

Advanced light Photomicrography *

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Abstract

Photomicrographs of phytoplankton in the literature often lack depth of field. This article describes how to overcome this disadvantage. Furthermore, there are some techniques to suggest how one can enhance contrast and resolution of the images. Essentially, you have to switch to digital photography with all its advantages to enhance the images by stacking, stitching and contrast correction. DIC is very useful to get higher contrast with low silicified objects. Short wave, monochromatic illumination can improve the resolution distinctly.

Introduction

Most National and International Publications of phytoplankton use photomicrographs to support the description and determination of their genus and species. Unfortunately, the objects to be photographed for a publication, are complex structures. This is especially true of radiolaria and many diatoms. Using a microscope for visual observation one is always changing the focus up and down to get an overview of the object. This is not practical with normal photography. When shooting an image you will see only parts of your specimen, which will be represented by the depth of field of the microscope objective. This means you may not see the entire object in a photomicrograph. The higher the numerical aperture (NA) of an objective, the smaller is the depth of field and less detail of the entire objects can be seen. This article will show how to overcome this problem and, in addition, how to increase the resolution of a photomicrograph.

Getting better depth of field and contrast

Physically it is not possible to enhance the depth of field of objectives without decreasing their magnification and resolution. Using digital photography, however, allows us to virtually overcome this problem. The highest resolution of the images will be maintained.

The solution consists of two steps:

First you shoot an adequate number of images with succeeding change of focus e.g. beginning with the highest focus and lowering it step by step. Next, you digitally process the so called Z-stack of images with appropriate stacking software [1]. This software overlays all the images and combines them into one, single, image of deep focus.

If one wants the highest resolution, the use of high aperture and magnification objectives is essential. This means a smaller field of view with respect to the size of the camera

sensor. In this case, it is possible to combine several stacked, partial images, of the object by so called stitching. Sticking software may be delivered with the software-package by your camera manufacturer or downloaded e.g. from Microsoft [2].

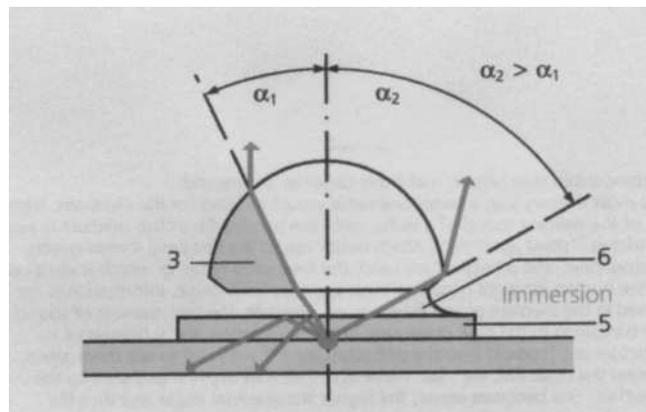
With low silicified objects, using Differential Interference Contrast (DIC), is very helpful.

Physical background to microscopical resolution

The resolution of a microscope depends, among other things, on the NA of the objective and the condenser. The NA of an objective is defined as:

$$NA = n * \sin(\alpha)$$

with α = half of the opening angle of the objective and
 n = refractive index of the immersion medium used between the objective and the coverglass of the microscope slide.



Besides the NA of objective and condenser, the wavelength of light is very important for the resolution. The resolution can be calculated by the formula [3] below:

$$d_o = 1.22 * \lambda / (NA_{obj} + NA_{cond})$$

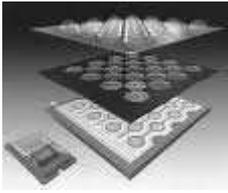
with λ = light wavelength.

If the objective and condenser NA is higher than 0.9 , both optics must be immersed with an appropriate oil to get the maximum resolution.

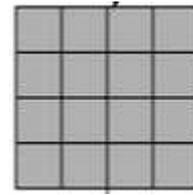
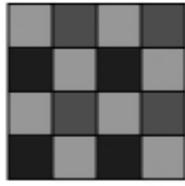
The above formula means: the shorter the wavelength, the better the resolution d_o .

The total resolution, however, is influenced by the camera chip with its pixel construction. The chip consists of a matrix of light sensitive sensors.

We must decide between digital colour cameras and black-and-white cameras. Besides the light sensitive pixel layer, the colour camera has an additional filter layer, the Bayer mask, which makes the sensors beneath to react to the filtered wavelength, only. A Bayer mask consists of 4 red, 4 blue and 8 green filters on an area of 16 pixels. The higher number of



Colour camera chip with Bayermask



BW camera chip

green filters is due to human eye's sensitivity to green. If you want to use monochromatic, violet or near ultraviolet light, colour cameras are useless because the blue filters will pass light blue only. Thus, you may only use dedicated Black & White cameras, which don't have a filter layer.

Total magnification of the intermediate image of the microscope must be optimised according to the pixel size of the camera chip and the resolution of the microscope objective. The resolution distance d_o should cover at minimum, 2 (Nyquist criterion) or better, 3 or 4 pixels.

Example:

- total magnification = $64 * 1.25$; pixel size = $4.6 \mu\text{m}$;
- NA of objective and condenser = 1.4; $d_o = 0.18 \mu\text{m}$ at wavelength of 420 nm
- d_o in the intermediate image = $14.4 \mu\text{m}$, thus d_o is covered by 3.1 pixel being optimal.

Enhancing the microscopical resolution in practice

With respect to the above mentioned basics you can optimize the resolution of your photomicrographs by use of:

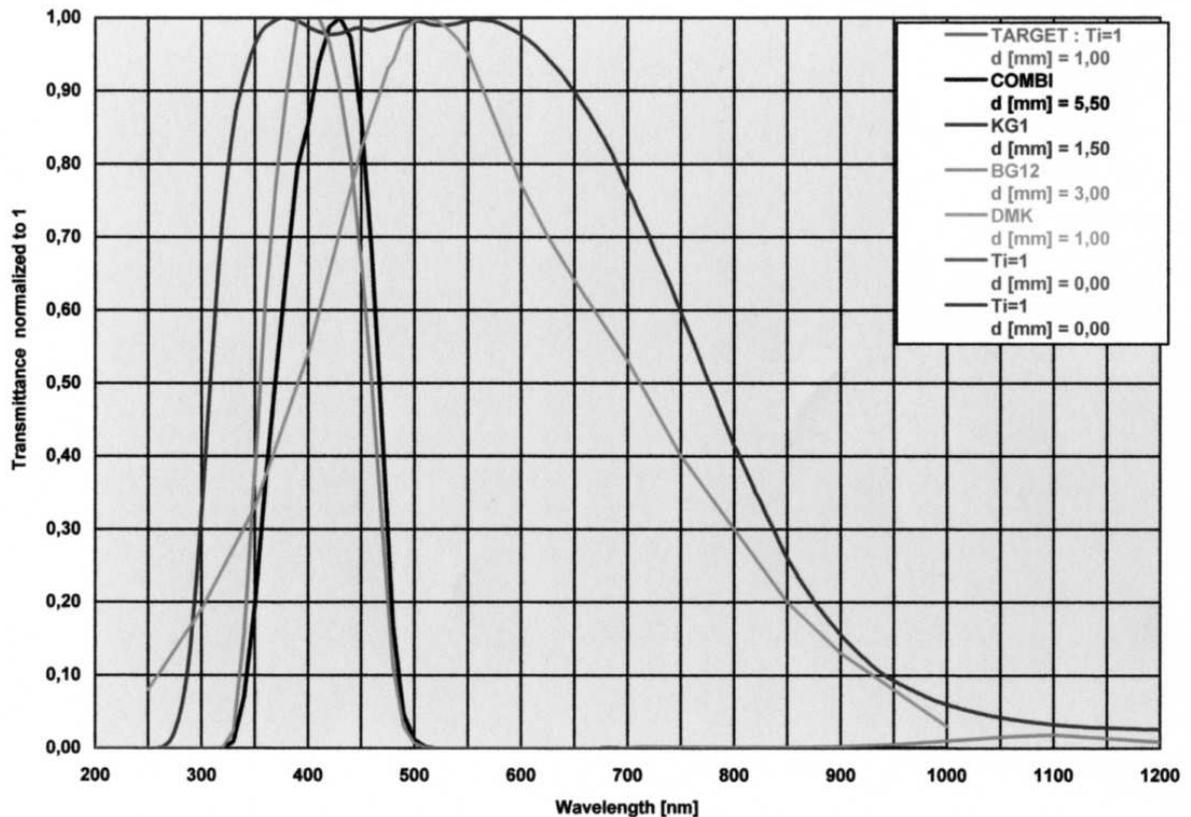
- objectives with highest available NA and a corresponding condenser
- digital Black & White camera
- nearly monochromatic light of a short wavelength enabling the objective to deliver pictures free of reasonable optical failures.

Light filtering

To get a nearly monochromatic, intense, short wavelength light, you have to use illumination with appropriate output of the preferred wavelength (Halogen, Xenon or LED lamp).

Choose a bandpass filter e.g. in the violet in combination with a heat filter, which attenuates the infra-red to which CCD-chips are reasonably sensitive. An example is shown in the graph below.

You will find the attenuation graphs of the two filters BG12 (violet) and KG1 (heat prevention) and the sensitivity of the BW camera (DMK). The intense, black curve, shows the resulting bandpass. Narrow bandwidth illumination is essential otherwise less resolved images of longer wavelengths will blur the higher resolved one.



With older microscope systems which have no infinity objectives, monochromatic light is unavoidable if you photograph the intermediate image. Objective types of 160 mm tubelength have an inherent constant colour magnification error which will be corrected by the eyepiece. That failure is true for infinity objectives too, but is corrected by the tube lens of that system.

Conclusion

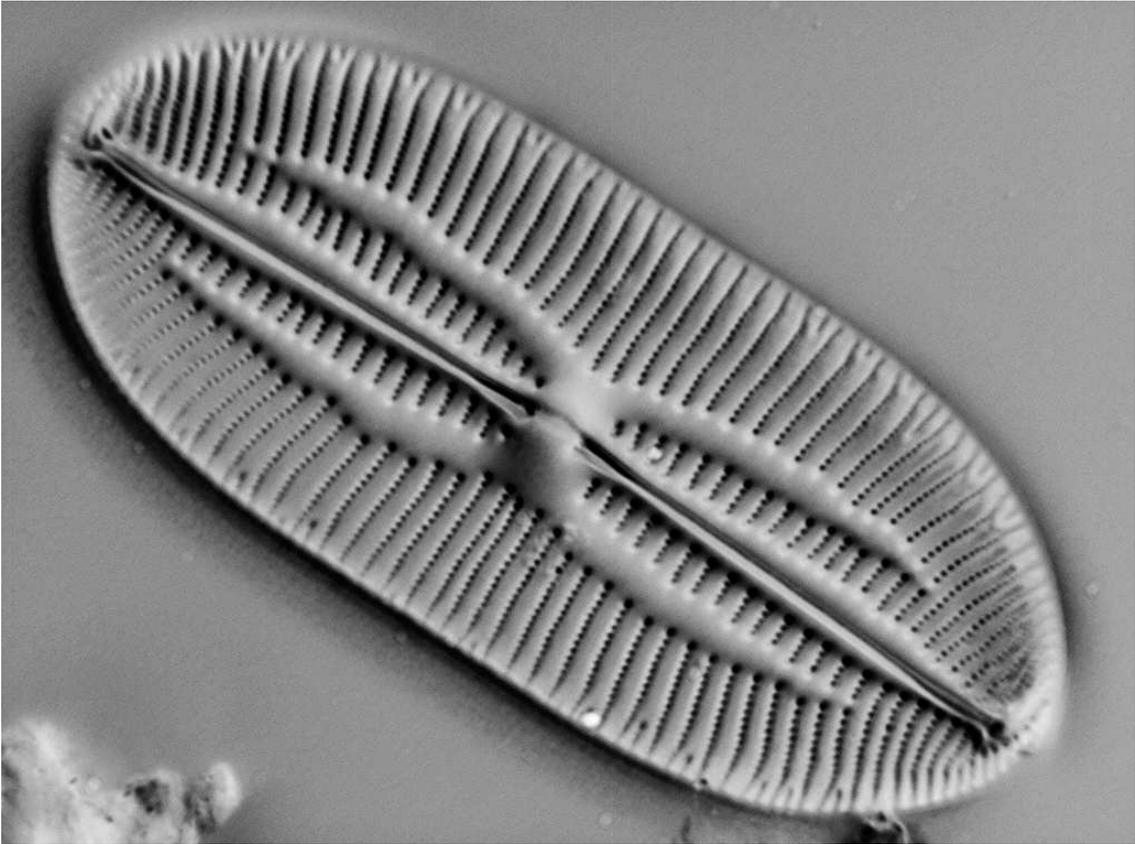
- The concept is based on digital photography.
- To overcome the small depth of field of high aperture objectives, combine several images at different succeeding focus by a stacking software.
- Combination of several stacked images by stitching software is necessary if the intermediate image of the object exceeds the size of the camera chip.
- For enhancing contrast, the additional use of Differential Interference Contrast is very helpful.
- With a BW camera, you are able to apply shorter, nearly monochromatic light to enhance the resolution.
- All these measures lead to high resolution and high contrast images of even large diatoms.

[1] CombineZP by Alan Hadley; www.hadleyweb.pwp.blueyonder.co.uk

[2] MS-ICE; <http://research.microsoft.com/en-us/um/redmond/groups/ivm/ICE/>

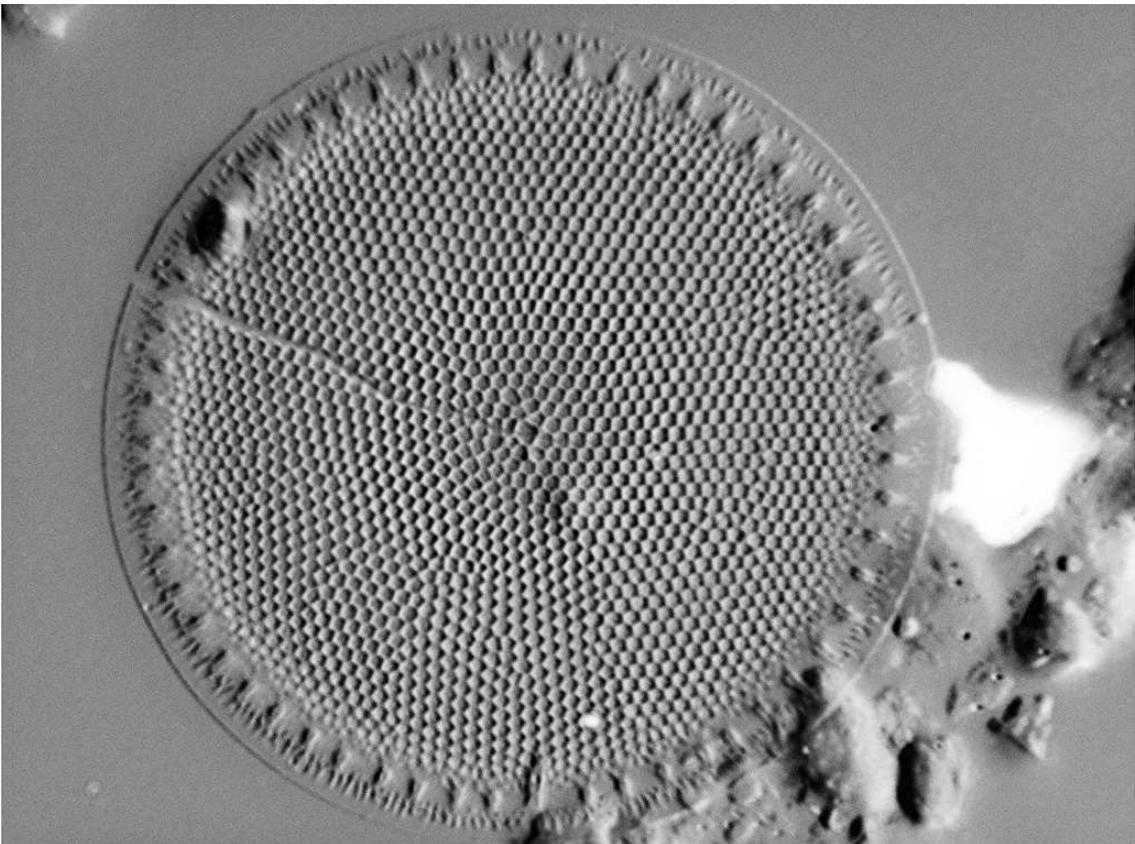
[3] Zeiss brochure: Microscopy from the very beginning,
www.zeiss.com/C1256B5E0047FF3F/?Open

* Published in the March 2011 issue of Micscape magazine; www.microscopy-uk.org.uk



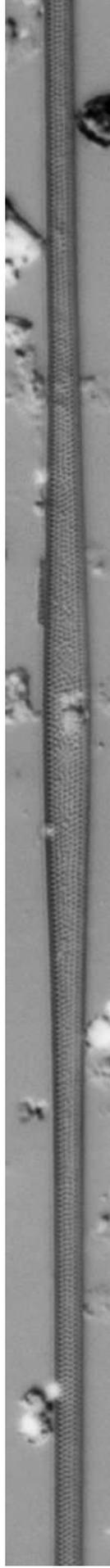
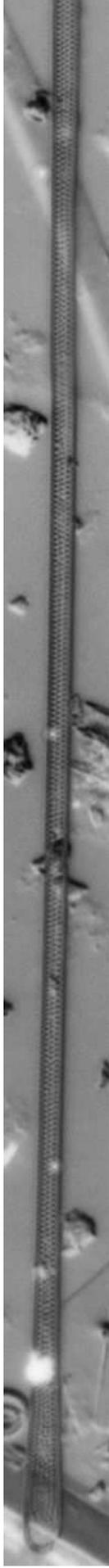
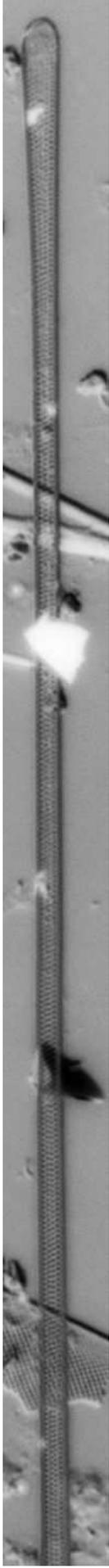
Lyrella abrupta

length: 46 μm



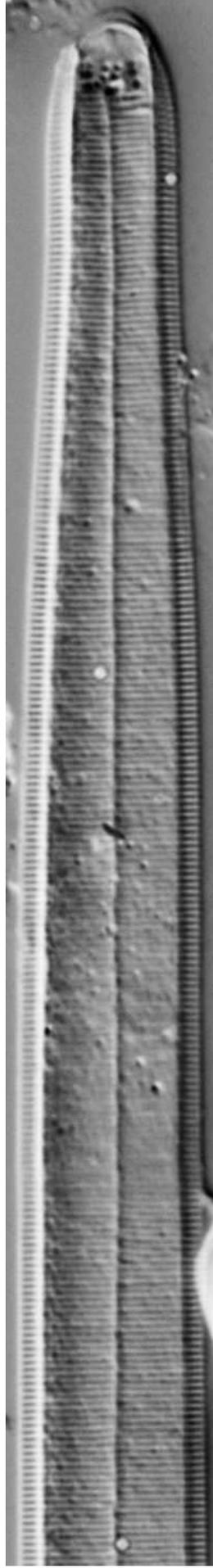
Thalassiosira sp.

diameter: 34 μm



Toxarium-hennedyvanum

total length: 245 μm

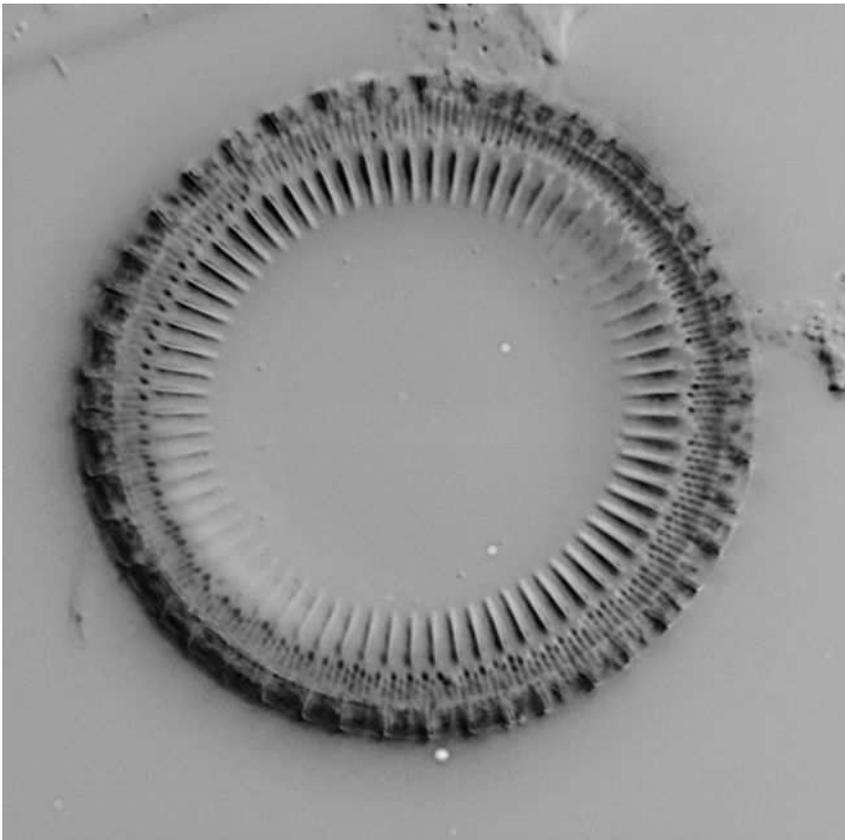


Hyalosinedra laevigata



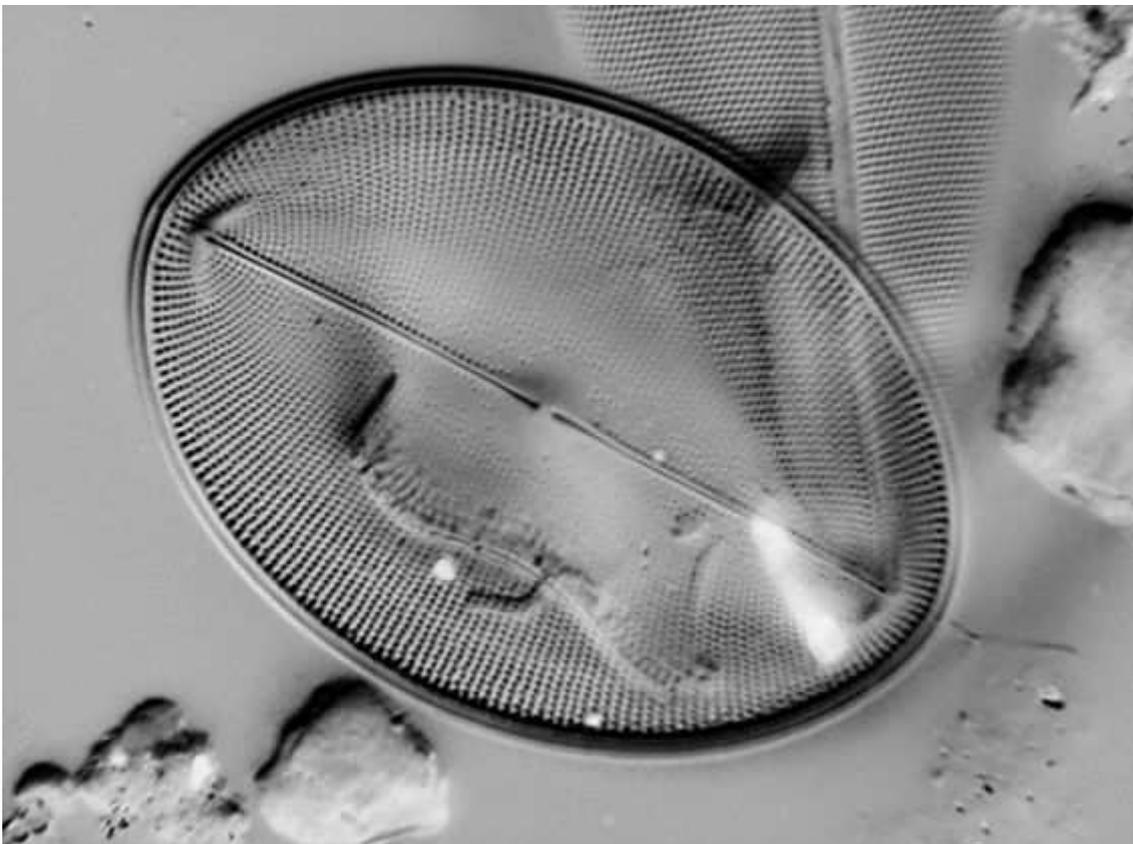
Surirella febigerii

length: 137 μm



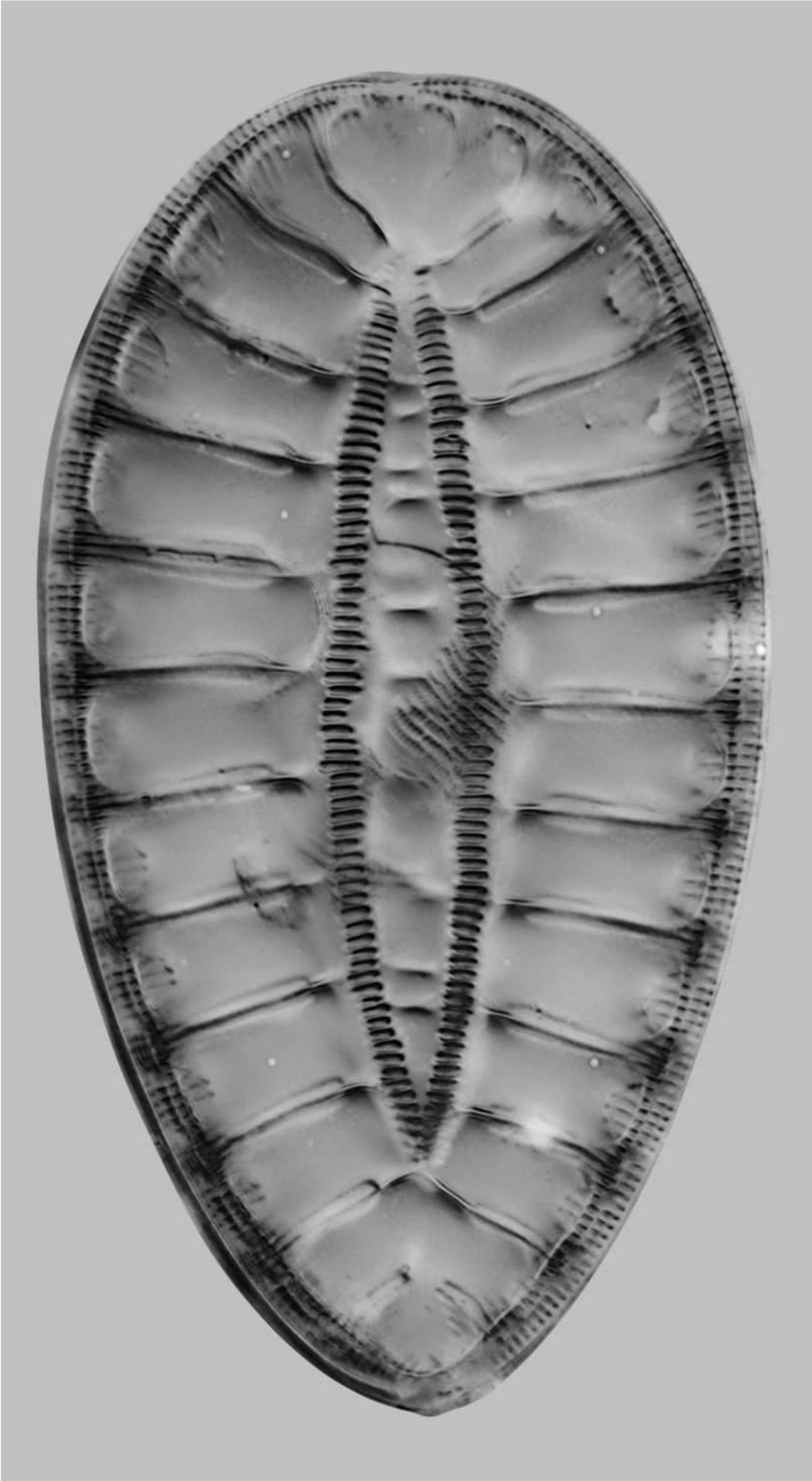
Paralia sulcata

diameter: 34 μm



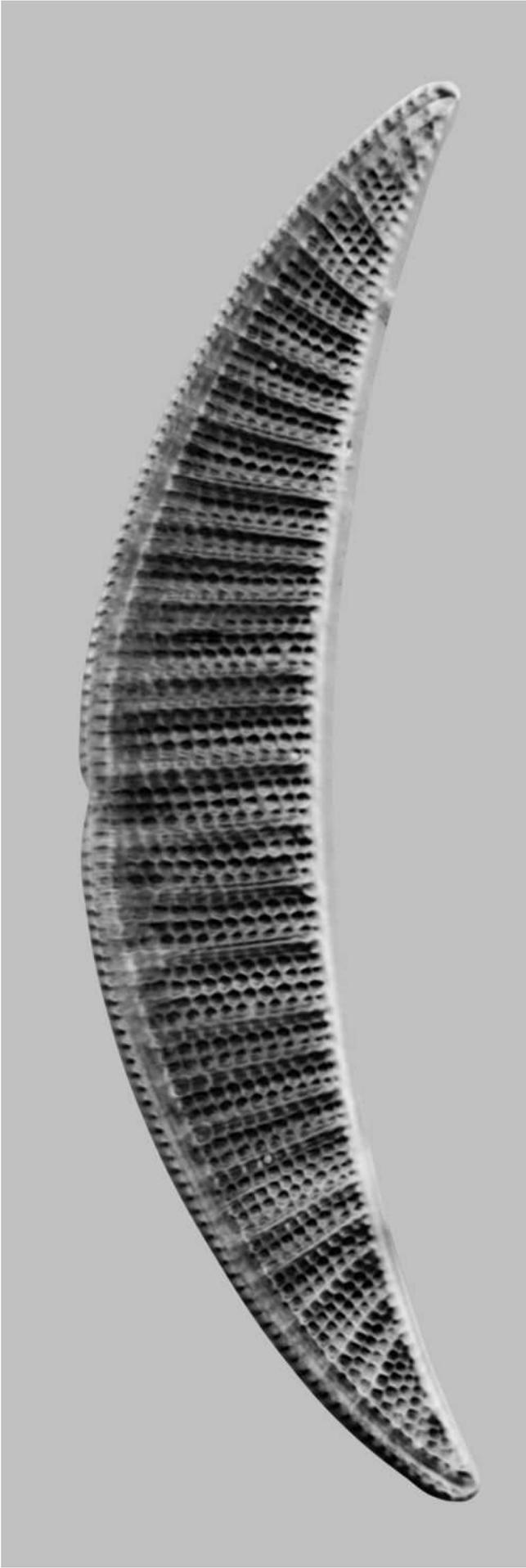
Cocconeis pellucida

length: 45 μm



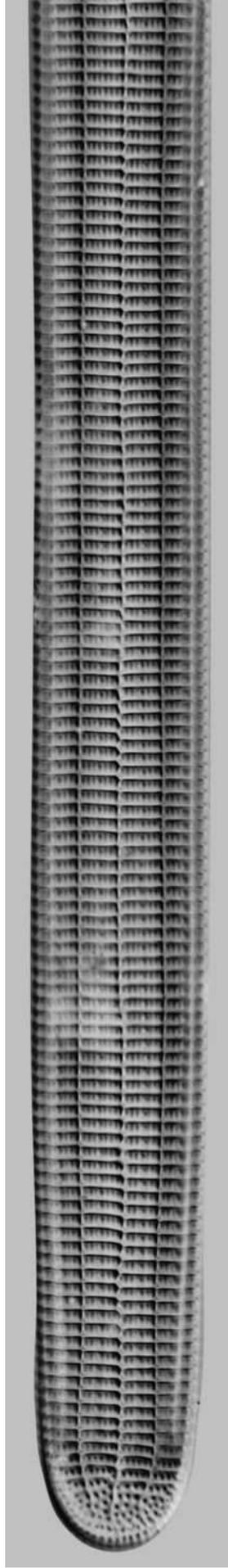
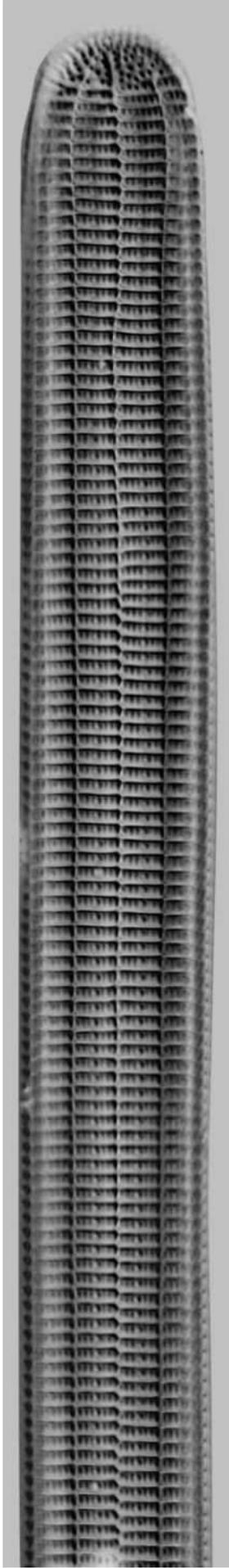
Surirella guinardii

length:h 88 μ m



Rhopalodia musculus

length: 85 μm



Ardissonia formosa details at apices

Total length: 520 μm

Explanation to the above photomicrographs

All photomicrographs were made with Differential Interference Contrast (DIC) at violet light and an objective Zeiss Planapo 63/1.4, 160 mm Tube length.

The camera to capture the images is a lensless USB Black & White one with remote control software. This equipment is very comfortable because you directly see the image on your PC-monitor while focusing. Triggering is done electronically.

The depth of field was achieved by stacking several images which number may vary between 5 and 50 or even more. This depends on the shape of the diatom.

In case the size of the diatom exceeds the size of the camera-chip, you have to stitch overlapping stacked images. That may increase the number of images to shoot drastically.

At the time being for stacking I use the software Combine-ZP from Alan Hadley and for stitching a software which was delivered with my compact camera from Canon. But surely there are many other softwares which will perform equivalent good results.