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A Technique for Making High-Resolution Megapixel Mosaic Photomicrographs of Nematodes

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Abstract: Multiple images of a whole nematode specimen were taken with a high power oil-immersion objective lens and joined together to form one high-resolution megapixel, mosaic photomicrograph of the entire specimen, with the use of a relatively new mounting technique made with a 4% water agar pad. The agar pad kept the specimen nearly level and lateral, and when amended with 10 mM sodium azide, this mounting technique gradually paralyzed the nematode in a natural pose to enable production of sharp, clear images. The individual photographs were joined together and merged into one very large, seamless image. These montaged images will be useful for teaching because the student has access to a virtual specimen that is mounted in the correct orientation, imaged with a research grade microscope, and preserved in a narcotized, living condition. Such specimen images can also serve as representatives of type and voucher specimens without the deterioration typical of real types. The files can be copied and viewed with a computer almost anywhere and at any time, rather than using a more cumbersome, limiting, and expensive microscope.

Key words: agar pad technique, digital micrographs, mounting nematodes, type specimens, voucher specimens.

Single photomicrographs of nematodes on film or in digital format are limited both by resolution of the objective and by the field of view (Piper, 2008). Observation of the entire specimen requires the use of a low-power dry objective that has limited resolution; whereas, improving the resolution with a higher power oil immersion lens limits the field of view. Therefore, detailed observation of a nematode requires a series of high-power observations along the entire length of the specimen. Fortunately, digital images can be stitched together to form one mosaic image that appears to be a single photograph. This photomicrograph can be viewed at various levels of magnification, allowing the viewer to zoom from a very low-power magnification of the entire specimen to a very high magnification of detailed structures of the nematode. These high-resolution micrographs of entire nematodes are very useful for recording nematode morphology for taxonomic studies, for training extension workers on the identification of nematode genera and species, and for teaching students about the morphology and taxonomy of nematodes.

The most commonly used techniques for mounting whole nematode specimens usually involve gently relaxing them over a hot flame in water, or pouring a hot fixative over them in a small amount of water. These techniques have the advantage of causing the specimen to form a typical shape that is sometimes useful in the diagnosis of the group, but heating can easily be under- or overdone where the nematode either remains alive, or the coagulation of proteins is so extreme that a loss of many structural details is evident. Furthermore, different techniques of supporting the coverslip, such as with rings made from sealants, wax, or nail polish, or with three small glass rods, are difficult to use, the specimens often do not lie in a lateral position, and they are not level. If the supports are too small, the specimens become flattened, and if they are too big, the specimens may float out of the horizontal plane.

Agar has been used to support the coverslip, essentially replacing the small glass rods used for traditional nematode mounts (Grewal, 1990), or for observing the vulva areas of cyst nematodes (Esser, 1988); however, the agar pad technique (Driscoll, 2008) has been used mainly by researchers working with *Caenorhabditis elegans* (Maupas, 1900) Dougherty, 1953. Specimens mounted on an agar pad are nearly always level and they lie in a lateral position. This agar pad technique was slightly modified to make preparations of nematodes that are level and lying in a natural lateral position (Fig. 1). The water agar prevents the nematodes from being flattened by the weight of the coverslip and it contains a narcotizing agent that causes paralysis instead of an unnatural death caused by heat or harsh fixatives. As a result, the specimens can be photographed at high resolution with very fine details, reminiscent of fresh, living nematodes (Figs. 2-3). Furthermore, students who have never mounted a nematode before usually achieve success on the very first attempt; whereas, other techniques require several days or weeks to master.

Montaged images of nematodes that have been narcotized in a living condition are mounted in the correct lateral orientation and photographed with a high quality, research-grade microscope. For nematode taxonomy and morphological research, these images can serve as representatives of type and voucher potentially are preserved in perpetuity. These images also can serve as voucher specimens for molecular and field research, and they can be duplicated and viewed on a computer screen, rather than a microscope, for extension workshops and training students in the classroom. High-resolution mosaic micrographs insure that every student has the opportunity to see the best, most representative specimen, photographed with a high quality microscope,

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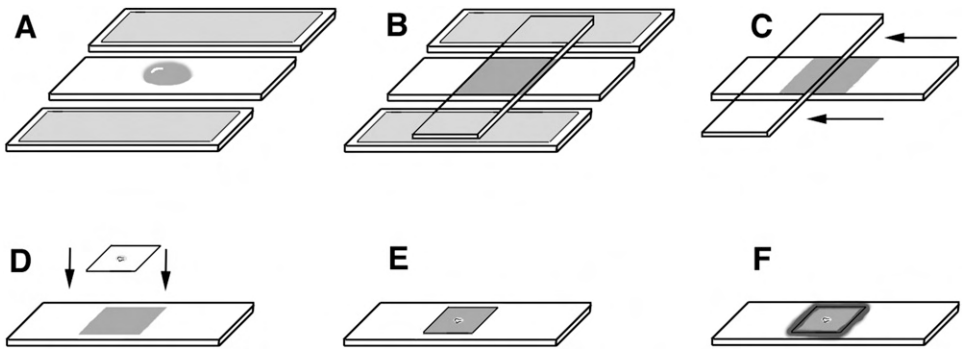

Fig. 1. Drawings illustrating the various steps of mounting nematodes in a level and lateral position utilizing the agar pad technique. A.) A 100-µl drop of melted 4% water agar is pipeted onto a glass slide surrounded on both sides by glass slides covered with a thin slip of card stock paper. B.) A second glass slide is placed on top of the melted agar so that a thin agar layer is formed between the two slides. C.) After the agar has solidified, one of the slides is gently removed, leaving a thin pad of agar on the other slide. D.) The agar pad is trimmed with a single-edged razor blade so that it is smaller than the coverslip. Specimens are placed in a small drop of water on a coverslip that is inverted to form a hanging drop. E.) The coverslip is placed in the proper position on the agar pad and left undisturbed so that the nematodes are not disoriented from their natural lateral position. F.) The coverslip is sealed in place with a generous layer of fingernail polish. After the sealant has dried, the specimens can be observed and photographed for several hours. (Redrawn from Driscoll, 2008)

as long and as often as necessary, and on his/her own personal computer. Furthermore, these specimens will be available for review and reference by the students for as long as they keep them on a computer or have access to them on a website.

Materials and Methods

Mounting nematodes that are level and lateral: Two glass slides covered with a thin card stock (Fig.1) were placed on a level laboratory bench on both sides of a third blank slide. A 100-µl drop of melted 4% water agar was pipetted onto the middle slide Fig. 1A) and a fourth glass slide was placed (at a right angle to the middle slide) on top of the agar to form a thin layer between the two slides (Fig. 1B). The top slide was placed carefully onto the agar in order to avoid the formation of air bubbles that could trap the specimens below the surface of the agar. A narcotizing agent such as sodium azide (NaN_3) (10 mM) was added to the melted water agar after it had cooled for a few minutes.

After the agar was solidified, the top slide was removed by gradually sliding it off the agar pad (Fig. 1C). The edges of the pad were cleaned with a single edged razor blade and the square was made slightly smaller than the coverslip. The pad cannot be kept for more than a few minutes because the agar will dry out and become unusable. Prepared pads may be kept for longer periods of time if they are stored in a moist chamber.

Several specimens were hand-picked into a small drop of water (containing 0.85% NaCl or KCl) on a square glass coverslip and inverted to form a hanging drop with nematodes suspended inside. The coverslip was gently placed onto the agar pad (Fig. 1D-E). Its edges were sealed with a generous coating of nail polish (Fig. 1F), taking care not to move the coverslip.

Photographing the specimen: The specimen was placed in the field of view and the nematode was examined to insure that it was lying in a lateral position that was nearly level, in a healthy physiological condition, immobile, and with morphology typical for the species. Nematodes that were still moving would become paralyzed in a few minutes. After the correct exposure was selected for the initial picture, the exposure was locked for all of the remaining images. A series of photographs along the entire length of the specimen were made, overlapping each new image by 30-50% of the previous image (Fig. 2A). The position of the camera was not moved in order to insure that the pictures were taken with the same orientation. Since the specimen was already nearly level, only the most minor adjustments to the focus were necessary in order to keep the medial plane of the specimen in focus. If certain morphological features occurred outside of the medial plane, the level of focus was changed and the photograph with that feature was imaged after the entire specimen was imaged. Depending upon the length of the specimen and the magnification, the entire nematode was photographed in a series of two to thirty or more images (Fig. 2A).

Stitching several digital photomicrographs into one mosaic: Mosaics of several photographs were composed manually with an image-editing software program (Adobe Photoshop CS6). Photoshop can also stitch the photographs together automatically. Several software programs that detect and assemble a series of photographs into one mosaic could have been used. Software that automatically selects and stitches mosaics from a series of photographs include Helicon Focus, Image J, Autostitch, PTgui, PanaVue ImageAssembler, Panoweaver, Photofit, Autodesk Stitcher, AutoPano Pro, Pixtra PanoStitcher, Panorama Plus, PhotoStitcher, and numerous others. Most of these programs are for the PC platform, but many can be used with the Macintosh platform, as well.

The photographs used in this report were taken with a Nikon D300 digital single lens reflex camera attached

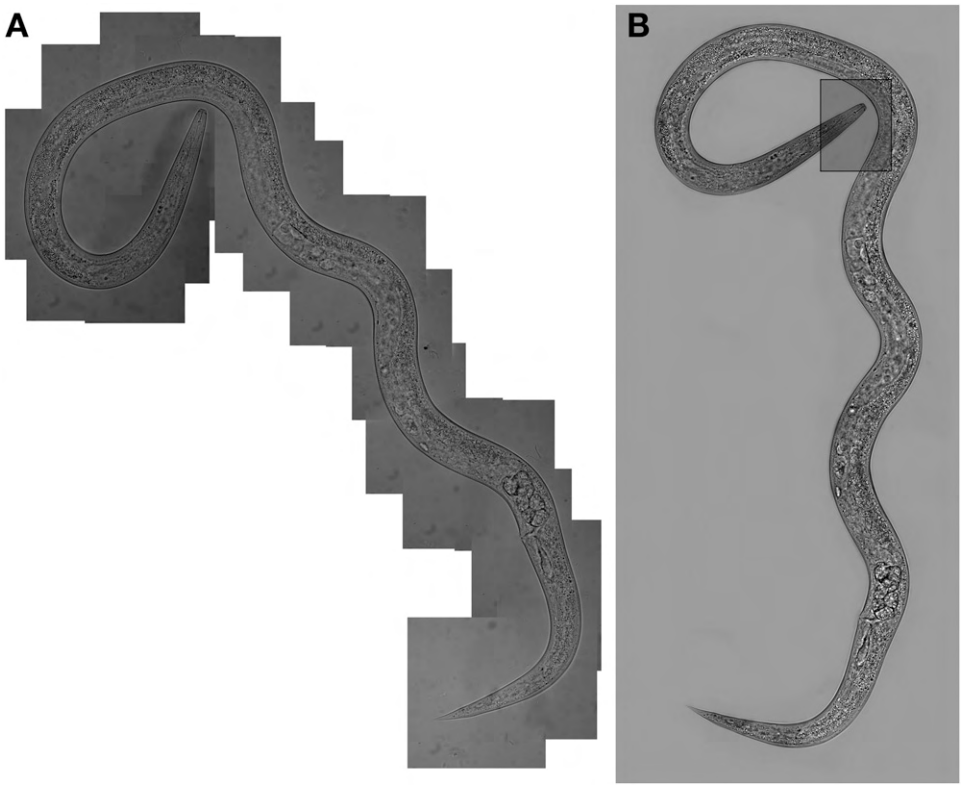

FIG. 2. A.) A high-resolution megapixel mosaic composed of 22 photomicrographs that are twelve megapixels each of a female of *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936 imaged with a digital camera attached to a plano-apo quality 63x oil immersion objective. B.) The mosaic image after the 22 individual photographs have been flattened into a single image and the background has been filled with a uniform color. The insert near the anterior end of the female has been printed at 300 dpi and presented in Fig 3.

with a T-mount onto a Leitz Dialux 22 research-grade bright field compound microscope with a 63x oil immersion lens with a 1.4 NA. For the example shown in this paper, 21 individual images were stitched by hand with Photoshop CS6 as follows:

1) All photos of the specimen that would be used to form the mosaic were opened in Photoshop CS6.
2) A starting photo was selected, usually the anterior end of the nematode.
3) The finished size of the mosaic was estimated and the canvas was enlarged to fit. The canvas could be increased in size as the image was formed or it could be cropped if it was too big for the final image.
4) The intermediate work was saved as a Photoshop file (.psd) to avoid accidentally overwriting the image file.
5) The adjacent photo forming the image was copied and pasted into the working Photoshop file.
6) The opacity of the pasted layer was adjusted to 50%.
7) The semi-transparent layer was moved into position to match the background image, and its opacity was restored to 100%.
8) The sharp, overlapping edge of the top photo was erased with the eraser tool set as a brush with soft edges.
9) Steps 6 - 8 were repeated for all remaining photos of the specimen (Fig. 2A).
10) Finally, a selection was created to crop the mosaic photograph into a rectangular shape that contained the entire specimen (Fig. 2B).
11) The specimen was rotated so that it was in a nearly vertical orientation.
12) All of the layers of the mosaic photomicrograph were flattened into one, and additional editing replaced the blocky, irregular background with the same color (Fig. 2B).

RESULTS

The resulting high-resolution megapixel mosaic photomicrograph of *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936 (Figs. 2-3) is the most detailed photograph that has been made of a whole nematode; it is more than 616 megapixels in size. At 300 dpi, the photograph is 80 cm wide by 194 cm long. An insert of the whole specimen shown in Fig. 2B is presented at 300 dpi in Fig. 3. Many details of the specimen are clearly visible including the morphology of the stylet, the opening of the dorsal pharyngeal gland, and the nucleoli and nuclei of the oocytes. The photograph of the entire specimen contains many additional morphological details. Because of the large size of the imaging sensor in modern cameras (12.8 megapixels in this case), these photographs

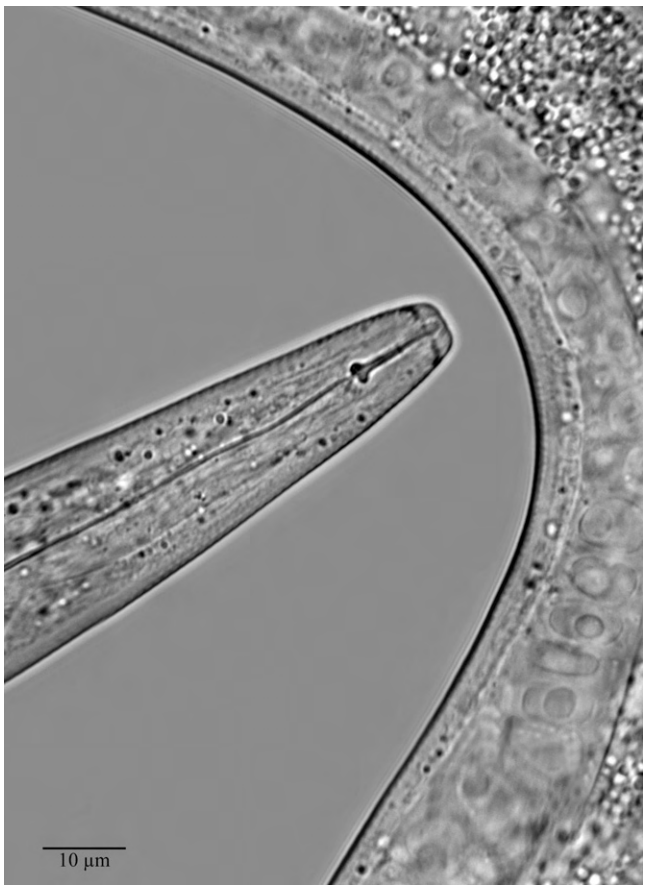

Fig. 3. A portion of a high-resolution megapixel mosaic photomicrograph of a female of *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936 printed at 300 dpi showing the details that are visible for the entire specimen when observed on a computer monitor or printed on a large format printer.

can be reduced in size by 50% and still retain many of the morphological details that were contained in the original image.

Discussion

High-resolution megapixel mosaic micrographs provide an image that is superior to corresponding single image micrographs because of the inherent nature of the optical objectives utilized in the two scenarios. In the single-shot image, the optical objective lens has a much lower lateral resolution than in the mosaic image where the objective lenses have a much lower numerical aperture and depth of field but a much higher lateral resolution. In this case for the tylenchid nematodes with rather simple morphology, the lower depth of field is not extremely important because the optical section through the middle of the specimen provides a significant amount of information about the morphology of the entire specimen. For those details that occur in different planes, supplemental images can be inserted into the background of the mosaic image, or they can be imposed at the correct location on the mosaic image by utilizing image-editing software. For other nematodes with more complex head morphology additional imaging techniques may be necessary to record important details such as video focusing through multiple focal planes (Anonymous, 2012; DeLey, 2002; 2012).

Poor quality images can be made with the techniques described in this paper. Good images depend upon a physiologically healthy specimen that is typical in morphology. The nematode must be properly anesthetized so that it will not move during the lengthy series of exposures; the microscope has to be of research grade and properly adjusted, maintained, and cleaned; the camera must be of high quality with a large sensor; and the mosaic has to be composed in a skillful manner. However, a well-done final image is vastly superior to any image taken with a single exposure.

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