Journal of Microscopy, Vol. 203, Pt 2, August 2001, pp. 208–213.
Received 3 July 2000; accepted 1 December 2000

3D light scanning macrography

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Key words. Digital, insects, macrography, macroscope, micromachining, morphometry, photomacrography, surface scanning, three-dimensional visualization.

Summary

The technique of 3D light scanning macrography permits the non-invasive surface scanning of small specimens at magnifications up to 200×. Obviating both the problem of limited depth of field inherent to conventional close-up macrophotography and the metallic coating required by scanning electron microscopy, 3D light scanning macrography provides three-dimensional digital images of intact specimens without the loss of colour, texture and transparency information. This newly developed technique offers a versatile, portable and cost-efficient method for the non-invasive digital and photographic documentation of small objects. Computer controlled device operation and digital image acquisition facilitate fast and accurate quantitative morphometric investigations, and the technique offers a broad field of research and educational applications in biological, medical and materials sciences.

Introduction

Pictorial representations of objects in the millimetre range require image recording techniques that are intermediate between macrophotography and scanning electron microscopy (SEM). In the search for an adequate imaging technique, several compromises have to be met; macrophotography offers an often insufficient depth of field, while SEM generally implies somewhat more expensive and heavy equipment, as well as the irreversible metallic coating of the specimen.

Light scanning photomacrography (LSP) is an attractive alternative for the noninvasive documentation of small specimens in a magnification range of up to 200×. LSP has been known for 40 years (Zampol, 1960) and has been the subject of several comprehensive reviews (McLachlan, 1964; Dale, 1982; Root, 1985; Sharp & Kazilek, 1990). LSP is based on the following principle: a specimen is moved through a very thin sheet of light whilst it is photographed by a camera whose focal plane coincides with the plane of the light sheet. In this way, the scanned specimen will gather light only when passing through the plane of focus, resulting in a selective – and focused – illumination of the specimen. Portions of the specimen that are not in the thin plane of focus are not illuminated and therefore remain unexposed. During this scanning process, the shutter of the camera is left open. The entire specimen is thus continuously superimposed onto the photographic film with a full depth of field. Because the sheet of light contains the complete spectrum of the visible light, the resulting picture is recorded in real colour.

The method has rarely been used in scientific research and several reasons can be invoked that could possibly have offset the advantages of light scanning. First, the different light sources used to form the illumination necessitate arduous adjustments and time-consuming tests and calibrations. Second, multiple light sources result in a rather cumbersome set-up. Finally, only a few film formats are available (e.g. 35 mm, 6 × 6 cm) and the use of a darkroom is usually required. To our knowledge, only one model of light scanning microscope is available commercially (Dynaphot, Irvine Optical Corporation, Burbank, CA, U.S.A.). Other methods have been developed that allow the monochromatic and tomographic 3D optical recording of small objects; for example, white light confocal microscopy (Juškaitis *et al.*, 1996) and confocal laser scanning macroscopy (Damaskinos *et al.*, 1995; Dixon *et al.*, 1995). The magnification power of these devices places them in the microscopic range, a range that is complementary to that of the 3D light scanning macroscope presented here.

A variety of light scanning devices has been described (Root, 1985; Sharp & Kazilek, 1990), in which the light sheet arises from one or more sources positioned at right angles to the

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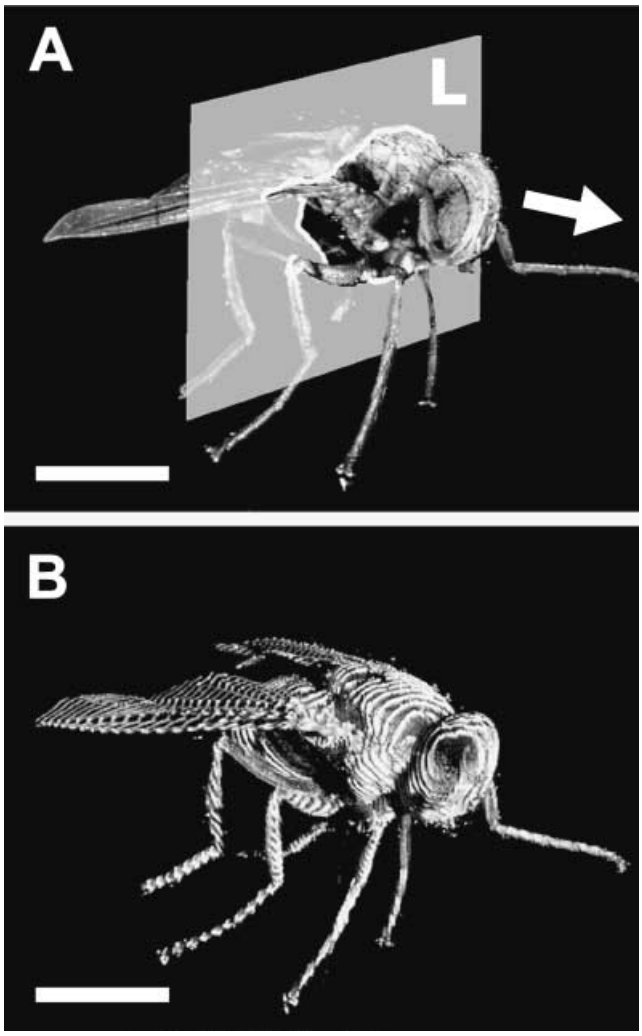

Fig. 1. Principle of light scanning macrography. (A) The specimen (a fly) is moved step-by-step through a thin sheet of light (L). The white arrow indicates the direction of the specimen's movement as well as the optical axis of the camera. The thin white line surrounding the fly is the only part that is captured by the camera at that instant of the scanning procedure. (B) Three-dimensional reconstruction of the object. In this example the specimen was moved in only 50 steps through the sheet of light. The 50 optical slices were subsequently reunited to form a single, three-dimensional image stack. This stack is shown here at an angle of view of 20°. For high-resolution 3D reconstructions, the steps must be smaller, typically counting from 200 to 300 slices. Using high scanning resolution, the original optical slice spacing becomes invisible. Scale bars: 2 mm.

optical axis of the camera. A critical factor in the quality of LSP is the design of the light sheet illuminating the specimen. Practically, a thin slit is etched, or carved, in a projecting slide. The slit has to be designed so that its focused image can be finely adjusted to precisely converge on the specimen. Ideally, high image quality requires the width of the light sheet to be smaller than the depth of field of the image-recording optics. If several slide projectors are used, then the different light beams have to be accurately adjusted in one plane and focused on the object. In conventional systems using non-digital image acquisition, the exposure time is regulated by the scanning speed of the object through the light sheet. In the system proposed here, exposure time is independent of scanning speed, a degree of freedom that helps reduce the influence of the vibrations inherent in the translational scanning of the specimen. The definite optical advantages of LSP prompted several developers to propose novel and elegant solutions for the design of the light sheet (Turner, 1986; Root, 1991). The use of a video camera in combination with a memory unit has been proposed, to replace the photographic film (Davidhazy, 1994).

The technique of 3D light scanning macrography (3D-LSM) presented here contributes to bridging the gap between traditional macrophotography and scanning electron microscopy. By attempting to take the best from two worlds, 3D-LSM permits the acquisition of colour, transparency and texture information in addition to providing axonometric 3D images with extended depth of field at an enlargement factor of up to 200×. 3D-LSM extends the functions of existing devices by providing a novel method of illumination, a linear optical geometry, and the versatile use of computer-controlled operations for digital (or classical photographic) image acquisition. The use of digital imaging technology, instead of classical photographic film, significantly improves the speed and accuracy of image acquisition, and opens the doors to the flexible world of three-dimensional digital image processing. It is within the context of our comparative studies on the structure and function of hearing organs in small insects that we developed an alternative technique for the rapid three-dimensional pictorial documentation of small objects.

The 3D-LSM device

Stepwise scanning and image acquisition

The continuous displacement of the specimen through the light sheet is advantageously replaced by a step-by-step displacement during which the image is recorded with a video or digital camera. In such a case, image acquisition is like that of a tomograph: it optically cuts the surface of the specimen into thin and contiguous slices (Figs 1A and B). The slices are then digitally reunited as an image stack, which allows the three-dimensional reconstruction of the scanned specimen. The result is a 3D image of the specimen, which combines an outstanding sharpness, an extended depth of field and, remarkably, a lack of perspective distortion. The image is in fact an exact axonometric three-dimensional representation of the object due to the fact that the distance from the object to the lens (or camera) remains constant for each optical slice of the object. This optical characteristic is particularly convenient for distortion-free measurements on small three-dimensional objects.

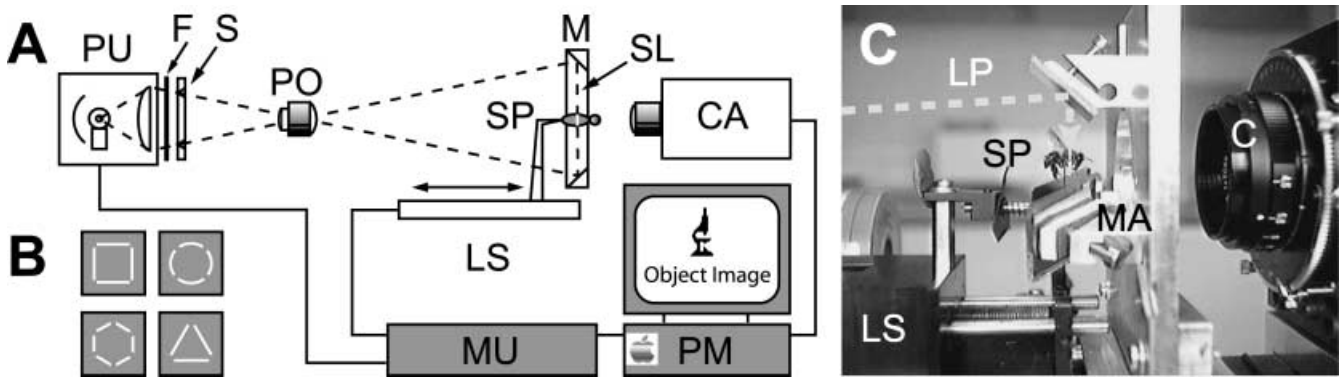

Fig. 2. Schematic overview of the light scanning unit. (A) The linear arrangement permits a significant reduction in size and makes the device portable. Projector unit with the light source (PU); colour-correcting filter (F); slit slide (S); projecting lens (PO); specimen (SP); linear displacement stage (LS); mirror unit (M); sheet of light (SL); camera system (CA). The CCD camera is connected to the video entry of a Macintosh Power PC (PM), which acquires and stores the sequential images by the Object-Image software. The same program also regulates the scanning procedure over a microprocessor-based control unit (MU) connected to the serial port. The dotted lines indicate the paths of the light beams. (B) Examples of slit patterns used for designing the light sheet incident on the object in combination with corresponding mirror arrangements. (C) Close-up view of the mirror arrangement (MA). Only the thin illuminated contour of the specimen (Honeybee, SP), which is mounted on the linear stage (LS), is recorded by the camera (C). The dashed line indicates the path of the light beam (LP), which is reflected by the mirror arrangement (MA).

Using a linear optical bench and linear positioning stage

One of the innovative elements of the 3D-LSM consists of the linear arrangement of its optical elements (Fig. 2A). Minimizing space and facilitating transport, this arrangement allows for a large degree of flexibility in the positioning of the optical components and provides an increased overall mechanical stability and positioning accuracy. The linear displacement of the specimen is achieved by a linear positioning stage (Fig. 2A). For the step-by-step scanning procedure, a positioning accuracy of at least 5 μm is desirable to ensure the smooth contiguity of the optical sections. The displacement of the positioning stage may elicit vibrations that can alter image quality. To minimize vibration image blur, the scanning algorithm was thus instructed to acquire the image only a second after the stage had stopped its step travel. Like other functions, such as shutter control and light on/off, the number and size of scanning steps and the total depth of scanning are regulated by a microprocessor based control unit (Fig. 2A). The control unit is connected to a Macintosh computer (serial port) and controlled by macro commands of the Object-Image software. This procedure ensures standardized and reproducible scans and measurements across different samples of a population of objects. All mechanical parts of the optical bench and the linear positioning stage, as well as the microprocessor-based control unit were custom-built.

Design of the light sheet

A crucial design feature of any LSM device is the system used to create a thin sheet of light for the illumination of the specimen. In the present device, the light source consists of a single, axially positioned, projector equipped with a conventional halogen light bulb (250 W, 12 V AC) (Fig. 2A). This projector illuminates a thin metallic slide in which a slit pattern has been carved by a combination of die-sinking and wire-cut electrical-discharge machining. In the case presented here, the pattern is an isosceles triangle, with slits of 40 μm in width (Fig. 2B). This slide thus projects three planes of light orientated at 60° to each other (Projection lens: Rodenstock Rogonar 80 mm, f: 5,6). The three light planes are reflected by three correspondingly orientated oblique mirrors so that they fall together in one single plane at the centre of the mirror arrangement (Fig. 2C). The resulting single light plane is thus perpendicular to the optical axis of the macrographic imaging system. For other requirements of illumination, this triangular slit pattern can be easily replaced by other polygonal patterns and their corresponding mirror arrangements. Unlike dual or triple light source configurations, the use of a single projector guarantees the constancy of luminosity and colour balance of all light beams and presents the advantage of requiring only a single calibration and adjustment session. The light temperature can be controlled by the use of colour correcting filters, which are placed in the projecting pathway.

Image recording optics

The image recording side of the device is a simple bellows construction. Depending on the application and enlargement required, different types of macro lenses, bellow extensions and camera or field holders can be used

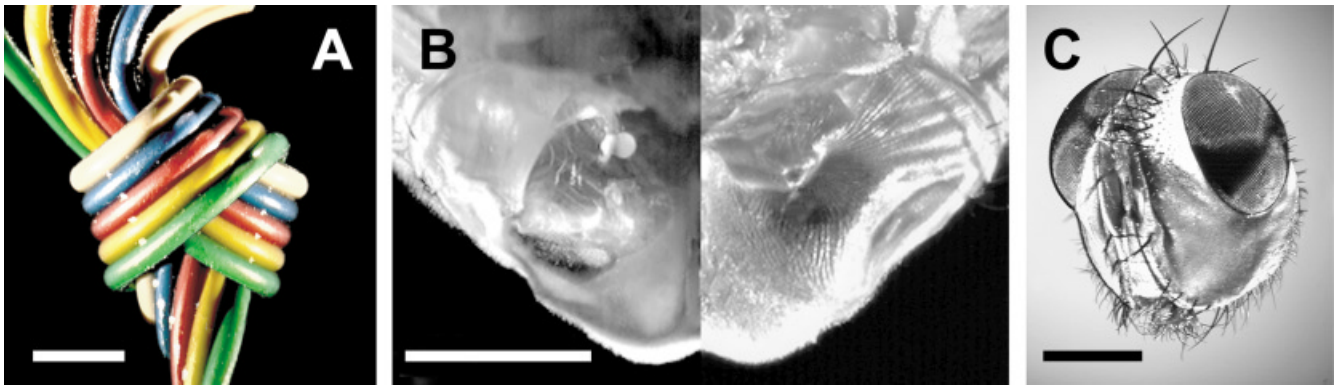

Fig. 3. Examples of 3D-LSM. (A) Knot in a mutifilament cable. The wide tonal range illustrates nicely the colour capabilities of the 3D-LSM device. Scale bar: 2 mm. (B) The tympanal hearing organ of the tachinid fly *Ormia ochracea* as an example for the selective recording of internal structures of translucent objects. On the left side the internal anatomy is made visible by simply omitting the first slices of the split image stack. On the right side, only the first slices are displayed. Scale bar: 500 μm. (C) Head of a sarcophagid fly (*Emblemasoma auditrix*). The black background inherent to the LSM method can be changed by adding a supplementary image of a back illumination of the object's silhouette to the image stack. Scale bar: 1 mm.

interchangeably. Our device is equipped with different video cameras (Hamamatsu C2400–75 for B/W, Sony XC003P for colour), but adapters allow us to convert the device to any small (35 mm), middle (6 × 6 cm) or large format (4′ × 5′) photographic image recording device. The device sketched in Fig. 2 provides magnifications between 1 and 200× using different macro lenses (Olympus 21 mm, Olympus 35 mm, Nikon 55 mm). Magnifications of up to 400× could also be obtained by using a much narrower slit pattern (10 μm). Although useful for the recording of very small objects, such high enlargements necessitate the use of microscope optics. The depth of field of such lenses is very small (< 20 μm) and therefore smaller than the available sheet of light (typically 20–60 μm). The present 3D-LSM is equipped with a removable lightproof cover that permits better optical adjustments and the elimination of stray light, which may illuminate the out of focus regions of the specimen and degrade the image quality.

Calibration of optical slicing

Several calibrations and reference measurements are necessary to assess enlargement factors, optimal step size to obtain contiguous optical slices, and evaluate image quality. For this purpose the known geometry of a round-headed sewing pin was scanned and reconstructed from a stack of 200 optical slices. The 3D reconstruction was then compared with the original sphere geometry. Using the same procedure, another important calibration was made to assess the distortion caused by the non-square pixel aspect ratio of many video cameras. In our case (Hamamatsu C2400–75, pixel ratio, 1 : 1.03), such distortion was detected and corrected by a re-calibration made at the level of the imaging software.

Three-dimensional image reconstruction, visualization and analysis

The visualization of three dimensional image information is widely used in confocal microscopy. In principle, similar programs and algorithms can be used for the 3D reconstruction and analysis of images acquired by 3D-LSM. For this purpose, the free scientific image software Object-Image was found to be very well suited to our needs (Extended version of the popular NIH-Image, Vischer *et al.*, 1994; http://simon.bio.uva.nl/object-image.html.) Object-Image allows fast 3D slicing through reconstituted image stacks, and also offers the possibility of marking transparent layers for measurement purposes. Additionally, the program supports standard video entry for AV-Macintosh computers (or other scientific frame grabbers), thus considerably simplifying image acquisition. In our case, the video camera was connected to the S-VHS video entry of a Macintosh 8600 PowerPC (with 256 MB RAM). The same software was used to monitor the entire scanning process via a serial port interface, controlling stepwise scanning and image capture, as well as perform 3D reconstructions and morphometric measurements. The 3D-visualization method of choice is that of the projection by brightest point proposed by Object-Image, which simply summates all pixel values throughout the image stack in a defined angle. This method is particularly suitable because LSM slices are generally constituted of thin illuminated surroundings in front of a black background (Figs 1B and 3A,B).

Beyond documenting the specimens photographically, the acquisition of pictures in a three-dimensional digital file format permits the quantitative evaluation of numerous morphometric parameters. Performing three-dimensional morphometric measurements directly on the image stacks is of particular attraction, and is not possible with conventional

Fig. 4. Anaglyph stereo representations (red: left eye, green: right eye) of the 3D-LSM recordings. (A) The fruit fly *Drosophila melanogaster*. 3D-LSM is the ideal instrument for the accurate documentation of phenotypes, which may be modified genetically. Scale bar: 500 μm (B) Example of a museum specimen of the tachinid fly *Ormia ochracea*. Scale bar: 2 mm (C) Detailed view of the eye of fly *Ormia ochracea*. This represents the upper limit of the magnification range of 3D-LSM in normal practice. Scale bar: 50 μm.

macrography and SEM techniques. Very conveniently, Object Image allows measurements of distances, angles and surfaces directly on a 2D and 3D slicer in a non-invasive manner and with a sub-pixel resolution. A further scientific advantage of this method of image analysis is the possibility to redefine the parameters measured at any time during the analysis and to let other investigators to perform independent controls in the form of blind measurements. In addition, as shown in Figs 4(A)–(C), the three-dimensional information of image files can also be represented as stereo anaglyphs. The anaglyph images were created with the Object-Image software by superimposing two brightest point projections of the 3D data set with an angle of 10° difference. Further imaging examples can be explored at http://www.unizh.ch/~dhuber.

Since, at the time of usage, Object-Image program (version 1.61) did not allow the direct handling of colour information, the three colour channels (RGB) of the video source were acquired and reconstructed separately and subsequently merged at the stage of final visualization. These operations were automated by additionally programmed macro procedures.

Limitations of the 3D-LSM technique

As in any other photographic techniques, the quality and quantity of light play an important role in the quality of the resulting image. Depending on the object to be scanned, the optimal degree of diffusion of the light may be different. In this respect, the geometry of the reflecting mirrors, as well as their reflective characteristics need to be taken into consideration when optimizing the illumination process. For instance, our own experience with numerous mirror configurations (Fig. 2B) and qualities indicates that circular mirrors (in conjunction with circular slit slides) are not suitable for translucent objects. In such a case, the convergence of each light beam at the optical axis creates a strong concentration of light at the centre of the object. Although it creates an interestingly eerie artistic effect, lighting from the core of the specimen prevents a clear definition of its surrounding, and results in a loss of information. The interchangeability of the projecting slide and mirror elements warrants the possibility of suiting illumination to the specimens considered.

Not all objects in the macrophotography range can be successfully scanned with the present LSM. Especially highly reflective or completely reflectionless objects are not well suited for LSM. Specimens with highly contrasted superficial tissue may be problematic too. In addition, special attention has to be paid to objects with translucent parts for which light penetrating inside the specimen may result in illumination, through a 'piping' light-guide process, of locations that are out of the plane of focus. Nevertheless, translucent parts may also be advantageous in some circumstances as they may permit the selective recording of internal structures. As shown in Fig. 3(B), internal structures can be recorded in 3D, and displayed by simply omitting slices of the image stack. Image background can also be altered by inserting a supplementary slice produced by diffuse back-illumination of the specimen's silhouette with the desired colour (Fig. 3C).

Actual and expected applications in science and technology

A multiplicity of methods allow the photographic documentation of macroscopic specimens and they all present their particular and respective advantages and disadvantages (Engel, 1968). Indeed, whilst photomacrography is relatively cheap and fast but offers only a limited depth of field, scanning electron microscopy provides unmatched picture resolution but is expensive, time-consuming and

more importantly irreversibly alters or destroys the specimen. Thus, the present method may be of great convenience in situations where the specimens are unique, fragile, or when the objects must undergo non-invasive quality control prior to their subsequent packaging or further use in the manufacturing chain.

Several fields of scientific and technological application can be envisaged for the 3D-LSM. In biology, the LSM technique has proved useful for the documentation of precious museum insect specimens (Fig. 4B). The technique was used, as in our research for instance, to observe the small hearing organs of flies, the three-dimensional morphology of which is crucial to their function. Beyond this pictorial description, the 3D-LSM images from numerous museum specimens were analysed morphometrically in a non-invasive way. This allowed the extraction of anatomical parameters with a high spatial resolution (micrometre range) otherwise inaccessible by conventional macrography. The technique was particularly adapted to the task because the specimens were often unique and precious, and therefore could not undergo a damaging SEM procedure. More generally, the three-dimensional image acquisition of insect collections in a digital format would also permit the creation of an electronic image database that could be consulted online without the risk of deterioration of historical specimens. Other scientific domains such as botany, palaeontology or geology provide a large field of different applications.

Further applications in the technological domain are currently explored. For micromachining and microtechnology industries, the implementation of 3D-LSM, optimized for scanning speed, is likely to be advantageous for rapid yet reliable quality control of microsystems. For example, the surface and general integrity of a three-dimensional micromechanical electronic microsystem can be assessed without requiring time-consuming SEM imaging. Further developments of 3D-LSM can be envisaged that would be adapted to serve better the needs of the expanding micromachining technology. The use of 3D-LSM in combination with rapid prototyping techniques (e.g. stereo lithography) is currently being tested.

Acknowledgements

Many thanks go to G. A. Haldimann for sharing his expertise on LSM, to R. Stidwil and M. Nakano for their advice on microscopy techniques and to M. Müller for reviewing the manuscript. The development of the 3D-LSM device was also encouraged and supported by several industrial partners (Georg Fischer AG, Schaffhausen, Agie AG Losone, Charmilles Technologies SA Geneva, and Sinar AG Feuerthalen). Additional financial support was provided by ETS High-Tech (Ville de Lausanne) and M. & V. Huber-Tissi. The research part of the project was supported by a grant from Swiss National Science Foundation to D.R.

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