

Thin laser light sheet microscope for microbial oceanography

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Abstract: Despite a growing need, oceanographers are limited by existing technological constraints and are unable to observe aquatic microbes in their natural setting. In order to provide a simple and easy to implement solution for such studies, a new Thin Light Sheet Microscope (TLSM) has been developed. The TLSM utilizes a well-defined sheet of laser light, which has a narrow (23 micron) axial dimension over a 1 mm x 1 mm field of view. This light sheet is positioned precisely within the depth of field of the microscope's objective lens. The technique thus utilizes conventional microscope optics but replaces the illumination system. The advantages of the TLSM are two-fold: First, it concentrates light only where excitation is needed, thus maximizing the efficiency of the illumination source. Secondly, the TLSM maximizes image sharpness while at the same time minimizing the level of background noise. Particles that are not located within the objective's depth of field are not illuminated and therefore do not contribute to an out-of-focus image. Images from a prototype system that used SYBR Green I fluorescence stain in order to localize single bacteria are reported. The bacteria were in a relatively large and undisturbed volume of 4ml, which contained natural seawater. The TLSM can be used for fresh water studies of bacteria with no modification. The microscope permits the observation of interactions at the microscale and has potential to yield insights into how microbes structure pelagic ecosystems.

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Introduction

The utilization of optically based remote sensing techniques in the ocean to explore small scale ecological questions to date has been almost nonexistent. This is especially true in the case of underwater microscopy, where we know of no technique, which would permit the study of underwater microbes in their natural setting. Although scientists are starting to unravel the details of the spatial distributions of plants in the ocean at scales of millimeters to centimeters [1,2] the spatial interactions of organisms at a resolution of microns to hundreds of microns are unknown. We report here on a system, which demonstrates first steps for achieving this goal.

Microbes play crucial roles in oceanic biogeochemical cycles through their activities in the production and decomposition of particulate organic matter [3,4,5]. However, microbial oceanographers are bound by technical constraints to study microbes in large samples, tens of milliliters to liters of seawater, which only provide average characteristics of "bulk water" and ecosystem behavior. Until recently, the pelagic microbial environment was perceived as being relatively homogeneous, and governed by diffusive processes. New discoveries suggest that pelagic microbes live in highly structured environments governed by detrital particles [6], gel-like transparent particles [7] and aggregated marine snow [8]. These particles result from microbial activity [9,10,11,12], and are speculated to structure the microenvironment through bacteria aggregation [13,14] and chemical gradients [15,16,17]. Bacteria *in situ* exhibit a spectrum of activity levels, from highly active, rapidly growing to dormant cells [18,19]. To understand the mechanisms that create variability in the distribution, diversity and activity of microbes, a technique that could image specimens in their natural state is very desirable.

A new imaging system is described here which can observe particles at a resolution down to microns or sub-micron that are suspended in a three dimensional transparent fluid. A large field of view, on the order $\sim 1\text{mm}^2$, is desirable to observe spatial interaction between bacteria and larger particles or organisms. An important requirement is that the system should be a true "remote sensor" in that the test volume would not be disturbed during specimen preparation or image collection. One way to achieve this goal is to contain the specimen in a much larger volume, on the order of milliliters. Then, image only the sub-volume under consideration, which would leave the immediate environment of the specimen undisturbed. Under these guidelines, as in our approach, the microscope requirements are a long working distance for the objective lens and the capability to both project light into and capture images out of this sub-volume. Finally, as an additional criterion we note that marine bacteria are capable of swimming very fast ($100\text{'s } \mu\text{m s}^{-1}$) [20]. It is therefore required that the technique be able to take a picture in a short time interval: several milliseconds. Examining the range of potential imaging techniques which could accomplish this goal leads one to consider several types of systems such as: conventional epi-fluorescence, confocal and deconvolution microscopy. Here we review some of their characteristics with special regard to the potential future evolution for an *in situ* underwater microscope, which is the desirable direction of such a system.

In examining the possible use of conventional microscopy, we note that the current practice in bacteria studies is to stain the bacteria with a fluorescent stain (e.g. SYBR Green I) [21] and then filter the bacteria onto a membrane. The particles are then observed using a standard epi-fluorescence microscope. Although these methods have been used for abundance estimation, we strongly suspect that filtering the sample substantially alters any of the spatial relationships that the organisms might have had *in situ*. This technique is therefore unsuitable for our goals.

Confocal microscopy is a tried and true technique [22,23] that has provided excellent images of three-dimensional specimens [24]. There are a number of commercial products that are available and likewise a large number of three-dimensional images exist that have been obtained with these systems. However, confocal microscopy involves scanning the scene on a

point-by-point basis, and is more adequate for non- (or slow) moving objects. So, for example, a state-of-the-art confocal microscope (e.g. Radiance 2100, from Bio-Rad) at its fastest scanning speed takes 250 ms to scan an image with a resolution of 512x512 pixels. Preliminary tests in our group, however, indicate that bacteria motility dictates a much shorter exposure time (on the order of 15 ms, as shown later). Furthermore, the introduction of a beam splitter and a pinhole in the microscope light path results in signal losses, which in turn requires longer exposure times (or slower scanning speeds) when the signal level is low. Nevertheless, in the development of the Confocal theta fluorescence microscopy method [25,26], researchers showed a significant improvement in axial resolution (factor of ~3) when the sample was illuminated perpendicular to the observation axis [27].

A faster confocal microscope (e.g. CARV System from Zeiss) does exist. At its current design, however, it is based on spinning Nipkow disk technology and uses broadband illumination (Halogen or Xenon). Although we have not made a direct comparison of this microscope with our own data (as below) at the least, the system has a degree of mechanical complexity, which makes it unappealing for the ultimate field applications. Secondly, confocal systems are well known for having poor optical efficiency. Methods that utilize light efficiently would seem to hold more promise for viewing smaller targets with less contrast.

An alternative technique, which seems suitable for looking within volumes as well, is Deconvolution Microscopy (e.g. DeltaVision - Applied Precision, Inc.). This instrument works by scanning a three dimensional volume via the collection of a set of serial sections each of which is in focus. The set of images are then combined into a three dimensional volume with consideration of the point spread function of the imaging system and by the use of computer deconvolution techniques. The details of the technology were critically explored in the late 1980's [28,29] and the main limitations of the technique are well known. An unfortunate aspect of deconvolution microscopy is that light from planes that are out of focus is still recorded by the system. This leads to a relatively large direct component level in the image background which decreases the effective dynamic range of the recorded scene and makes it more difficult to sense smaller, lower contrast particles, as demonstrated below.

Nevertheless, deconvolution processing (or image restoration) is a valuable tool applicable to images acquired with a standard microscope (e.g. MicroTime from VayTek Inc.), which may be incorporated to enhance image-quality in the system proposed here as well. Standing wave fluorescence microscopy (SWFM) [30,31] is another technique that provides excellent axial resolution (~50 nm), however SWFM is best suited for very thin, fixed samples. Note also that there have been other modern advancements in microscopy, which are not reviewed here [32]. Although some of these systems may have some capability in satisfying the requirements considered above, the new design presented here is extremely simple and has the capability of retrofitting existing conventional microscopes with only a new illumination system.

Material and Methods

The quality of an image viewed through a microscope objective is mainly determined by the sharpness of the objects that are in focus and the amount of background noise in the scene. In standard epi-fluorescence microscopy the excitation light uniformly illuminates the samples. The observed image is then characterized by the microscope objective's depth of field (δ), defined as the thickness of specimen in focus under the microscope objective lens. When imaging particles whose size is smaller than δ , those located within δ will be in focus while particles that are above and below δ will appear blurry and contribute to the background noise in the image. In order to improve this situation and to design a geometry that seems simple enough for eventual sea-going deployment we propose a novel modification. The new design uses a well-defined thin laser light sheet, positioned in the microscope field of view such that only the area defined by δ is illuminated. The advantages of thin light sheet

microscopy are two-fold: First, it concentrates the light only where the light is needed, thus maximizes the efficiency of the excitation source. Second, and most important, this technique minimizes the level of background noise while at the same time maximizes image sharpness. Since particles that are not located within δ are not illuminated, they do not fluoresce. This outcome is similar to the concept of the Two-Photon Fluorescence microscope [33] but we believe that our implementation is far less complicated and most importantly - does not require scanning. A prototype system that demonstrates the capability of the new design was built and a pilot study was performed on cultured microbes and natural seawater samples.

Thin Light-Sheet Microscopy (TLSM) prototype system: Figure 1 shows an overall schematic of our proposed system and illustrates our concept and the necessary components.

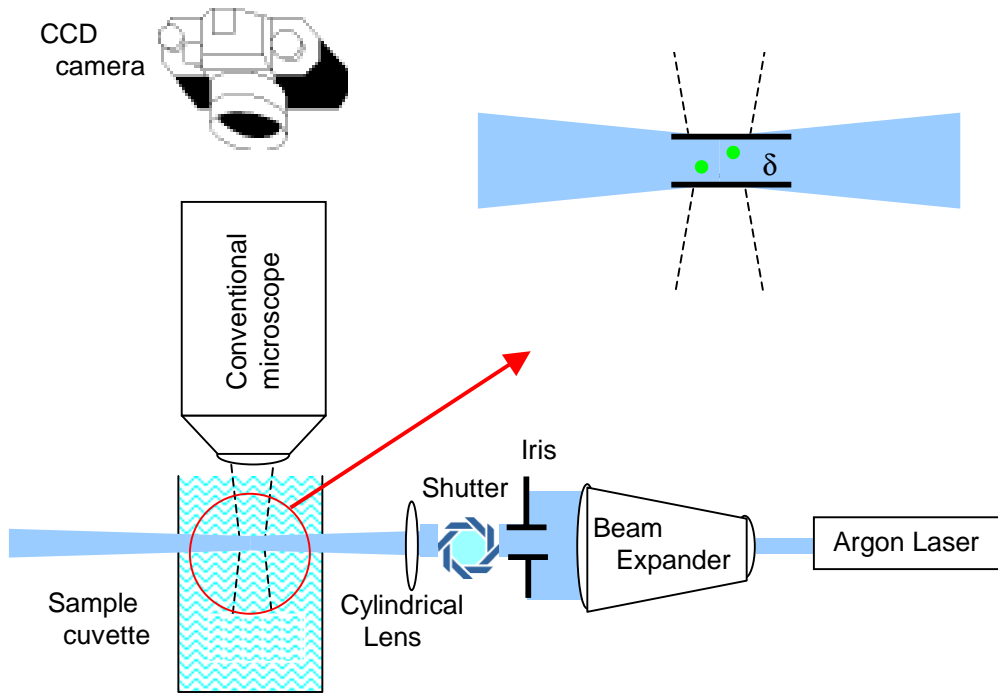


Figure 1: Thin light sheet microscopy (TLSM), schematic diagram.

A thin beam of light is projected into the field of view of the microscope at the appropriate focal plane. In the case that the light sheet is thinner than the depth of field of the microscope all of the specimens that are illuminated are in sharp focus. Moreover, in contrast to the deconvolution microscope, there is not a gradient of defocused images, which are superimposed on one another since our target is not to illuminate the sample beyond the depth of focus of the objective lens. An estimate for the thickness of the desired beam is first required. To calculate δ use [34]:

$$\delta = \frac{n \lambda_0}{NA^2} + \frac{n e}{NA M}$$

where n is the medium refraction index, λ_0 is the light wavelength (in vacuum), NA is the objective's numerical aperture, e is the smallest distance that can be resolved by a detector located in the image plane of the microscope (for the case of a CCD sensor, e is the spacing between pixels, or pixel size [35].) and M is the total magnification of the system. We used a 10x objective with $NA = 0.22$, which coupled with the microscope's internal optics results in total magnification $M = 125$. Let $n=1.33$ for seawater, and $\lambda_0 \approx 540$ nm. A state-of-the-art CCD camera (e.g. Roper CoolSnap HQ or Hamamatsu Orca ER) has $e \approx 10$ μm , yielding $\delta \approx 17$ microns. This is our target for the light sheet thickness.

Due to physical constrains, the cylindrical lens had to be positioned more than 100mm away from the microscope. We thus used a lens with a focal length $f=150$ mm (Coherent, 23-7479). The adjustable iris (SM1D12 from ThorLabs, Inc.) diameter was set at $\varnothing 6.5$ mm, yielding $f/\# = 23.08$. The beam spot of a diffraction-limited lens can be calculated using [36]:

$$d = K \cdot \lambda \cdot f/\#$$

where K is a constant dependent on the pupil illumination. $K = 1.69$, which approximates uniform illumination yields $d_{1/e^2} = 21$ microns, which the expected width for our light sheet. d_{1/e^2} is defined such that the intensity cutoff is set to 13.5% ($1/e^2$) of maximum intensity. We used an argon ion laser (Nexel 95, 488nm) in conjunction with a 10x beam expander (BE10 from ThorLabs, Inc.). The iris was positioned in the expanded laser beam to select a uniform section, producing a 0.4W, near-planner collimated beam.

In order to measure the thickness of the laser beam, a custom setup was used. A quick survey of laser beam analyzers (e.g. BeamStar, Ophir Optronics, Inc.) revealed that the power density of the direct beam at its focal point (approximately 300 W cm^{-2}) will overwhelm the optical sensor (a CCD chip) of such a diagnostic system. Furthermore, these systems offer resolution of no better than about 10 microns. Hence we decided to build a custom system to measure the beam thickness. We used a 2-axis micro-positioning translation stage (with a resolution of 1.0 micron; ST1XY-D from Thorlabs, Inc.) to position a 4.0 micron core-diameter fiber optics (FS-SN-3224 with $NA=0.12$ from 3M) such that the fiber optics end travels through the tested beam. Light emerging from the fiber was recorded with a Photo Multiplier Tube (PMT; Hamamatsu, HC-125) which measured light intensity as the fiber traveled through the beam at various cross-sections. Note that the 4.0 micron diameter of the fiber and its angle of acceptance act as a "spatial smoothing filter", thus the measured results are expected to be slightly larger than the actual beam size. To estimate the true beam size we modeled the measured beam thickness as a convolution of a normal distribution function (simulating the laser beam shape) and a trapeze-shaped filter (simulating the fiber entrance). By performing numeric deconvolution (using MATLAB software [37]) we were able to estimate the measured result by isolating the "filter effect".

Figure 2 shows the outcome of this analysis. The measured beam thickness, d_{1/e^2} , was less than 25 microns for approximately 1mm in length. By applying the deconvolution analysis described above, the beam thickness was corrected to 23 microns - very close to theoretical value that we calculated (21 microns). We conclude that, when well positioned, the thin part of the light sheet can uniformly illuminate the entire field of view while only slightly extending beyond the microscope depth of field. We also note that this measurement was done in air while the final experiment will be done in seawater. Nevertheless, we expect little to no deflection of the beam in the cuvette, as the light is practically perpendicular to the face of the cuvette at the point of entry.

We next incorporated the light sheet to the microscope, and the thin portion of the beam was positioned such that it covered the depth of field of the objective lens. Images were captured with MetaMorph software (version 4.63) through a Roper CoolSnap HQ CCD camera mounted on an Olympus BH-2 microscope that was fitted with a Zeiss 10x objective

($NA=0.22$). Coupled with the microscope's internal optics, the total magnification was $M=125$.

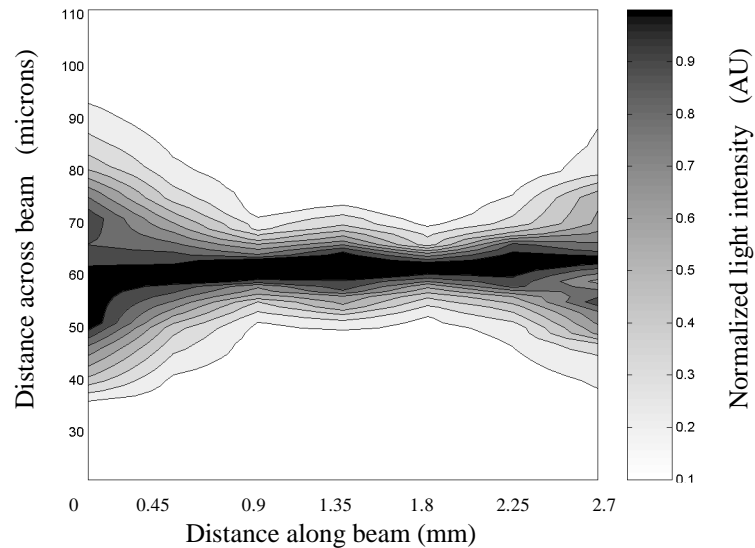


Figure 2: Thin light sheet – beam characteristics.

The high power laser beam used in the prototype (0.4W) caused local heating in the sample, which introduced heat convection (thus bulk water motion) in the 4ml glass cuvette. This intrusive local heating is inevitable as long as the beam is projected into the water due to the high concentration of optical power at a small spot. However, this effect can be minimized. To shorten the exposure time of the sample to local heating we placed a shutter in the beam pathway and synchronized it with the camera. The sample is only exposed to the laser beam while the shutter is open and the camera captures an image, thus the “preheating” of the specimen is avoided. The very short exposure time required in our application, (~ 15 ms), suggests that even at a relatively high scanning rate of one scan per second, the “on” duty cycle when the sample is exposed to the illumination light is very low (0.015), and subsequent heating is minimized.

Testing the TLSM. Several experiments were conducted to test and demonstrate the potential capabilities of the system. The first experiment was done to determine if bacteria are visible with the system with its relatively low magnification. Seawater was collected off Scripps pier on 10 July 01 and filtered through a $3.0 \mu\text{m}$ polycarbonate filter (Poretics, Livermore, CA, USA). In this sample we expect primarily bacteria-size or smaller (viruses) organisms to be present, but smaller protists may also pass through the filter. Sub-aliquots were stained with SYBR Green I (Molecular Probes, Eugene, OR, USA), a nucleic acid stain, and transferred to a glass cuvette. Images were recorded using the TLSM. To test if we could quantify bacteria with the TLSM, we compared it with the standard filtration method for the enumeration of bacteria. A parallel sample of the $3 \mu\text{m}$ filtrate was stained with SYBR Green I and filtered onto a $0.2 \mu\text{m}$ polycarbonate filter, then mounted on a microscope slide for standard enumeration [21].

To demonstrate the capability of viewing and documenting different microbes (e.g. bacteria, protozoa and phytoplankton) that cover a variety of sizes and a relatively large

viewing area (approximately 850×1100 microns = 0.93 mm^2), unfiltered seawater was stained with SYBR Green I and observed with the TLSM. An additional experiment was performed to further characterize the intrinsic advantages of the TLSM for resolving individual bacteria in suspension. Four different motile marine bacterial isolates were grown in ZoBell broth (5 g of peptone and 1 g of yeast extract in 1 liter of seawater that were filtered through a GF/F filter – glass microfiber filter with nominal pore size of $0.7 \mu\text{m}$) overnight and then pelleted and washed with $0.22 \mu\text{m}$ filtered autoclaved seawater (FASW). The cells were resuspended in the FASW and incubated for two days. During this time the isolates' size was reduced from $\sim 1\text{-}2 \mu\text{m}$ to less than $1 \mu\text{m}$, closer to that of bacteria in seawater assemblages. Samples were stained with SYBR Green I. We first simulate epi-fluorescence microscopy by uniformly illuminating the sample with an expanded, uniform laser beam with a diameter of $\text{\O}6.5\text{mm}$ (the cylindrical lens in Figure 1 was removed). Next, the exact same setup and the same sample were used with one exception: the addition of the cylindrical lens, which concentrates the expanded beam and places the thin sheet of light precisely at δ . Camera setup remained constant in both configurations.

Results

Results are presented in the following figures, which demonstrate the resolving capability of the TLSM. Individual bacteria were clearly discernible (Figure 3a). We empirically determined the optimal exposure time to be 15 ms, as this exposure time captured enough light for detection and provided discrete identification of the bacterial cells with little blurring.

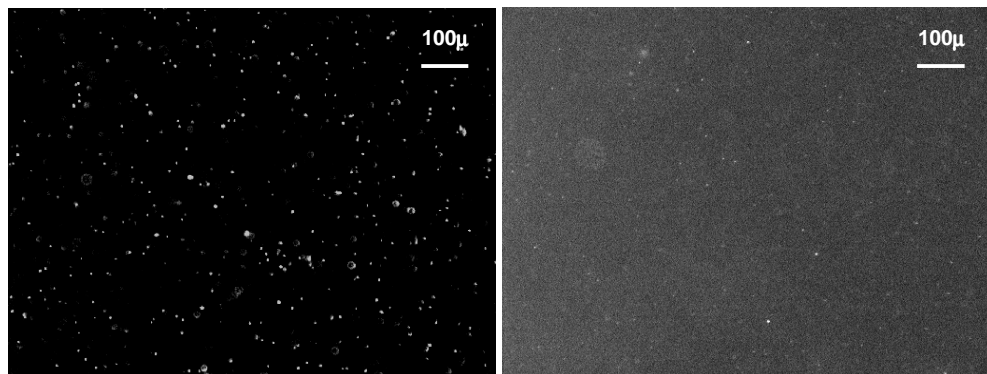


Figure 3 (a): Sample illuminated with the thin light sheet illumination. (b) Same sample with a broad beam illumination. Marine bacterial isolates in $0.22 \mu\text{m}$ filtered seawater, stained. Particle size cannot be scaled due to sensor blooming. Particles appear about 1.7 times larger than their actual size.

To verify that the number of bacteria observed in the image was similar to the actual bacteria abundance in the sample, we compared parallel natural seawater samples. Standard epifluorescence microscopy enumeration gave bacterial abundances of $12.2 \times 10^6 \text{ ml}^{-1}$, while $8.1 \pm 0.8 \times 10^6 \text{ ml}^{-1}$ bacteria were measured from the images captured by TLSM. This discrepancy may arise from several possible sources: shadowing of one bacteria by another, merging of neighboring bacteria that look like a single dot in the image, and lack of detection of bacteria that were so small that the exciting illumination was not sufficient to generate enough fluorescence photons for detection by the CCD camera.

We examined the advantage of illuminating the sample with a thin sheet of light versus uniform illumination (similar to the illumination concept in standard epi-fluorescence). Figure

3a was captured with the thin sheet of light placed exactly at δ , while Figure 3b was captured using a $\varnothing 6.5\text{mm}$ laser beam to uniformly illuminate the sample. Images taken with the thin light sheet in place (e.g. Figure 3a) were clearly sharper than when the samples were illuminated uniformly (e.g. Figure 3b) and individual bacteria could easily be discriminated. The density of bacteria visible in Figure 3a ($7.8 \text{ bacteria/cm}^2$) was 2.2 times higher than in Figure 3b ($3.6 \text{ bacteria/cm}^2$), determined by counting visible bacteria per unit area in the images collected (data collected from 40 images), and closer to the actual number as determined by standard enumeration.

To investigate the TLSM's ability to observe organisms of different sizes, we examined total seawater stained with Sybr Green I. Organisms of several size scales were visible (Figure 4). We used a standard epi-fluorescence microscope with higher magnification to study parallel samples (not shown) and concluded that the largest objects ($\sim 50 \mu\text{m}$) are likely to be dinoflagellates, specifically *Lingulodinium polyedrum*. Organisms with sizes on the order of $\sim 10 \mu\text{m}$ are heterotrophic flagellates, while the $\sim 1 \mu\text{m}$ organisms are predominately bacteria. The large organisms in our images appear approximately 1.7 times larger than their true size due to sensor blooming. Although an undesirable effect, we "endure" the sensor saturation from larger organisms in order to detect the fluorescence signal emerging from the much smaller bacteria.

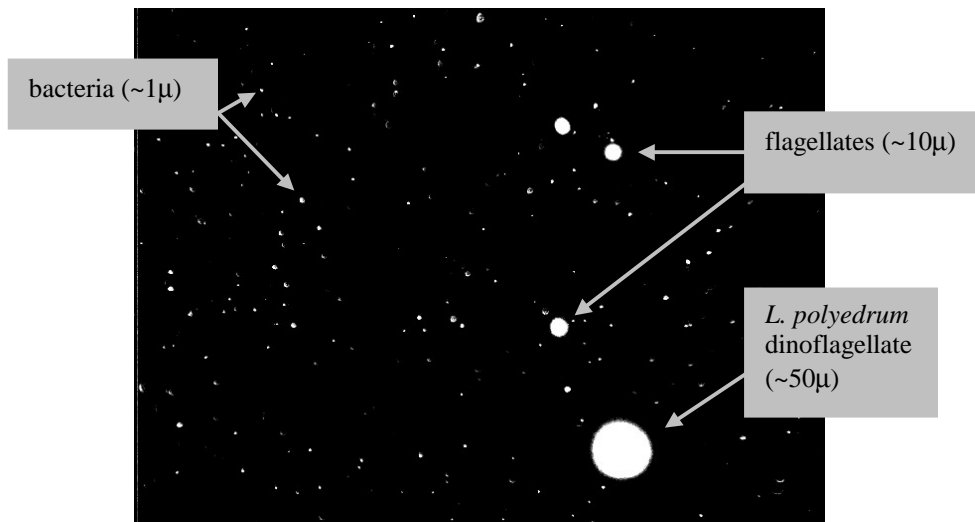


Figure 4: Different size organisms imaged with the TLSM of total seawater. Particles appear about 1.7 times larger than their actual size.

Discussion

Using standard optical components, a thin sheet (approx. 23 micron thick, 1 mm long, 6.5mm wide) of blue (488nm) laser light was produced, diagnosed and positioned precisely at the location of the depth of field (δ) of a standard microscope. The benefit of such a system is its sharp contrast images that result from delivering excitation light only to the portion of the sample that is in focus. The advantages of such a system over existing microscopy techniques for observing microbes suspended in seawater are its simplicity and high concentration of excitation power. A prototype system was presented here to demonstrate the potential use of the technique as instrumental in the study of marine bacteria in their natural environment.

Figures 3 and 4 clearly show the capability of the system to detect, discriminate and document organisms ranging from micron size (bacteria) to large particles (50 μm dinoflagellate), using SYBR Green I as a fluorescence marker. Note that although the diameter of the diffraction-limited spot for a 0.22 NA lens is about 3 microns (first dark ring of the point spread function), smaller bacteria can be detected, since they act as fluorescence point sources that emit photons and are bright enough for the CCD camera to detect. The bacteria density estimated from those visible in the images captured by our system ($8.1 \pm 0.8 \times 10^6 \text{ ml}^{-1}$) are lower, but reasonably close to the number that was enumerated by standard techniques from parallel samples ($12.2 \times 10^6 \text{ ml}^{-1}$). Images recorded by the TLSM showed sharper images and produced estimates of bacteria density 2.2 times higher (and closer to the actual number) compared with images that were captured using uniform illumination from the same sample.

Natural fluorescence was also clearly detectable to a human observer through the microscope oculars. Natural autofluorescence from chlorophyll *a* was visible in red ($\sim 680 \text{ nm}$) and easily differentiated from the green fluorescence emission of the SYBR Green I marker. In order to record and distinguish between the natural (red) and artificially marked (green) pigments, digitally, the camera/filter setup should be modified. Replacement of our black and white camera with a color one should permit us to image this color differences.

The new microscope technology presented here has the potential to make fundamental contributions to microbial oceanography, by making accessible an essentially unexplored realm of marine ecosystems to quantitative studies. Studies of microbial distribution and interactions at the microscale should yield novel insights into how microbes structure pelagic ecosystems. Such knowledge should contribute to an integration of the roles of marine microbes in mechanistic models of the ocean's biogeochemical dynamics and ecosystem structure and function.

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