THE USE OF INTERFERENCE REFLECTION CONTRAST IN THE EXAMINATION OF DIATOM VALVES

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Even though scanning electron microscopy (SEM) is now needed to identify some species of diatoms, the majority of identifications and quantification of these organisms in ecological works is accomplished with a light microscope, using transmitted light optical methods. In this paper we demonstrate the use of interference reflection contrast (incident light) for the examination of diatoms, a method that significantly improves the resolution of structural detail, and therefore, identification of diatom taxa with light microscopy. Using incident light we were routinely able to distinguish between structures that were close to the theoretical limit of resolution for visible light, and that were not resolvable with such standard transmitted light techniques as phase contrast and differential interference contrast (DIC). Light microscopes with epi-illumination light paths can be easily and inexpensively outfitted to use this simple technique.

Key index words: Bacillariophyceae; diatoms; incident light; interference reflection contrast; resolution

Abbreviations: DIC, differential interference contrast; IRC, interference reflection contrast; LM, light microscopy

Diatoms have been utilized for decades as bioindicator organisms (e.g. Hustedt 1939, Patrick 1977), and are now invaluable as tools for determining historical changes in aquatic ecosystems (Birks et al. 1990, Hall and Smol 1996, Siver et al. 1999). Over the last decade, paleolimnological inference models utilizing diatom remains have been instrumental in reconstructing historical conditions in individual lakes (Lott et al. 1994), estimating changes in water chemistry over broad regions (e.g. Hall and Smol 1996, Siver et al. 1999), defining effective target conditions for lake restoration efforts (Brenner et al. 1993), and in evaluating lake recovery following management efforts (Anderson and Rippey 1994).

The most critical and useful bioindicator work requires identification at the species or subspecific taxonomic levels, which requires examination of cleaned frustules. Historically, delineation of diatom species has, and continues to be, based largely on morphological characteristics of the frustules (Round et al. 1990). Common characteristics used to separate taxa include, but are certainly not limited to, overall valve shape, symmetry and size, details and arrangement of structures such as areolae, strutted processes, spines, and raphes, and the density of structures such as areolae, striae, and keel punctae.

In the vast majority of ecological and paleolimnological works that utilize diatoms as bioindicators, the specimens are critically identified and enumerated using light microscopy (LM) (Bennion et al. 1996, Hall and Smol 1996, Siver et al. 1999). Although SEM is becoming increasingly needed to make proper identifications of some diatom taxa, most of the quantification data are collected with LM, and presumably will be for the indefinite future (Round et al. 1990, Siver et al. 1999). Thus, the ability to distinguish between taxa with a light microscope remains a key component of many diatom studies. To maximize the contrast between the siliceous diatom valves and the glass coverslip when viewing diatoms with light microscopy, the valves are usually embedded in a medium with a refractive index different from glass, and observed with either phase contrast or DIC optics (Round et al. 1990). Despite the use of such contrast enhancing techniques, many structures are difficult or impossible to resolve with LM.

We describe a simple and inexpensive light microscope method, interference reflection contrast (IRC), that utilizes incident light for examination of diatom valve structure. The IRC method yields resolution of structures close to the theoretical limit for visible light, and often far superior to what is attained using transmitted light optics. We further demonstrate how this technique can improve the study and identification of diatoms with LM, and explain how the technique can be easily incorporated into such studies.

MATERIALS AND METHODS

Configuration of the microscope. All observations were made using a Leica DMR microscope outfitted with bright-field, phase-contrast, and DIC optics. All images were taken with a Sony DKC-ST5 digital camera using PL Fluotar 100X/1.3 oil-immersion lenses. Except for slight modifications to brightness and contrast, no digital enhancements were made to the micrographs. The Leica DMR was further equipped for observation using IRC by simply adding two items:
1) A lamp housing with a standard 12 volt 100 watt halogen bulb (Leica model #1150470, ca. $1,000) was fitted onto the epi-illumination path.
2) A Smith reflector (Leica model #11555000, ca. $800) was installed in the epi-illumination path.

Several additional points are worth noting when configuring the microscope for IRC optics. First, an oil-immersion lens is required to ensure that the medium between the lens and the top of the coverglass has the same refractive index as glass. Second, in our studies we obtained similar images using a phase-contrast lens as we did with a bright-field objective; the phase ring in the objective lens did not prevent us from obtaining quality images using IRC. Third, we emphasize that special lenses with wave plates, polarizing filters, a high energy mercury lamp, and computer enhancement software are not required for inspection or for obtaining quality images using the IRC technique. Fourth, we most often reduced the opening of the field aperture on the incident light path to a diameter slightly greater than the specimen to reduce glare from reflected light not associated with the formation of the image.

**Specimen preparation and observation.** Only cleaned specimens of diatom remains from the surface sediments of ponds were used in this study. Surface sediments were taken from ponds on Cape Cod, MA, using a Grew gravity corer and prepared according to Siver (1999). Samples of cleaned diatoms were affixed by air drying onto a coverglass and then mounted in either Hyrax® or air onto a glass microscope slide. Specimens should be attached to the coverglass, and not on the glass slide, prior to mounting in a medium; that is, specimens areolae within a given striae were clearly and consistently resolved (Fig. 1C). However, using IRC optics, the elongated areolae within a given striae were clearly and consistently resolved (Fig. 1C).

**RESULTS AND DISCUSSION**

**Resolution of structure.** *Navicula radiosa* var. *tenella* (Bréb ex Kütz.) Grun., and closely related species, are raphé-bearing taxa with striae consisting of elongated or slit-like areolae that are more or less parallel with the apical axis of the valve (Fig. 1). We examined five populations of *N. radiosa* var. *tenella* from Cape Cod lakes using both SEM and LM. Based on over 100 measurements (ca. 20 valves per lake) made with SEM, valves of this taxon consistently had a mean of four elongated areolae per micron, or one every 250 nm, within a given striae (Fig. 1A), close to the theoretical limit using visible light optics (Bradbury 1984). Using transmitted light optics we were unable to resolve the elongated nature of the areolae on literally hundreds of specimens from all of the populations (Fig. 1B). However, using IRC optics, the elongated areolae within a given striae were clearly and consistently resolved (Fig. 1C).

**Use of the interference reflection contrast method in routine diatom analysis.** Improvements in resolution of structure, similar to what we documented for *N. radiosa* var. *tenella*, were observed for many different diatom taxa (e.g. Fig. 2), and as a result, we have incorporated its use into routine analysis of diatoms for both biotic survey and paleolimnological work. In particular, the improved resolution of detail offered by IRC has significantly aided our analyses of diatoms in three basic ways: (a) being able to identify more taxa to the species level with LM and to distinguish between potentially different morphotypes; (b) estimating the density of structures per unit distance, such as the number of striae per 10 µm, and; (c) understanding the true nature of the surface anatomy of taxa, especially of rare species in samples with many different organisms, so they could be located more easily for study with SEM.

**Improving the identification of species and morphotypes with LM.** We will illustrate three examples, using species from the genera Fragilaria, Aulacoseira, and Staurosira, where the IRC method was useful in the identification and/or separation of taxa during routine examination with LM. Our first example deals with small specimens of the genus *Fragilaria*. We identified two very small (often less than 4 µm in length) *Fragilaria* taxa in the Cape Cod lakes that were difficult, and most often impossible, to separate with transmitted light optics. Using SEM, and based on characteristics given in Williams and Round (1987) and Krammer and Lange-Bertalot (1991), we identified the two taxa as follows. *Fragilaria construens* var. *venter* (Ehrenberg) Grunow [also known as *Staurosira construens* var. *venter* (Grun.) Hamilton], had striae composed of small, closely spaced, and relatively circular areolae, and spines that originated near the edge of the valve.
between the striae, the width of the axial region on our specimens of *F. construens* var. *venter* varied from being quite small to about 1/3 the width of the valve (Figs. 2J–L). The second taxon best fits the description of *Fragilaria elliptica* Schumann given by Krammer and Lange-Bertalot (1991); this taxon also had striae composed of circular areolae, but the areolae were much larger and spaced further apart than those of *F.*
construens var. venter. Most of our specimens of *F. elliptica* had relatively small axial regions, but within the range observed for our specimens of *F. construens* var. *venter*. Although according to Krammer and Lange-Bertalot (1991) spines on specimens of *F. elliptica* may originate either between or within the striae, our specimens of *F. elliptica* had spines that only originated within, and not between, the striae. On many specimens of both *Fragilaria* taxa, as is often the case with sediment samples, the spines were broken, leaving only remnants of the bases.

Despite the clear distinction between the two taxa as observed with SEM, we were often not able to separate them with transmitted light optics, but we could routinely with IRC (compare Figs. 2J–K with 2L, and 2M with 2N). With IRC, but not transmitted optics, we consistently resolved the larger and more widely spaced areolae on specimens of *F. elliptica* (Fig. 2N). In addition, we could easily determine that the spines originated from within, and not between, the striae. Although the close spacing of areolae on *F. construens* var. *venter* prevented us from resolving them even with IRC, we could distinguish this taxon from *F. elliptica* based on the narrower nature of the striae and the position of the spines between the striae; this was true even on specimens where only the bases of the spines remained (Fig. 2L).

Characteristics of linking spines is a common feature used in delineating species of *Aulacoseira* (Haworth 1988, Siver and Kling 1997). However, the true nature of spines on specimens of *Aulacoseira* is often not resolved using LM, resulting in the use of SEM for more accurate identifications (Siver and Kling 1997). The linking spines of specimens of *Aulacoseira lirata* (Ehrenberg) Ross in Hartley, known to be spatula to cruciform in shape (Haworth 1988), is one character used to distinguish this species from other taxa in the genus. Although we were never able to fully resolve the structure of the linking spines with bright-field, phase-contrast, or DIC optics (e.g. Fig. 2O), we were routinely able to distinguish spine detail with IRC (Fig. 2P and the insert), and thus identify this taxon with LM.

The areolae comprising the striae of *Stauroneis ancesps* *t. gracilis* (Ehrenberg) Brun are often, but not always, observed to be lacking around the central portion of the valve. The lack of areolae, which was not always evident with transmitted light optics (Figs. 2A–B), was clearly and routinely observed with IRC (Fig. 2C). Because we have been able to effectively separate the two morphotypes using IRC, we have found that each morphotype appears to grow under different environmental conditions; this separation has improved the use of these taxa as bioindicators.

**Estimating the number of structures per unit distance.**

Common characteristics used to identify diatoms often include an estimation of the number of structures per unit distance (e.g. Krammer and Lange-Bertalot 1991). Estimating the number of striae per 10 µm can often be a difficult task, especially on small specimens with numerous striae. Our ability to resolve, and therefore estimate, the number of striae per unit distance has significantly improved, because we have incorporated the use of IRC into our analyses. In the example of *Sellaphora pupula* (Kutz.) Mereschk (also known as *Navicula pupula* Kutz.) presented (Fig. 2H–I) the striae were resolvable with transmitted optics, including phase contrast (Fig. 2H), but they were more clearly resolved and more easily enumerated across the entire valve surface using IRC (Fig. 2J). Because the resolution of striae on many diatom species is significantly improved, we routinely make density estimates using IRC.

**Understanding the nature of the surface structure.**

In our examination of diatoms in Cape Cod lakes and ponds we have encountered a number of small specimens, often less than 10 µm, identified as *Navicula pseudoscutiformis* Hustedt (Fig. 2F). Most of the specimens appear to have circular areolae, although a few appear to also have more elongated areolae. In the specimen illustrated with phase contrast (Fig. 2F), the nature of the individual areolae cannot be fully resolved. However, with IRC, it is clear that the specimen has only circular-shaped areolae (Fig. 2G). Thus, the concept of the surface structure of *Navicula pseudoscutiformis* valves based on the IRC image (Fig. 2G) is quite different than the image formed using any of the transmitted light methods, but very similar to what is actually observed with SEM. Using IRC to understand the true nature of the surface structure of organisms such as *Navicula pseudoscutiformis*, and other taxa such as small *Cocconeis* specimens (Figs. 2D–E), has allowed us to more reliably find and examine the same taxa with SEM, and thus link the SEM observations with phase contrast and DIC images.

The increased resolution achieved using IRC is the result of a number of variables. Resolution of structure is reduced with phase contrast optics because the light ring utilizes only a fraction of the condenser aperture. With DIC, the condenser aperture can be set equal to that of the objective aperture, but periodic structures with a pitch equal to the shearing distance of the DIC prism combination will be suppressed. In addition, images formed using any transmitted light method represent the sum of all light diffracting features of the specimen in the z-axis, including superimposed out-of-focus planes (Rochow et al. 1966). IRC is not subject to such limitations. With IRC, the objective lens serves as both the condenser and image forming lens, and the resulting image is an interferogram of the surface topography, not a combination of diffraction patterns due to light passing through the specimen. As a result, images observed with IRC often appear as “miniature” SEM images.

In conclusion, because the IRC method described herein yields resolution of structures close to the theoretical limit using visible light, it is an effective and valuable tool for examination of diatoms. The IRC technique is especially useful in studies that require routine identification and enumeration of cleaned diatom specimens with LM. We believe that not only will
the identifications of many specimens significantly improve by using IRC, but that the worker will spend less total time processing samples due to the improved ability to distinguish between taxa. Configuring a light microscope that has epifluorescence capabilities for IRC optics is simple and inexpensive, and allows the user to instantly switch between standard transmission and IRC optics. In addition to enhancing our ability to resolve the structure of diatoms, we have also noted significantly improved resolution of siliceous scales from synurophytes. We challenge investigators working on other types of algae to examine their specimens using this technique.

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