IMPROVING DEPTH OF FIELD RESOLUTION FOR PALYNOLOGICAL PHOTOMICROGRAPHY

Antoine Bercovici, Alan Hadley, and Uxue Villanueva-Amadoz

ABSTRACT

Optical microscopy continues to be the preferred method for imaging in paleopalynology. While usefulness of other tools, such as the scanning electron microscope, is not questioned, the ease of use and timely results of optical microscopy remains unsurpassed. However, obtaining good quality photomicrographs requires the use of the highest magnifying power objectives available, which are inevitably associated with very limited depth of field. To avoid the need for multiple photomicrographs in order to fully describe each palynomorph, a software solution for reconstructing depth of field is proposed. This solution allows for keeping the main advantages of high magnifying power objectives (better resolution and improved contrast) while suppressing their main weakness. In addition, photomicrographs published using depth of field reconstruction have a more natural appearance, similar to when directly viewed with the eye under the microscope. While this paper deals primarily with the usage of depth of field reconstruction for the enhancement of palynological photomicrograph, the technique can be applied similarly to many other paleontological and geological objects as well.

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INTRODUCTION

Optical microscopy remains the preferred method for imaging in paleopalynology. The study of palynomorphs, for both taxonomic description and quantitative data extraction from counts, strongly relies on the observation of palynological assemblages mounted on microscope slides using traditional techniques of preparation (Doher 1980; Wood et al. 1996; Traverse 2007). With the improvement of optical microscopy over time, imaging of details on transparent samples (which until then had often produced poor quality results) was made possible by optical means, without the use of chemical/dying techniques (Pluta 1989; Slayter and Slayter 1992; Davidson and Abramowitz 2002), which alter the subject in some way. These innovations include various contrast enhancing techniques (Hoffman 1977; Abramow-

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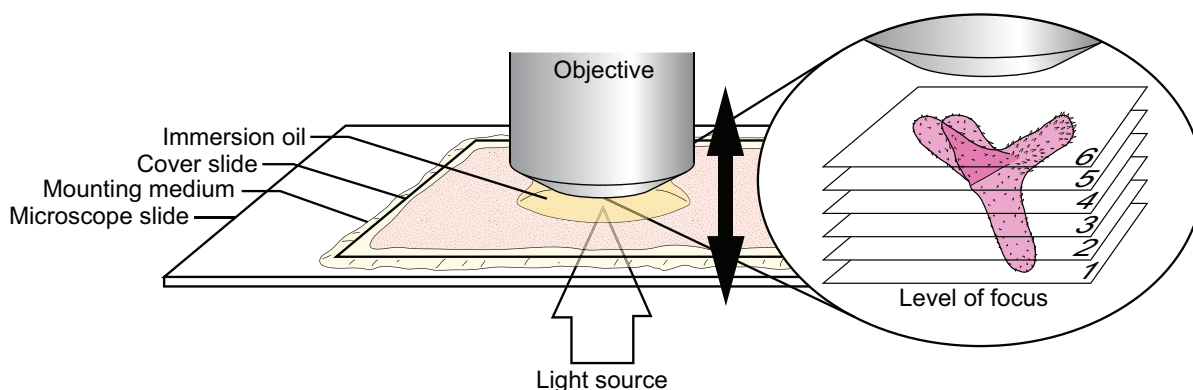http://palaeo-electronica.org/2009_2/170/index.html

FIGURE 1. Residue resulting from the standard processing of rock samples is mounted on a microscope slide using a mounting medium of appropriate refractive index. The slide is observed using transmitted light microscopy (with DIC if available), using the more powerful objective with immersion oil. Varying the focus adjustment of the microscope allows one to cross successive optical sections (planes 1 to 6 are examples) across the palynomorph.

itz 1987; Bradbury and Evennett 1996; Davidson and Abramowitz 2002) and more especially differential interference contrast (DIC) microscopy, invented in the mid 1950s (Allen et al. 1969; Nomarski 1955). For palynology, DIC allows the observation of very minute ornamentation of the exine not visible under regular brightfield microscopy.

However, optical microscopy suffers from resolution limitations. Horizontal resolution limitation (the resolving power between two points occurring in the plane perpendicular to the optical axis) is often given and is an easily understood parameter as it directly translates into maximum attainable magnification of an object. Additionally, optical systems are also characterized by their ability to resolve along the optical axis, which is termed axial resolution. The second resolution, measured in a plane parallel to the optical axis, is known as the depth of field (Pluta 1989; Slayter and Slayter 1992; Davidson and Abramowitz 2002). Practically, depth of field represents the distance that separates the nearest object plane in focus to the farthest object plane which is simultaneously in focus (Davidson and Abramowitz 2002). In microscopy, this distance is very short and typically measured in microns. This very limited depth of field does not present an issue for flat objects such as thin section, but objects preserved in three dimensions (such as palynomorphs), with a greater thickness than the depth of field for the objective. In such cases it is only possible to see a single optical cross section of the object at a time, and the entirety of the object can only be reconstructed as a mental image by constantly varying the focus adjustment of the microscope (Figure 1).

Both axial and horizontal resolutions are driven by the numerical aperture of the objective, but in different ways. As the axial resolution decreases, the horizontal resolution increases with the numerical aperture. Therefore, objectives with higher numerical apertures give more contrast and higher magnification but a lower depth of field, thus it is necessary to select an appropriate trade-off between these quantities. Traditionally this trade-off is solved in photographic descriptions of palynomorphs by including a general view of the specimen taken at a lower magnification to avoid depth of field problems, and a series of pictures at higher magnification to highlight details, or to expose different views of the same specimen as separate optical sections. While the inclusion of multiple pictures for a single palynomorph is necessary and commonly used for taxonomically oriented publications (such as for description of new species), it is rarely done in publications involving description of palynological assemblages for biostratigraphy.The amount of space required for depicting detailed palynological plates can be quite high for large assemblages, and multiple views of a single palynomorph for identification is not practical. As a result, palynomorphs are depicted as a single lower resolution photograph that may not show all the necessary details for easy identification and verification.

To circumvent this problem, dedicated software solutions may be employed to reconstruct a single image from multiple optical sections, each containing only parts of the object in focus. This technique allows obtaining the advantage of higher magnification and contrast levels given by the high-
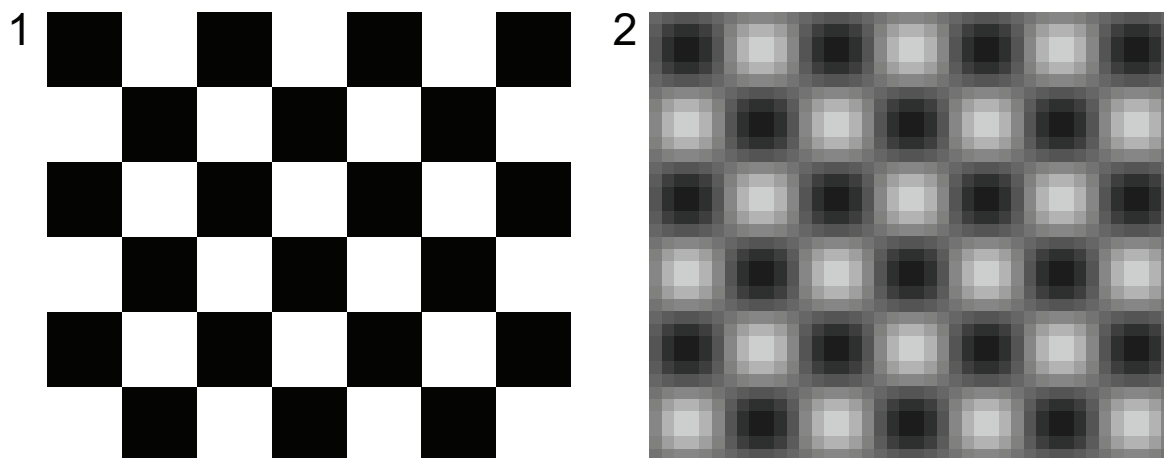
FIGURE 2. Digital image of a checkerboard pattern 1: in focus, 2: out of focus.

est magnification objectives, whilst eliminating the limited depth of field issue.

RECONSTRUCTING DEPTH OF FIELD BY SOFTWARE PROCESSING

The solution relies on a software algorithm capable of reconstructing a full image based on a stack of multiple individual pictures. There are two popular image processing techniques based on image stacks: the first is called image stacking and consists of using multiple image of an object taken in the same conditions. Image stacking is effectively working on limitations due to the imaging system (and especially digital camera sensors) by combining all of the separate images into a single image with reduced noise, defects, and increased resolution. This reconstruction technique, very popular for astronomical photography, should not be confused with the second image processing technique called focus stacking. Focus stacking uses a stack of images taken in different focus conditions in order to reconstruct a complete image of the object.

Both commercial and free open sourced software incorporate or were specifically developed to apply image stacking and/or focus stacking on a set of images. A summary of commonly available software is listed in Table 1 along with the operating system on which it can be installed, as well as the type of license for its usage (free or commercial). For the purpose of this paper, focus stacking was tested on each of the listed software to compare the quality of the end result image. However, keep in mind that the majority of this software is designed for use by photographers using the "macro" mode of their camera. While this is compatible for use with opaque paleontological and geological objects, translucent palynomorphs may be only partially reconstructed as two overlapping areas (e.g., the proximal pole and the distal pole of

TABLE 1. List of available software with image stacking and/or focus stacking capabilities.

Software	Author	Licence type	Usage	Linux/Unix	MacOS	Windows	Image Stacking	Focus Stacking
ALE	David Hilvert	Open source, GPL	Free	Yes		Yes	Yes	Yes
AutoPano Pro	Kolor	Proprietary	Commercial	Yes	Yes	Yes	Yes	
CombineZM	Alan Hadley	Open source, GPL	Free			Yes		Yes
DeepSkyStacker	Luc Coiffier	Freeware	Free			Yes	Yes	
DpxView Pro AZ	DeltaPix	Proprietary	Commercial			Yes		Yes
Extended Depth of Field (ImageJ plugin)	Alex Prudencio	Proprietary	Free for research use	Yes (Java)	Yes (Java)	Yes (Java)		Yes
Helicon Focus	Danylo Kozub	Proprietary	Commercial		Yes	Yes		Yes
Image Pro Express	Media Cybernetics	Proprietary	Commercial			Yes	Yes	Yes
Image Pro Plus	Media Cybernetics	Proprietary	Commercial			Yes	Yes	Yes
ImageJ	Wayne Rasband	Open source, GPL	Free	Yes (Java)	Yes (Java)	Yes (Java)	Yes	
PhotoAcute	Almalence Inc.	Proprietary	Commercial		Yes	Yes	Yes	Yes
Photoshop CS3 Extended	Adobe Inc.	Proprietary	Commercial		Yes	Yes	Yes	
Photoshop CS4 Extended	Adobe Inc.	Proprietary	Commercial		Yes	Yes	Yes	Yes
Picolay	Heribert Cypionka	Freeware	Free			Yes	Yes	Yes
RegiStax	Cor Berrevoets	Freeware	Free			Yes	Yes	
SAR Image Processor	Saruzinsky	Shareware	Commercial			Yes	Yes	Yes
Stack Focuser (ImageJ plugin)	Michael Umorin	Open source, GPL	Free	Yes (Java)	Yes (Java)	Yes (Java)		Yes

a spore) can appear in focus at a different level. For this reason, among all of the available software listed in Table 1, only CombineZM (Hadley 2006) will be discussed herein. This particular software was adapted to work on translucent objects and give significantly better results for palynological use.

The algorithm behind the "magic"

Several methods can be adopted for the image processing required to complete depth of field reconstruction. The method used by CombineZM consists of identifying zones within each frame that appear to be in focus. These zones are subsequently merged while out of focus areas are ignored. Identification of zones in focus is determined by using the amount of details visible in a frame, such as edges and dots: when parts of the object are out of focus, details will appear blurred or will not be seen at all. Details on a picture are usually recognizable as zones with well-defined edges and strong contrast, a characteristic that tends to disappear if out of focus. In a digitized image, this translates into the amount of variation that can be observed among neighboring pixels. To illustrate this, Figure 2 shows a checkerboard pattern imaged in the focus plane (1) and out of focus (2). Indication that the checkerboard pattern A is actually in focus is identified by the well-defined edges of each black and white square (representing a maximum variation of 100% (white) to 0% (black) on the edge of each squares). The checkerboard pattern in B depicts a much more gradual variation in the value of each adjacent pixel's brightness. The algorithm used in CombineZM uses this principle and quantifies the variations in the values of adjacent pixels, then sums these quantities thus giving each pixel a score. The areas around the pixels with the highest scores are then merged to form the composite image.

Method of reconstruction

CombineZM provides a set of two predefined routines (macros), each using a different method for reconstructing depth of field. These methods can be customized by editing the macros, (e.g. the sharpness and/or contrast of the resultant images can be adjusted to attain better results depending of the equipment used). Correct operation of the program requires the original pictures to be normalized; the reconstructed image consists of an overlay of pieces of the individual original frames, all of the individual frames must be normalized to the same value of contrast, brightness and colour.

Also, the object of interest must be scaled at the same size ratio and the same position on each frame in order to be rendered correctly. CombineZM can perform all of these adjustments automatically using the top frame of the stack as the reference for adjusting all other pictures. Thus it is necessary to ensure that the brightness/contrast ratio as well as the colour balance is adequate on this first frame. Once normalized, two processing macros are proposed:

- Do stack

This macro selects areas that are identified to be in focus and uses them to reconstruct the object by stacking them on top of each other. Adjacent patches from different frames are merged into a composite image. However, this macro does not merge details that reside on different frames at the same location. Due to this limitation, details on the front, within, and on the back of transparent objects may not appear in the final reconstructed image.

- Do weighted average

This macro uses the weighted average of corresponding pixels in the stack. The weighting factor used is the score produced by summing the difference between each pixel and its neighbours on the same frame. As translucent objects such as palynomorphs create a challenge for the "Do Stack" macro above (having parts of the object that can overlap), this algorithm may be more suitable in dome cases. This macro overcomes the problem outlined earlier, however, its drawback is that because several frames may contribute to the image at each location there may be some loss of sharpness, some fogging, and the merging of objects that in reality are far apart along the optical axis.

Guidelines for photography

Regarding the operations conducted during software processing, a number of guidelines need to be followed during acquisition of the individual frames of the stack. Particular attention should be made to use the same settings for image acquisition on each frame. Even if the CombineZM software is able to correct disparities between frames, it is always preferable to keep them at a minimum. The camera should be set to a manual mode in which both white balance and exposure time can be kept identical for all pictures. Additionally, attention should be paid to avoiding possible motion of the subject or of the optical setup (especially if the camera is mounted on the top of a long optical tube

subject to vibration) between frames. Remote shutter release (electronic or cable) and mirror lockup (for single-lens reflex cameras [SLRs]) can help to prevent these vibrations.

The number of optical section pictures required for a particular stack is not critical, as long as at least each frame contains the focus interval to be reconstructed in the stacked image. However, there is no need to take pictures at close intervals, or to take duplicated pictures, as they do not add extra data and may alter the final image. Ideally, pictures should be ordered starting at the uppermost frame (processed first by CombineZM), to the lowermost frame. With the advances of digital photography, digital cameras, or even high-end SLRs, are commonly mounted directly on microscopes, and provide sensor resolutions in the tens of megapixels range. While creating large, ten-megapixel images may be useful for certain situations, processing a series of such images requires a tremendous amount of memory and processing power on the host computer running CombineZM. Not only will the processing time exponentially increase, but the quality of the output decreases as camera resolution increases (close examination of these images usually reveals that edges and details are less sharp at the pixel level). It is preferred to downsample such large images to a more acceptable value of five, two, or even one megapixel. Lowering the resolution not only speeds up the processing, but also artificially improves the identification of details, as the reduced number of pixel artificially increase the difference between neighboring pixels located at the edge of each detail. Remember that at a standard printing resolution of 300 dpi, a five megapixel picture of a palynomorph occupying the entire field of view of the camera represents an actual size of 22 x 16.5 cm, which surpasses the size requirements for publication, so downsampling images should not be a concern.

Batch processing

In addition to the CombineZM software, an extra plug-in called CZBatch was developed as a way to automatically apply a predefined set of operations to a group of stacks. Each group of frames representing a stack needs to be collected in an individual folder prior to running CZBatch. While the batch processing plug-in can dramatically speed up the processing of large numbers of stacks of pictures, it is recommended to carefully control frames that are chosen as input for each stack.

Post-processing

Resulting stacked pictures can be post-processed prior to assembling a palynological plate. Because of the stacking technique, all areas of the palynomorph now appear in focus, and the margins are sharp and clear, which allows for easy removal of unwanted background around each palynomorph. Several palynomorphs can then be assembled on a single plate with minimum lost space and on a more desirable, totally white background. On occasions the stacking process may introduce undesirable artifacts such as halos next to some edges, smearing of reflected and refracted highlights, trails of dots left when a single "bad pixel" or "dust spot" on the camera sensor appears in a different place on each frame after resizing and alignment, and erroneous patches often caused by noise in the original frames. Such artifacts can be easily edited manually in an image editing program.

RESULTS AND EXAMPLES

As illustrations of the results obtained by the use of depth of field reconstruction on actual palynomorphs, pictures depicting selected specimens from two different palynofloras are presented. The first palynoflora is the latest Cretaceous (Maastrichtian) Hell Creek flora from southwestern North Dakota, USA. Abundant literature on the description of the palynological assemblage and species exists (Funkhouser and Evitt 1959; Stanley 1965; Leffingwell 1971; Tschudy 1971; Jarzen 1977; Sweet 1986; Jerzykiewicz and Sweet 1986; Hotton 1988; Srivastava 1994; Sweet and Braman 2001) and are summarized in the work of Nichols (2002) and Nichols and Johnson (2002). This particular flora is located within the northern *Aquilapollenites* biogeographical domain (Herngreen and Chlonova 1981), with the occurrence of easily recognizable pollen grains of the triprojectate complex. The second palynoflora used is the mid-Cretaceous (Albian - Cenomanian) palynoflora of the region of Teruel, northeastern Spain. Also, references on these palynological assemblages can be consulted for the original descriptions (Menéndez Amor and Esteras Martín 1964; Médus 1970; Cabanés and Solé de Porta 1986; Querol and Solé de Porta 1989; Solé de Porta et al. 1994; Solé de Porta and Salas 1994; Peyrot et al. 2005, 2007a, 2007b). This palynoflora corresponds to the *Cerebropollenites* biogeographical domain. However, there is a significant influ-
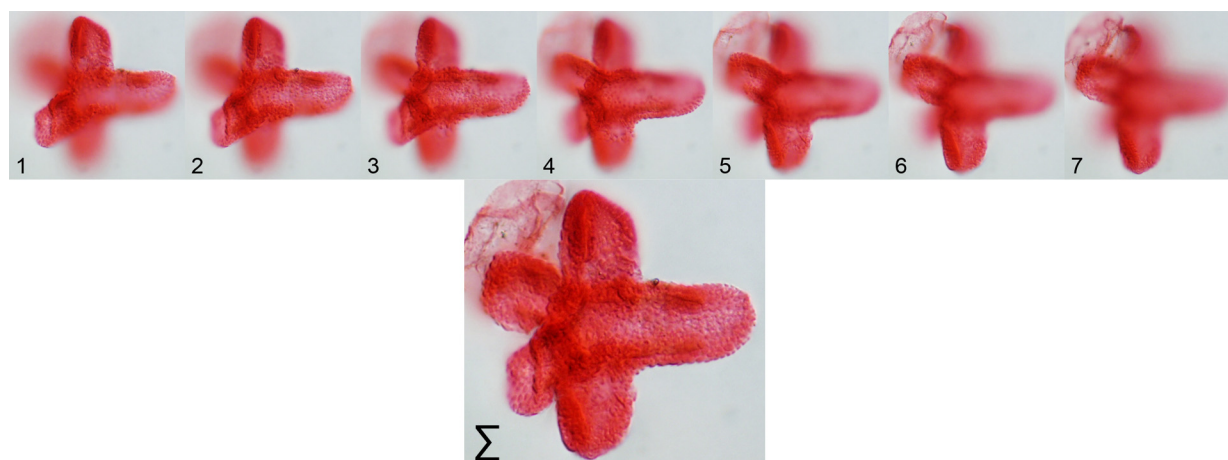
FIGURE 3. Example of reconstruction for *Aquilapollenites quadricretaeus* Chlonova 1961 (48 µm, dyed, Hell Creek), a pollen grain with complex three dimensional structure. The final reconstructed image is the result of "Do Stack" on all seven optical sections.
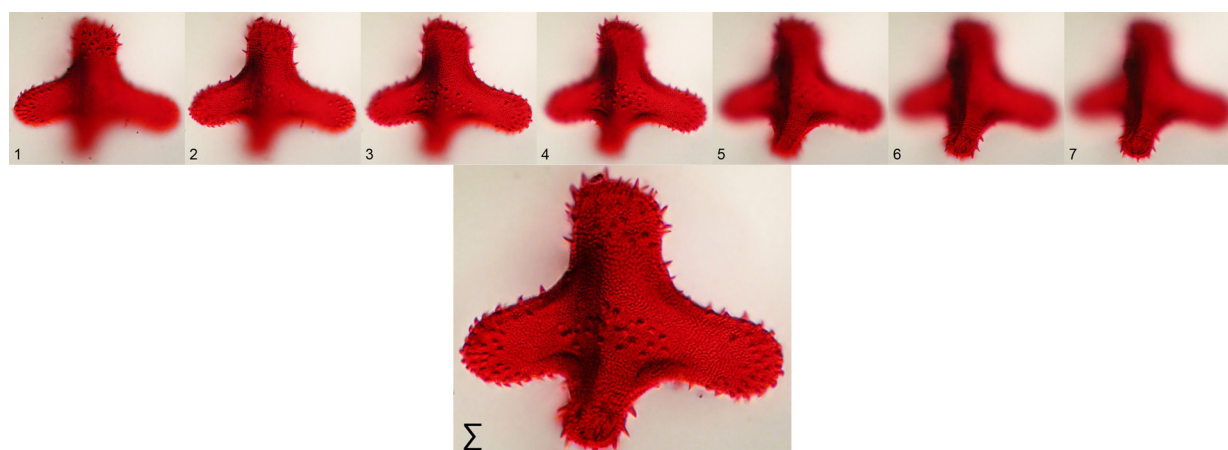

FIGURE 4. Example of reconstruction for *Aquilapollenites attenuatus* Funkhouser 1961 (75 µm, dyed, Hell Creek), a pollen grain with complex three dimensional structure. The final reconstructed image is the result of "Do Stack" on all seven optical sections.

ence of the Northern Gondwana Province (Elaterate biogeographical domain) as well.

These palynofloras display a great diversity of morphological types with excellent preservation quality, which makes them ideal candidates to evaluate the performance of depth of field reconstruction for each case. Each palynomorph is depicted on a series of pictures representing the individual optical sections used for reconstruction from bottom to top. The reconstructions are shown at the end of the sequence as well and commented below.

- Example of Figures 3 and 4 shows two triprojectate pollen grains (*Aquilapollenites quadricretaeus* and *Aquilapollenites attenuatus*) from the Hell Creek flora. The very complex three-dimensional structure makes them spectacular and convincing candidates for depth of field correction. Using the "Do Stack" macro efficiently combines all of the frames into a single comprehensive image preserving all the details and ornamentation of the exine.

- Depending on the mounting medium used for preparing the palynological slides, palynomorphs can more or less frequently be situated in an unfavorable position, not lying flat in the plane perpendicular to the optical axis. For example, Figure 5 (*Nyssapollenites* spp.) shows that the use of depth of field reconstruction is very efficient in compensating for this problem.
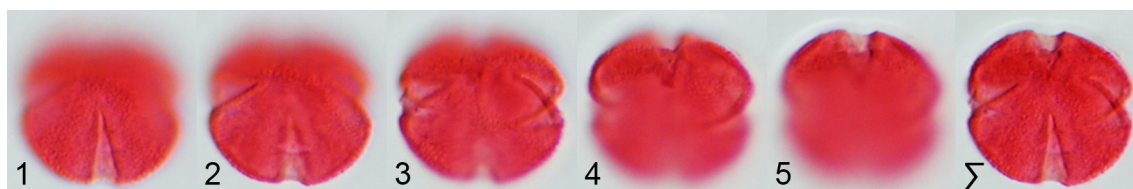
FIGURE 5. Example of reconstruction for *Nyssapollenites* spp. (25 µm, dyed, Hell Creek), this particular specimen is not mounted flat on the cover slide. The final reconstructed image is the result of "Do Stack" on all five optical sections.
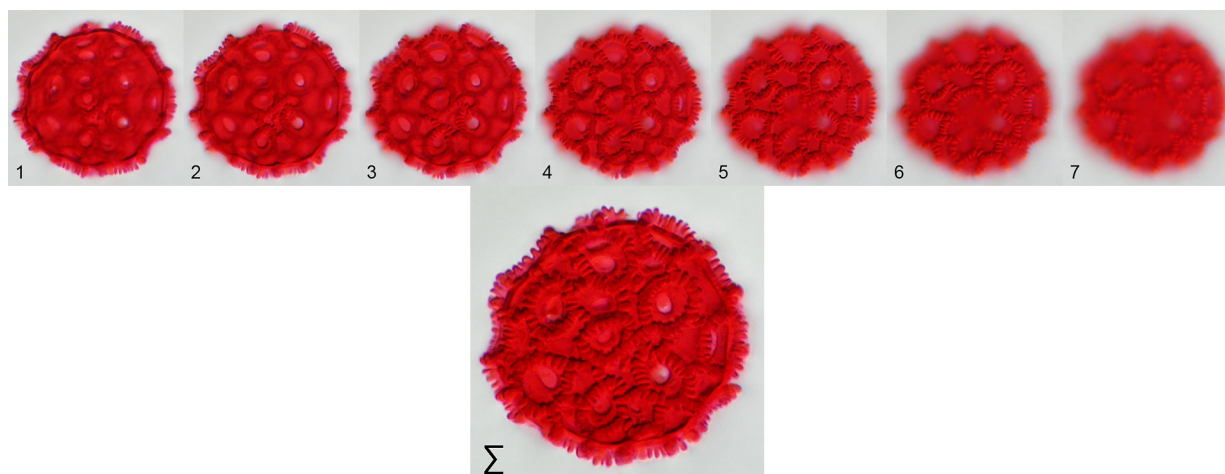

FIGURE 6. Example of reconstruction for *Erdtmanipollis cretaceus* (Stanley 1965) Norton in Norton and Hall 1969 (42 µm, dyed, Hell Creek), a pollen grain with crotonoid ornamentation. The final reconstructed image is the result of "Do Stack" on all seven optical sections.

- Palynomorphs with very developed external sculptures also benefit from depth of field reconstruction. Four examples are given, 1) *Erdtmanipollis cretaceus* (Figure 6) with a wellpronounced crotonoid sculpture, 2) *Retitriletes* spp. (Figure 7) with a reticulate scultpure, 3) *Wodehouseia spinata* (Figure 8) with echinate sculpture, and 4) *Aequitriradites* spp. (Figure 9) with reticulate-spinulose sculpture. More care should be taken in the selection of input frames for processing. Variable results can be obtained when patterns tend to overlap. The use of only a limited number of optical sections may help.

- Figures 10 to 13 depict a series of palynomorphs with a different structure between the proximal and distal poles. Here, the "Do Weighted Average" macro was tested on all optical sections. As can be seen, it is not now possible to distinguish ornamentation that occurs on the proximal pole from that occurring on the distal pole. Separating the input images into two sets may be necessary to describe some palynomorphs fully without presenting a false image of everything stacked in a single plane.

OTHER RELATED TECHNIQUES

While this paper represents the first mention of the use of such software for paleopalynology, other superior imaging techniques do exist. The most familiar is of course the scanning electron microscope (SEM), which uses a focused beam of electrons for analyzing the specimen. The possibility of magnification with larger depth of field makes it a very desirable and useful tool for palynology (Ferguson et al. 2007). While it is evident that electron microscopy is an important tool for palynological morphological analysis, its use is limited by a number of factors. The preparation techniques for the samples differ completely from those routinely used for optical microscopy. Pollen grains must be mounted on a metallic stub and metallized by ion sputtering prior to observation. This implies that samples previously mounted in a permanent medium on a microscope slide cannot be analyzed under SEM.
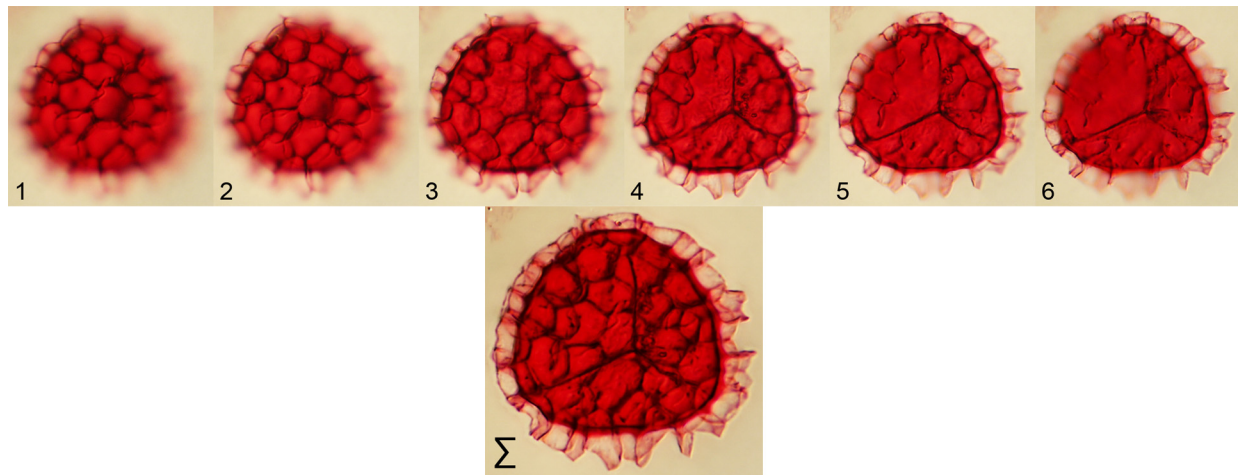
FIGURE 7. Example of reconstruction for *Retritriletes* sp. (45 µm, dyed, Hell Creek), a spore with complex ornamentation. The final reconstructed image is the result of "Do Stack" on all six optical sections.
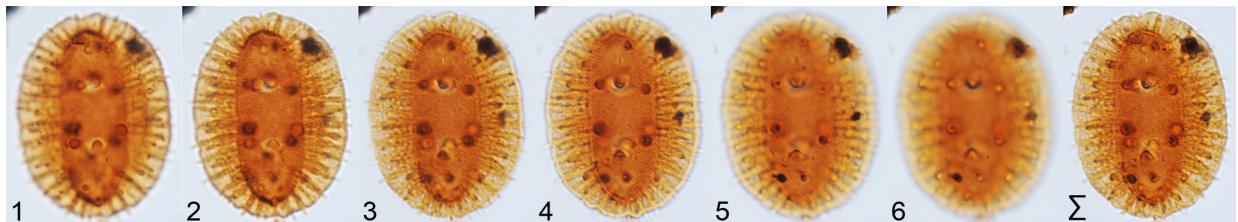

FIGURE 8. Example of reconstruction for *Wodehouseia spinata* Stanley 1961 (80 µm, undyed, Hell Creek), a pollen grain with complex ornamentation. The final reconstructed image is the result of "Do Stack" on all six optical sections.
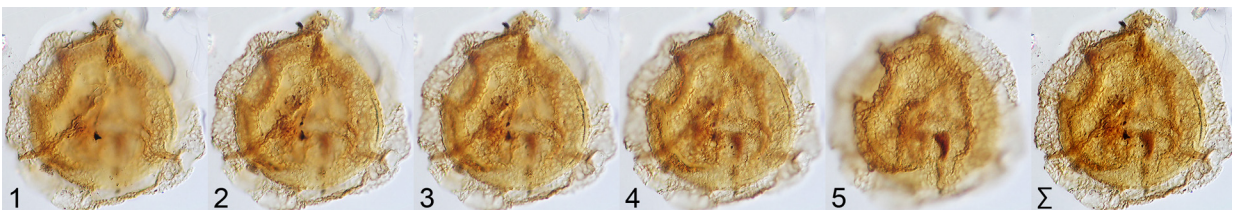

FIGURE 9. Example of reconstruction for *Aequitriradites* spp. (93 µm, undyed, Utrillas Formation), a spore with complex ornamentation. The final reconstructed image is the result of "Do Stack" on all five optical sections.
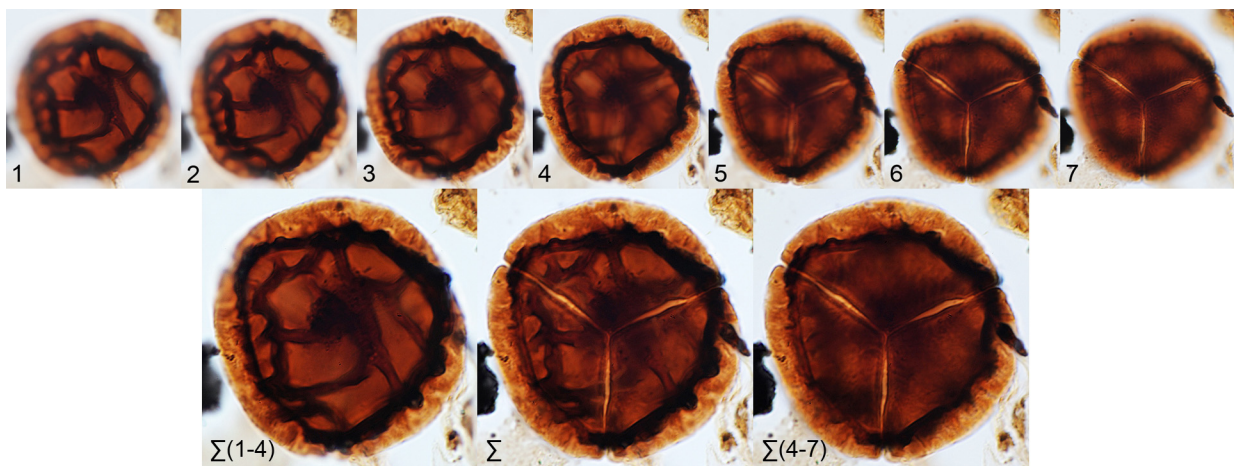

FIGURE 10. Example of reconstruction for an Unidentified liverwort spore (60 µm, undyed, Hell Creek), with different morphological features on proximal and distal view. The final reconstructed images are the result of "Do Weighted Average" for the complete reconstruction, and of "Do Stack" for the reconstructions using only a subset of the original optical sections.
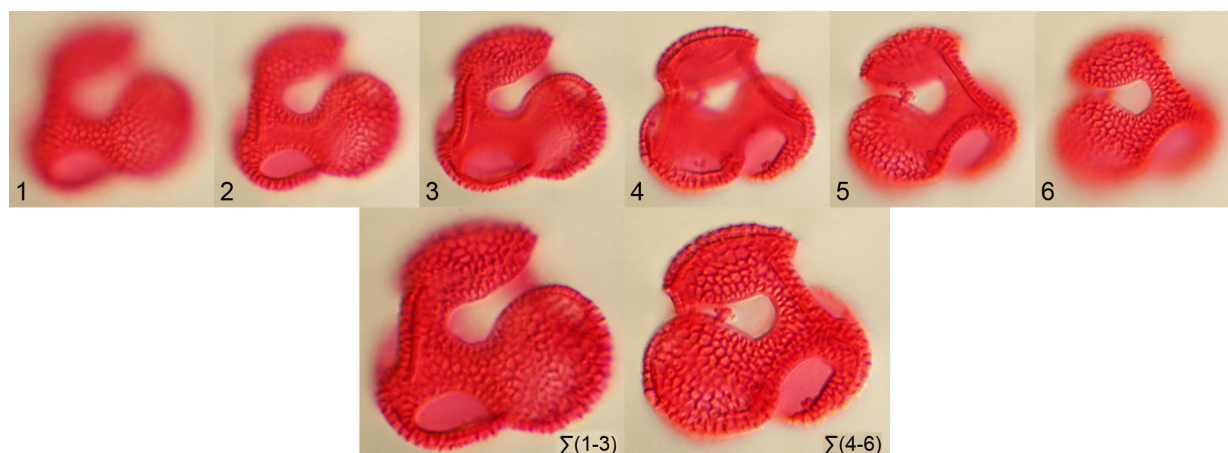
FIGURE 11. Example of reconstruction for *Libopollis jarzenii* Farabee et al. 1984 (33 µm, dyed, Hell Creek), with different morphological features on proximal and distal view. The final reconstructed images are the result of "Do Stack" on selected optical sections.

FIGURE 12. Examples of reconstruction for *Cibotiumspora juncta* (30 µm, undyed, Escucha Formation), with different morphological features on proximal and distal view. The final reconstructed images are the result of "Do Stack" on selected optical sections.
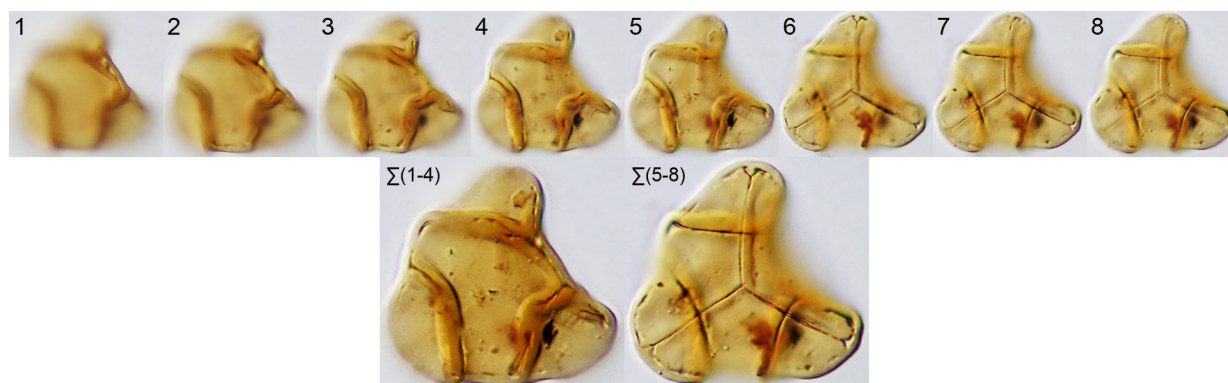
Another lesser known tool is the confocal laser scanning microscope (CLSM). This microscopical technique, derived from optical microscopy, allows for the imaging of very thin optical sections (at a micrometer scale) (Cogswell and Sheppard 1992; Sheppard and Shotton 1997; Claxton et al. 2005). These optical sections are not only very thin, but also do not present any out of focus zone corresponding to the parts of the object above and below the focus plane. Computer reconstruction from these thin sections allows for very precise three- dimensional reconstruction that can be viewed from different angles. This approach is valuable as it represents a non-invasive way of re-imaging and re-investigating type collections. Use of CLSM for paleopalynology on the Duxbury (1983) Albian dinoflagellate cysts collection was part of a project undertaken by the Natural History Museum of London (Feist-Burkhardt et al 1998; Feist-Burkhardt and Pross 1998).

However, both electron microscopy and confocal laser microscopy require the use of expensive heavy items of equipment, which may not be routinely available in any palynological laboratory. In contrast, optical microscopes are part of the standard equipment, and optical photomicrographs are still the standard for comparison. CombineZM, and similar software, can be routinely used as a convenient way to depict palynomorphs in publications, but they do not pretend to replace more advanced imaging methods.

CONCLUSIONS

This method was verified to be a great enhancement to the imaging of palynomorphs under optical microscopy. As taxonomic nomenclature can change over time, and possible differences in the identification among different palynologists can occur, illustration may be the only
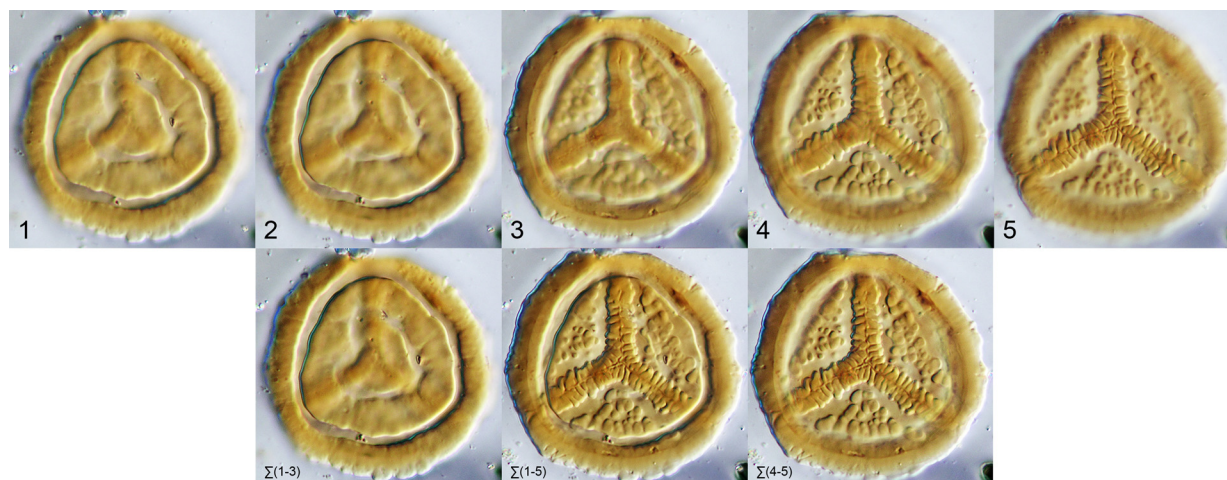
FIGURE 13. Example of reconstruction for *Taurocusporites segmentatus* (46 µm, undyed, Utrillas Formation), with different morphological features on proximal and distal view. The final reconstructed images are the result of "Do Weighted Average" for the complete reconstruction, and of "Do Stack" for the reconstructions using only a subset of the original optical sections.

way to validate or verify published data years after the publication date. Thus, it cannot be overemphasized how important it is to use the best possible figures for palynomorphs, in association with the publication of palynological taxonomic lists. Not only does the software allow for reducing the number of photomicrographs necessary to describe palynomorphs, it also provides a more natural appearance to the depicted palynomorphs, more like that seen under the optical microscope. This improvement is obvious and helps with the recognition of specimens on a palynological slide by comparison with published taxa as printed in a journal. However, it should be noted that as a main limitation to this method, three-dimensional structures are collapsed into a two-dimensional image, which can result in one feature obscuring another, or two or more features being merged.

Additionally, while this type of image processing has proved to be very helpful for paleopalynology, the method can be extrapolated in any other case where depth of field reconstruction is needed. This technique could scale to others geological or paleontological objects such as microfossils (foraminifers, microvertebrates, and many others) observed under the binocular microscope, up to the largest dinosaur bones photographed with a camera stand or macro lenses.

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