

## **AMOR – A NEW SYSTEM FOR AUTOMATED IMAGING OF MICROFOSSILS FOR MORPHOMETRIC ANALYSES**

Michael W. Knappertsbusch, Daniel Binggeli,  
Andreas Herzig, Lukas Schmutz, Sebastian Stapfer, Claude Schneider,  
Jean Eisenecker, and Lukas Widmer

### **ABSTRACT**

A new robot for automated microfossil orientation and digital imaging called AMOR is presented. AMOR (from Automated Measurement system for shell mORphology) was built in collaboration between the Natural History Museum in Basel (NMB), the University of Applied Sciences Northwestern Switzerland (FHNW), and the Geological-Paleontological Institute (GPI) of the University of Basel. AMOR is capable of automatically positioning, orienting, focussing, zooming, and imaging isolated microfossils that are mounted in standard multicellular micropaleontological slides. This device is useful for the efficient collection of morphometric data for evolutionary studies from digital microfossil images. It is superior to manual data collection because of higher speed and improved level of precision and reproducibility.

Michael W. Knappertsbusch. Natural History Museum Basel, Geology Department, Augustinergasse 2, 4001-Basel, Switzerland [michael.knappertsbusch@unibas.ch](mailto:michael.knappertsbusch@unibas.ch)

Daniel Binggeli. University of Applied Sciences Northwestern Switzerland (FHNW), School of engineering, Institute for Automation, Steinackerstrasse 5, 5210-Windisch, Switzerland [daniel.binggeli@fhnw.ch](mailto:daniel.binggeli@fhnw.ch)

Andreas Herzig. Swisslog AG, 5033 Buchs, Switzerland

Lukas Schmutz. Siemens Schweiz AG, Global Services Industries, GSI CIS1, Freilagerstrasse 40 8047-Zürich, Switzerland [lukas.schmutz@siemens.com](mailto:lukas.schmutz@siemens.com)

Sebastian Stapfer. University of Applied Sciences Northwestern Switzerland (FHNW), School of Engineering, Mecatronics trinational, Gründenstrasse 40, 4132-Muttenz, Switzerland [sebastian.stapfer@fhnw.ch](mailto:sebastian.stapfer@fhnw.ch)

Claude Schneider. Institute of Geology and Paleontology, Department of Geosciences, University of Basel, Bernoullistrasse 32, 4056-Basel, Switzerland [Claude.Schneider@unibas.ch](mailto:Claude.Schneider@unibas.ch)

Jean Eisenecker. University of Applied Sciences Northwestern Switzerland (FHNW), School of Engineering, Institute for Automation, Steinackerstrasse 5, 5210-Windisch, Switzerland [jean.eisenecker@fhnw.ch](mailto:jean.eisenecker@fhnw.ch)

Lukas Widmer. Rossbergstrasse 10, 5222 Umiken, Switzerland [lutz.widmer@gmx.ch](mailto:lutz.widmer@gmx.ch)

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**Figure 1.** Motorized binocular and 4-axis gliding and tilting stage of AMOR (Automated Measurement system for shell mORphology).

## INTRODUCTION

Morphometry is an important method in taxonomic paleontology. In combination with paleoenvironmental, geochemical, and molecular analysis of species, traditional morphometry aids investigation about evolution of species through time and space, and so allows the observation and understanding of the history of biodiversity on earth. Despite microfossils being particularly interesting subjects for the study of evolution, morphometry remains underrepresented in micropaleontological research because it is time-consuming to collect quantitative data when the tiny shells must be properly oriented prior to measurements in order to guarantee comparable results.

During recent years, several products for automated routine investigations under light microscopes have come to the market, including motorized planar gliding stages for transmission light microscopes (Ludl, Märzhäuser, and other brands). Such systems are widely used in optical screening of medical preparations or in micropaleontology for embedded microfossils like diatoms, calcareous nanofossils or palynomorphs (Bollmann et al.

2004). Other automated systems are capable of imaging and measuring randomly oriented isolated microfossils under a moving camera (Schmidt 2002; Bollmann et al. 2004). These devices move the objects into the light path of the camera system, but suffer from lacking 3D-orientation of microfossils into a standard measurement position. There exist novel non-destructive imaging systems like micro X-ray CT (see e-foram Stock at Tohoku University Museum, URL <http://webdb2.museum.tohoku.ac.jp/e-foram/>) to model microfossil surfaces and internal structures but, apart from high prices, these devices are not suitable for automated multiple object screening.

Traditional morphometry requires that microfossils be moved into a standard position under a binocular. An extended morphometric investigation with thousands of specimens takes too long for a typical research project. In order to facilitate our own research on morphological variability and evolution within the Neogene planktonic foraminifer *Globorotalia menardii*, an orientation robot for isolated shells in reflected light called AMOR (Automated Measurement system for shell mORphology) was built (Figure 1). Preliminary results about the morphological variability of menardiform globorotalids, which still are based on manual data collection using a digital imaging system, are given in Knappertsbusch (2007) and in Brown (2007).

Steps toward instrument-assisted microfossil orientation for imaging started using a hemispherical stage: a simple manually driven two-axis tilting stage (Stage I, see Knappertsbusch 2002), a motorized version of it (i.e., Stage III: Knappertsbusch et al. 2006), and subsequent improvements on the motorization, including the construction of Stage IV, an automated multi-axis stage built by students at FHNW in 2005, succeeded by Stage V built by one of the authors (Binggeli) in 2006. Upon software integration with the motorized microscope, Stage V became part of AMOR (diploma thesis of Herzig and Schmutz 2007) and AMOR2 (diploma thesis of Stapfer 2007). AMOR2 was further improved mechanically (work of C. Schneider) and extended to become the current version AMOR3 (bachelor thesis of Widmer, 2008). Although application of AMOR requires special slide preparation and pre-orientation of the microfossils, routine imaging is faster with higher precision than using the original hemispherical stage.

AMOR3 automatically moves a pre-oriented microfossil sitting in a faunal slide under the microscope lens, tilts it in x and y directions into a pre-



**Figure 2.** Hemispherical stage. Microfossils are mounted on a standard multisquared faunal slide, one specimen per field. The slide is fixed on a hemispherical body of polystyrene that sits on a ring-shaped basin.

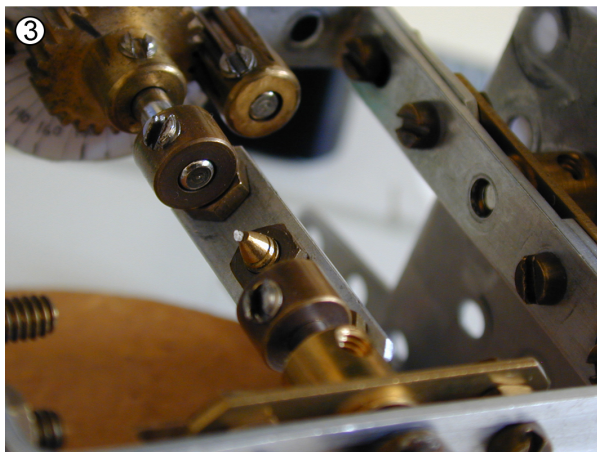
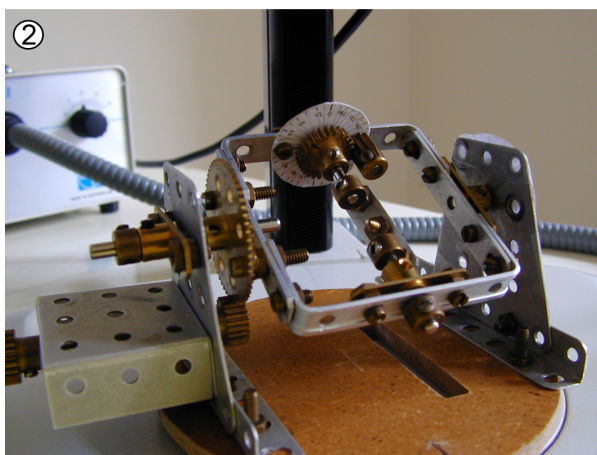
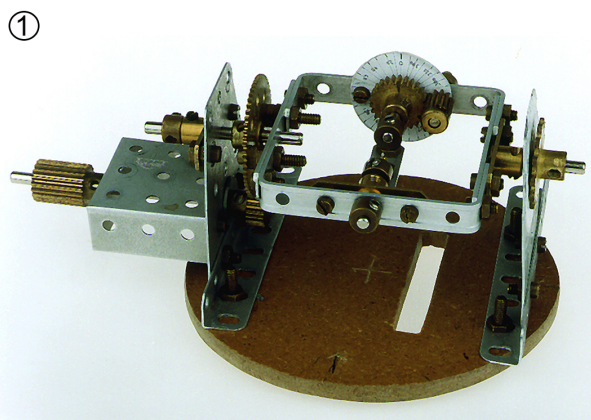
defined orientation, focuses and rotates the image where necessary, automatically zooms to the optimal magnification, and then takes a digital image to store it to disc. This robot allows for rapid handling of standard micropaleontological slides having 36 or 60 fields but custom slides can be handled as well. It produces a series of images that can later be batch processed using a suite of morphometric programs described in Knappertsbusch (1998, 2004).

### History of Stage Development

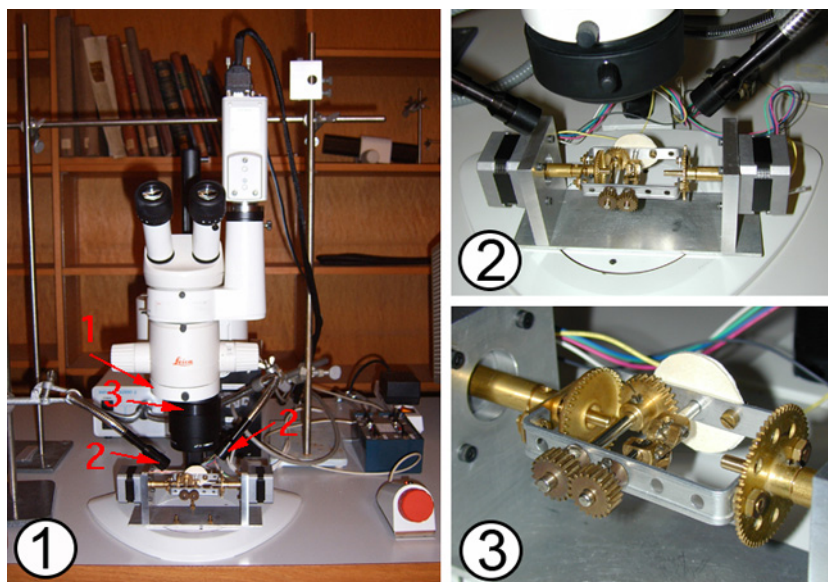
The first tool for the orientation of isolated microfossils under a binocular was a hemispherical stage (Figure 2).

While observing the microfossil through the binocular microscope or on a computer monitor, the investigator needed to manually move the specimen as precisely as possible until a desired orientation was attained. This monotonous manipulation of microfossils gave way to the design of a mechanical stage (=Stage I), that can be placed under the binocular microscope for imaging a single specimen. This device was first realized with a STOCKYS© toys set for children (see Figure 3.1-3.3).

The advantage of Stage I is that tilting in x and y directions can be controlled using goniometers, a prerequisite for the construction of stereographic virtual reality representations of microfossils under



**Figure 3.** 3.1, Stage I, the precursor of AMOR. It allows controlled tilting in x and y directions of a single microfossil. Gears are necessary in order to optimize freedom for tilting under the limited space of the microscope objective. A single foraminifer is mounted on the tip of a screw. 3.2, Detail of Stage I. 3.3, Close-up showing the tip of screw with a foraminifer mounted.



**Figure 4.1.** Motorized Stage III in working position. The two stepping motors on either side of the stage are driven either via a PC or independently by an extra controller box. They allow for precise positioning of single specimens under a microscope. Due to its compactness, Stage III is portable and can be applied in external micropaleontological collections or museums. **4.2**, Close-up of Stage III. **4.3**, Detail showing gears of Stage III. The minimized geometry is illustrated for optimum usage under a binocular microscope; a tilting range from  $-90^{\circ}$  to  $+90^{\circ}$  can be attained, which is ideal for 3D-imaging of a microfossil under incident light.

incident light (Knappertsbusch 2002). In Stage I the two perpendicular tilting-axes ( $x$  and  $y$ ) are adjusted in such a way that the microfossil remains within the focal range of the microscope. This is achieved by two perpendicular frames, which are suspended in a cardanic geometry. In order not to touch the microscope objective, the inner frame ( $x$ -tilt) is moved by gears and co-axially (parallel) driven with the outer ( $y$ ) tilting frame. This design is very compact and gives maximum freedom for tilting under the limited space underneath the microscope objective.

The next major advance was the motorization of the two tilting axes (Figure 4.1-4.2). The motorization was accomplished using two stepping motors, which can be controlled via a computer (Knappertsbusch et al. 2006). Because each step of the motor is converted into small steps of angular increment, positioning of specimens is more precise than in Stage I.

Stages I through III are designed for the orientation and imaging of a single specimen under a binocular microscope. For statistical morphometric studies, however, an apparatus for multiple specimens in micropaleontological faunal slides (Figure 5) was needed.

This goal was achieved with AMOR Stage IV (Figure 6) in collaboration with students of the Uni-

versity of Applied Sciences of Northwestern Switzerland (FHNW).

AMOR Stage IV was thus the first motorized and automated gliding and tilting stage for standard micropaleontological gridded faunal slides under a binocular microscope; however, due to disturbing oscillations while imaging Stage IV required improvement. Stage IV was redesigned by and programmed to AMOR Stage V (Herzig and Schmutz 2007, see Figure 7.1), then extended to AMOR2 (Stapfer 2007), and recently improved to AMOR3 (Widmer 2008).

## DESCRIPTION OF AMOR3

### Microscope Setup, Illumination, and Digital Imaging

A Leica MZ6 binocular microscope is equipped with a zoom-body allowing for continuous magnifications from 0.63x through 4x. On the camera tube a 3CCD color camera from Sony (DXC 390P) is mounted via a 1x C-mount connector piece. The camera is connected to a commercial IMAQ 1405 frame-grabber from National Instruments. The frame grabber card is inserted into a desktop PC operating under Windows2000. The microscope body is mounted on an AX-stand from Leica, which enables the microscopist to choose

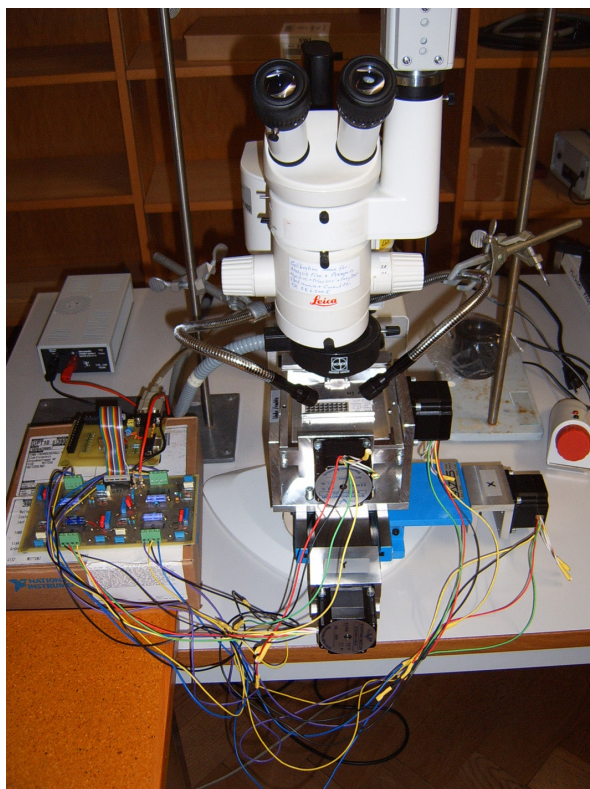


**Figure 5.** View on a standard micropaleontological faunal slide with globorotalids mounted in keel position.

between stereo- and mono-vision. In stereo-vision both light beams of the binocular microscope allow for 3D-perception of the microfossil. In the mono-vision mode the microscope is mechanically shifted sideward by the amount of parallax, and only a single light beam is directed to the camera tube. Here, the position for mono-vision is applied for digital imaging because the mechanical parallax correction compensates for the lateral shift of digital images that otherwise appear on the computer monitor during vertical movement of the microscope for focusing (see Knappertsbusch 2002). The vertical movement of the microscope is motorized using the MST 39 Motorfocus system from Leica. A software autofocus has been implemented in AMOR, which is described further below.

Illumination is critical for automatic positioning and imaging of microfossils under reflected light (Brown 2007). Lateral illumination (through swan-neck fiber-optic lamps) gives high contrast but often produces disturbing shadows. Alternatively, ring-illumination produces homogeneous light, with the effect that topographic features appear with rather low contrast in the image. Experimentation has shown that incident illumination (side and ring

light) causes reflections from the uneven bottom of the slide, which is problematic during automated particle identification. Such difficulties can be avoided by using cross-polarised incident light (Bengtson 2000). In our system a standard ring-illumination from Volpi holds a polarizing plate, which filters the light entering the microscope. The emanating light passes through a second, ring-shaped analyser plate, which can be rotated to produce extinction under cross-polarized conditions. Especially in the case of calcitic shells of planktonic foraminifera, which are often slightly translucent, cross-polarized illumination is fortunate for the following reasons: It enhances the contrast between shell and background while removing disturbing reflections from the background. It so strongly facilitates thresholding and image segmentation for specimen recognition. Insertion of polarizing filters into the light path, however, reduces light intensity, especially when magnification increases. In order to compensate for light loss, polarized ring-illumination and polarized sideward illumination were combined; shadows otherwise occurring disappear to a great extent when using polarizer caps on the swan-necks.



**Figure 6.** Binocular and Stage IV, the first motorized version having a 4- axis gliding and tilting stage designed for standard micropaleontological multi-squared faunal slides. Control of this stage occurs via a simple application under LabView. Electrical circuit boards, drivers, and controllers were all custom-designed for this study.

#### Four Axis Stage

The four-axis motorized stage (=Stage V) is central to AMOR. It holds and moves the microscope slide into a desired orientation (Figure 7.2).

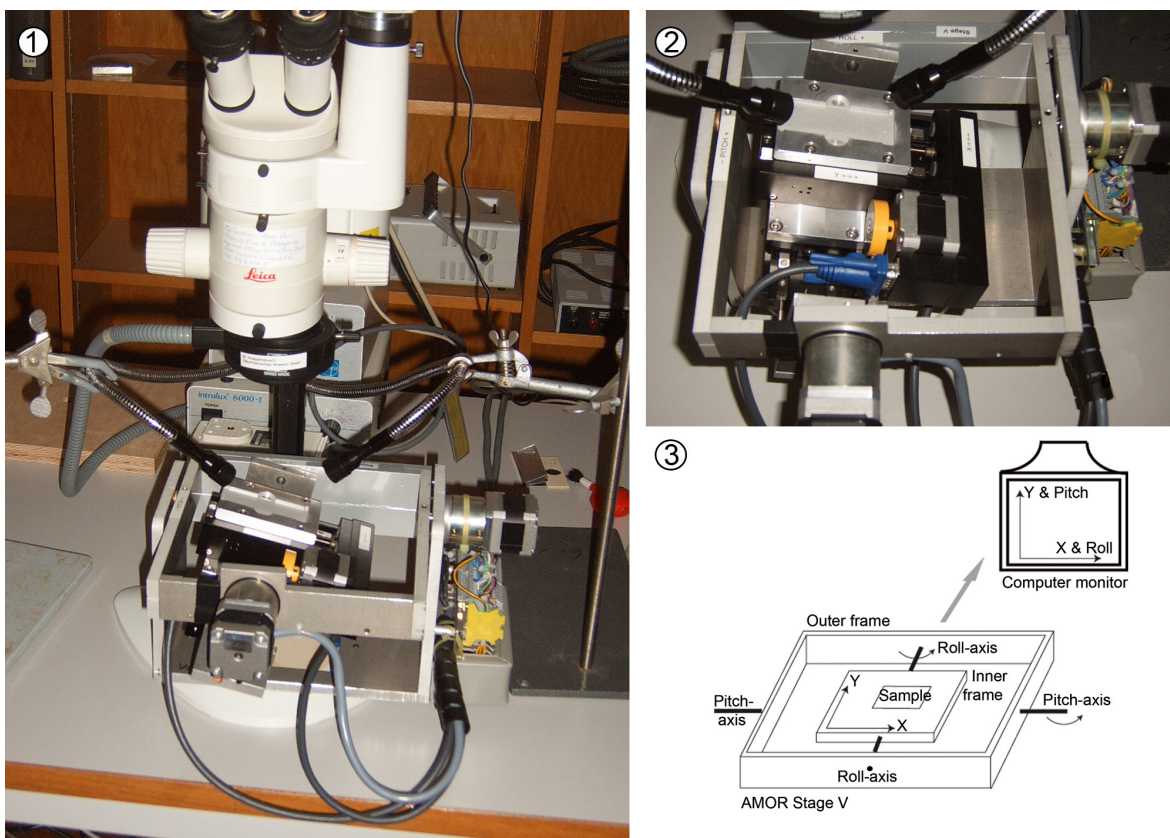
The slide is held by a metal plate with a rectangular cavity, in which standard micropaleontological gridded faunal slides from PASI (P.A.S.I.s.r.l., Torino, Italy) can be inserted. The slide holder resides on two perpendicular precision linear stages (model LTM 60P from OWIS GmbH), through which the slide can be shifted in the same plane (range in X-direction: from -20 mm through +20 mm, maximum resolution 312nm; range in Y-direction: from -25mm through +25mm, maximum resolution 312nm). This “inner frame” is cardinally mounted in the middle of an outer and larger frame, which can be tilted in two perpendicular directions producing a “pitch- and roll movement” of the slide, similar to the geometry in Stage I (Figure 7.3).

The two motors for the “pitch” and “roll” movements are stepping motors from Nanotec. In order not to collide with microscope parts, the range is limited from  $-30^\circ$  through  $+30^\circ$  (the working freedom for the MZ6 binocular using a 1x achromatic lens when a ring-light with 9 cm diameter is attached underneath the lens is 6.5cm at a magnification position of 4.0x). The resolution of these tilting motors is  $0.007^\circ$ . They are controlled through a specially manufactured controller box. This “stepping box” is fed with digital signals for motion control by commercially available data acquisition boards (DAQmx 8.6) from National Instruments, which are inserted in the PC.

#### Motorized Zoom

In earlier versions of AMOR an unfavorably high magnification during tilting sometimes caused a specimen to leave the imaging frame on the monitor, which means loss of control of the specimen. In such a case AMOR interrupted positioning. In order to avoid such situations the zoom-knob of the magnification body of the microscope was motorized and integrated into the AMOR program (Figure 8), and specimens are automatically re-centered with respect to the imaging window if necessary.

There exist few motorized zoom motors on the market (for example from [www.asiimaging.com](http://www.asiimaging.com)), but they were not compatible to our Leica MZ6 geometry without major modifications. For this reason an automated zoom was designed and programmed in LabView. A two-phase stepping motor (model CSK266-AT from Orientalmotor GmbH, resolution  $1.8^\circ$  at full-step) was found to fulfill the requirements, which is connected with the AX stand of the microscope. The connection between motor-axis and zoom-drive of the microscope is created with a flexible coupling (Toolflex) from KTR (KTR-Kupplungstechnik AG in Zürich). The electronics for the automated zoom-control is housed in a second external “Zoom-Box”, which is fed by a second motion control card in the PC. This extra PCI card was necessary because the first card for moving the stage axes could not handle more than four axes. For a full zooming cycle, i.e., from 0.63x through 4.00x the motor uses about 200 steps. Two electrical push-button switches are attached on either side of the zoom-axis in order to stop zooming at the lower and upper end of the zoom range. Because the magnification per motor increment increases following an exponential law (see Supplement #6 in Knappertsbusch 2004), the sen-



**Figure 7. 7.1**, AMOR with Stage V developed by Binggeli in 2006 and programmed by Herzig and Schmutz (2007). PC and stepper-box are not shown in this figure. **7.2**, Close-up of AMOR Stage V to illustrate the metal plate to hold slides. Inside the outer frame (Pitch) there is the inner element (Roll) holding the two precision gliding stages (X-translation and Y-translation). Note polarizer caps on tips of swan-necks. **7.3**, Schematic representation of AMOR Stage V illustrating pitch and roll axes.

sitivity is greatest at the higher-end of the magnification range.

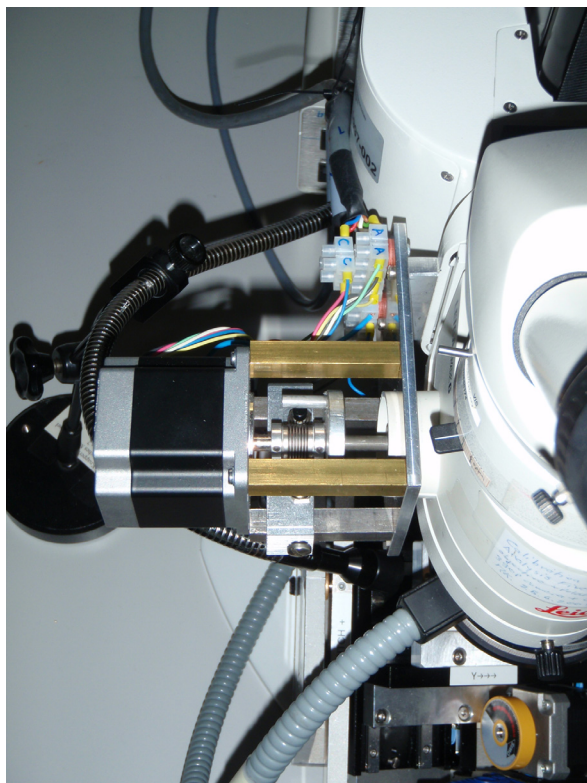
Only through addition of this motorized zoom to the system it is now possible to sequentially scan all fields of the slide and automatically orientate all specimens in the slide. The motor-zoom can be software enabled or disabled during initialization of AMOR, which maintains the functionality of the tilting stage under manual zoom operation in case the motorized zoom fails.

#### System Integration and Orientation Control

System integration of AMOR was achieved with LabView 8.5 from National Instruments, a graphical software development environment common in industry for machine vision and motion applications. The program (also called AMOR) consists of a logical sequence of so-called VIs (virtual instruments), which are factory designed routines and functions to solve a special task like generation of a standard input current signal to drive a stepping motor. Orientation control of

microfossils was achieved by using the VIs of NI-Vision module of LabView. AMOR can be used to orient a disk-shaped foraminifer like *Globorotalia menardii* in two basic positions: Keel-view and umbilical or spiral view. In the keel position, the specimen “stands” on its periphery with the primary aperture facing upward. In the umbilical or spiral position the umbilical or spiral side is facing upward, respectively. Before application specimens must be fixed in an approximately oriented position, which needs to be done manually using some water soluble glue under a binocular microscope.

The coordinate system of AMOR is Cartesian and refers to the rectangular grid-lines drawn on the slide: X denotes the translation to the left or right, Y denotes the translation in perpendicular direction in the plane of the slide. On the computer monitor these movements correspond to image translations in left/right directions and up/down directions, respectively. Tilting follows a similar scheme: Roll causes movement of a specimen on



**Figure 8.** Motorized zoom attached to the microscope as developed by Stapfer (2007) and after improvement of mechanical fastening by Claude Schneider.

the computer monitor in direction of the X axis (i.e., left, right), and pitch causes movement of a specimen on the monitor in direction of the Y axis (up and down, see Figure 7.3).

The perfect orientation of the shell in *keel view* is attained when the following three conditions are met (Figure 7.3). (1) The enclosing area of the test during tilting about the roll-axis is minimal [“roll condition”]. (2) The longest axis in keel view during tilt about the pitch-axis reaches a maximum. This pitch-condition is necessary in order to keep the apertural face of globorotalid shells in upward position, which functions well in the case of menardi-form globorotalids, our main study object. (3) The longest axis in keel view is vertical in the imaging window [“rotational condition”]. While pitch- and roll-conditions are achieved through physical specimen tilting and real-time image calculation of area and length measurement, the rotational condition is achieved through soft-image rotation with the function “Autorotate” (see further below). These conditions were evaluated through experimentation. For other foraminiferal species the ideal conditions for positioning must first be defined and tested. Orientation in umbilical or spiral view of the test is easier

because then the perfect position is attained when the surface area of the shell reaches a maximum.

### Autofocussing

Autofocussing is a prerequisite for automated object orientation. Two ways for obtaining a focused image were implemented: A coarse focus selects for the highest variance within an active region of interest (ROI) of the image. A well-focused image has many details with many grey-levels in a narrow area and hence shows a high variance. This method worked well at lower magnifications, where the depth of field of the parfocal zoom-lens system of the Leica MZ6 is quite wide. A parfocal microscope objective stays in focus when magnification is changed from a higher power objective to a lower power objective. At higher magnifications, when incident light becomes more attenuated and the narrower depth of focus causes less strong local variances, a different algorithm (fine focus) was implemented. This is a minimizing function for the surface area of the object at perfect focus conditions. It is based on our observation that at higher magnifications a bright object appears in focus as soon as its surface area (defined by the area inside of the periphery of the particle) becomes minimal. When the object moves out of focus (“under- or overfocus”) the surface area increases continuously before it fades away.

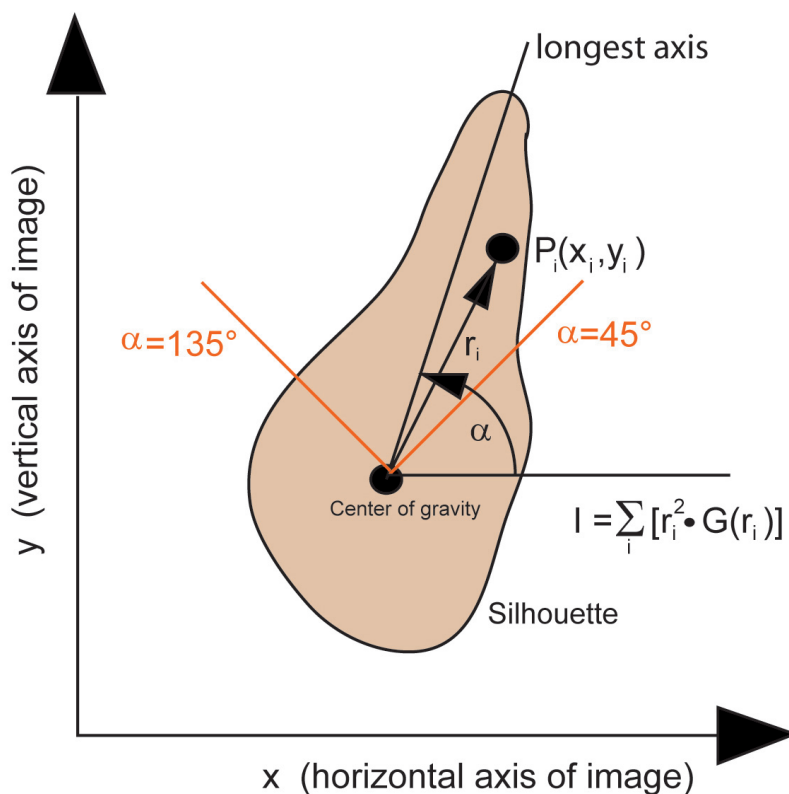
### Autorotate

Autorotate applies to shells in keel view and is a software function to rotate the silhouette of the shell into vertical orientation (i.e., with the largest axis being parallel to the vertical side of the imaging window). This step is necessary for later outline analysis of the shells. In AMOR vertical orientation is achieved by minimizing the momentum of inertia of the thresholded particle (see Figure 9). This method is applicable here because specimens are manually pre-oriented in the slide in north-south orientation as much as possible. For ideal orientation of the specimen function Autorotate then requires only a small amount of rotation of the image (usually less than about 5 degrees, depending on how well the specimens were placed north-south in the slide).

### Slide Calibration

Experience has shown that there is some variability in position and size of the array of grid fields in commercially available faunal slides, even if they come from the same manufacturer. Without correction, this variation prohibits reliable automated





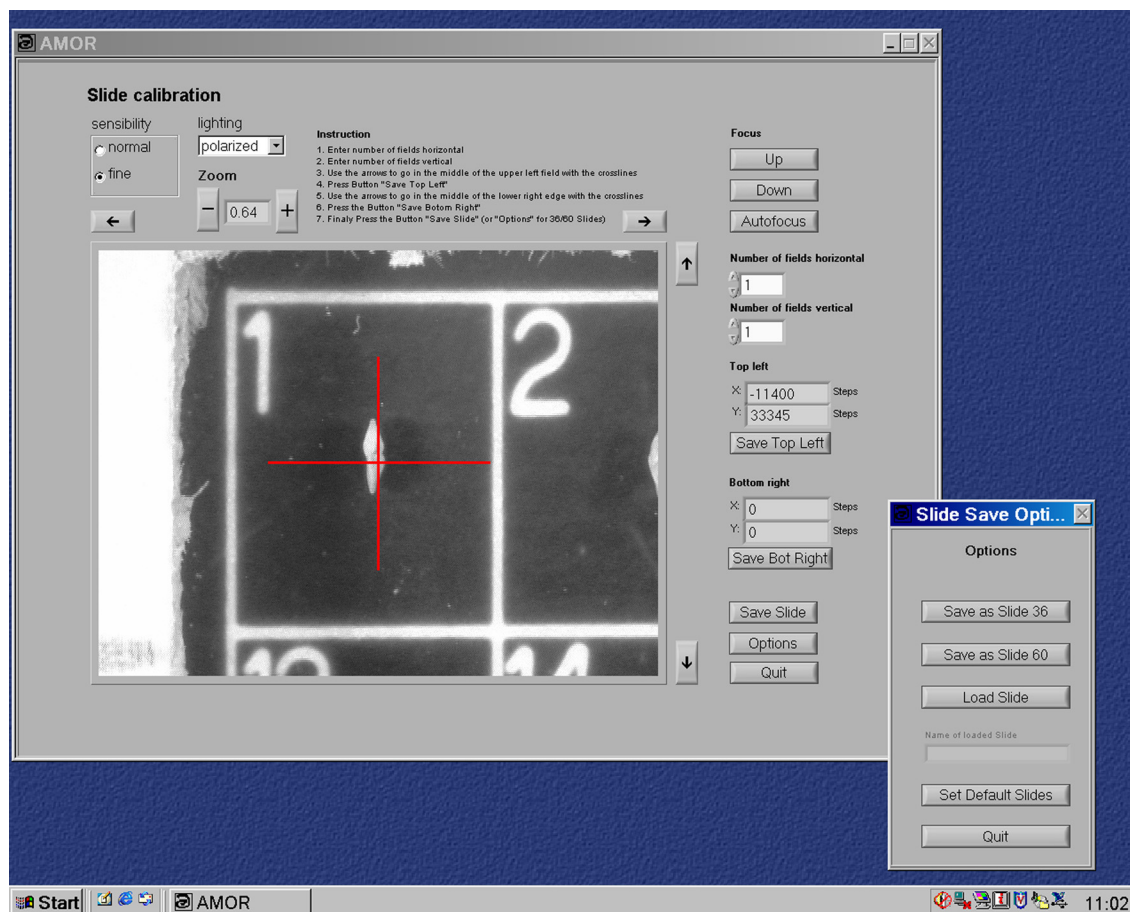
**Figure 9.** Momentum of inertia ( $I$ ) applied to vertically orientate the particle on the computer monitor. The vector  $r_i$  denotes the vector from the point of rotation (=center of gravity) to a pixel with grey level  $G_i$  at image coordinates  $x_i$  and  $y_i$ . In a binarized image  $G_i$  is 255 (white) inside the object and 0 (black) outside the object. Alpha is the angle between the longest axis of the object and the x-axis of the image. The momentum of inertia is a periodic function of alpha: It is minimal if alpha is  $0^\circ$ ,  $90^\circ$ ,  $180^\circ$ ,  $270^\circ$ , etc. (i.e., if the longest axis is parallel to one of the coordinate axes). The momentum of inertia  $I$  reaches a maximum if alpha is  $45^\circ$ ,  $135^\circ$ , etc. (i.e., if the longest axis is in a diagonal position). Due to the pre-orientation of the shells in the slide, the longest axis is always located in the upper sector between the two red lines. .

positioning. In order to avoid this difficulty, a special slide calibration function was implemented in AMOR3 (Widmer 2008). This function enables the user to assign the characteristics of a particular slide to an external file (see Figure 10). These characteristics include the number of columns and rows of fields of the gridded slide and the center coordinates of the first and last fields in the slide. This information can later be re-loaded into the memory of the program, if needed. In this manner, any kind of gridded faunal slides with standard dimensions of 75 mm x 25 mm (=size of the cardboard), but independent of the number of engraved fields, can be applied.

#### Autocharacter Function

Another difficulty with earlier versions of AMOR was that occasionally the white numbers

that are engraved in each field of a faunal slide were mistaken as a microfossil. Such misidentification led to failure during automated orientation. In version 3 of AMOR this difficulty was remedied through implementation of a character recognition and blending function (Widmer 2008). The fix was possible using character recognition algorithms embedded in the library of LabView's Vision module. With AMOR3 the user is also no longer confronted with the additional problem, that in some faunal slides the numbering is located in the upper left corner of each square (such as in slides with 60 fields) while in other types the numbering sits in the lower right corner of each square (for example in faunal slides with 36 fields). In this way the frequency of microfossil misinterpretation could be reduced to about one failure per 100 fields.



**Figure 10.** Panel for calibration of a slide. By moving the slide left/right or up/down (using the arrows) the red cross is placed into the center of field 1 and its coordinates stored to an external text file. This procedure is repeated with the last field of the slide. By indication of the number of rows and columns in the slide the center of each field is calculated, which helps to quickly locate or center a specimen whenever necessary.

### USING THE STAGE

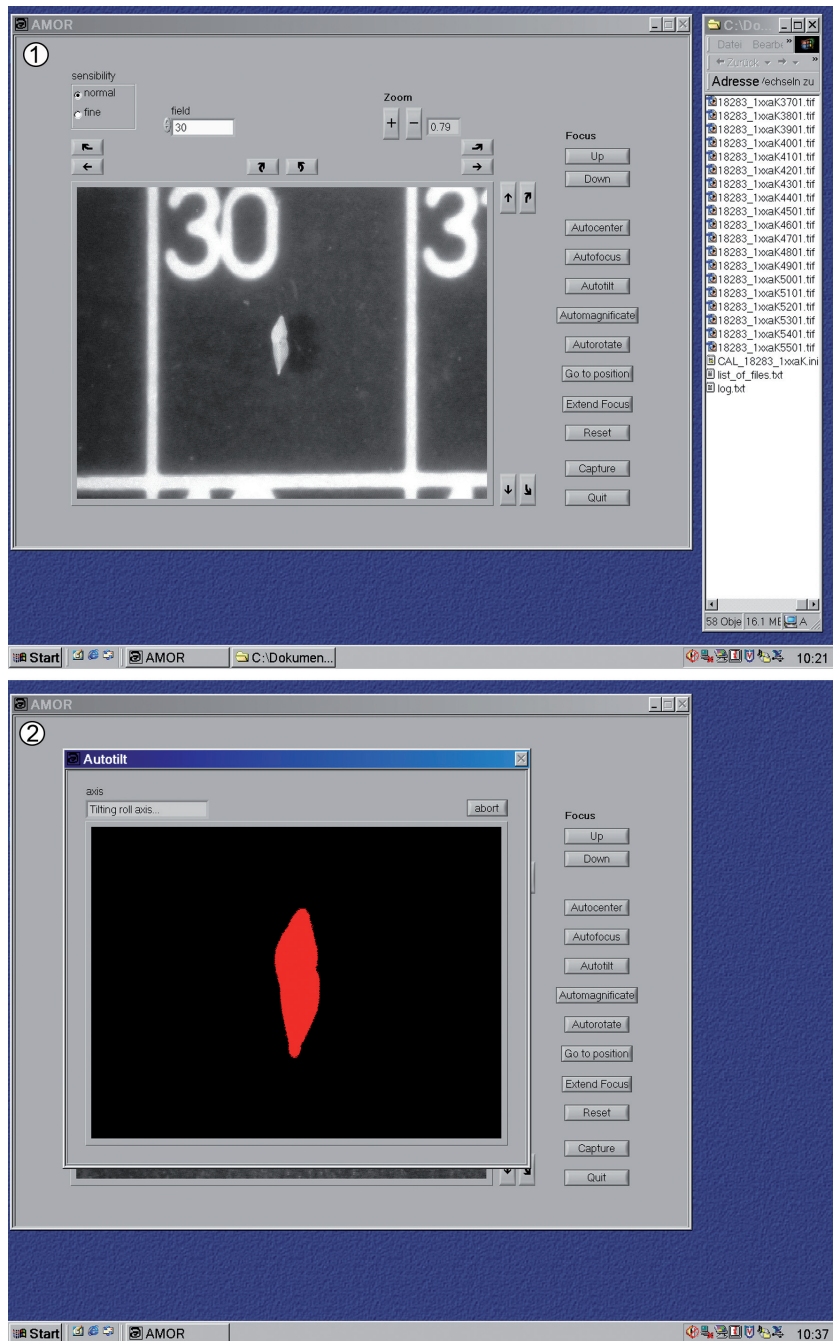
AMOR3 recognizes the presence or absence of a microfossil in a field of the slide only when the specimen resides in or close to the center of a particular field. For this reason, slides must be prepared accordingly, and specimens must be located near the center of a field in the slide. The second prerequisite for a successful operation of the program for keel-view analysis is that specimens are pre-oriented in approximate keel position. This conditioning was chosen in order to keep the complex problem of specimen orientation as simple as possible.

#### Single-Specimen Mode and Automeasurement Mode

The AMOR software has two modes: A single-specimen mode, where individual specimens can be addressed manually (i.e., by driving the x and y gliding and tilting axes by mouse-clicks), and an

auto-measurement mode for automated batch orientation and imaging of specimens in a slide. In the single-specimen mode, functions like specimen centering, up and down movements of the focus, autofocussing, zooming, translational and tilt movements, image rotation, and image capturing can be launched in any desired sequence (see Figure 11.1-11.2).

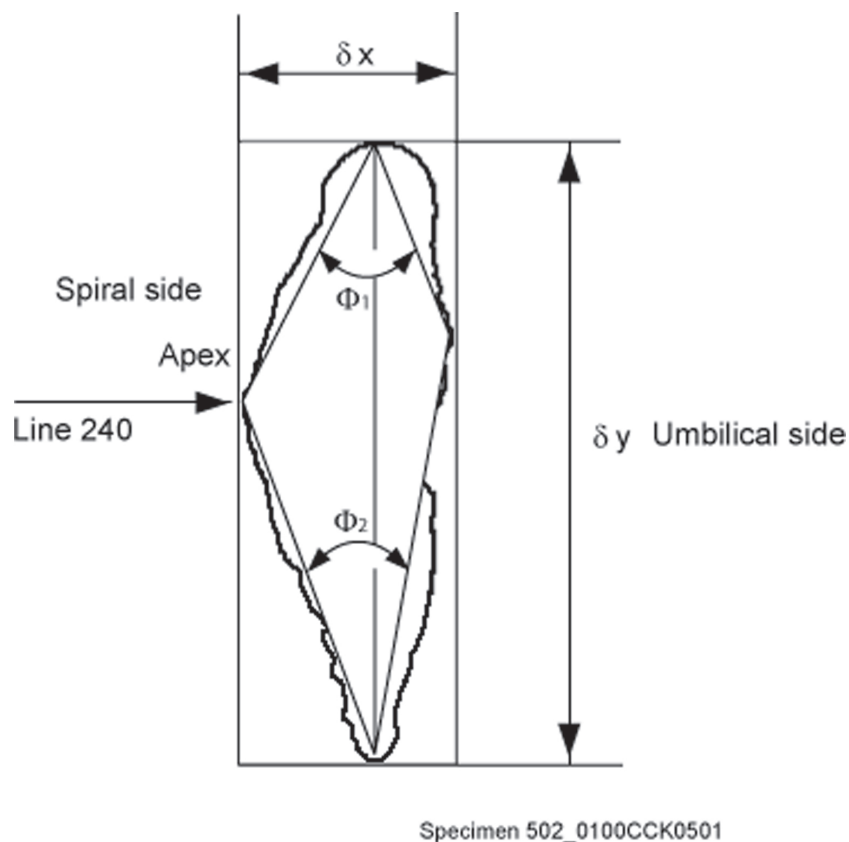
In the auto-measurement mode these steps are automated. In this mode, a special panel allows flagging specimens individually for automated treatment of a selected range of fields in the slide. Alternatively, the entire slide can be processed at once. Also in this mode individual functions can be selected by check-boxes. Examples are the inclusion or exclusion Autorotation (keel view only, see above), or the manner how the software shall respond to runtime-errors (i.e., continue or interrupt for manual interaction). Upon launching the auto-measurement mode, the system moves to the first



**Figure 11. 11.1**, Software operating mode of AMOR in single measurement mode. The various buttons are needed to drive the stage in X and Y directions, and to activate pitch and roll movements. Other buttons are needed to manually focus up and down, and activate the autocenter, autofocus-autotilt, autorotate, and image capture functions. On the right-hand side, the directory is shown, where images and the log file are stored in. The log file records settings during a measurement cycle. **11.2**, Active window of AMOR while tilting a menardiform specimen into the optimum position in keel view.

field that was previously selected, autocenters the specimen, focuses automatically, starts the pitch and roll tilting movement until the ideal position is attained, zooms up to the maximum possible magnification, focuses again, rotates the specimen ver-

tically (only possible in keel view), captures the image, and saves it to a disc into a pre-defined directory. Grey-level images are written in Tiff format and have a size of 640 x 480 pixels, with 256 grey-levels per pixel. Thereafter, the system



**Figure 12.** Outline of a *Globorotalia menardii* in side (keel) view and designations for morphometric parameters that can be extracted with MorphCol routines (Knappertsbusch 2004 and 2007). DeltaX=spiral height, DeltaY=axial width, Phi<sub>1</sub>=upper keel angle, Phi<sub>2</sub>=lower keel angle. Line 240 indicates the Y-position (in pixels) in a 640 x 480 pixel image with 256 grey levels, where outline extraction begins.

advances to the next field and repeats the sequence until all fields are treated. In addition to the images produced by the scan, a text file is generated to record the image names to reduce the scanning time per slide movement from one row to the next occurs in a meander-like manner. Next to the images a text file is generated, which records the image names together with the corresponding magnifications for each image taken. This "list of files" can later be used in combination with external MorphCol programs (Knappertsbusch 2004) to automatically extract outline coordinates and to convert them from pixels to micrometers. During operation, a logging file records the successful treatment of each field, the magnifications, and the tilting positions. If an error occurs, it is logged as well, and the system abandons the actual field to advance to the next field.

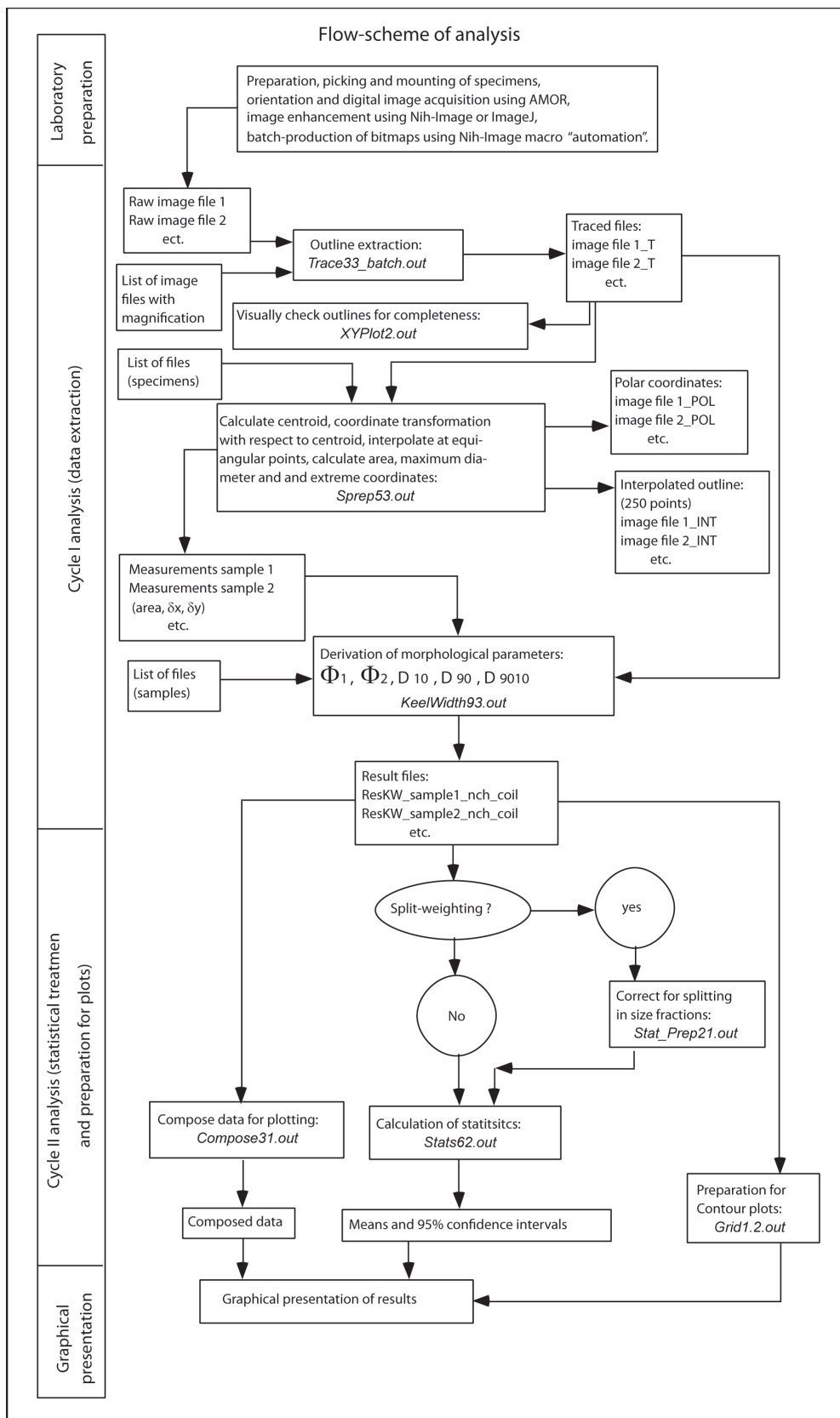
For a slide containing 60 specimens in keel position and with the motor-zoom function enabled, AMOR needed 86 minutes to process in auto-measurement mode. If the motor-zoom is disabled (in

this case the magnification remains constant throughout scanning of the entire slide) the system is even faster. This rate of image collection is fast and outcompetes any experienced manually operating person.

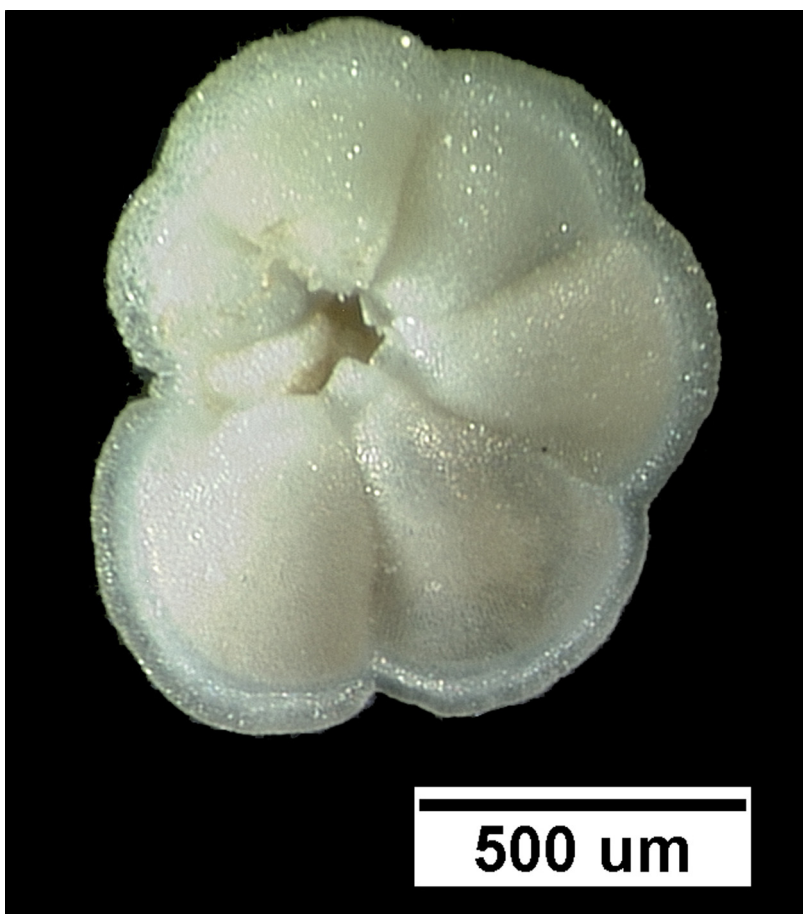
#### Image Collection and Post-Processing

Upon termination of AMOR3, the user has a collection of grey-level images with foraminiferal shells in perfect keel or spiral/umbilical view together with the necessary information about the magnification taken for every image.

The collected grey-level images need to be further processed to outline coordinates in micrometers, from which geometric parameters like size or shape descriptors can be derived. In our case outline analysis of the shells in keel view has been proven to contain the most useful shape information (see Figure 12). There exist a great number of software packages in the public domain (see, for example, the morphmet discussion list) for this task. In our case a suite of self-written Fortran pro-



**Figure 13.** Flow-scheme for post processing of images for the extraction of outlines and morphometric data (see Knappertsbusch 2007).



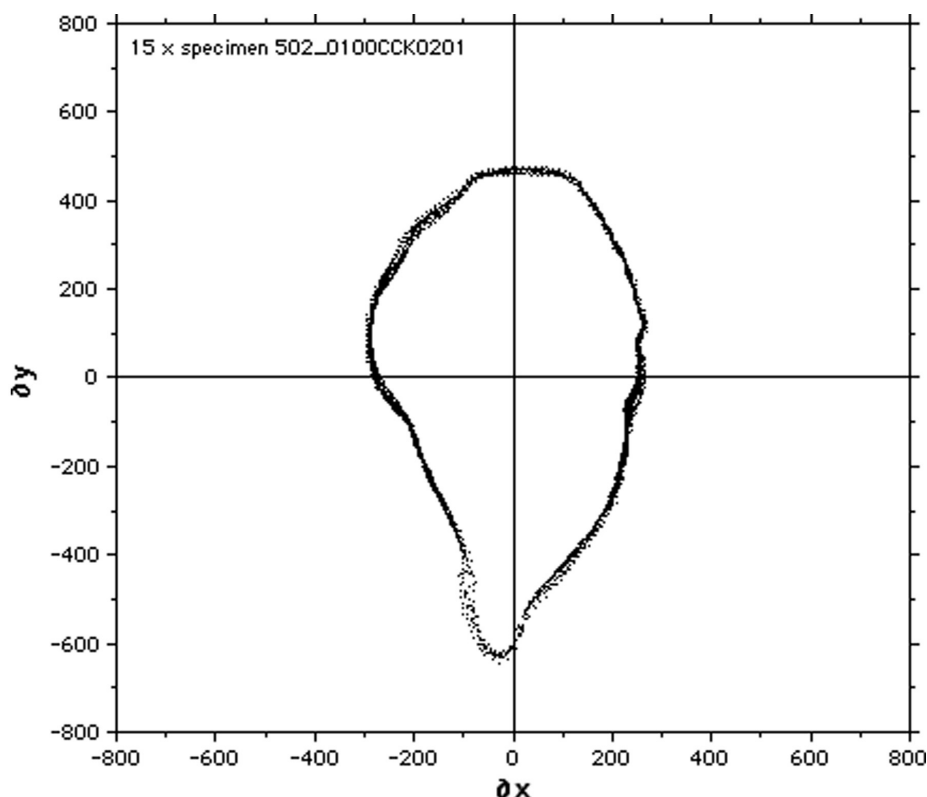
**Figure 14.** A specimen of the planktonic foraminifer *Globorotalia menardii* in umbilical view. Sample DSDP 68-502-1H-cc, 0-4cm, size fraction 500-1000 micrometers, 1/64 split, specimen 502\_01\_\_CCU1703. Image taken with Leica MZ6, Planapo 1x objective, magnification set to 2.00x, cross polarized light, processing with AutoMontage software from Syncroscopy using an image sequence of 30 vertically stacked images across the focal depth (see Knappertsbusch et al. 2006). The scale bar represents 500 micrometers.

grams is used, which are described in Knappertsbusch (1998) and Knappertsbusch (2004), in combination with public domain software NIH-Image or ImageJ from Wayne Rasband. A special macro “automation” written in NIH-Image (see Brown 2007) for a listing) helps to quickly batch-process grey-level images to binary black and white images prior to outline extraction (note: binary images could, theoretically, already be exported to disc during the real-time image processing while orienting the specimens. This bears potential for abbreviating the entire process of image collection and postprocessing in the future. At this stage of development, this possibility has not implemented yet in order to keep development steps (i.e., image collection vs. post-processing) as easy as possible. Figure 13 illustrates a work-flow scheme for the post-processing for *Globorotalia*

*menardii* (Figure 14) applied in Knappertsbusch (2007).

#### **Precision and Repeatability of the System**

The quality of morphometric data after imaging with AMOR depends on many factors: Illumination, image quality, thresholding and segmentation in binary images, precision of the x and y translational and tilting motors, correct camera calibration (i.e., pixel to micrometer conversion as a function of magnification), calibration of the microscope magnification (i.e., precision of the mechanical drive of the zoom and the translation of the zoom-movement into a numerical magnification factor). All these factors were carefully checked prior to routine applications, under controlled conditions of a darkened room (changing roomlight may alter the light conditions) and always using the same single specimen.



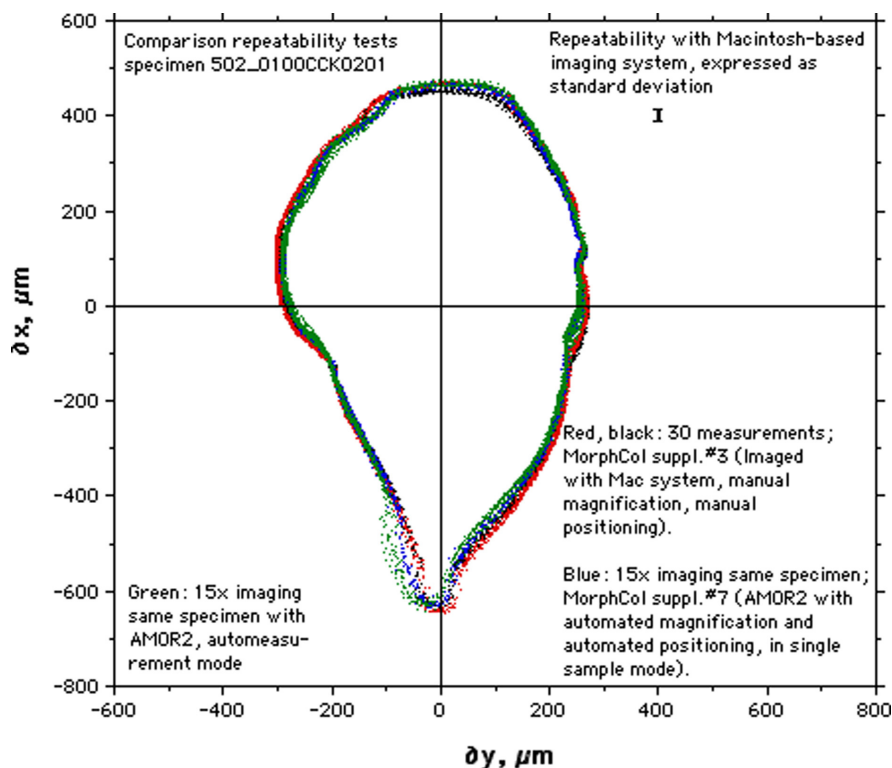
**Figure 15.** Repeatability test of AMOR: Overlay of the outline coordinates from 15 times orienting and imaging the same specimen (#502\_0100CCK0201) in keel view under the same conditions and using AMOR in single-object measurement mode (with the Auto-zoom enabled). DeltaX (in micrometers) is the spiral height, DeltaY (in micrometers) is the axial width of the shell in keel view.

In AMOR thresholding can be adjusted during initialization by setting the illumination, diaphragm, and the polarizers. For better control, the diaphragm and the polarizers were labelled using a millimetre-paper stripe, so that identical settings can be reproduced. Too low illumination caused the binary image of the specimen to be eroded. However, it was observed that above a certain threshold this influence vanished. The camera calibration was performed using a 1 mm scale with 10 micrometer subdivisions. Calibration must be carried out for every objective used, at every magnification in horizontal and vertical directions in order to compensate for the various influences of chip-size of the camera and the properties of the frame grabber. Whenever the imaging system is modified (i.e., new objectives, different type of zoom-body or microscope, digital camera, C-mount, frame grabber, or another computer system) calibration must be renewed. The influence of polarizing filters (if inserted in the light path of the microscope) did not cause any changes in magnification factors, but caused a slight lateral shift of the image on the

computer monitor that does not affect morphometric measurements.

The magnification of the zoom-body of the Leica MZ6 as a function of the rotation angle of the zoom knob follows an exponential relationship. This relationship was found empirically by attaching a millimetre-paper stripe around the zoom knob and so measuring the angular positions at the indicated magnifications (i.e., 0.63x, 0.8x, 1.0x, 1.6x, 2.0x, 2.5x, 3.2x, and 4.0x). The detailed results of these experiments are described in supplements No. 6 and 7 of MorphCol (Knappertsbusch 2004). Applying this relationship allowed the calculation of magnifications at any zoom-position when using the stepping motor.

Figures 15 and 16 illustrate the precision of AMOR after repeated orientation, imaging, processing, and extraction of outlines to cartesian coordinates of the same single specimen for several times: In Figure 15 the test specimen was 15 times oriented, imaged, and processed to outlines in auto-measurement mode and using the motorized zoom. In Figure 16 three different repeatability tests are compared to each other: In Test 1 the



**Figure 16.** Comparison of repeatability experiments: Tests for manual positioning with a hemispherical stage and through a Macintosh-based digital imaging system versus automated positioning and imaging using the new AMOR system. The experimental setup and processing are described in supplements #5, #6, and #7 of MorphCol (see Knappertsbusch 2004). All outlines represent the same single specimen as shown in Figure 15. Delta X is the spiral height, Delta Y is the axial width of the shell in keel view. Explanation of colors: Red and black silhouettes (30 outlines in total) were taken with a Macintosh-based imaging system and a different camera and were manually positioned into keelview using the hemispherical stage. Blue silhouettes (orienting and imaging the same specimen 15 times) were generated using AMOR in single-specimen mode and with the auto-zoom enabled. Green silhouettes (orienting and imaging the same specimen 15 times) were generated with AMOR in auto-measurement mode. The obvious deviations of outlines in the lower left quadrant of the graph occurred because the specimen was removed from the slide after completion of the (earlier) Macintosh-based tests (red and black outlines) and later re-mounted to the measurement slide for doing the AMOR-based tests (blue and green outlines).

same specimen was oriented 30 times by hand using the hemi-spherical stage shown in Figure 1 and using Nih-image software on a Macintosh-based digital imaging system. In Test 2, the same specimen was automatically oriented 15 times using AMOR2 and with the automated zoom enabled, but in single-specimen mode. In Test 3 of Figure 17, the test specimen was oriented using AMOR2 and with the automated zoom enabled, but in auto-measurement mode. In general, there is reasonable agreement of the test series among and between each other.

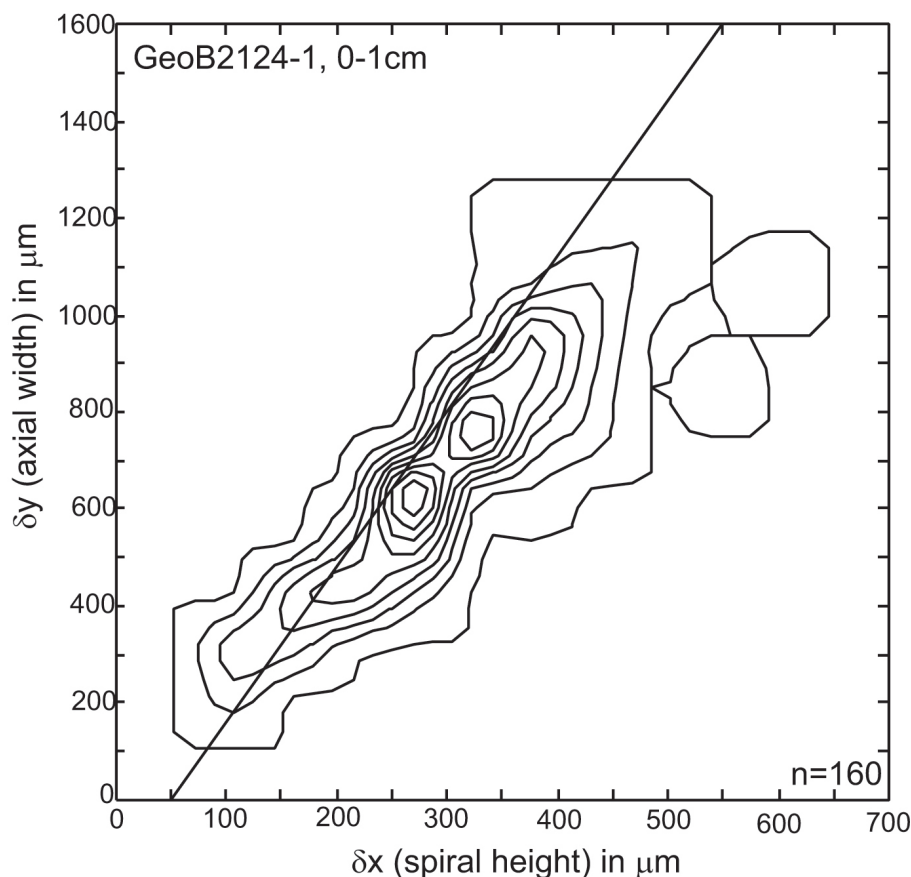
Statistical analysis of the areas in keel view from 38 orientation measurements done for the same specimen have shown that the standard error with respect to the mean area in keel view is 0.233% and 3.438% with respect to the difference

between the minimum and maximum area of the 38 investigated shells (which is  $0.0263\text{mm}^2$ , see MorphCol supplement No. 8, Knappertsbusch 2004).

### EXAMPLE

It is not the scope of this paper to present and analyze large sets of morphometric data, but an example that can be obtained with AMOR shall be given. *Globorotalia menardii* (Figure 14) is an extant, Neogene tropical planktonic foraminifer, which shows considerable morphological variation biogeographically and since the upper Miocene (Knappertsbusch 2007). Figures 17 and 18 illustrate contoured bivariate frequency diagrams of spiral height versus axial length measurements of Holo-





Grid-cell:  $\Delta x=50\mu\text{m}$ ,  $\Delta y=100\mu\text{m}$   
 Contour interval=2 specimens per gri-cell

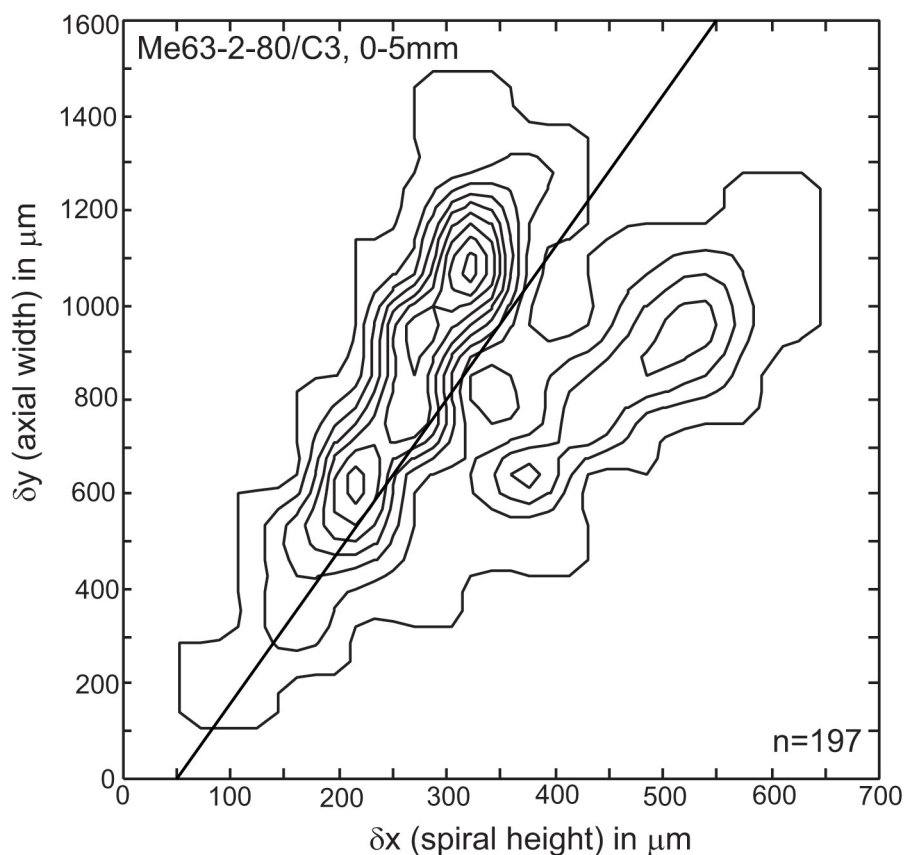
**Figure 17.** Example 1 of a bivariate frequency distribution of spiral height (Delta X) versus axial width (Delta Y) from 160 individual specimens of *G. menardii* in keel view from Holocene sample GeoB2124-1, 0-1cm and imaged with the new system of AMOR. The sample comes from the South Atlantic (Lat. 20.96° S / Long. 39.56° W, water depth 2003 m). The grid-cell size for counting frequencies was 50 micrometers in direction of Delta X (spiral height) and 100 micrometers in direction of Delta Y (axial width). The contour intervals are two specimens per grid-cell. The diagonal line was derived from several Pleistocene sample horizons in the Caribbean Sea (see Knappertsbusch 2007) and indicates a best visual separation between *G. menardii cultrata* (above line) and *G. menardii menardii* (below line). In individual – sometimes unimodal - samples such as the present one, however, there may be overlapping areas of one frequency mode with the other field, showing that numerical taxonomic subdivisions are always artificial. In this example the majority of menardiform globorotalids belong to the *G. menardii menardii* mode.

cene representatives of *G. menardii* from two surface sediment samples in the Atlantic Ocean. The images for these measurements were generated using the auto-measurement mode of AMOR: the outline extraction and analysis of morphometric data were obtained using the methods described in Knappertsbusch (2007).

## CONCLUSIONS AND OUTLOOK

Automated specimen orientation and image collection with AMOR opens new possibilities for

obtaining extended morphometric foraminiferal data sets for evolutionary studies. Replacement of a human operator by an automat reduces variability of measurement and human error. The speed of specimen throughput is enhanced with AMOR, although the results must always be critically supervised. Application of AMOR to record morphological shell change through geological time and along geographic or (paleo-) climatological gradients gives way for statistical analyses at greater sampling density than before. It allows for a



Grid-cell:  $\Delta x=50\mu\text{m}$ ,  $\Delta y=100\mu\text{m}$

Contour intervals=2 specimens per grid-cell

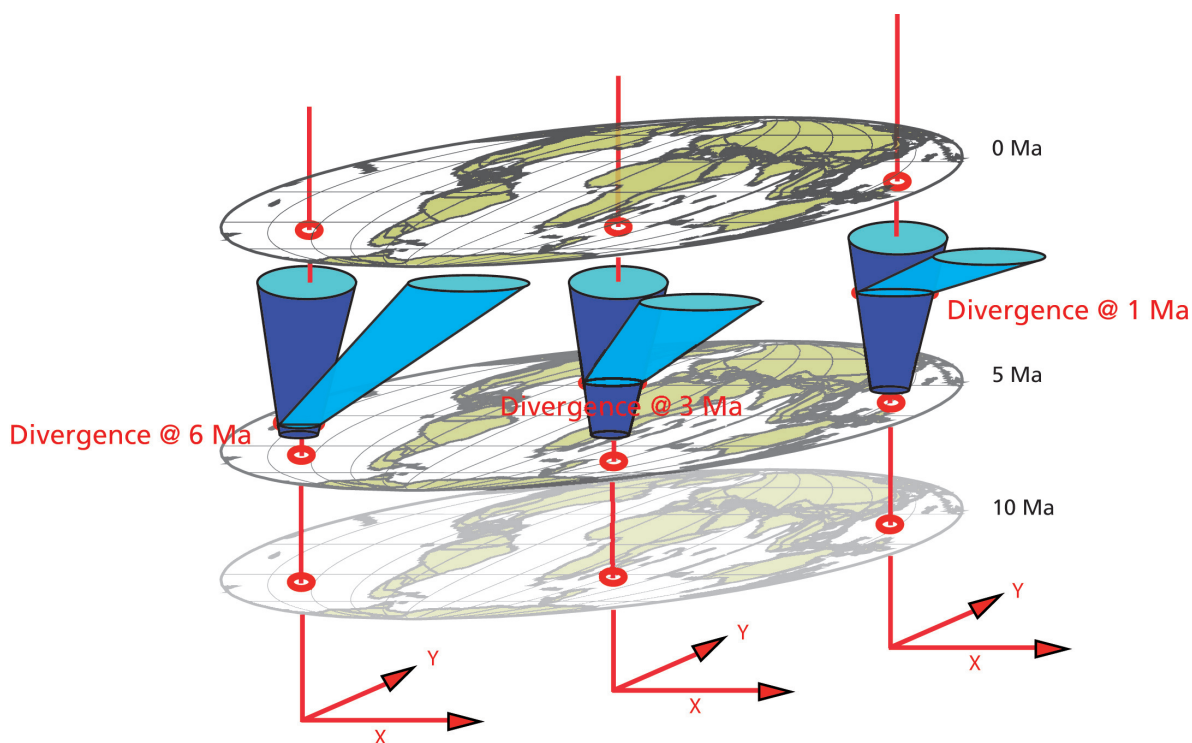
**Figure 18.** Example 2 of a bivariate frequency distribution of spiral height (Delta X) versus axial width (Delta Y) from 197 specimens of *G. menardii cultrata* (above line) and a mixture of *G. menardii menardii* and *G. tumida* (below line) in keel view from Holocene sample Me63-2-80/C3, 0-5mm and imaged with the new system of AMOR. The sample comes from the Guinea Basin (collected within the framework of project DIVA II of Deutsches Zentrum für Marine Diversitätsforschung (DZMB) during Meteor-cruise 63/2, station 80, lat.  $0^{\circ} 50.0' \text{ N}$  / long.  $5^{\circ} 35.0' \text{ W}$ , waterdepth 5138m). Same grid-cell size and contour intervals as in Figure 17. In this example the distribution is clearly bimodal.

more complete exploration of the morphological speciation and divergence patterns and ecophenotypic variation buried in the sedimentary record on a global scale (concept of “*evolutionary exploration*”, see Figure 19). Until present AMOR was extensively tested using the *Globorotalia menardii* foraminiferal plexus, but other foraminiferal species groups can be analysed with the system as well.

Apart from micropaleontological applications, AMOR may have potential for quality control of microscopic particles in all sorts of powders (washing powder, cosmetic powder, concrete powder), beads, sands, plant seeds, pigments, etc., i.e., in cases where orientation plays a role in discrimination of shape parameters.

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**Figure 19.** Concept of "evolutionary exploration", i.e., the mapping of the history and geography of morphological changes and divergence patterns. The blue diagrams between the time-planes represent idealized phylogenetic reconstructions in the bivariate morphospace defined by morphological characters  $x$  and  $y$  for menardiform globorotalid shells. Only with an efficient automated imaging system like AMOR such an endeavour can be realized.

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