Portable Confocal Microscope Reveals Fossil Hominid Microstructure

Timothy G. Bromage, Alejandro Perez-Ochoa, Alan Boyde
1. New York University College of Dentistry, NY, USA
2. Dept Paleontology, Universidad Complutense de Madrid, Spain
3. Dental Biophysics, Queen Mary University of London, UK

INTRODUCTION
Most fossil specimens are either translucent or, if they are surface reflective, are not flat. In both cases, light interacts with the sample over a considerable vertical range and is reflected (or the fluorescent light emanates) from a thick layer. We have found a solution in the development of portable confocal microscopy for the evaluation of rare and unique early hominid fossils.

The principle of the confocal scanning light microscope (CSLM) is to eliminate the scattered, reflected or fluorescent light from out-of-focus planes. Only light originating from the plane of focus of the objective lens contributes to image formation at the several conjugate focal planes (intermediate, eye point, image recording device) in deference to light that is eliminated from all out-of-focus planes. In practice, an illuminated spot in the plane of focus is scanned across the field of view and an image is compiled. CSLM thus differs from conventional light microscopy, where light from the focus plane of the objective lens, as well as from all out-of-focus planes across the entire field of view, is observed. The history and various technical achievements in confocal microscopy are summarized in Boyde [1].

We employed a CSLM based on the Nipkow disk technique [2], described in detail by Petran and Hadravsky [e.g. 3] and first commercialized in the early 1980s. The Petran and Hadravsky design uses a so-called two-sided disk; the specimen is illuminated through an array of pinholes on one side of the disk while detected through a conjugate array of pinholes on the other (via a number of delicately aligned mirrors). Applications of this technology to bone and tooth microanatomy were demonstrated by Boyde et al. [4]. Another Nipkow disk design employs a single-sided disk in which the illumination and detection pinholes are the same [5]. This latter design trades slightly improved quantum efficiency for a robust construction able to tolerate our relatively extreme portable applications.

MATERIALS AND METHODS
A one-sided Nipkow disk (Technical Instrument Co. K2S-BIO confocal module, Zygo Corp., Sunnyvale, CA) was specifically configured to contend with challenging imaging problems such as those encountered in paleoanthropology (Figure 1) [6]. Like other confocal scanning light microscopes, the image derives from the plane in focus, thus eliminating the fog due to the halo of reflected, scattered or fluorescent light from all elements in the sample above and below the plane of focus, which is otherwise confounding image content in conventional light microscopy.

An interesting feature of the single-sided disk design by Kino [5] is the solution taken to suppress internal non-image-related reflections that are otherwise a significant problem in this type of system: the classical method of illuminating with polarized light to stop that light reflecting from within the optical system (e.g. from optical hardware within the body of the microscope), but not the useful light reflecting from the specimen and returning through the objective lens. Linear polarizing light filters and a single quarter-wave plate are employed for this purpose. This design reduces the number of mirrors in the light path and the alignment of the optics is not so critical, which makes the instrument very robustly constructed and able to tolerate transport and relatively rough handling (e.g. as checked-in baggage for air travel).

The microscope configuration included several other features critical to our research.

Figure 1:
The system in use observing a juvenile Australopithecus africanus facial skeleton (Taung Child, ca 2.5-3.0 m.y., at the Palaeoanthropology Research Unit, Department of Anatomy, University of the Witwatersrand, South Africa.)
Consideration was given to obtaining objective lenses with relatively long working distances (i.e. around 20 mm). Often we had little control over the geometry of broken fossil bone surfaces examined under remote field or museum conditions, and so we had to be prepared to image through long Z-height positions to avoid mechanical interference between the bone and the objective nosepiece. Objectives chosen included a 10× lens (19-mm working distance; Thales-Optem Inc., Fairport, NY, USA) and a Mitutoyo 20× lens (20-mm working distance; Mitutoyo Asia Pacific Pte Ltd, Singapore). Flexibility in magnification was achieved by both the introduction of a Thales-Optem 0.5× or 1.9× CCD adapter or by converting the fixed magnification optical assembly described above into a zoom system, which involved the introduction of a Thales Optem 70X2 zoom module (1-7×) between the K25-B10 module coupler and the manual coarse/fine focus module. For fully automated image acquisition, we motorized the 2 focus.

Complete automation in all X, Y, and Z axes was implemented onto the Portable Confocal Microscope. This included a KP35 motorized precision micro-stepping X-Y stage (Semprex Corporation, Campbell, CA, USA) and a Vexta 2-phase Z-axis stepping motor (Oriental Motor USA Corp., Torrance, CA, USA). Integrated XYZ movement was performed by an Oasis 4i PCI stepper motor controller board for XY stage and Z focus. A three-axis trackball/mouse control of XYZ axes allowed manual stage and focus movement to aid real-time viewing.

A JVC KY-F1030U 6-pin IEEE 1394 digital camera connected by an RGB camera economics on weight at 470 g and, because it has a 6-pin IEEE 1394 connection, does not require a separate power supply. The camera contains a 1/2" colour progressive-scan interline CCD containing 1360 × 1024 output pixels, operating at 7.5 frames per second. The 300 W Lambda LS xenon arc lamp (Sutter Instrument Co., Novato, CA, USA) transmits a flat and intense beam of light via a liquid light guide, operates at wavelengths suitable for both fluorescence and white light illumination, is robustly constructed with a pre-aligned lamp, and is economically packaged and light-weight, housing its own power supply in one 26.7 × 24.1 × 25.4 cm cabinet at 4.8 kg.

A Shuttle XPC SB52G2 with a Pentium 4 Intel processor and Windows XP (Shuttle Computer Group Inc., Los Angeles, CA, USA) supported fully automated image acquisition. The Shuttle XPC harbours a small form factor at 30 × 20 × 18.5 cm and has a lightweight aluminium chassis; the entire PC weighs only 3.5 kg. The computer has PCI expansion cards slots, a 6-pin IEEE 1394 port, a CDRW-DVD drive, front and rear USB ports, and many other common PC features. A reasonably lightweight and thin standard 1024 × 768 15" monitor (Dell Inc., Round Rock, TX, USA) was chosen for our real-time viewing.

The microscope returns image detail from a very thin optical plane at and immediately below the object surface (1-50 µm, depending upon specimen characteristics). To obtain two- or three-dimensional projections from a surface which is anything but perfectly flat, potential fields of view must be compiled from a through-series of captured images at all optical planes represented in the Z-axis. Computerized control over image acquisition using Syncroscopy Auto Montage software (Syncroscopy Inc., Frederick, MD, USA) permitted an even and fully representative image of either a pseudo-planar field of view or a three-dimensional reconstruction of surface or sub-surface details. For more extensive automated XY image montaging, Syncroscopy Montage Explorer (Syncroscopy Inc., Frederick, MD, USA) software was employed, which can operate in ‘3D mode’ to acquire all pertinent Z focal planes.

The stand and system integration was provided by GT Vision (Frederick, MD, USA). The stand is simple and lightweight. Composed of aluminium, it includes an upright cylinder, containing within a lead screw operable from above, which drives the Nipkow disk module platform up or down and serves as a coarse focus adjustment. The cylinder inserts into a sleeve at the base from which two hollow rectangular feet slide forward and rotate out at any angle appropriate for the balance of weight and required workspace. The platform rides on a bearing, conveying the Nipkow disk module in any rotational position.

The entire microscope assembly fits into two suitcases (Pelican Products, Inc., Torrance, CA, USA), automatically switches between 110V and 220V electrical supplies (only the Nipkow disk motor requires optional 110/220V adapters), and may be set up and tested within one hour of arrival at museum locations.

**RESULTS**

Several applications of the Portable Confocal Microscope have been tested at African repo-
sitories of early hominid skeletal remains. There is much interest in obtaining details of hominid enamel microanatomy from fractured surfaces, but such surfaces are rarely forgiving and the resolving power has been wanting. Figure 2 illustrates a 3D image of a complex naturally fractured surface from a Paranthropus robustus molar. Figure 3 presents the same field of view after the adherence of a glass coverslip to the surface with glycerin. Through-focus imaging of this topographically complex surface revealed a plane view of enamel increments (e.g. striae of Retzius coursing from upper left to lower right). Overlain onto this image is an Auto Montage colour-coded relief map of the original surface for comparison.

Examination of intact enamel surfaces provides excellent reflection images, though it is also useful to combine this with sub-surface imaging. Figure 4 represents such an image of an early Homo molar in which through-focus images include both surface incremental features (perikymata) as well as subsurface enamel prism details. Because reflected light conditions are intriguingly different from the properties of transmitted light, the polarizing and quarter wave filter arrangement employed for reducing unwanted internal reflections results in the conditions necessary to generate a circularly polarized light (CPL) image [7]. For this reason birefringence associated with collagen fibre orientation in bone may be examined [8].

Figure 5 presents a CPL image of the cross section of an Australopithecus afarensis femur (‘Lucy’). The laminated concentric structures are vascular canal spaces surrounded by lamellar increments of bone formed during the incorporation of a new blood vessel. This process of internal remodeling is a natural process occurring with age. As bone lamellae are laid down, the orientation of their contained collagen is seen to be either relatively parallel with the cross-section of bone (appearing perpendicular to the cross-section of bone (appearing dark), images such as these may be used to interpret the manner in which the bone was used in life. This image derived from the anterior cortex is relatively ‘dark’, indicating that this cortex was better adapted to resist tensile forces in life.

It is useful to know something of the distributions of cell spaces in bone (osteocyte lacunae), particularly when reconstructing the developmental history of a bone whose surface has been damaged during fossilization and cannot be evaluated. Bone cells align with the prevailing collagen fibre orientation, which varies as a function of bone growth pattern, thus 3D images of cell spaces in fossil bone illustrates something of its developmental history. Figure 6 presents a 3D image of Australopithecus afarensis facial bone lacunae, whose regular orientation is an indication that the bone surface above was originally forming (not resorbing, as it would be in this location in modern humans) during growth.

**DISCUSSION**

While the improvement over conventional light microscopy in imaging thin sections may not be too important, the improvement made by the Portable Confocal Microscope for the examination of the surface layers of bulk samples is nothing short of revolutionary. Even if images cannot be obtained through a given thickness, the convenience factor of not having to produce a thin section as a prerequisite for excellent optical microscopy is a very great advantage in our research.

Two microscopes are in service to date. The first (PCM-1K2) was described by Bromage et al. [6]; it is automated in Z and operates a note-book-based PC monochrome image acquisition system (Figures 2 and 3 were obtained with this system). This microscope is dedicated to specific long-term projects (e.g. dissertations). The microscope described here (PCM-2K2; Figure 1) is fully automated in X, Y and Z, and offers the maximum potential of this technology for the imaging and analysis of fossil bone and tooth specimens at remote locations. With development of the Portable Confocal Microscope the potential for non-destructive mineralized tissue research on rare and unique early hominid remains is great.

**CONCLUSIONS**

The Portable Confocal Microscope is the first instrument to offer superb analytical light microscopy of early hominid skeletal material. Limitations over the handling and transport of rare fossils have motivated the development of this technology, but it is as well suited to applications as diverse as art conservation, forensics and the space sciences, i.e. wherever and whenever the microscope must go to the place and the subject.

**REFERENCES**


2. Nikpaw, P. Elektrisches Telekop. Patentschrift 30105 (Kaiserliches Patentamt, Berlin), patented 06.07.1884, 1884.


