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C. Greaves and J.B.R. Eamer

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Critical review

A. Normandeau

L.T. Dafoe

Authors

C. Greaves (cameron.greaves@nrcan-rncan.gc.ca)

*Geological Survey of Canada
1 Challenger Drive, P.O. Box 1006
Dartmouth, Nova Scotia
B2Y 4A2*

J.B.R. Eamer (jordan.eamer@nrcan-rncan.gc.ca)

*Geological Survey of Canada
1 Challenger Drive, P.O. Box 1006
Dartmouth, Nova Scotia
B2Y 4A2*

Present address:

*Department of Earth and Environmental Science
Acadia University
12 University Avenue
Wolfville, Nova Scotia
B4P 2R6*

Correction date:

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Focus stacking for cataloguing, presentation, and identification of microfossils in marine sediments

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Abstract: Microfossils represent an important part of studying past depositional environments and determining ages for the strata they are found within. The key to ascribing paleoenvironmental interpretations to the sediments in which a microfossil is found is accurate identification of the microfossil. A number of techniques can be used to identify microfossils, including ones that use key features, morphologies, and characteristics from imagery acquired using a scanning electron microscope. A low-cost, efficient alternative method is digital photography of optical microscope images. This technical note presents a method for acquiring photos of microfossils and two methods for compiling them into high-resolution images using focus stacking. The process is described in four main steps: image acquisition, exportation, focus stacking, and annotation.

Résumé : Les microfossiles constituent un élément important de l'étude des milieux de dépôt du passé et de la détermination de l'âge des strates dans lesquelles ils se trouvent. La condition fondamentale pour attribuer des interprétations paléoenvironnementales aux sédiments dans lesquels se trouve un microfossile est l'identification précise du microfossile. Un certain nombre de techniques existent pour identifier les microfossiles, dont celles qui utilisent les principaux traits, les morphologies et les caractéristiques révélés par des images acquises au microscope électronique à balayage. Une méthode alternative peu coûteuse et efficace est la photographie numérique d'images au microscope optique. Cette note technique présente une méthode pour acquérir des photos de microfossiles et deux méthodes pour compiler celles-ci en images haute résolution à l'aide de l'empilement de mises au point. La façon de procéder comporte quatre étapes : l'acquisition d'images, l'exportation, l'empilement des mises au point et l'annotation.

Corresponding author: J.B.R. Eamer (jordan.eamer@nrcan-rncan.gc.ca)

INTRODUCTION

Marine microfossils are organisms or skeletal elements that require a microscope for analysis or identification because of their small size (usually <1 mm) (Witkowski et al., 2015). They play an integral role in marine geology. They are used to correlate stratigraphic facies (e.g. Fensome et al., 2019), to provide the material necessary for absolute age determination (e.g. Lister et al., 2015), for deriving paleoenvironmental indicators (e.g. Pieńkowski et al., 2013), for developing data on modern and ancient oceanographic processes (e.g. Bradley et al., 2012) and on sedimentary processes (e.g. Mudie and Yanko-Hombach, 2019), and for geochemical methods such as isotopic studies (e.g. Bohaty et al., 2012), among other uses.

Developing useful data sets from microfossils extracted from a geological sample requires accurate identification of the taxonomy of the fossil. This is done using microscopy and, increasingly, using digital methods. With advances in technology related to microscopy and imagery, the digitization of microfossils viewed under an optical microscope (as opposed to images generated using a scanning electron microscope) enables researchers to share, publish, and discuss microfossils inexpensively and efficiently. Digitization has also enabled the development of efficient tomographic techniques for generating 3-D images from several 2-D optical images, important for deriving more information from a fossil (Sutton, 2008).

Improvements in image processing software, and the increasing ubiquity of software developed for this purpose, have led to opportunities to collect higher quality digital imagery from optical microscopes. In particular, adding a third dimension through serial focusing (Sutton, 2008) or focus stacking (e.g. Kibby, 2019) is a simple and effective way to produce '2.5-D' imagery of microfossils. Systems automating both photography and focus stacking for high-resolution imagery exist (Chen et al., 2019), but they are expensive and ideal for digitization of material from archival collections (Thorpe and Zimmerman, 2019). A study by Miller et al. (2015) described a cost-effective technique for photographing conodont fossils using a petrographic microscope. Here, we present a similar method developed using free and simple software and a standard laboratory optical microscope/camera setup. The material used for demonstration below includes mixed benthic foraminifera sampled for radiocarbon dating from archival sediment core 90031-0061PC (Thibaudeau and Currie, 1991) held in the GSC-Atlantic Marine Geoscience Collection (core data are available from the NRCan expedition database: https://ed.marine-geo.canada.ca/cruises_e.php).

METHODOLOGY

Preparing samples

Selected subsamples from the 90031-0061PC core were wet-sieved to greater than 150, 125, and 63 μm fractions and dried on filter paper. A fine-tipped paint brush was used to isolate microfossils from sediment on picking trays. Representative microfossils were picked for photography. Each microfossil was centred on a tray with a black background.

Acquiring images

Images in this technical note were acquired on a Zeiss Axio Scope A1™ in reflected light brightfield configuration using the Zeiss AxioCam 512™ colour camera with 1 \times lens magnification and variable objective focus (10–20 \times). The AxioCam captures all images to a proprietary RAW .czi file, which can be exported to TIFFs while preserving most of the important image metadata. The Zeiss microscope comes with free software, ZEN lite™, which is used for acquisition and exportation (ZEN lite is available at <https://www.zeiss.com/microscopy/en/products/software/zeiss-zen-lite.html>).

To acquire imagery, first align the microfossil on a photography tray, ensuring that key features for identification (such as the aperture of the foraminifer) are facing up. Next, focus and centre the sample under the microscope. Launch the ZEN lite software. The workflow for photo acquisition is shown in Figures 1 and 2. Turn on 'Live View' (red label 1 in Fig. 1) and ensure that the microfossil is centred under the camera. Adjust the 'Microscope Components' setting (label 2) to match the camera model and the objective focus that the microfossil is being viewed under. Under the 'Camera' setting, set 'White Balance' to 'Auto' (label 3). For file saving, set up a directory folder under 'Tools' > 'Options' > 'Saving' and select the destination path for the files to be written to. Once the edge of the specimen is brought into focus, make note of the tick mark that corresponds to the fine-focus dial position (edge). Next, bring the very top of the specimen into focus and make note of the fine-focus value (top). The difference between these two readings will be the number of pictures needed: each picture will be taken with the lens 4 μm closer to the stage (one increment on the fine-focus drive, Fig. 2) than the previous focal depth. The focal depth slices will define components of the Z-stack for the composite image (Fig. 3). To acquire the first image of the stack, start with the very bottom edge of the microfossil in focus and click the 'Snap' button in ZEN lite (label 4 in Fig. 1). Turn the dial counterclockwise, moving to the next fine-focus increment. Repeat this process until the top of the sample is reached. For microfossils without significant relief, twice as many focal depths (photos at half-increments) may be necessary within the same fine-focus drive distance.

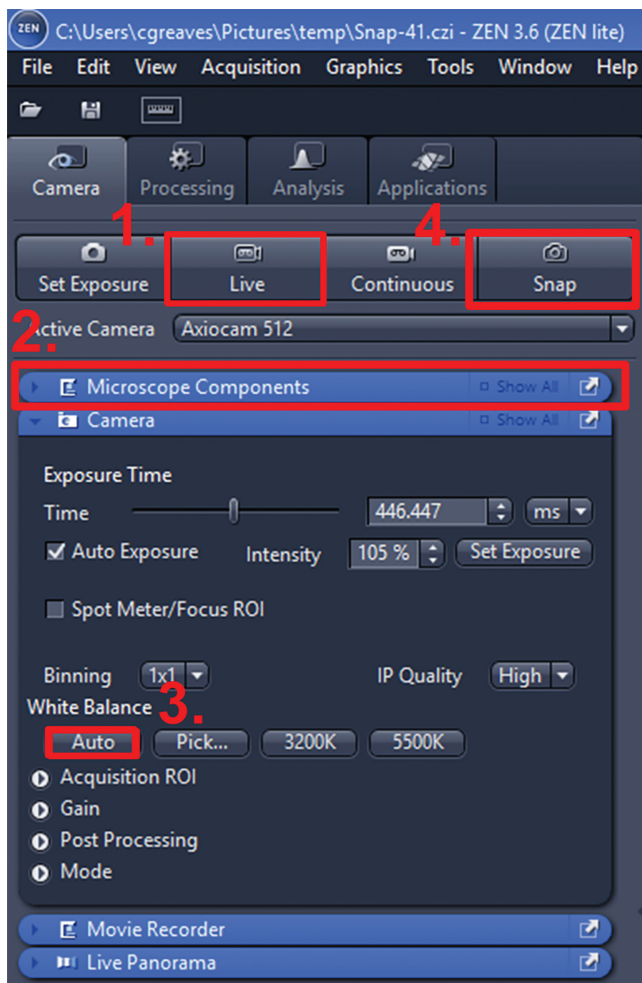


Figure 1. ZEN lite (3.6) image acquisition workflow.

Exporting .czi files to TIFFs

A batch export of .czi files can be completed in the ZEN lite (3.6) software, detailed in Figure 4. Select RAW files under 'Processing' (red label 1 in Fig. 4) > 'Batch' (label 2) > '+Add...' (label 3) and then assign the folder directory. To assign the batch export parameters select 'Batch Method' (label 4) > 'Image Export' > 'Parameters.' Additional settings are visible under 'Show All' (label 5) in the top right of the 'Parameters' field. Create a name for the export parameters (label 6). For recommended settings, select 'File Type' > 'TIFF' (label 7), select 'Original Data' (label 8), and unselect 'Create Folder' (label 9). It is easiest to save the files to the same folder by selecting 'Use Input Folder as Output Folder' (label 10). By default, these parameters will only be edited for a single image and will need to be assigned to the other images in the listing. Select the image from the listing and select 'Copy Parameters' (label 11) and highlight the rest of the image list by checking the 'Select All' box (label 12) in the top left of the 'Batch Processing' window. Select 'Paste Parameters' (label 13) and click 'Run Selected' (label 14) to



Figure 2. Zeiss Axio Scope microscope fine-focus dial, indicating drive direction for increasing the height of focus. Photograph by C. Greaves. NRCan photo 2022-445

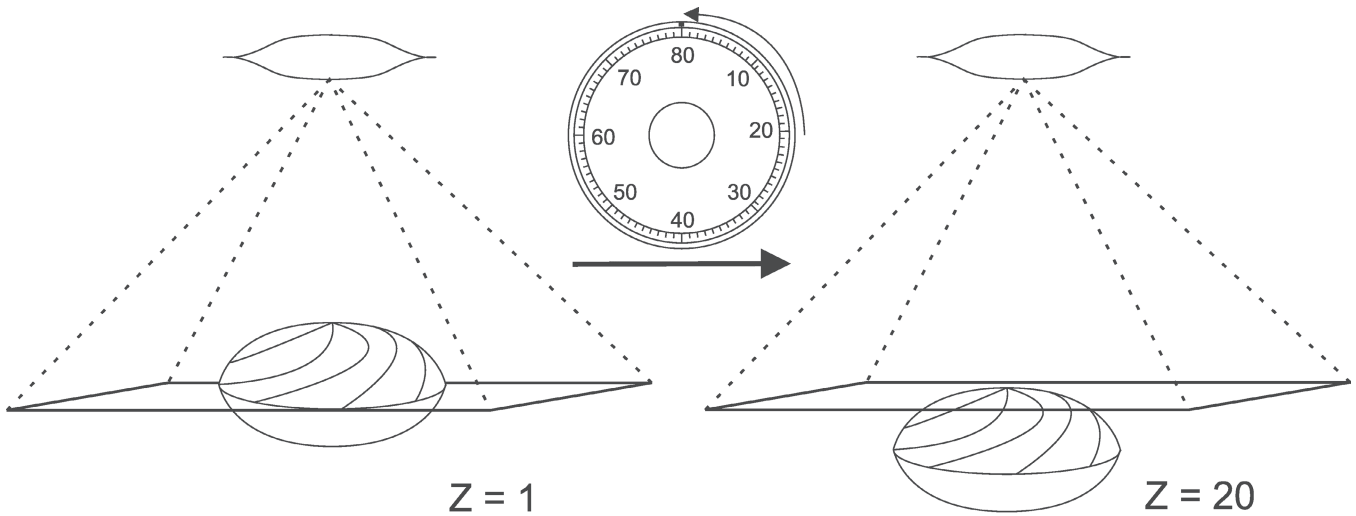
execute the batch export. Note that photos acquired using a microscope other than a Zeiss can still be batch exported in the TIFFs through the Zen lite software.

Focus stacking

Focus stacking can be done using a variety of software. For this note, Teledyne Lumenera Infinity Analyze 7™ (available at <https://www.lumenera.com/support/microscopy/drivers-downloads.html>) and ImageJ (Fiji) (Schindelin et al., 2012) using the Extended_Depth_Field.jar (Forster et al., 2004) plugin (from <http://bigwww.epfl.ch/demo/edf/>) were used. Note: the former is freeware; the latter is open source. The Infinity Analyze software has a user-friendly interface with an efficient workflow; however, sharper results have been produced from using ImageJ.

Focus stacking in Infinity Analyze 7

Focus stacking is highlighted in Figures 5 and 6. Open up the TIFFs using 'File' > 'Open' and select the directory for the folder with the images. Once all the images are loaded into Infinity Analyze, locate the focus stacking tool: 'Combine' > 'Focus Stacking' (red label 1 in Fig. 5). A prompt window will open with all the files available in the bottom pane. Select all these images and drag and drop them into the 'Focus Stack' pane (label 2). Keep all default settings and hit 'Preview' (label 3). This will generate a stacked



*Microscope stage is moved down by one fine-focus value for each Z-slice

Figure 3. Illustration of focal-depth increments (Z-slices) in relation to the microfossil, showing the fine-focus for the edge on the left and the fine-focus for the top on the right. Each tick mark increment represents a fine-focus value for photo acquisition.

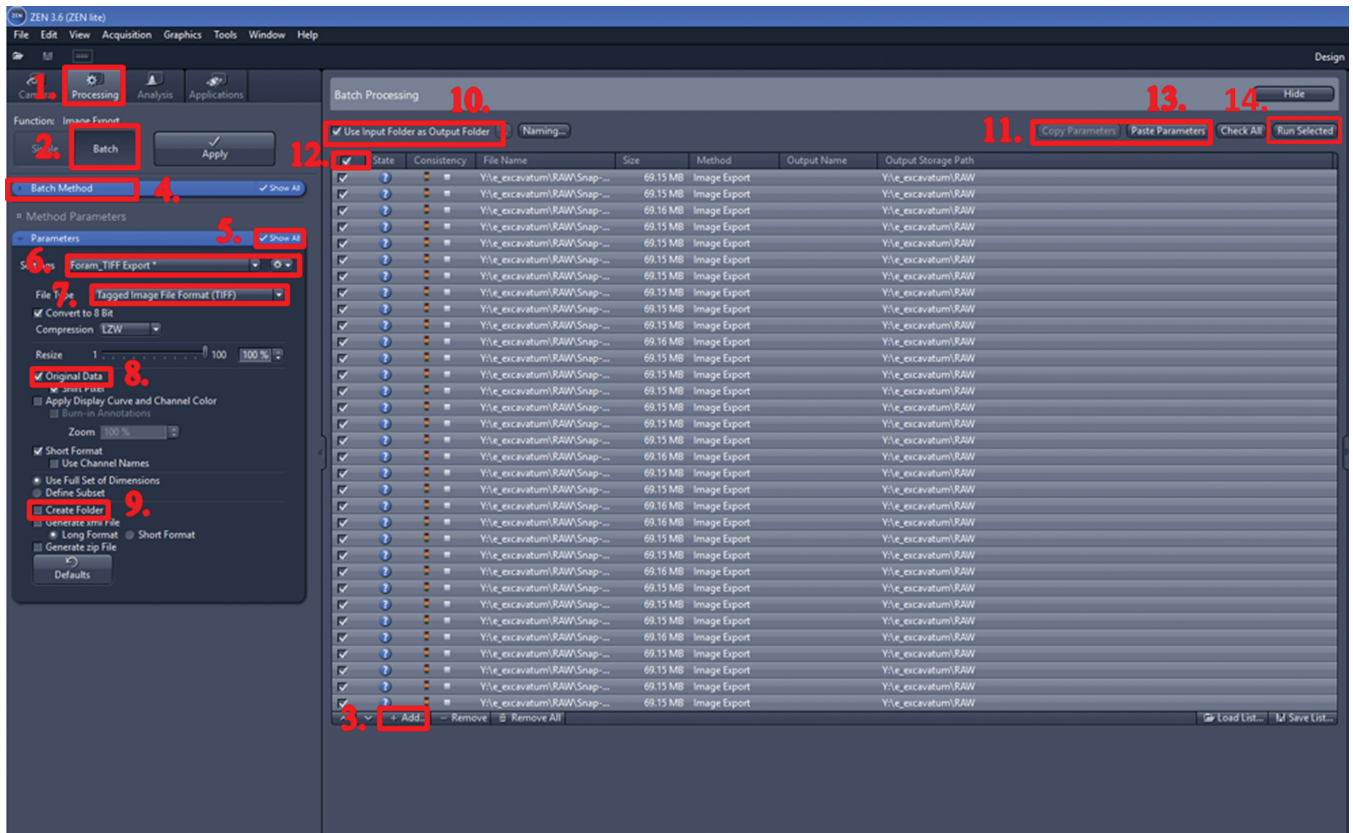


Figure 4. Workflow for exporting .czi files to TIFFs in ZEN lite (3.6).

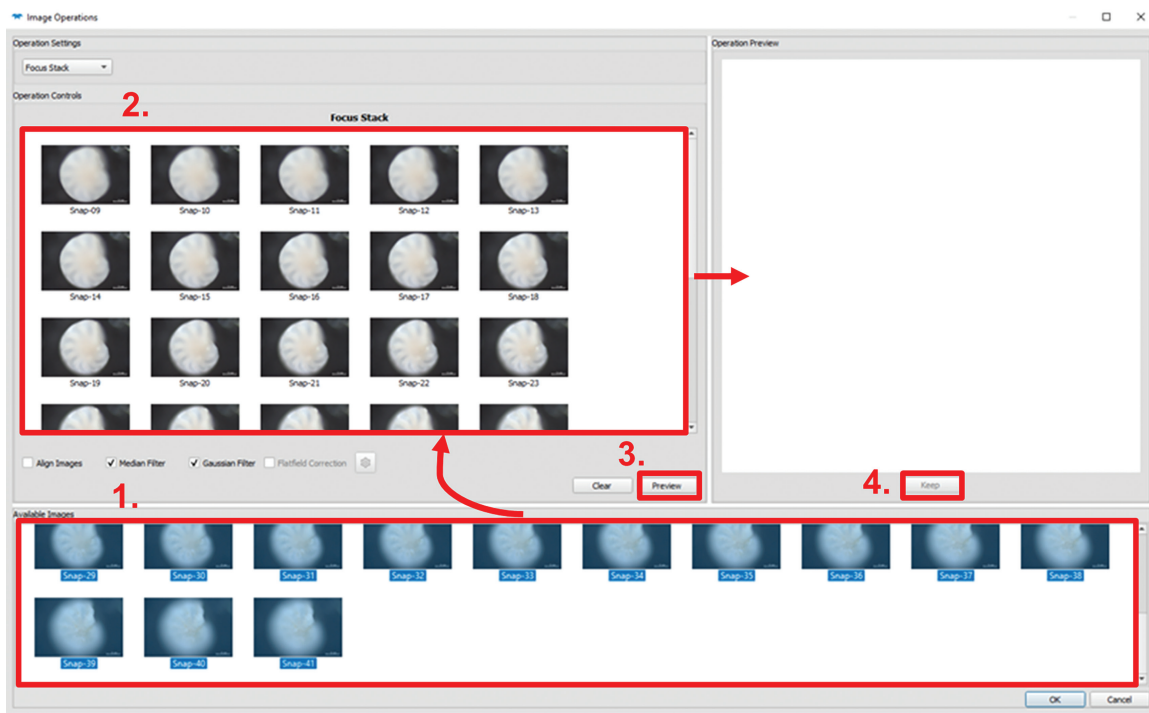


Figure 5. Focus stacking workflow for Infinity Analyze 7.

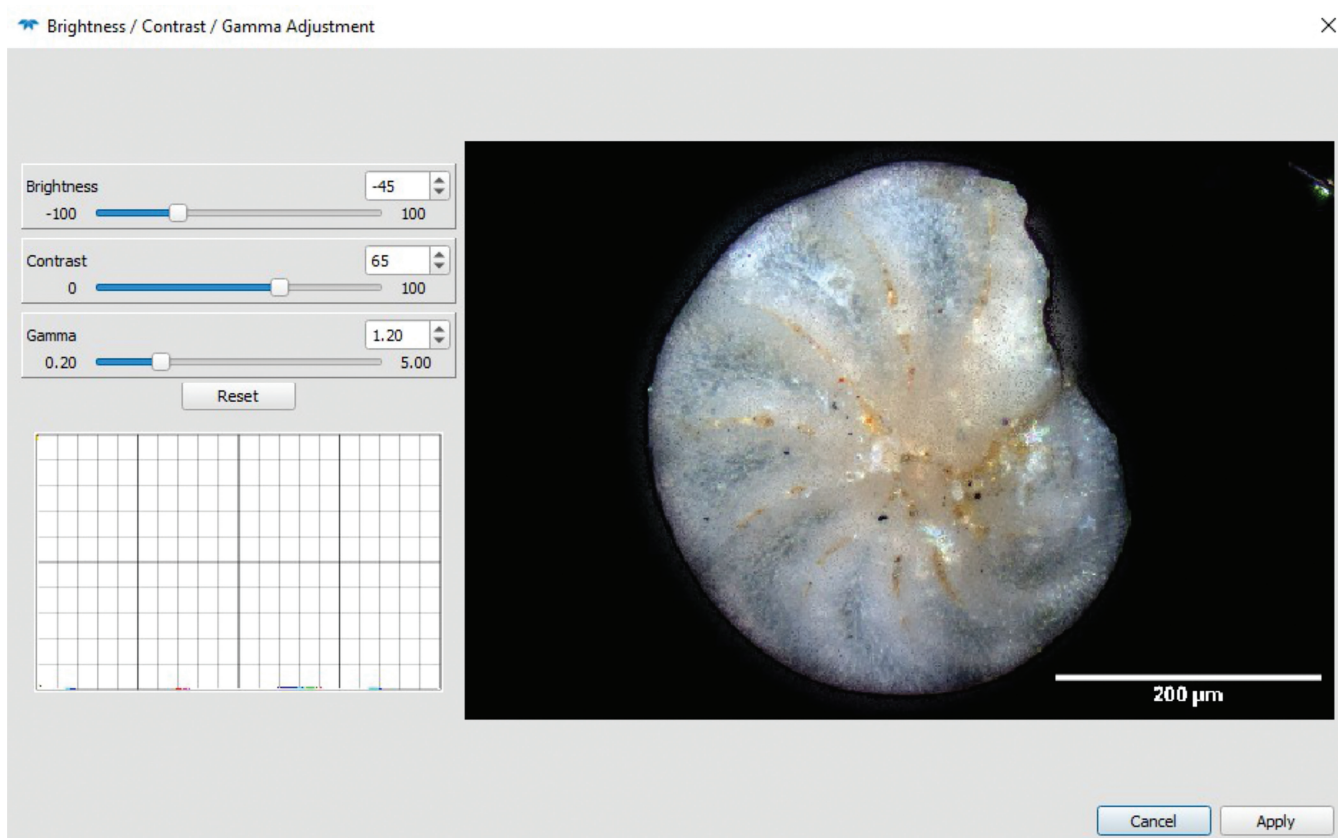


Figure 6. Image adjustments for focus-stacked image in Infinity Analyze 7.

image, and if results are satisfactory, select 'Keep' (label 4). The generated image will be a bit blurry and require adjustment. Under 'Adjust,' select 'Brightness / Contrast / Gamma'; recommended settings used in this note for sharp images are 'Brightness' = -50 to -40, 'Contrast' = 50 to 65, and 'Gamma' = 1.00 to 1.20 (Fig. 6). Important note: other image processing tools can be found under 'Enhance,' but these should only be used with careful consideration, as they change the data of the original image.

Focus stacking in ImageJ (Fiji) with TIFFs

To start, install the plugin `Extended_Depth_Field.jar` into ImageJ by opening the folder with the installation of ImageJ. Under the folder named 'Plugins,' drag and drop the `Extended_Depth_Field.jar` plugin and restart ImageJ to ensure the installation takes effect. To generate a stack in ImageJ, go into 'File' > 'Import' > 'Image Sequence.' In the 'Import Image Sequence' window, select 'RGB' (red-green-blue) image type and keep the other default settings. ImageJ will assemble these photos into a stack; image stacking may take a few minutes to complete, depending on available memory on the computer. Once finished, type 'EDF' in the ImageJ search bar, which will result in 'EDF Easy Mode' becoming available under the 'Commands' line. Select this and click 'Run.' Accept the defaults of this tool (Fig. 5) and run it, which may take a few minutes. After the focus-stacked image is finished, it can be fine-tuned under 'Image' > 'Adjust' > 'Brightness/Contrast' (Fig. 5). The sharpest images can be created by lining up the slope of the line with the histogram maxima. Once set, save the focus-stacked image as a TIFF under 'File' > 'Save As' > 'Tif.'

Focus stacking in ImageJ (Fiji) with RAW .czi files

Install the plugin `Extended_Depth_Field.jar` in the ImageJ plugin folder (as described above) and ensure that your current version of ImageJ has Bio-Formats installed. If not, you will have to install it (available at <https://www.openmicroscopy.org/bio-formats/downloads/>). This is the same process as for the EDF plugin. To stack the .czi images, type 'Bio-Formats' in the search bar and select 'Bio-Format Importer' under the 'Commands' tab. Run the command, and you will be prompted with a file directory. Choose the folder where the .czi files can be found and select 'OK.' An additional window will open, 'Bio-Formats Import Options,' for which the following options should be selected: under 'Stack viewing' select 'Hyperstack'; under 'Dataset organization' select 'Group files with similar names,' and under 'Colour options' select 'Composite.' Accept all other defaults and press 'OK.' The program will assemble a hyperstack and will split the colour channels into RGB, which multiplies the photo stack by three. Once the stack is loaded, select 'Image' > 'Colour' > 'Stack to RGB,' which will merge the colour channels

back to a composite colour image. Under the search tab, type 'EDF' and run the 'EDF Easy Mode' command and click 'Run.' A prompt will open with a 'Speed/Quality' slider and a height-map regulator ('Height-map reg.') slider. Accept the defaults and click 'Run,' which may take a few minutes (Fig. 7). After the focus-stacked image is finished, fine-tune it under 'Image' > 'Adjust' > 'Brightness/Contrast.' The sharpest images usually will be found by aligning the slope of the line with the histogram maxima (Fig. 8). Once set, save the focus-stacked image as a TIFF under 'File' > 'Save As' > 'Tif.'

Annotating

Determining scale

Most microscope cameras should have a system that writes the scaling metadata onto the image in the acquisition software. Resolution tends to be expressed in pixels per inch for general photography; however, scientific photography will report resolution in pixels per micron. To check the resolution of an image in ImageJ, open one photo from the stack and select 'Image' > 'Show Info' (Fig. 9).

Setting scale

In ImageJ search for 'Set Scale' and run the command. A window prompt (Fig. 10) will ask for the distance in pixels per known unit of length; microns can also be entered as micrometres (μm). The aspect ratio should always be left as 1.0; to keep the scaling the same for all images select 'Global.'

Adding scale bar

In ImageJ search for 'Scale Bar' and run the command (Fig. 11). The default scale bar is horizontal, and the length can be defined in microns. All adjustments can be seen on the image. Recommended settings used in this technical note for 0.16 microns/pixel resolution is 200 μm width, a line thickness of 25 pixels, and a font size of 100. All annotations can be permanently fixed to the image by unselecting the 'Overlay' option. This will allow the scale bar to be seen outside of a technical image viewer such as ImageJ. To save the final image select 'File' > 'Save As' > 'Tif' (Fig. 12). For compressed copies of the base file, export to JPEGs.

CONCLUSION

Photographing microfossils at varying focal depths is a rapid, inexpensive process that uses focus stacking to develop high-resolution images. The process can be subdivided into image acquisition, exportation, focus stacking, and annotation.

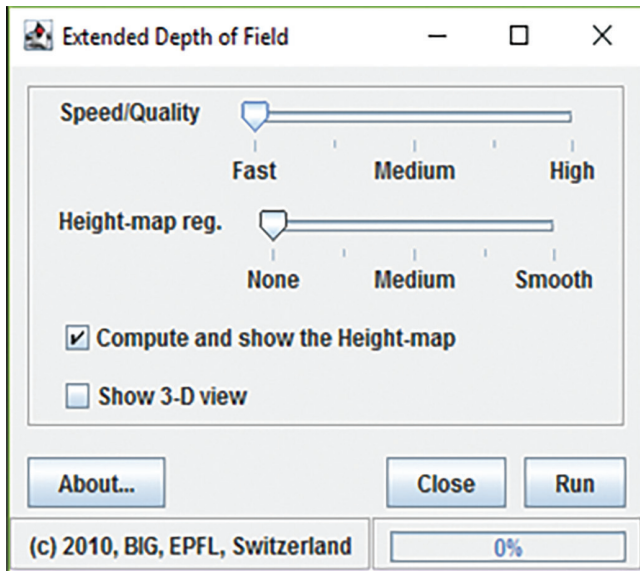


Figure 7. Focus stacking using Extended_Depth_Field.jar plugin.

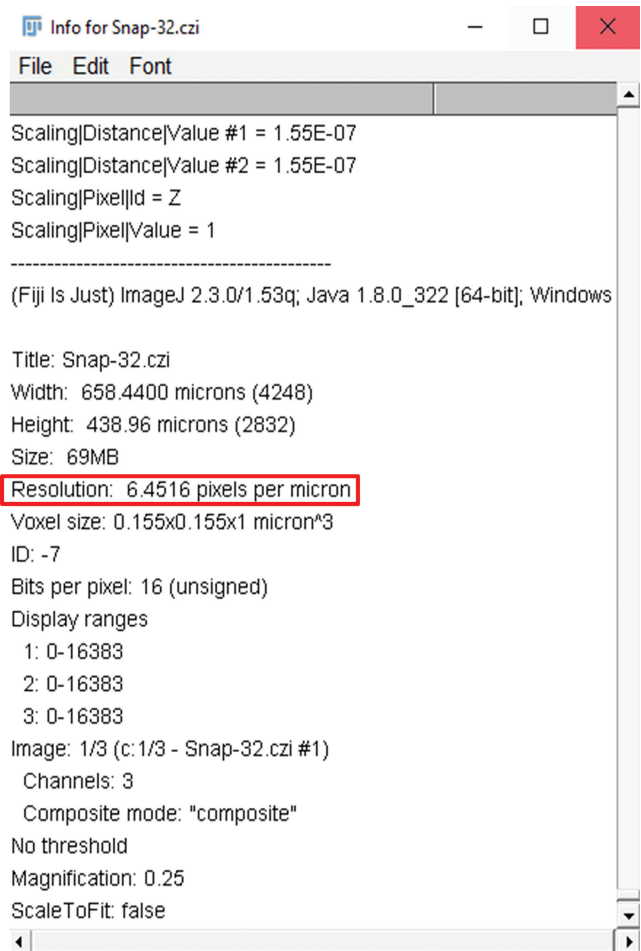


Figure 9. ImageJ image information tab with image resolution entry highlighted.

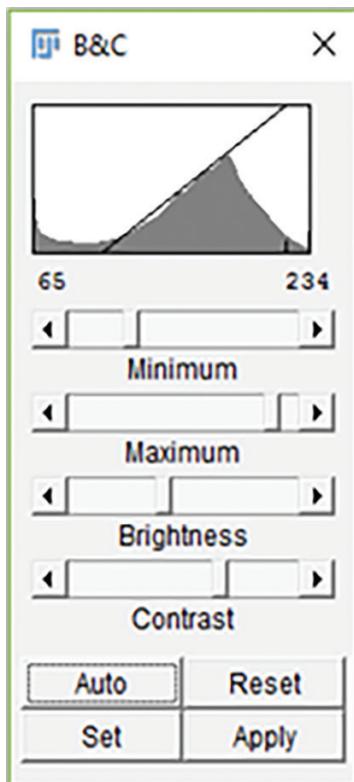


Figure 8. Image adjustment of brightness and contrast in ImageJ.

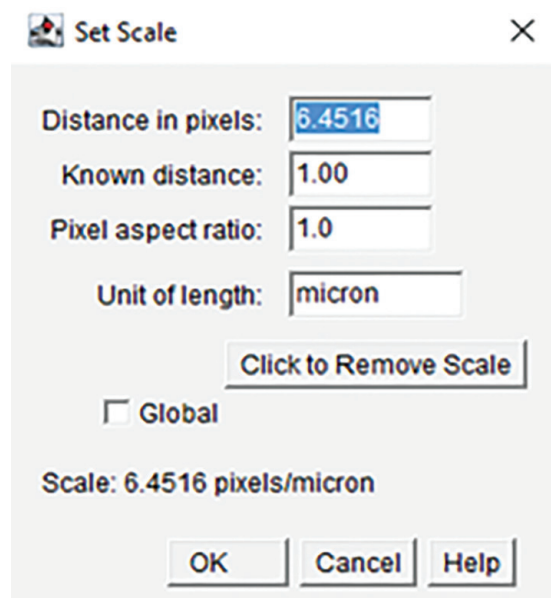


Figure 10. ImageJ 'Set Scale' command configuration window.

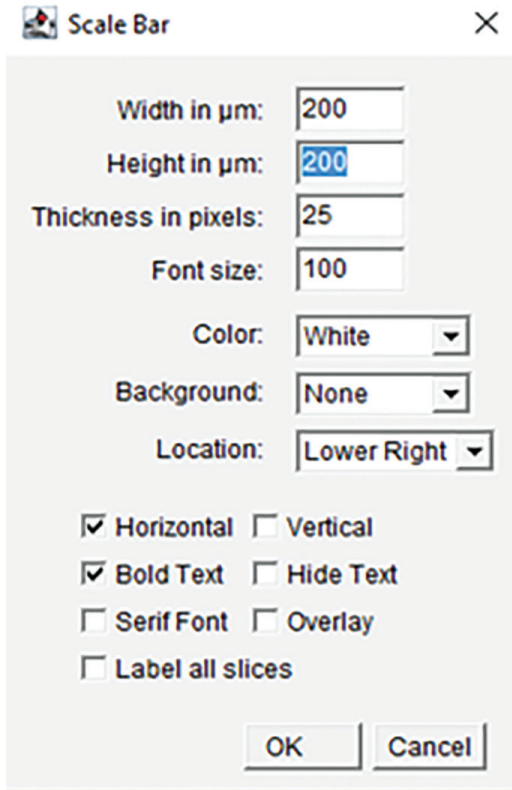


Figure 11. ImageJ 'Scale Bar' window.

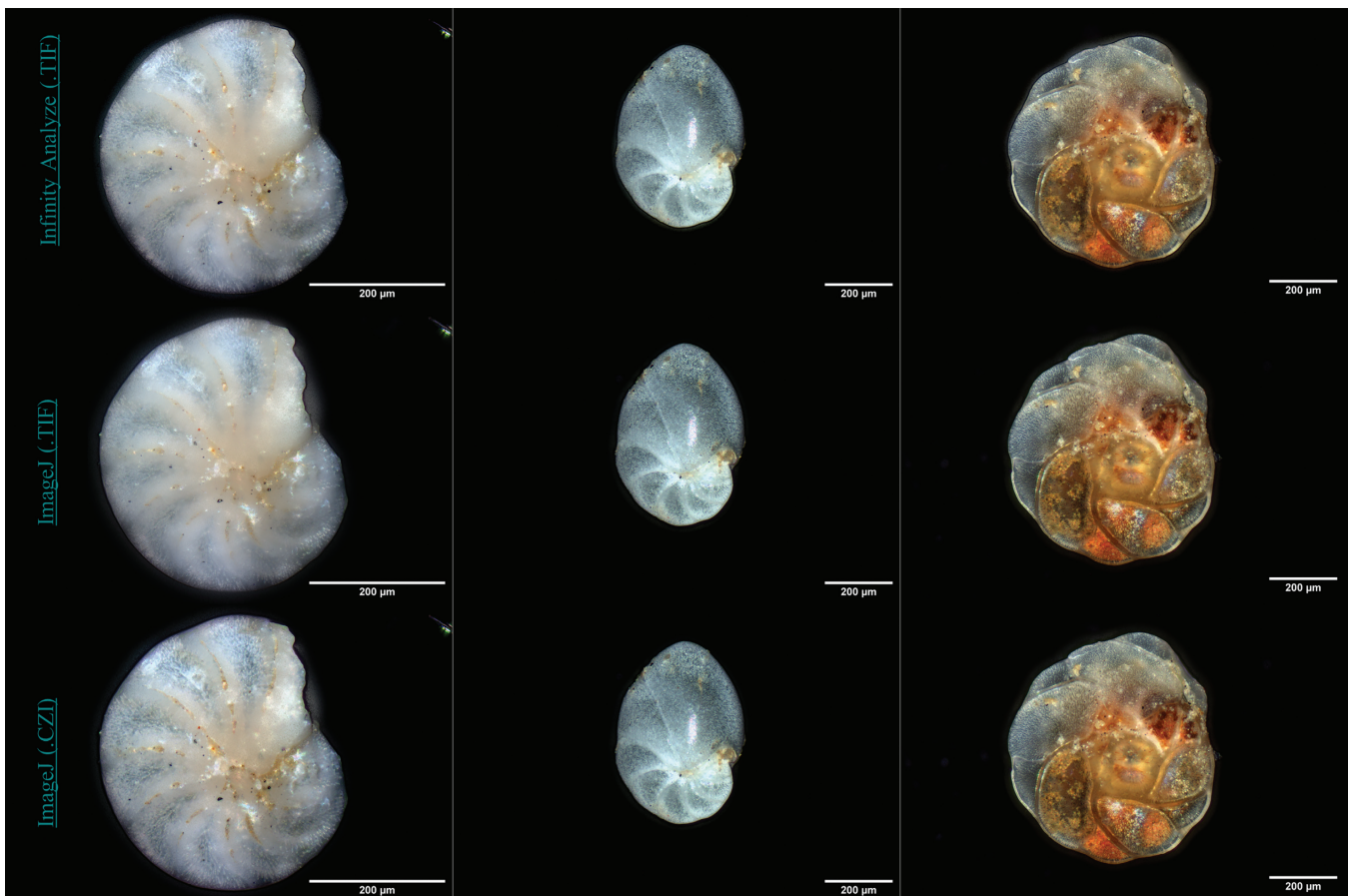


Figure 12. From top to bottom: focus-stacked images (focus stacks) using Infinity Analyze 7, focus stacks using TIFF exports in ImageJ, and focus stacks using RAW .czi files. Both stacks in ImageJ used the Extended_Depth_Field.jar plugin (Forster et al., 2004).

The acquisition process consists of capturing photos at 4 µm fine-focus increments from bottom to top, each of which has focused slices of the fossil. To maintain image fidelity, it is key to batch export these images into an uncompressed file format (.czi or TIFF was used in this note). Once exported, these images can be assembled into a Z-stack, where they can be algorithmically assembled into a composite image using focus-stacking software (e.g. ImageJ or Infinity Analyze). Lastly, photo viewer application tools can be used to display annotated information, such as a scale bar, on the image.

The procedure highlighted in this technical note will allow researchers to more easily share valuable information that is rich in detail to better communicate and answer questions relating to paleoenvironment, paleogeography, and paleoecology.

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