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POSITIONING AND ENHANCED STEREOGRAPHIC IMAGING OF MICROFOSSILS IN REFLECTED LIGHT

M.W. Knappertsbusch, K.R. Brown, and H.R. Rüegg

ABSTRACT

We present an advanced digital microscope imaging system for isolated microfossils under reflected light. It combines sophisticated commercial software for generating images at improved focal depth with a new motorized two axis XY-tilting stage developed by ourselves for viewing microfossils under various angles of observation. The system is used for the construction of animated scenes in color or stereographic (anaglyph) views of isolated microfossils, such as foraminifera, ostracods or radiolaria. The speed and precision of the system is good enough to allow the systematist to collect images for animated digital micropaleontological type specimen- and reference atlases with three-dimensional perception.

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INTRODUCTION

Until recently the production of well focused images from a 3-D microscopic object was challenging and required extensive manual handling of microscope settings and digital image composition. Creation of animated 3-D visualizations requires a series of in-focus images of various angles of view. The necessary labour is therefore multiplied by the number of focal planes needed for a single image position.

Whereas the usage of stereo or anaglyph images is already a recognized technique in paleontology and micropaleontology (Purnell 2003, Gatesy et al. 2005 and references therein), the generation of animated scanning electron microscope (SEM) illustrations of microfossils was first applied by Lyons and Head (1998). Images taken with an SEM are usually devoid of unsharp focus and need no special treatment. In 2002 Knappertsbusch presented an inexpensive method to com-

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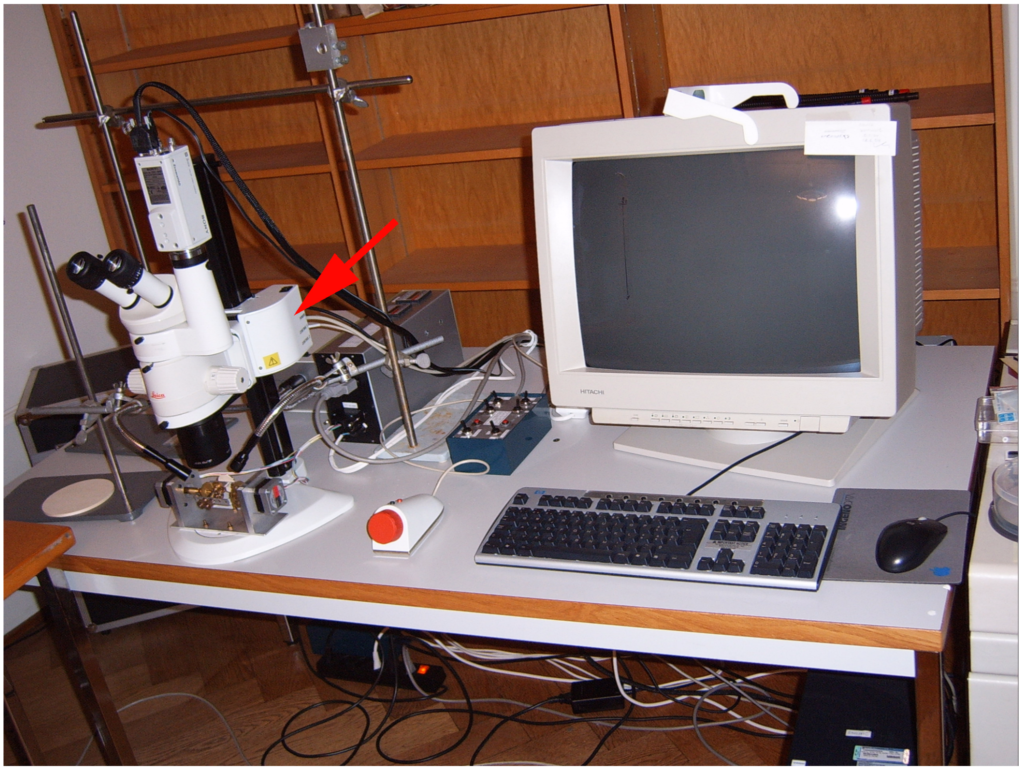

Figure 1. Microscope and hardware setup. Red arrow points to motorized focus.

bine animated image sequences of microfossils with improved stereographic vision under reflected light, yet requiring an unfavorable amount of effort to produce them. Here, we present an improved technique using sophisticated digital image processing and focussing software, better illumination techniques, and a new motorized two-axis specimen tilting stage. These techniques allow to improve access to type reference materials to a greater audience via the internet. They are also useful for micropalaeontological teaching and specialist training, and during public demonstration tours in a museum.

This technical note addresses the various working groups that are currently engaged in establishing web-based palaeontological information systems such as CHRONOS (http://www.chronos.org), or are involved in GeoInformatics programmes (http://www.geoinformatics.org/), or are future practicing micropalaeontologists onboard research vessels, like the *Chikyu* of the Integrated Ocean Drilling Program (http://www.iodp.org). In this context, this paper demonstrates the potential of these new techniques for future online illustration and documentation of microfossil holdings in museum collections.

MATERIALS AND METHODS

Microscope setup and hardware. For imaging a 3 CCD color video camera from Sony (model DXC 390P) is used. It is mounted on a Leica MZ 6 binocular microscope with a Leica AX stand for stereoscopic and monoscopic vision, a zoom magnification changer, and a motorized focus control (Figure 1). The camera produces images at a resolution of 752 (horizontal) by 582 (vertical) pixels. The camera and the motor focus are connected to a PC running under Windows 2000 that is equipped with an internal four-axis (OASIS-4i) controller board from Objective Imaging. The OASIS-4i board allows, next to controlling the motorized microscope focus, the connection of three additional devices that can be driven by stepping motors.

Digital imaging software. The imaging system runs with AutoMontage Pro version 5.01a software from Syncroscopy (Synoptics Group), http://www.syncroscopy.com/syncroscopy/am.asp. It automatically captures images at a series of predefined focal depths during a vertical scan of the microscope. The software allows the user to select the number of vertical increments at which images are taken. Vertical scanning of images through the focal range and alignment of images is very fast.

For example, image capturing from 50 vertical levels the system requires no more than about 80 seconds, and image alignment and montaging them takes another 45 seconds, which is a great advance over the system described in Knappertsbusch (2002). This system is useful for the construction of stereographic anaglyph images from microscopic objects without stepped surfaces that would occur if too few focal planes are included. AutoMontage allows the user to choose from a variety of algorithms for image montage, which enables one to adapt to the different nature of surface roughness of the objects.

Improved illumination techniques. Direct illumination can introduce disturbing reflections from the slide, on which the object is mounted. Bengtson (2000) and authors referenced therein demonstrate the usage of cross-polarized light to be very effective for enhancing contrast in digital imaging fossils. In our case, a polarizing filter is placed in the light path of the microscope objective, and a pair of polarizing filter caps is fixed on the arm tips of a swan-neck fiber optics (Figure 2). If a near vertical illumination body is attached to the microscope, a single polarizing filter plate can be glued to the microscope end of the fiber-optics cable in order to arrive at the same effects. Twisting the fiber-optics cable allows one to adjust the polarized light to extinction if necessary. These arrangements filter out glare from the background or from the object itself, and at the same time enhance the contrast between the object and the background dramatically. Tests have confirmed that insertion of a polarizing filter in the light path of the microscope does slightly shift the image reproduced on the computer monitor, but has no effect on the magnification of the image.

High magnification causes loss of light due to increased attenuation of the light, which can be compensated by stronger illumination. Particularly high constrast of calcareous microfossils against a dark background is obtained if sideward illumination is applied. This contrast enhancement occurs because calcareous microfossils are often slightly translucent, which causes differential extinction under crossed polarizing filters, similar to birifringent minerals in petrographic thin sections.

Determination of the depth of focus as a function of magnification. Extended focus images are built from a stack of images at focal planes ranging from the object's base plane to its top. Focal planes represent volumes with a thin but finite thickness depending on the magnification and/or the numerical aperture used. For image composition at extended focus the neighbouring focal volumes must have a certain overlap in order to fully cover the object's topography. Theoretically, the ideal number of images is obtained at the Nyquist criterium, e.g., if the vertical thickness of the focal volumes Δz is half the depth of focus (Meyer, personal commun., 2003). The depth of focus of our imaging system is therefore estimated with an equilateral triangular glass prism where a straight line was scratched in the surface (Figure 3). While placing the prism under the microscope the depth of focus (DOF) is estimated by measuring the horizontal projection ΔX of the line that appears in focus at various magnifications:

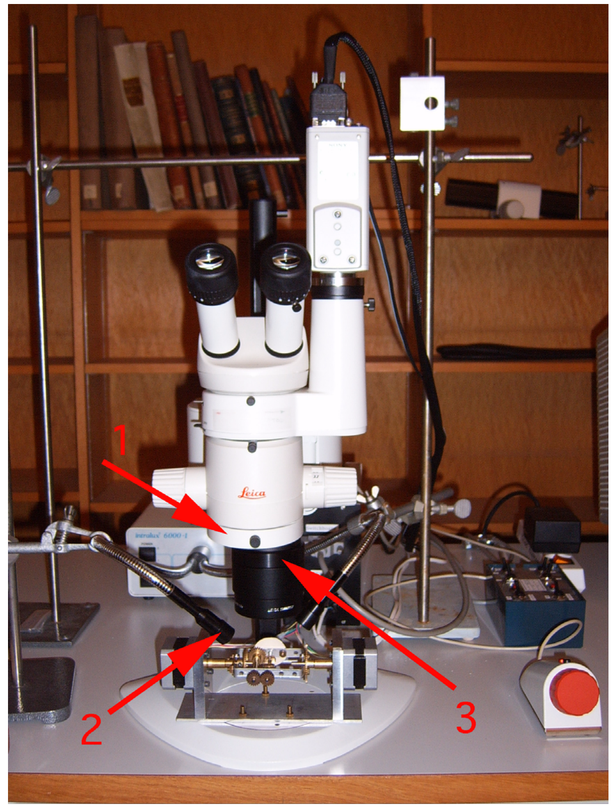

Figure 2. Details of the microscope (numbered red arrows): 1. AX-stand, which allows coaxial vertical movement of the microscope-camera unit without lateral shift on the computer monitor (lateral shift occurs when the geometry of the light path is oblique with respect to the focus movement axis). 2. Polarizer caps on tips of swanneck arms. 3. Analyzer plate is inserted between objective and magnification body.

$$\text{DOF (in µm)} = \Delta X * \tan 60°$$

Δx is given in micrometers. It can be estimated from the distance measured in pixels on the computer monitor by multiplication with a calibration

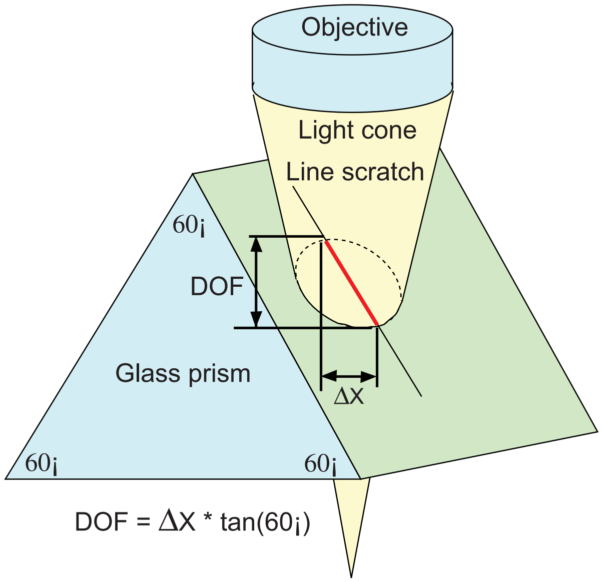

Figure 3. Determination of the depth of focus: Geometric relationship between the depth of focus (DOF) and its horizontal projection Δx that can be measured. The red portion of the line scratch appears in focus.

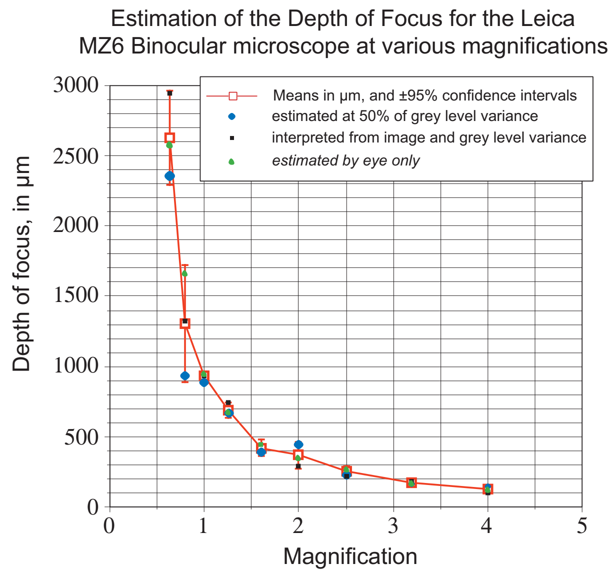

Figure 4. The depth of focus as a function of magnification for the Leica MZ 6 binocular using the 1x achromatic objective lens from Leica.

factor, which converts pixels into micrometers for a particular magnification.

Ideal vertical step interval for optimum focus montaging. Figure 4 illustrates the relationship between the depth of focus and magnification for the Leica MZ 6 binocular with a 1x achromatic objective lense inserted. It shows a mean depth of focus range between 120 to 170µm if a typical magnification is set between 3.2x and 4x at the magnification changer body of the microscope. The ideal vertical step interval of acquiring images at these magnifications is between 60 to 70 µm. If another magnification changer body and/or a different objective are used (for example the magnification changer body of the Leica MZ 12 and/or a 1x apochromatic lense) the ideal vertical step interval needs to be estimated separately.

A PORTABLE MOTORIZED XY-TILTING STAGE

In Knappertsbusch (2002) a simple universal stage was applied to generate movie sequences from microfossils in reflected light at varying X- and Y-tilt angles. The mechanics allows for tilt not less than about 3° intervals between adjacent positions, which, however, must be performed manually, and which is not very precise. Here, a revised, coaxial gearing geometry is presented, and the stage is equipped with two programmable Vexta stepper motors from Oriental Motor GmbH (http://www.orientalmotor.de/). More precise tilting of specimens in x and y directions is now possible at intervals of 1.8° or more from one image position to the next (see Figures 5A and 5B). Motorizing the stage enables the user to repeat imaging at sequences of angular tilt with high precision and guarantees good reproducibility of the results.

The XY-tilting stage can be operated in two alternative ways: In the first modus the stage is directly connected to SK1 port of the internal OASIS-4i four axis controller, which is needed for the vertical (Z) focus control of the binocular microscope in AutoMontage. The OASIS-4i controller is a PCI compliant plug-in board from Objective Imaging (http://www.objectiveimaging.com/) that provides control of up to four micro-stepping motors. By using the OASIS-4i controller the motors of the XY-tilting stage can be accessed via the OASIS.EXE program. This is a PC standalone application supplied by Objective Imaging together with the board. This method requires alternating between the AutoMontage and OASIS.EXE programs because the Z-stepper (which drives the motorized focus of the binocular) and the x,Y-tilting stage access the same hardware and cannot run simultaneously without a major reprogramming of AutoMontage (see Appendix).

In the second modus, the stage is connected to an external device, which we designed and manufactured (Figure 6), and uses SG8030JY controllers from Vexta (available from Oriental Motor GmbH, http://www.orientalmotor.de/). In this modus the OASIS-4i plug-in board is only needed if the

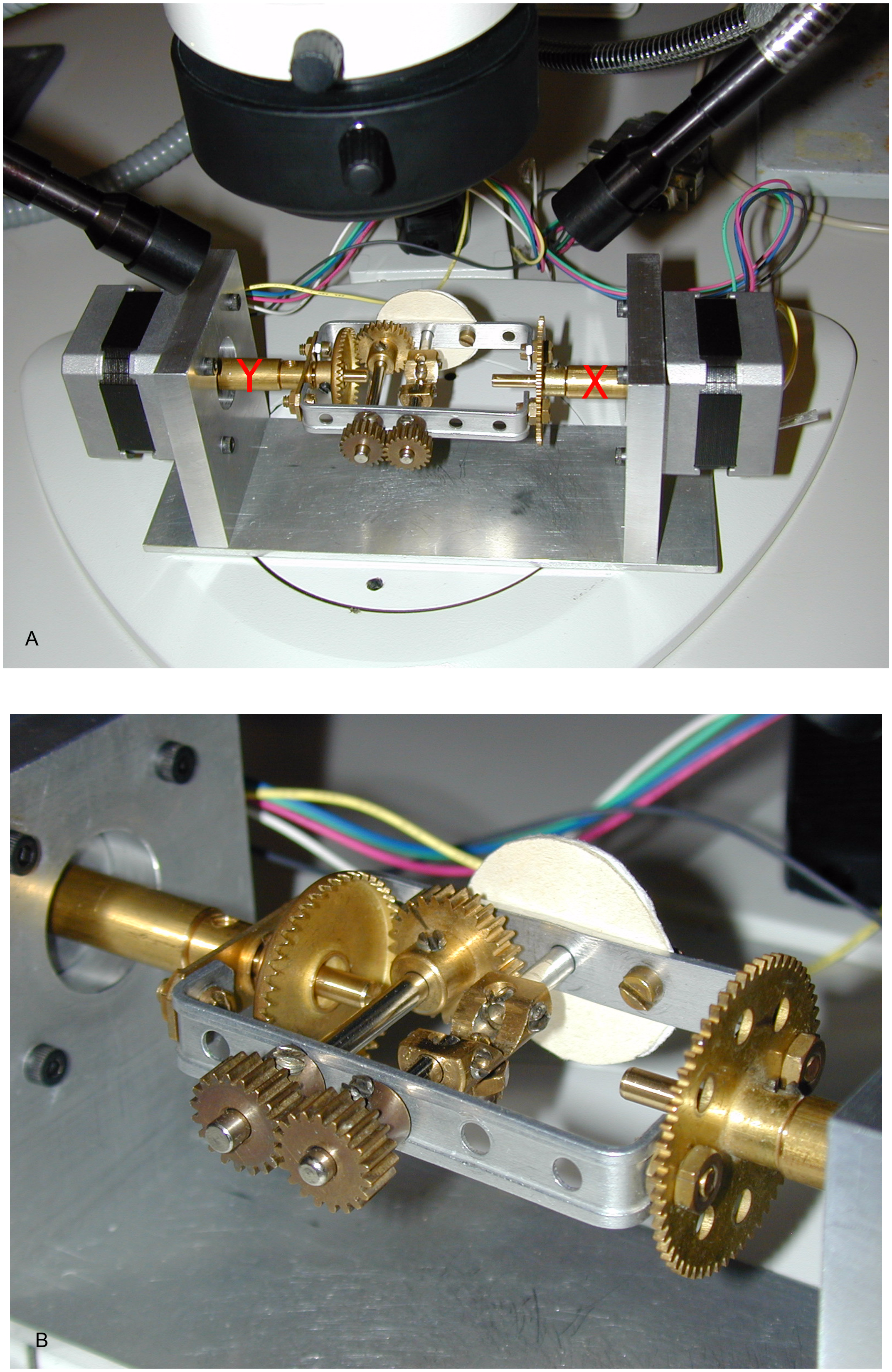

Figure 5. Two-axis tilting stage for isolated microfossils. The two stepper motors are sideways fixed allowing co-axial drive of two perpendicular tilting axes. Red letters X and Y indicate the X- and Y-tilting axes, respectively.

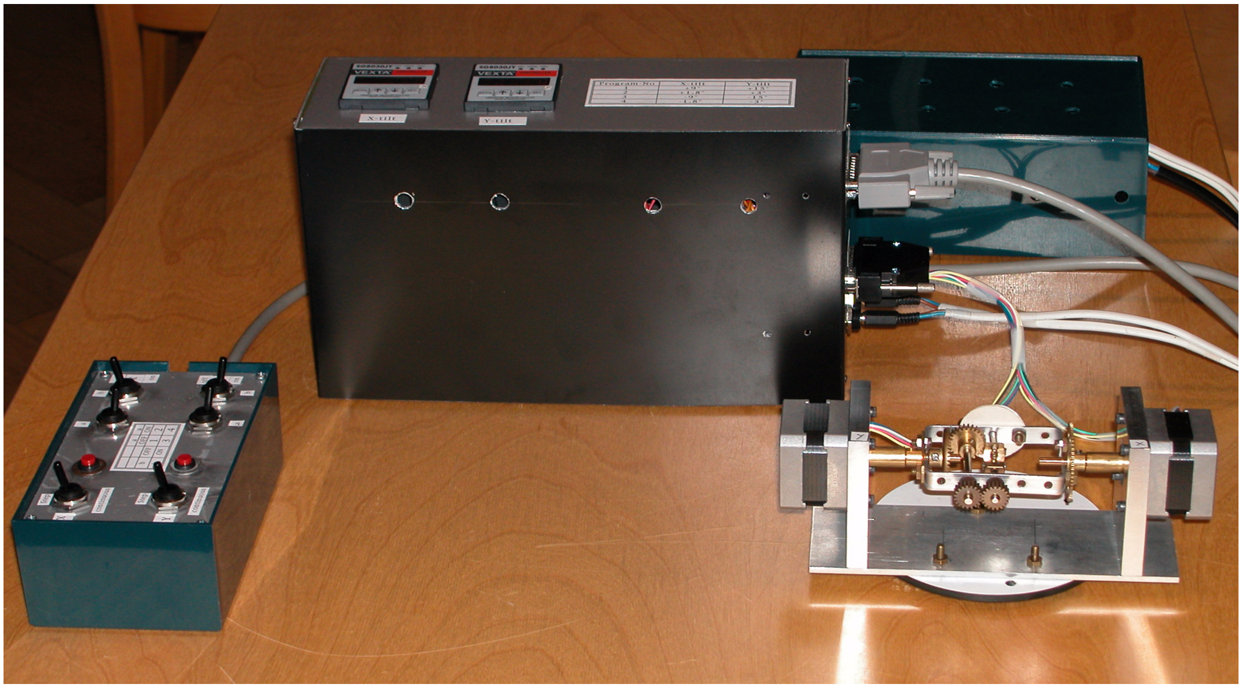

Figure 6. Components of the XY-tilting stage. The large box in the center of the image contains the two programmable Vexta controllers for the X- and Y-axes stepping motors. This component can be connected to the PC for controlling of the motors via software. Alternatively, it can be connected to the logic switch-box for manual control of the motors (small blue box to the left side of the image). This second combination is entirely independent from any computer and makes the stage portable. The switch-box allows the user to select between forward/backward and slow/fast movement of the motors. When pressing the red contact switches, motors move by a selected number of steps. The blue box in the background contains the power supply.

operator decides to use AutoMontage as the imaging software (e.g., for automated focus (Z)-control of the binocular microscope). A logic switchboard is connected to the external controller allowing to select four programs for each axis: Forward and backward movement, fast (=several steps) and slow motion (=1 step). The angular increments for both tilting axes can be programmed separately. Motion of the stage is triggered manually by pushing a button for either the x or the y direction. This design makes the stage easily portable, so that it can be used under any binocular microscope without being dependent on any specialized computer hardware.

EXAMPLES

Microfossil specimens are mounted with water-soluble glue on a sharpened screw tip in the center of the stage. Alternatively, specimens can also be fixed on a very thin glass rod using Ecyanoacrylate adhesive to increase the angular range for taking images. Figures 7 through 10 illustrate montaged color and anaglyph movie scenes from specimens of various planktonic foraminifers generated with the above-described equipment[1]. Figures 7.1 and 7.2 show a specimen of an uncoated *Globigerinoides ruber* from a Late Pleistocene sample. Figures 8.1 and 8.2, 9.1 and 9.2, and 10.1 and 10.2 are re-illustrations of the holotypes of *Globorotalia multicamerata*, *Globorotalia pertenuis*, and *Globorotalia menardii gibberula*, in color view and anaglyph view, respectively. Figures 8.1 and 8.2 (*G. multicamerata*) is an uncoated specimen. Figures 9.1 and 9.2 (*G. pertenuis*) and Figures 10.1 and 10.2 (*G. menardii gibberula*) are gold-coated specimens showing an unfavorably high reflective surface if no polarizing filters are applied. In Figure 10.1 and 10.2 a green filter was inserted at the front end of the fiber-optics cable (in addition to partially crossed nicols) to enhance the surface texture of the shell. The settings for creating the anaglyph images (in AutoMontage Pro under the menu Anaglyph) were red/green for 'Mode', and a separation value of 10.0 (the separation value allows for modification of the 3-D percep-

1. Interactive image files are available from the PE website at this URL: http://palaeo-electronica.org/2006_2/reflect/index.htm

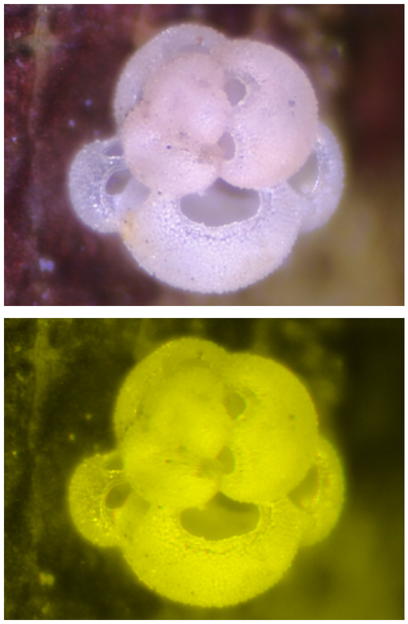

Figure 7. *Globigerinoides ruber* d'Orbigny, 1839. Sample: Box core Ki 10, 25-30cm, Station 15 (YOS). Vicomed I Cruise, Eratosthenes Seamount, Eastern Mediterranean Sea. (Lat. 33° 44.0' N, Long. 32° 45.2' E, 930m water depth). Age: Late Pleistocene. See Table 1 in Knappertsbusch (1993). Interactive QuickTime movie showing a specimen of *Globigerinoides ruber* produced with the described technique and equipment. Images were taken at 9° intervals (every 3 pulses in y direction) from -90° through +90°, and using vertical stacks of 50 images per position for extended focus construction in Auto Montage. Click within the images, and drag the mouse cursor horizontally to obtain various views of the object. Scale bar indicates 200µm. **7.1** (left): Color image, **7.2** (right) Anaglyph movie of the same specimen. Use red-green glasses in order to perceive the three-dimensional effect of the anaglyph image while moving it. Scale bar indicates 200µm.

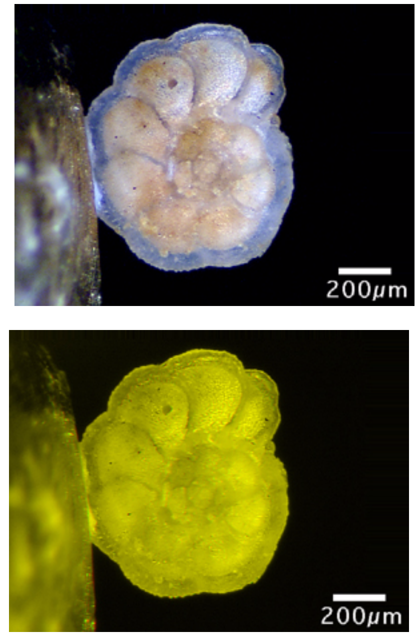

Figure 8. *Globorotalia multicamerata* Cushman and Jarvis, 1930, holotype. Interactive QuickTime movie, uncoated specimen. **8.1** (left): Color movie. **8.2** (right): Anaglyph movie of the same specimen. Scale bar indicates 200µm. Click into images and drag the mouse cursor horizontally. In order to experience the three-dimensional effect, watch the anaglyph image with red-green glasses. Illustrated with the permission of the Smithsonian Institution, Washington D.C.

tion and can manually be varied from 4 to 40). For alignment of neighbouring images Adobe Photoshop (displacement filter) and ImageJ version 1.2.3 (http://rsb.info.nih.gov/ij/) are applied. For movie composition from the image series Quick Time Virtual Reality Authoring Studio 1.0 from Apple is used (see Knappertsbusch 2002).

Imaging, montaging, and creating anaglyph images of a specimen in a given position occurs in a few tens of seconds. Depending on the number of tilting steps, production of a series of stacks of images at various angles of degrees may take between half an hour to an hour. More time is needed for alignment of neighbouring montaged images with Adobe Photoshop or ImageJ, so that a smooth movie can be composed. Depending on how precisely the specimen was mounted and oriented into the focal point under the microscope beforehand, this step can take several hours (depending on the number of images to be aligned). The subsequent conversion of the image series into a Quick Time movie is fast again. It took about one day for each movie presented in this technical note to be created.

OUTLOOK

While planar XY-tilting stages are readily available on the market, tilting stages for screening objects under a light microscope from various points of view are of very limited availability and currently only at prohibitive costs. With the increased need of digital documentation of microscopic objects, such a system will eventually become more in demand in the future. If motion control is automated and integrated with software capable of extended focal imaging, the speed to generate 3-D interactive movies can be enhanced. Implementation of multiple image alignment functions to match images from one tilting step to the next would make the tedious operations in Adobe Photoshop obsolete. Another step towards interac-

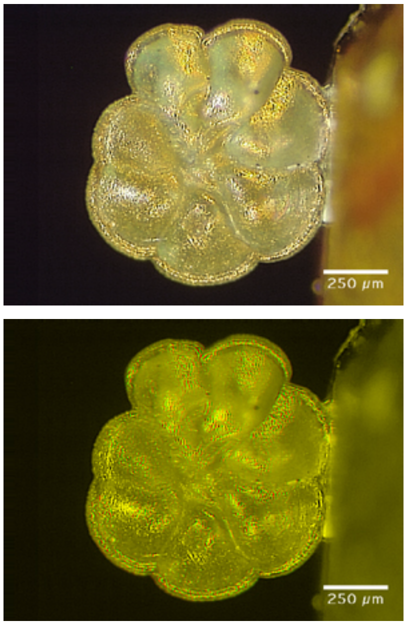

Figure 9. *Globorotalia pertenuis* Beard, 1969, holotype. **9.1.** Left: Interactive QuickTime movie of the holotype of *Globorotalia pertenuis* Beard. Gold coated specimen. **9.2** (right): Anaglyph movie of the same specimen. Scale bar indicates 250 µm. Click within the images, and drag the mouse cursor horizontally. In order to experience the three-dimensional effect, watch the anaglyph image with red-green glasses. Illustrated with the permission of the Smithsonian Institution, Washington D.C.

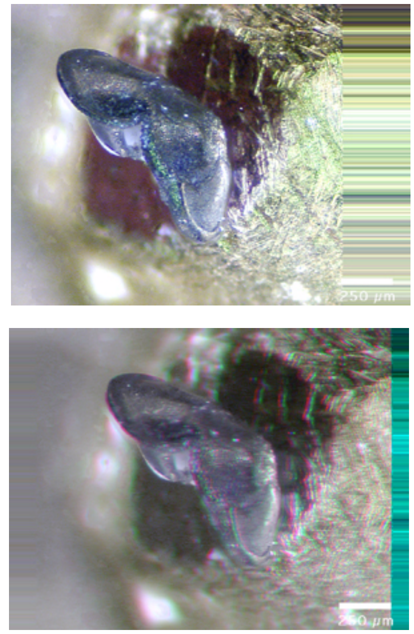

Figure 10. *Globorotalia menardii gibberula* Bé, 1977, holotype. **10.1** (left): Interactive QuickTime movie of the holotype of *Globorotalia menardii gibberula* Bé. Gold coated specimen. **10.2** (right): Anaglyph movie of the same specimen. Scale bar indicates 250 µm. Click within the images, and drag the mouse cursor horizontally. In order to experience the three-dimensional effect watch the anaglyph image with red-green glasses. Illustrated with the permission of the Smithsonian Institution, Washington D.C.

tive virtual microscopy may be achieved by using high-resolution images for movie construction. Virtual slides are already in use in telepathologic or general tele-teaching systems (Glatz-Krieger et al. 2003 and Glatz-Krieger et al. 2006). Zoom image server (ZIS) technology allows smooth scrolling through virtual slide boxes. Advanced ZIS technology may eventually be developed in the future to navigate and zoom through sequences of virtual slide boxes taken at various orientations in an interactive movie.

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REFERENCES

Beard, J.H. 1969. Pleistocene paleotemperature record based on planktonic foraminifers, Gulf of Mexico. *Transactions of the Gulf Coast Association of Geological Societies*, 19:535-553.

Bengtson, S. 2000. Teasing fossils out of shales with cameras and computers. *Palaeontologia Electronica*, Vol. 3, Issue 1; 4:14 p, 7.7 MB; http://palaeo-electronica.org/2000_1/fossils/issue1_00.htm

Bé, A.W.H. 1977. An ecological, zoogeographic and taxonomic review of Recent planktonic foraminifera, vol. 1, p. 61. In Ramsay, A.T.S. (ed.), *Oceanic Micropaleontology*. Academic Press, New York.

Cushman, J.A. and Jarvis, P.W. 1930. Miocene foraminifera from Buff Bay, Jamaica. *Journal of Paleontology*, Vol. 4, No. 4, pp. 353-368.

d'Orbigny, A. 1839. Foraminifères, pp. 1-224. In de la Sagra, R. (ed.), *Histoire physique, politique et naturelle de l'île de Cuba*. Arthus Betrand, Paris.

Gatesy, S.M., Shubin, N.H., and Jenkins, F.A. Jr. 2005. Anaglyph stereo imaging of dinosaur track morphology and microtopography. *Palaeontologia Electronica*, Vol. 8, Issue 1; 10A: 10p, 693KB; http://palaeo-electronica.org/paleo/2005_1/gatesy10/issue1_05.htm

Glatz-Krieger, K., Glatz, D., Mihatsch, M.J. 2003. Virtual slides: high quality demand, physical limitations, and affordability. *Human Pathology*, Vol. 34, Issue 10, pp. 968-974.

Glatz-Krieger, K., Spornitz, U., Spatz, A., Mihatsch, M.J., and Glatz, D. 2006. Factors to keep in mind when introducing virtual microscopy. *Virchows Arch.*, 448:248-255.

Knappertsbusch, M.W. 1993. Geographic distribution of living and Holocene coccolithophores in the Mediterranean Sea. *Marine Micropaleontology*, 21:219-247.

Knappertsbusch, M.W. 2002. Stereographic virtual reality representations of microfossils in light microscopy. *Palaeontologia Electronica*, Vol. 5, Issue 1; 11p, 1.1MB; http://palaeo-electronica.org/paleo/2002_1/light/issue1_02.htm

Lyons, P.D. and Head, L. 1998. QuickTime VR: a powerful new illustrative tool for micropaleontological research. *Palaeontologia Electronica*, Vol. 2, Issue 2; 12 p, 220KB; http://palaeo-electronica.org/1998_2/lyons/issue2.htm

Purnell, M.A. 2003. Casting, replication, and anaglyph stereo imaging of microscopic detail in fossils, with examples from cConodonts and other jawless vertebrates. *Palaeontologia Electronica*, Vol. 6, Issue 2; 11p, 419KB; http://palaeo-electronica.org/paleo/2003_2/rubber/issue2_03.htm

APPENDIX

Procedure to create a series of montaged images at varying angles of tilt by using the OASIS.EXE program (method 1)

1.) Start the AutoMontage program, generate a live image, and disable the z-stepper control.

2.) Start the OASIS.EXE program, position the specimen as desired by driving X- and Y-tilt motors with mouse clicks.

3.) Return to AutoMontage (click into the image), and activate the z-stepper control. Generate a stack of images, align them, make a montage image, and save the images to disk. Thereafter disable the z-stepper control.

4.) Repeat steps 2 to 4 with the next following tilting positions.