intermediate squares. Generally the total number of cells in the central large square is counted. To obtain the total number of cells in this

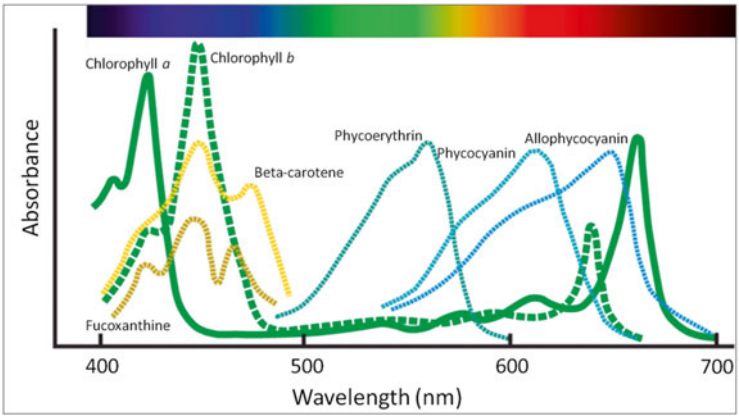

Fig. 26 Absorption spectra for various algal pigments

large square, the number of cells in each of the 25 intermediate squares are counted, recorded then added. The volume of central large square is $1 \times 1 \times 0.1$ mm $= 0.1$ mm^3. Since 1000 mm^3 (0.1 mm$^3 \times 10^4$) $= 1$ ml, multiplication of average cell counts in the central large square by 10^4 will give the cell density per ml. Hence 10^4 is the chamber conversion factor (for improved Neubauer standard hemocytometer).

4.2.2 Electronic Particle Counter (e.g. Coulter Counter) and Flow Cytometer

A Coulter counter is a device for counting particles suspended in electrolytes. In this method an algal suspension is placed inside an electronic particle counter, within which algae are passed through a tiny orifice. This orifice connects the two compartments of the counter which contain an electrically conductive solution. As each alga passes through the orifice, the electrical resistance between the two compartments increases. This generates an electrical signal which is automatically counted. In addition, flow cytometry can also be used to count unialgal samples.

5 Techniques for the Study of Algal Pigments

The major pigments in algae comprise chlorophylls, carotenoids and phycobiliproteins. These pigment molecules absorb light only in the wavelength range of 400–700 nm, this range is referred as photosynthetically active radiation (PAR). Each of these pigments has evolved to absorb only certain wavelengths of visible light and reflect the wavelengths they cannot absorb. Each type of pigment can be identified by the specific pattern of wavelengths it absorbs from visible light, which is the absorption spectrum (Fig. 26). Chlorophyll absorbs light in blue and red region.

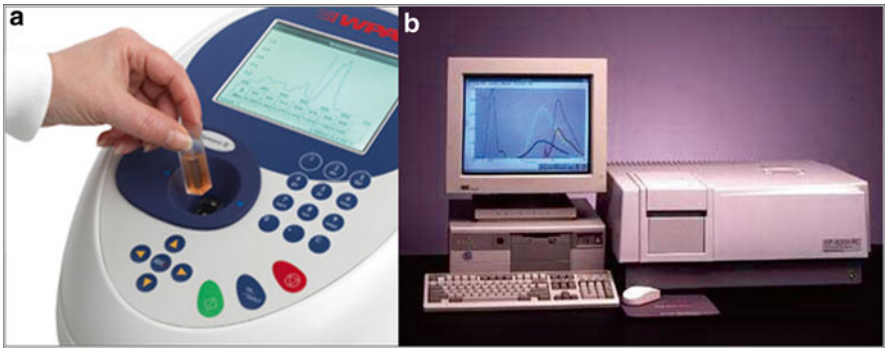

Fig. 27 (**a**) Spectrophotometer, (**b**) Fluorometer

Chlorophyll does not absorb green light; it reflects green light, hence appears green. Carotenoids absorb light energy in the blue-green and violet region. They reflect yellow, red, and orange light, hence appear yellow red or orange to us.

5.1 Spectrophotometric Determination

The types of pigments present in an alga can be determined by using a spectrophotometer (Fig. 27a). A spectrophotometer can differentiate the wavelengths of light a pigment can absorb. Spectrophotometer measures the intensity of transmitted light to determine the absorbance of the sample at that particular wavelength. By extracting pigments from algae and placing these samples into a spectrophotometer, we can identify which wavelengths of light an alga can absorb. Chlorophyll *a* is the major photosynthetic pigment of algae. It has been commonly used as an indicator of biomass or primary productivity. The spectrophotometric methods are routinely used to study the pigment composition in various algal species. However, the spectrophotometric method is not very sensitive and needs a large sample volume. In addition, an appropriate empirical formula for chlorophyll must be chosen (Jeffrey and Humphery 1975; Porra et al. 1989).

5.2 Fluorometric Method

Fluorometry is the measurement of fluorescence. A fluorometer excites the solution and can measure the emission. No two pigments can have the same fluorescence signature (excitation and emission spectra). This principle makes fluorometry an extremely specific analytical technique. The fluorometric method (Fig. 27b) is extensively used for the quantitative analysis of chlorophyll *a* and phaeopigments (non-photosynthetic pigments which are the degradation product of algal

chlorophyll pigments). Phaeopigments are usually formed during and after the formation of marine phytoplankton blooms). In vivo fluorescence measurements allow real-time monitoring of phytoplankton distribution (Fig. 1c, d).

High precision liquid chromatography (HPLC) can be used to discriminate and quantify pigments directly from water samples. HPLC provides detailed information about the composition of phytoplankton communities based on indicative carotenoids (i.e., peridinin for Dinophytes and alloxanthin for Cryptophytes) and some other marker pigments. It is worthwhile to consider the most appropriate technique. At present HPLC is the method of choice for the analysis of marker pigments and for achieving accurate measurements of Chl *a* and a variety of accessory pigments in the extracts of algae.

5.3 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a technique used to separate, identify and quantify the algal pigments in a mixture. High performance liquid chromatography (HPLC) pigment method provides accurate chlorophyll *a* data along with extensive information about the composition of algal communities (Mantoura and Llewellyn 1983). This method is based on the assumption that different algal classes include specific marker pigments. For instance, fucoxanthin is the marker pigment for bacillariophyta (diatoms), zeaxanthin for cyanobacteria (blue-green algae), and chlorophyll *b* for chlorophyta (green algae) (Stauber and Jeffrey 1988; Millie et al. 1993; Jeffrey and Vest 1997).

HPLC apparatus consists of high pressure solvent pumps, sample injector, chromatography column, detectors and a data recording and processing unit (computer) (Fig. 28a, b). In this technique solvent pumps are used to pass a highly pressurized solvent (mobile phase) containing the sample through a specially designed column filled with a solid adsorbent material (stationary phase). All components in the sample interact somewhat differently with the adsorbent material, resulting into different flow rates for various components. This leads to the separation of different components as they get eluted from the column. The time taken for a specific compound to pass through the system (starting from the column inlet up to the detector) is known as its retention time. The detector provides the output to a computer equipped with data acquisition software where the data is recorded and analyzed (Fig. 28c). The resulting chromatogram is used for the identification and quantification of a particular compound (Fig. 28d). The presence or absence of certain pigments helps in differentiating the major algal groups present in natural waters (Bidigare et al. 2005). Therefore HPLC has proved to be a powerful tool to study the processes that have an effect on phytoplankton pigment pool.

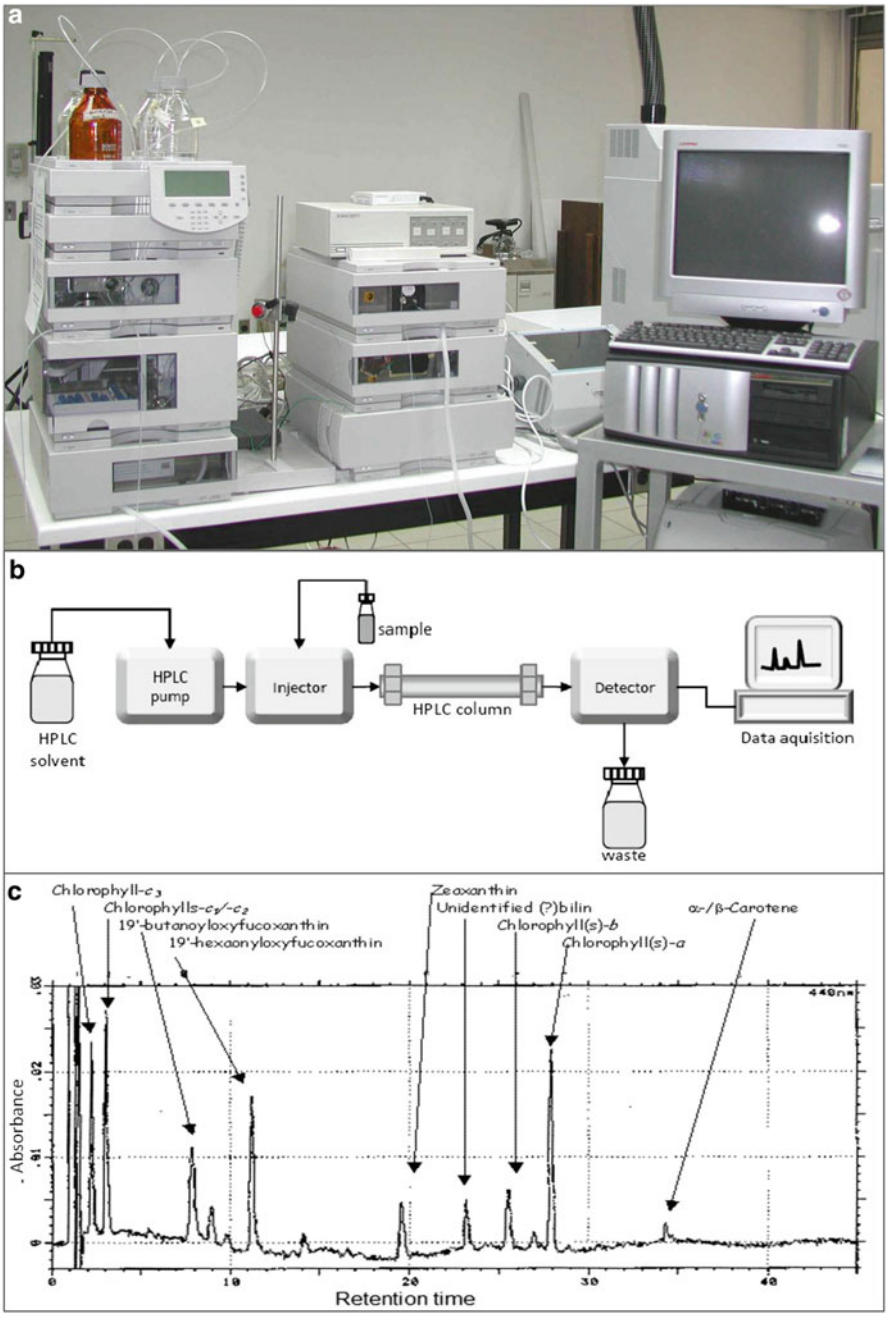

Fig. 28 Pigment analysis using high-performance liquid chromatography (HPLC)

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