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UV photomicrography of diatoms

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An historical survey of the photographic documentation of diatoms is followed by a description of a modern technique of photomicrography in ultraviolet light. With commercially available equipment, this yields the ultimate detail obtainable with a light microscope. The technique can be especially helpful in the examination (e.g., for typification) of specimens in slides with a low refractive index mounting medium, if no original material remains for scanning electrom microscopy studies.

Keywords: *ultraviolet light, ultimate resolution*

Introduction

From the very beginning of diatom studies it was clear that diatoms yield low-contrast images and, combined with their fine structure, this made them challenging objects to visualize and depict. The diatom frustule is colourless and transparent, and the light microscopy (LM) image mainly contains information resulting from differences in optical path length (phase shift between rays of light passing through the diatom and background, respectively). Neither the eye nor the camera records differences in phase, unless these are very major, at which point they become visible as slight differences in brightness.

High refractive index (RI) mounting medium leads to greater differences in optical path length and was used by nineteenth-century diatomists to obtain better contrast. Some of these mountants were rather unmanageable (e.g., α-monobromonaphthaline, a liquid with an RI of 1.66) and poisonous (Realgar, RI = 2.4). In general, such preparations are unstable, often becoming useless over time. A stable mountant used in the nineteenth century was styrax (a natural resin), but its RI was not high enough ($\sim$1.6) to yield high-contrast images.

The photomicrographic illustration of diatoms became feasible in the late nineteenth century. An early example was a diatom atlas published by Fritsch & Müller (1870). Cost may have been the reason why it was a long time before photomicrographs supplanted drawings in printed publications. As late as the 1960s, Norman I. Hendey was unable to document certain findings because the publisher did not allow a single additional plate for reasons of cost (N.I. Hendey, pers. comm. to F.A.S. Sterrenburg, 1989). Even today, publishers ask their authors to pay for colour plates themselves!

Despite the problems in imaging, photomicrographs of diatoms produced in the late nineteenth century were sometimes surprisingly good (Fig. 1). Actually, the limitations of the photographic materials of that time were advantageous for the photomicrography of diatoms. The negatives were large (9×12 cm or 4×5 inches) so that emulsion grain was no problem. Spectral sensitivity was restricted to shorter wavelengths so that resolution was good and the effect of chromatic aberration over a wide spectral range was reduced. These plates also had strong contrast.

The main problem was illumination: the photographic plates available were very insensitive and compact low-voltage high-intensity light bulbs had not yet been invented. Unless one used an electric arc light – a rather forbidding contraption – this meant very long exposures. The data written on the back of the print reproduced in Fig. 1 specify an exposure time of no less than 28 minutes! However, such long exposure times permitted a helpful photographic trick. Many diatoms show marked three-dimensional relief while the depth of field of the LM is very small. During very long exposures, the focus of the microscope could be carefully adjusted to give a sharp image of the higher as well as the deeper layers of the diatom frustules — as has probably been done for Fig. 1, judging by the image. With the modern illuminators and sensitive films used up to 2000 or so, exposures took mostly $<$1 s and such 'focusing through' became impossible and was forgotten. Comparable results only became possible again when digital photomicrographs could be processed with stacking software, often very helpful in photomicrography of diatoms.

An important reason for a nineteenth-century diatomist to publish photomicrographs was to furnish proof of

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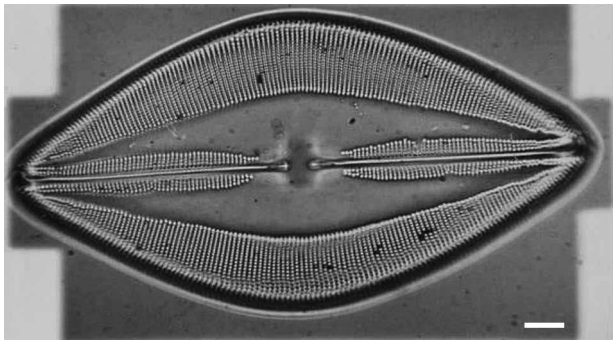

Fig. 1. A photomicrograph made by the Dutch diatomist J.J. Kinker, ca. 1885. Data written on the reverse of the print: '*Navicula hennedyi* var. *schleinitzii* Janisch, Galapagos, exposure 28 min'. Scale bar = 5 μm.

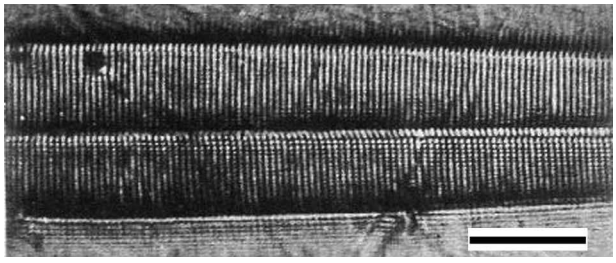

Fig. 2. Photomicrograph of *Amphipleura pellucida* in Realgar taken by Van Heurck with the special Zeiss 1/12′ α-monobromonaphthaline immersion objective, as shown in Carpenter & Dallinger (1901). Scale bar = 5 μm.

personal success in visualizing particularly fine diatom structure. In a quest for the ultimate in microscopic resolution spanning some two decades, the wealthy Flemish industrialist Van Heurck co-operated with the Carl Zeiss firm and received an exotic objective from this manufacturer in recognition of his assistance. This objective was an apochromatic immersion using α-monobromonaphthaline as the immersion fluid. A photomicrograph by Van Heurck of *Amphipleura pellucida* Kützing mounted in Realgar as resolved by this equipment was published in Carpenter & Dallinger (1901) and a scan is shown here in Fig. 2. However, Van Heurck's images have rightly been criticized because, despite the extraordinary investment, the results were not good. Resolution is only partial and the use of extreme oblique illumination resulted in serious artefacts – strong diffraction lines around the valve. For a slide of this diatom mounted in Realgar, better images can be obtained with ordinary objectives. Although the resolving power of this special objective was high (numerical aperture (NA) = 1.6, compared with the NA = 1.4 of the best modern objectives), only a few such objectives were ever made because it was impractical. For example, it required a special condenser with an equally high NA and slides and coverslips of dense flint, RI = 1.7. Further information can be found in Gerlach (2009).

The development of optical contrast-enhancement in the form of phase contrast (after World War II) or interference contrast (around 1960) brought a welcome improvement, although fairly high refractive index mountants are still preferred. Even with ordinary optics, however, markedly improved results can be obtained by using extreme annular illumination and polarized light, also in combination (Sterrenburg 1978, Oku 2004). Still, there are diatom species whose identities are difficult or impossible to ascertain in LM because their structure is too fine for visualization. The introduction of the scanning electron microscope (SEM) in the 1960s meant a quantum leap in resolving power and for the first time revealed the actual structural complexity of diatoms.

It would seem, then, that photomicrographic documentation of diatoms no longer poses problems, but this is not entirely true. A typical example would be the case where a taxon must be typified using a nineteenth-century slide that yields only poor images because of the mountant's low RI and where no original material can be retrieved to verify the taxon's morphology in a new isotype slide of better quality (mounted in a medium of high RI) or in SEM. In such cases microscopy in ultraviolet (UV) light may be helpful.

UV microscopy

The physics of image formation in the LM implies that resolution is inversely proportional to the wavelength of the light used for image formation. In the early decades of the twentieth century, experiments were therefore carried out with using UV light, which could theoretically almost double the resolution, as pointed out by Abbe in the late nineteenth century (see Gerlach 2009, p. 450). Köhler (1904) designed photomicrographic equipment that was manufactured by Zeiss and described its application to diatom studies (Köhler 1909). The many problems encountered prohibited application on a large scale. UV sources were expensive, short-lived and temperamental. The glass used for microscope lenses and slides is opaque to short-wavelength UV so that no image can be formed – special quartz condensers, slides, coverslips and quartz or mirror objectives were necessary. Finally, UV is invisible so that the image could not be focused by visual examination. In some experiments, 100 photomicrographs (on plates or film!) had to be taken wholly at random to obtain a single sharply focused image, as a review in Beck (1938) describes. In a publication that would have deserved much wider attention, Kingma Boltjes (1947) described surprisingly good results obtained with relatively long-wavelength UV (365 nm) using ordinary (glass, not quarz) optics and an elegant focusing trick.

With the introduction of electronic equipment (image converters, TV monitors) in the 1960s, some of the problems were solved (see Françon 1967). Nevertheless, because of its technical and financial complications, UV microscopy never became popular, in contrast to UV

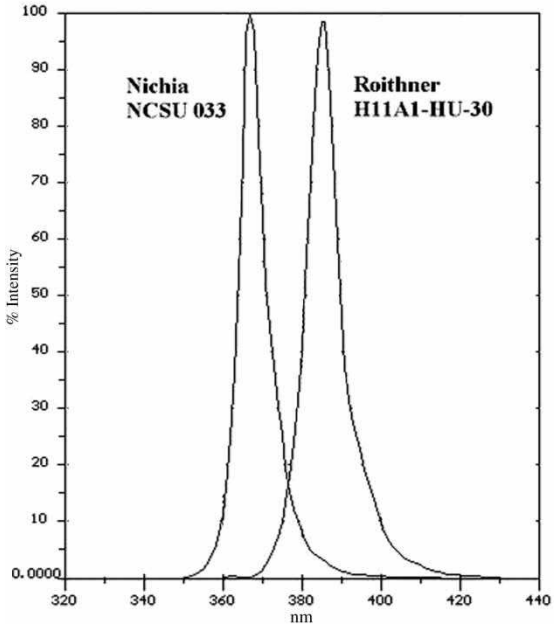

Fig. 3. Spectral emission curves of the Nichia NCSU 033 and Roithner H11A1-HU-30 light-emitting diodes tested.

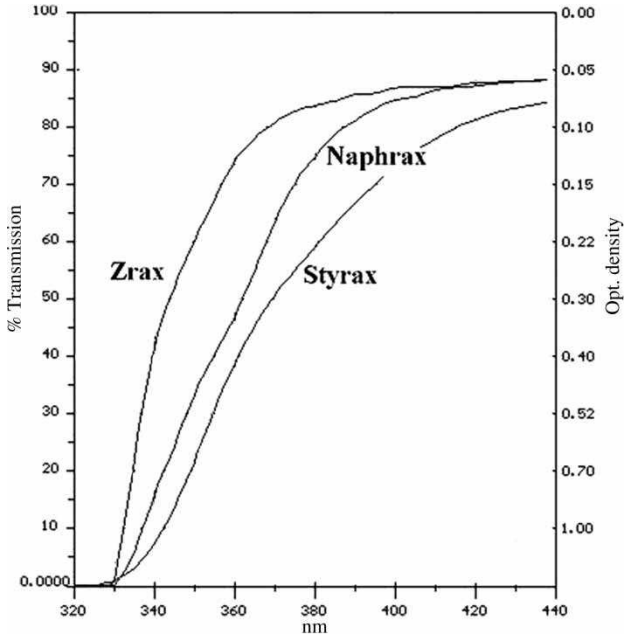

Fig. 4. Transmission curves of three mountants tested.

fluorescence microscopy, which has become a routine method of examination. Thanks to modern technology, UV microscopy has now become more practical.

Instrumentation

In a series of investigations (Höbel 2009) a modern approach to UV microscopy was developed, using commercially available equipment as follows.

Light source

Light-emitting diodes (LEDs) have a very long life, a high efficiency, produce minimal heat and are available with an emission spectrum peaking at 365–385 nm – relatively long UV wavelengths. Two suitable UV LEDs tested were: Roithner H11A1-HU-30 and Nichia NCSU 033. The measured spectral emission curves are shown in Fig. 3. The Nichia LED is so powerful (laser Class 3B) that under no circumstances should its UV light reach the observer's eyes. A regulated power supply with current limiting corresponding to the LED specifications is required. The illuminator of the microscope can easily be adapted by substituting the LED for its low-voltage bulb, with some mechanical adaptation.

Optics

At the relatively long UV wavelengths used, the usual glass slides and coverslips were found to cause only minor losses due to absorption. As regards microscope optics, the fewer glass components (lenses, prisms) the better. Flat-field objectives (planapochromats) are thus not suitable, as they cause marked absorption.

Mounting mediums

Various mountants used for diatoms (Naphrax, Styrax, Zrax) were examined and found to be sufficiently transparent at these UV wavelengths (Fig. 4).

Camera

Colour digital cameras are not suitable because they contain an internal filter that blocks UV; a monochrome camera is necessary. Several astrophotographic monochrome cameras with good sensitivity in the UV are available. These connect to the PC via the USB 2.0 port so that focusing can be carried out on the monitor screen. The camera used yields 12-bit files instead of the usual 8-bit files, offering a far greater number of grey-scale values.

Software

The camera was controlled by the dedicated K3CCD® software program, image files are handled by Registax®. For further processing (e.g., brightness, contrast), one of the many available image-processing programs can be used.

Procedure

For visual observation and focusing, the image on the PC monitor is sufficiently clear, but it has a high noise level ('grainy' image, Fig. 5). The imaging procedure used,

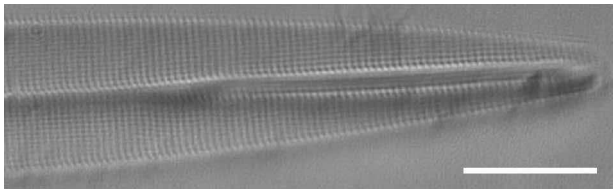

Fig. 5. *Amphipleura pellucida*, mounted in Caedax. A single UV image from the AVI file shows poor contrast, but sufficient for focusing. No software processing. Scale bar = 5 μm.

comparable with that in astrophotography, is complex, therefore:

A series of images is taken, the number depending on the 'visibility' of the diatom: for 'contrasty' diatoms 100 images may be sufficient, for 'difficult' diatoms up to 600 images may be required. These serial exposures, each of 1/10th to 1/50th second long, are automatically taken through the corresponding K3CCD® software control settings and result in an AVI file ('AVI-image' file).

1 With identical settings, but with the object removed, the same number of exposures results in another AVI file ('AVI-background' file).
2 Both the 'AVI-image' and 'AVI-background' files are first separately averaged with Registax® and this results in two separate data files: 'Image' and 'Background'. This suppresses the noise ('graininess') in the images.
3 With suitable image-processing software (e.g., 'Image-Pro'®), the 'Background' file is then subtracted from the 'Image' file. This removes artefacts like irregular illumination or interference from dust particles in the optics. The result is a clean image.
4 Brightness and contrast can then be suitably adjusted by one of the popular image-processing programs.

The image-processing methods described (averaging, stacking, background subtraction) can, of course, also be applied to photomicrographs in visible light.

Results

All the UV images in this study were taken with a Leitz Apochromatic 90/1.4 objective, a UV-LED Nichia at 365 nm and Lumenera SKYnyx® monochrome camera. Digital processing was as described above.

Amphipleura pellucida (Fig. 6)

This has been the 'classic' test object for oil immersion objectives for over a century. With 40 areolae in 10 μm, and sometimes more, it is near the limit of resolution of LM. In addition, the frustule is very delicate, so that mountants with high RI are necessary to obtain sufficient contrast. The photograph presented here, however, shows a specimen from an old slide mounted in Caedax, a medium with a low

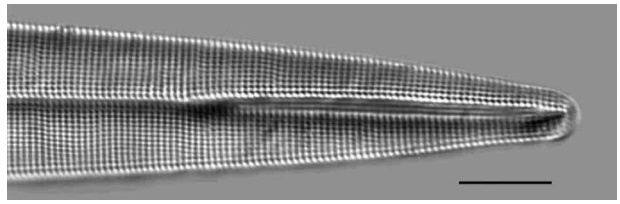

Fig. 6. *Amphipleura pellucida*, mounted in Caedax, UV light. Final result of combination of 500 AVI images. Scale bar = 5 μm.

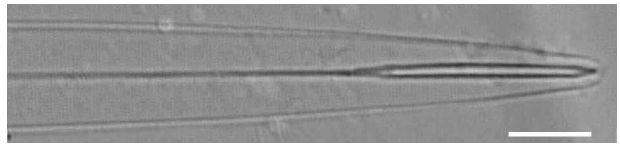

Fig. 7. *Amphipleura pellucida* in Caedax, strictly centrally illuminated brightfield image in white light. Because of the unsuitable mountant, the image is extremely poor. Objective 90×, NA 1.4, no software processing, camera 10 Mp. Scale bar = 5 μm.

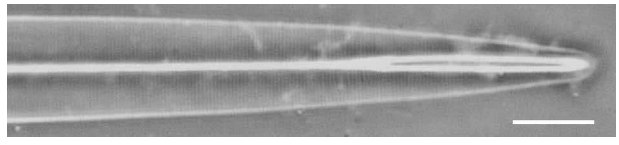

Fig. 8. *Amphipleura pellucida* in Caedax, Anoptral phase contrast in white light. This was found to give the best contrast but resolution is only partial. Objective 100×, NA 1.3, no software processing, camera 10 Mp. Scale bar = 5 μm.

RI (1.56) totally unsuitable for diatoms (Höbel 2009). In brightfield with strictly central illumination, contrast is very poor, as shown in Fig. 7. The visual image shows even less contrast. In the original image file (500 kb), transverse striae are faintly visible. The best visibility is obtained with Anoptral phase-contrast (Fig. 8), which shows resolution of the transverse striae and (in the original 500 kb file) some indication of 'dots'. The shortcomings of Caedax as a diatom mountant are obvious, however. In UV (Fig. 6), resolution is excellent (note also the raphe slit) and some digital processing yields good contrast. The result (a combination of 500 exposures) is representative of what can be achieved with old slides mounted in a medium of low RI, such as are found in museum collections.

A comparison with the field emission scanning electron microscope (FESEM) image of *A. pellucida* (Fig. 9) shows that the procedure described here does not produce artefacts in the image.

Gyrosigma sp. (Figs 10–13)

This diatom, or specimens similar to it, has probably been called *Gyrosigma tenuissimum* (W. Smith) Griffith & Henfrey in various publications, but it is definitely incompatible with *Pleurosigma tenuissimum* Smith as presented in

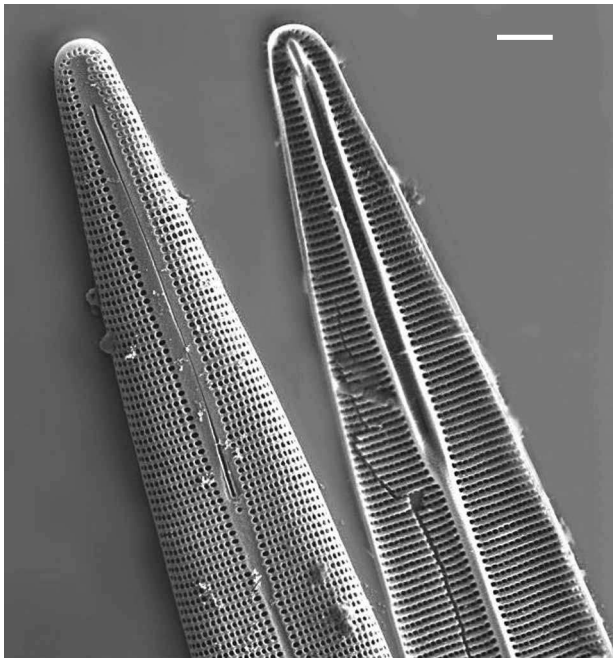

Fig. 9. *Amphipleura pellucida*, FESEM image of exterior and interior of two valves. Comparison with Fig. 6 shows that the UV image does not contain artefacts. Scale bar = 2 μm.

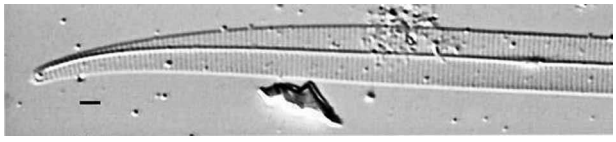

Fig. 10. *Gyrosigma* sp., differential image contrast, planapo oil immersion 63×, NA 1.4, visible light. Courtesy of W. Herwig. Longitudinal striae not resolvable. Scale bar = 2 μm.

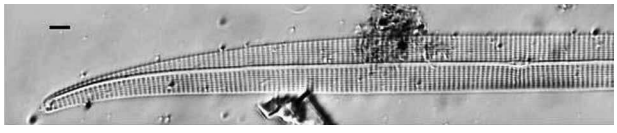

Fig. 11. *Gyrosigma* sp., UV image. Clear resolution of areolae. Scale bar = 2 μm.

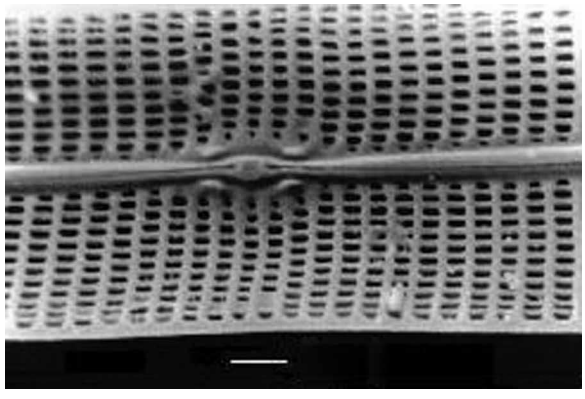

Fig. 12. *Gyrosigma* sp., SEM. Note oblong areolae. Scale bar = 1 μm.

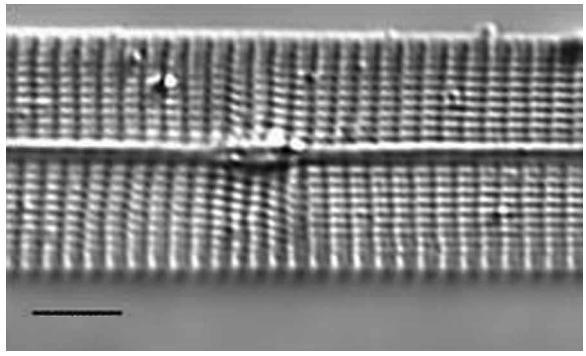

Fig. 13. *Gyrosigma* sp. Detail of UV image. Comparison with Fig. 12 shows that no artefacts are introduced by the procedure. Scale bar = 2 μm.

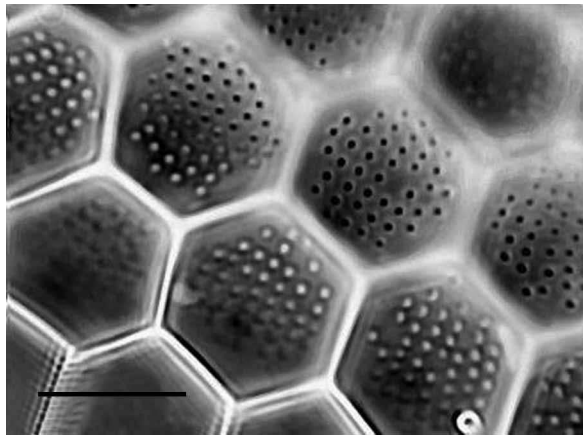

Fig. 14. *Triceratium favus*, UV image. Note '3D effect'. Scale bar = 5 μm.

Smith (1853), because this publication specifically mentions ('flexure considerable') and depicts a raphe-sternum and valve contour that are distinctly curved throughout, whereas both are linear except at the very apices here. Also, Smith (1853) describes the striae as 48 in 0.001 inch (∼19 in 10 μm), whereas the transapical striae of the diatom in question have a density of ∼25 in 10 μm and the longitudinal striae cannot be resolved even with the ultimate in modern optics (Fig. 10). No type material of Smith's species appears to have survived. UV microscopy (Fig. 11) of the same specimen as in Fig. 10, in material from the Mediterranean (leg. W. Herwig) yields a longitudinal stria count of ∼44 in 10 μm. The SEM image (Fig. 12) is of a matching (in LM) specimen, also from the Mediterranean. Note that the oblong shape of the areolae in the SEM image is faithfully reproduced in the UV image and no artefacts are present (Fig. 13).

Triceratium favus Ehrenberg (Fig. 14)

The perforations in the hymenes covering the hexagonal cells of this species are measured in nanometres. Although they can just be visualized in visible light, the UV image

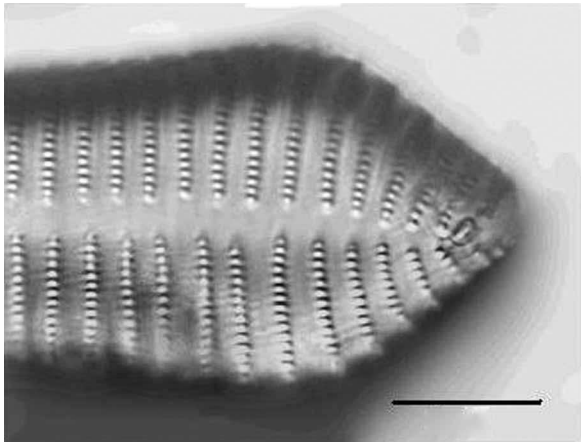

Fig. 15. *Fragilaria dilatata*, UV image. Note rimoportula. Scale bar = 5 μm.

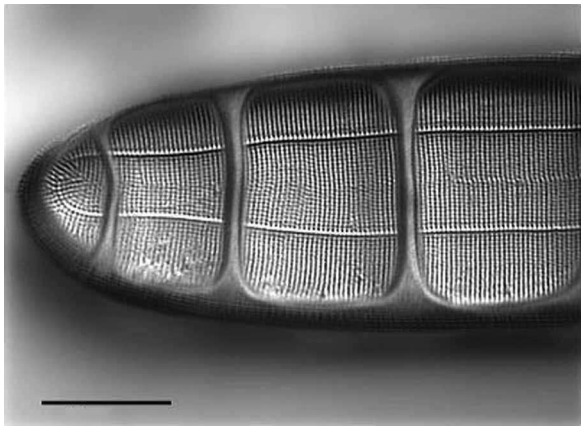

Fig. 16. *Climacosphenia moniligera*, UV image. Note '3D effect'. Scale bar = 10 μm.

is much more representative of the overall morphology as seen in SEM.

Fragilaria dilatata (Brébisson) Lange-Bertalot (Fig. 15)

Formerly called *Synedra capitata* Ehrenberg, this species shows excellent resolution of the areolae and the rimoportula in UV light.

Climacosphenia moniligera Ehrenberg (Fig. 6)

With clear resolution of the areolae and stacking of separate exposures, the UV image almost begins to resemble a SEM image.

Bacillaria paxillifer (O.F. Müller) Hendey (Fig. 17)

Raphe slit, areolae and cribra are clearly visible thanks to high resolution and stacking of exposures.

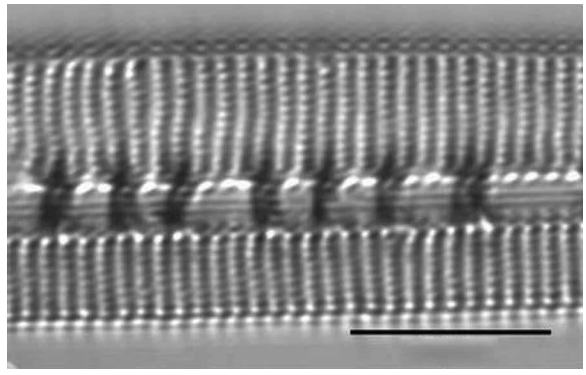

Fig. 17. *Bacillaria paxillifer*, UV image. Note raphe slit. Scale bar = 5 μm.

Conclusions

Archibald (1984) stressed that good illustrations are essential for diatom publications and showed that incorrect autecological conclusions have resulted from faulty identification. The drawings that dominated the taxonomic literature until the second half of the twentieth century may not be sufficiently reliable to serve as the type – although this is permitted by the rules of the International Code of Botanical Nomenclature (Greuter 1988). Despite advances in optics such as differential image contrast (DIC), there remain many diatoms whose delicate structure makes identification difficult. This is especially the case when taxa have to be typified in slides mounted in media of low RI, e.g., nineteenth-century slides era. In such cases, a modern approach to UV microscopy may assist in iconographic documentation of diatoms when no original material can be retrieved for SEM study. A caveat is necessary, however: intense UV may cause darkening of some mountants. This was observed for an old slide with unknown mountant, but other mountants tested, from Canada Balsam to Naphrax and Zrax did not show such deterioration. If no preliminary testing can be done for this phenomenon, all risks can be avoided by using blue light instead of UV. Suitable blue LEDs are widely available and the procedure is exactly as described above. Although resolution will be somewhat less than with UV, the results will be far superior to ordinary photomicrography.

Acknowledgement

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References

Archibald R.E.M. 1984. Diatom illustrations – an appeal. *Bacillaria* 7: 173–178.

Beck C. 1938. *The microscope. Theory and practice*. R. & J. Beck, London. 264 pp.

Carpenter W.B. & Dallinger W.H. 1901. *The microscope and its revelations*. J. & A. Churchill, London. 1181 pp.

FRANÇON M. 1967. *Einführung in die neueren Methoden der Lichtmikroskopie*. G. Braun, Karlsruhe. 332 pp.

FRITSCH G. & MÜLLER O. 1870. *Die Sculptur und die feineren Structurverhältnisse der Diatomaceen mit vorzugsweiser Berücksichtigung der als Probeobjecte benutzten Species*. Abtheilung. I. Zwölf Tafeln mikrophotographischer Abbildungen. O. Müller, Berlin.

GERLACH D. 2009. *Geschichte der Mikroskopie*. Verlag GmbH, Frankfurt/Main. 1045 pp.

HÖBEL P. 2009. Brechwertmessungen an Diatomeen – Technik und neue Messungen. *Mikrokosmos* 2: 98–102.

GREUTER W. (Ed.) 1988. *International Code of Botanical Nomenclature: adopted by the Fourteenth International Botanical Congress, Berlin, July–August 1987*. Koeltz Scientific Books, Koenigstein. 328 pp.

KINGMA BOLTJES T.Y. 1947. Some remarks on microphotography. *Antonie van Leeuwenhoek* 12: 232–242.

KÖHLER A. 1904. Mikrophotographische Untersuchungen mit ultraviolettem Licht. *Zeischrift für Mikroskopie und Mikroskopische Technik* 21: 129, 273.

KÖHLER A. 1909. *Aufnahmen von Diatomeen mit ultraviolettem Licht*. Jahrbuch für Photographie und Reproduktionstechnik, Halle a.S. 60–67 pp.

OKU O. 2004. A new method for visualization of diatom striae by using annular illumination and polarized light. *Bulletin of Plankton Society of Japan* 51: 25–33.

SMITH W. 1853. *A synopsis of the British Diatomaceae*, Vol. 1. J. van Voorst, London. 89 pp.

STERRENBURG F.A.S. 1978. Enhancing the visibility of diatoms. *Microscopy* 33: 384–389.