Photogrammetric Record, 14(79): 59–84 (April 1992)

REALLY CLOSE UP! SURVEYING SURFACES AT SUB-MICROMETRE RESOLUTION: THE MEASUREMENT OF OSTEOCLASTIC RESORPTION LACUNAE

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(Paper read at the Technical Meeting of the Society on 19th February, 1991)

Abstract

The authors discuss systems for the three dimensional measurement of microscopic features, in particular the pits made by isolated bone resorbing cells (osteoclasts) in test substrates with flat surfaces; such measurements may be used to assay the work done by these cells. Confocal light microscopy, with its very shallow depth of field, seems to be replacing scanning electron microscopic stereophotogrammetry as the preferred method for such measurement.

INTRODUCTION

IF close range photogrammetry involves the measurement of the faces of cathedrals or people, then the work discussed here is really close up; it deals with small volumes of tissue which need high resolution microscopes to show the required order of detail. The hard mineralised tissues of the mammalian body have sufficiently rigid surfaces to make microscopic surveying a sensible approach to the solution of discrete problems concerning their formation and destruction. In particular, photogrammetric methods using electron microscopic imagery have been employed in the elucidation of the mechanism of mammalian dental enamel development (Boyde, 1966 and 1973; Lester and Boyde, 1987), of the topography of the mature human enamel surface (Boyde, 1971) and relating this to the sizes of the bacteria inhabiting this surface (Boyde and Williams, 1971), and in measuring the work done by the cells which resorb bone and other mineralised tissues (Boyde, 1968; Boyde and Jones, 1979). The special instruments and methods which had been employed in this work to that date were reviewed in this journal by Boyde and Ross (1975). This paper summarises some of the progress made since that date.

SCANNING ELECTRON MICROSCOPY AND STEREOPHOTOGRAMMETRY

The basic requirement in the field of investigation in which photogrammetry is applied to microscopic features is to make topographic maps with a vertical resolution of 0·1 μm or even 0·01 μm. This accuracy can be achieved by obtaining the images by tilting the object in a scanning electron microscope (SEM) to achieve two (Boyde, 1968, 1970a and 1973; Howell and Boyde, 1972; Maune, 1973; Nagaraja, 1974; Kato *et al.*, 1977) or more (Simov, 1989) defined views, or by moving the effective origin of the raster in the SEM by electrical or magnetic deflection of the electron beam in addition to that normally required for scanning (Boyde *et al.*, 1972; Boyde 1974c and 1975). The SEM can be regarded as a low aperture, large depth of field camera, giving central or perspective projection at lower magnifications and a close approximation to parallel projection at higher magnifications. There are imperfections in the SEM due to variations in magnification across the field of view (Maune, 1973; Howell, 1978a and b). The

focal length of magnetic lenses changes with the current supplied to the lenses. The energy (wavelength or "colour") of the electrons changes with the accelerating voltage. Constancy in these parameters depends upon the design of stabilised voltage and current power supplies. In spite of these possible difficulties, the SEM has provided good solutions to real problems (Boyde and Ross, 1975).

Other investigators have potential applications which lie outside microscopic norms. At one end of the scale, there are specimens which need to be examined at such a high magnification that there is either nothing to resolve or not enough resolution to resolve it, considering the volume element from which the signal arises. At the other end, there are low magnification, broad field problems, where light optical solutions would usually be better advised, were it not for the fact that there are analogous problems in real light optical systems. Mapping a wide field whilst maintaining X, Y and Z resolution has to be done in small sections; the problem is how to register these adjacent fields.

Digital Readout from Stereocomparators

The analogue analytical devices previously described (EMPD1 and EMPD2: Boyde, 1974a; Boyde and Ross, 1975; Howell and Boyde, 1984) provided excellent solutions for the description of shape, but did not provide numerical solutions for the derivation of volumes, areas and angles. The affordable, new developments in SEM stereophotogrammetry are based on the facts that (a) there are more and better devices which will feed X, Y and ΔX co-ordinates to a microcomputer, which can be left to provide a good solution using the correct geometrical assumptions, and (b) microcomputers have become ever better and ever cheaper. For example, Howell (1981) fitted linear encoders to a Hilger and Watts medical stereoscope with parallax measuring facility (Boyde, 1966; Boyde and Jones, 1979). A similar commercial development has been made by Cartographic Engineering Ltd. for the (originally Hilger and Watts SB 180) folding mirror stereoscope.

RS3, built in 1980, was a tri-axis stereocomparator for electron microscopic photogrammetry ((Boyde, Jones *et al.*, 1983; Boyde, Ali *et al.*, 1983; Howell, Boyde *et al.*, 1986), designed specifically for the examination and measurement of stereopair scanning electron micrographs. RS3 had rotary encoders driven by the manual shift of the photocarriage to register the co-ordinates of image features in the two members of the stereopair. The raw data were passed to a Sharp MZ80K microcomputer programmed for the reduction and conversion of these data to derive real three dimensional values (Boyde, Ali *et al.*, 1983 and 1984). This prototype instrument was designed with the specific purpose of establishing an assay system to measure the volumes of the pits excavated by osteoclasts in test substrates, mainly slices of dentine or bone. It was the core of the first system which allowed large amounts of data to be collected without the tedium of manual data entry (Boyde, Ali *et al.*, 1984 and 1985; Howell and Boyde, 1984).

RS3 was eventually replaced by SFS3, again a completely new design of multi-axis recording stereocomparator (Ross, 1986) in which X, Y and ΔX co-ordinates were output directly to a microcomputer, and again conceived specifically to deal with the osteoclastic resorption lacuna problem. The SFS3 at University College London (UCL) was interfaced to a Sirius microcomputer (Boyde, Howell and Franc, 1986).

SFS3 was designed to speed up the work, to which end precautions were also taken at the stage of recording the stereopairs. Both members of the pair were recorded at the same magnification, carefully centered and under the same electron optical conditions. The need for mechanical refocussing (Boyde, 1970b) was avoided by bringing the specimen on to the eucentric plane of a six axis goniometer specimen stage for the Cambridge S4–10 SEM (Houghton *et al.*, 1971). The tilt axis was parallel to the frame direction, now defined as Y, to ensure that parallaxes could only arise in X. In order to save on photographic costs, the original SEM film was used for measurement and a clip was made to hold the cut 35 mm negatives without any need to permanently mount them. The strategy for measuring the pits involved scanning lines across these features in a zig zag horizontal raster and was designed

to take account of the special features of SFS3 as well as the problem at hand (Boyde, Howell and Franc, 1986).

The introduction of these purpose built digitising stereocomparators led to a very considerable increase in the efficiency of acquiring precise measurements of resorption lacunae, providing the first reliable data on this subject. Pits excavated by osteoclasts from different species, at different time intervals, on different substrates and under the influence of different drugs and hormones (Figs. 1–6) were investigated (Boyde, Ali *et al.*, 1983; Boyde and Howell, 1983; Ali *et al.*, 1984; Boyde, Howell *et al.*, 1984; Delaisse *et al.*, 1987; Jones, Boyde *et al.*, 1986 and 1991; Jones and Boyde, 1988; Taylor *et al.*, 1990).

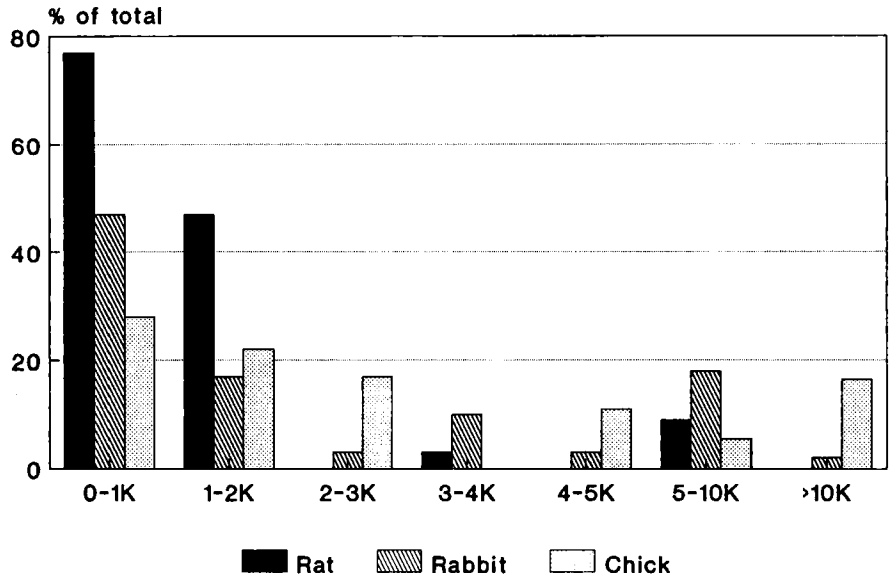


FIG. 1. Histograms showing the frequency distribution of the volumes (in thousands of μm^3) of resorption pits made by osteoclasts from pre-hatch chicks and neonatal rats and rabbits in 24 hour cultures on sperm whale dentine. Measurements made by SEM stereophotogrammetry using Ross Instruments SFS3.

From the methodological viewpoint, perhaps the most important finding was that it was necessary and correct to measure in three dimensions. Others who later adopted the idea of planting osteoclasts on resorbable substrates had been deterred by the complications of three dimensional measurement and even of the use of SEM. Rather than volumes (or depths), the areas or numbers of the pits have most often been measured. The real three dimensional measurements showed excellent correlations between volume and area within any one experimental situation (Fig. 4) and indicated that such correlations were much better than those between depth and area or depth and volume (Fig. 5). However, the relationship (Fig. 6) sometimes differed considerably between experimental and control groups (Jones *et al.*, 1986 and 1990; Delaisse *et al.*, 1987; Taylor *et al.*, 1990), and it would be unsafe to assume in advance that this was not so (Sato and Grasser, 1990; Hunter *et al.*, 1991; Evely *et al.*, 1991).

Stereological Stereometry

Part of the barrier to the introduction of three dimensional measurement is the cost of instruments and operator training. Apparently colleagues in other laboratories did not rate the value of the data sufficiently highly. The notion of "stereological stereometry" was introduced to satisfy the need for economy in data acquisition and processing from SEM stereopairs (Boyde, 1986). It is equivalent to

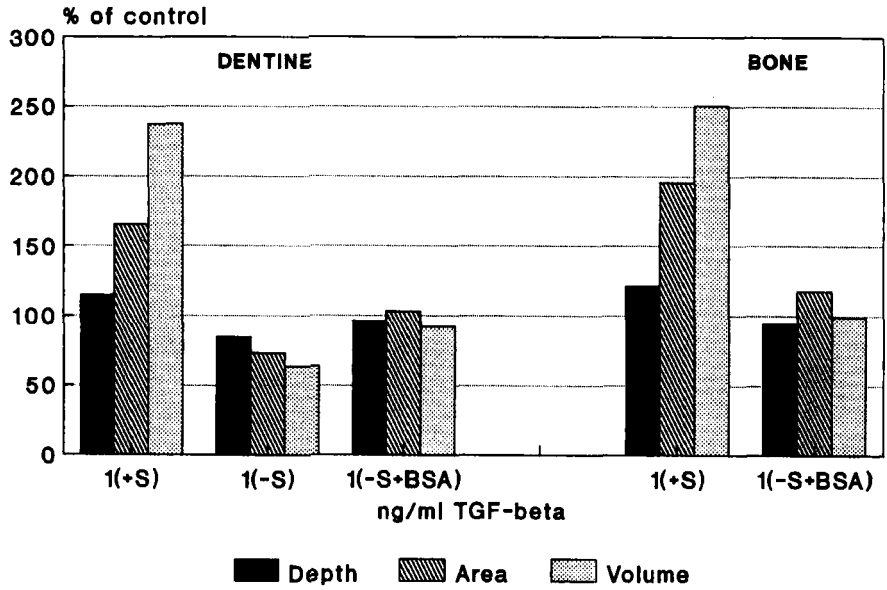


FIG. 2. Depths, plan areas and volumes of resorption pits made by chick osteoclasts in dentine or bone in medium with 1 ng ml^{-1} transforming growth factor, TGF-beta; and with (+S) or without (−S) added 10 per cent serum or added 1 per cent bovine serum albumin (+BSA). Mean values for the TGF-β cultures are shown as their percentage of control values. Measurements made by SEM stereophotogrammetry using Ross Instruments SFS3.

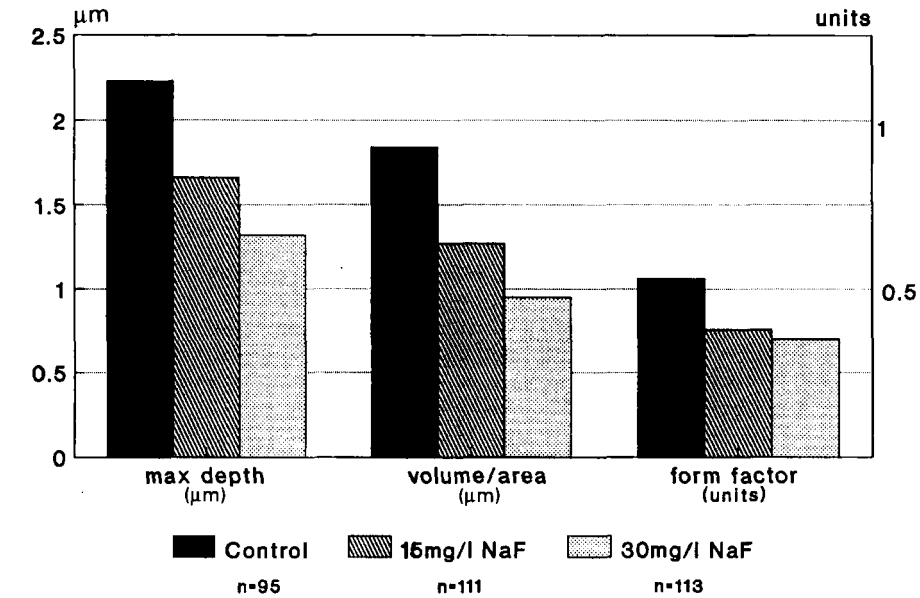
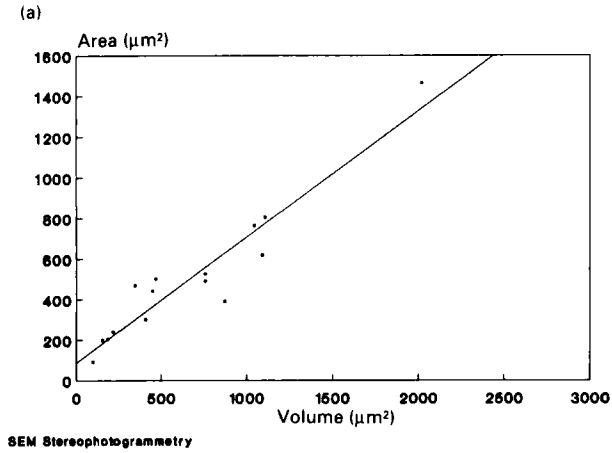
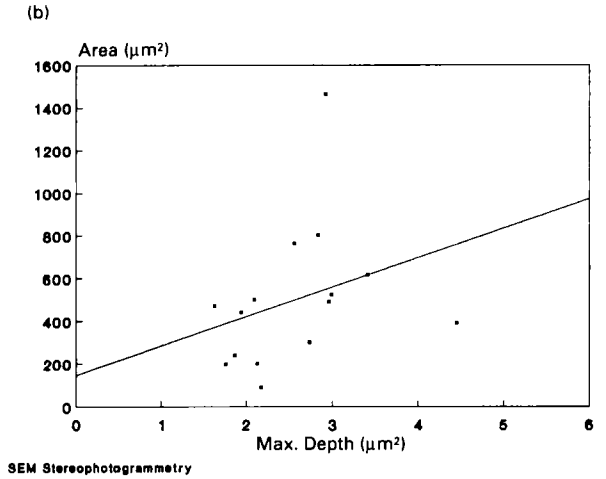


FIG. 3. The effect of the addition of fluoride to the medium upon the shape of the resorption pits made by pre-hatch chick osteoclasts on slices of sperm whale dentine in 24 hour culture. A dimensionless shape factor ($[V/0{\cdot}67\pi]^{0{\cdot}67}/[A/\pi]$) returns the value 1 for a hemisphere. The means of the maximum depths and mean depths (volume/area) are given in micrometres. Measurements made by SEM stereophotogrammetry using Ross Instruments SFS3.





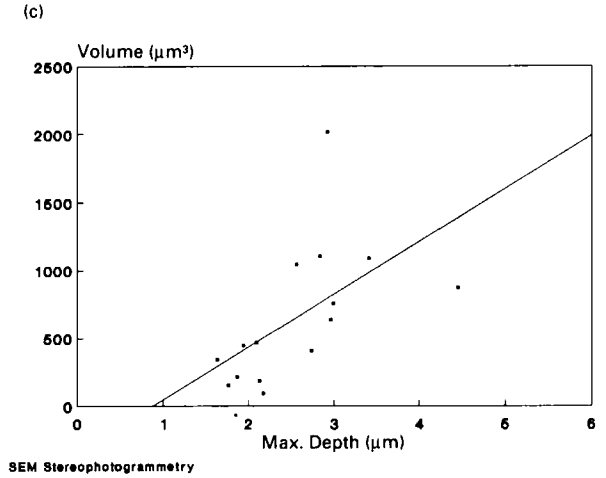


FIG. 4. Scatterplots of (a) area:volume (b) depth:area and (c) depth:volume measurements of resorption pits made by deer antler osteoclasts in dentine during 48 hour culture on dentine. Measurements made by SEM stereophotogrammetry with Ross Instruments SFS3.

measuring the volumes of pits by determining the number of balls or bricks which will fit in (Fig. 7). Walsh *et al.* (1991) describe an equivalent light microscope-based method.

Automated Analysis of Digital Scanning Electron Micrographs

Scanning electron microscopes can now be operated by digital scan generators with the signal level recorded at each pixel as a digital value. Such images in computer frame store can then be analysed automatically (Alff, 1987; Holburn and Smith, 1979; Koenig *et al.*, 1987; Tovey, 1978). Software for automated analysis of stereopairs has been produced commercially (for example, by Link Systems, in the UK and Noran Instruments and Princeton Gamma Tech in the USA; the software sold by Link contains a specific solution to the resorption pit measurement problem). This approach has the advantage of transferring feature matching and parallax measuring tasks to a machine, but unless the microscope stage is also controlled fully automatically, there is still considerable operator skill involved. Such automated matrix searching routines are enormously speeded up by a good vertical registration of the two images. Automated methods fail and give spurious values on occasion. These are easily seen as anomalous spikes in an analogue display and can be removed by manual editing. The software needs to be made smarter to recognise spikes or pits as aberrant features which should not be included in the data analysis.

Slope from Intensity: Use of Backscattered Electron Signal Difference

Surfaces with no cliffs (undercuts or overhangs) may be reconstructed by determining the slope from measurements of the difference in the signal obtained from two or more detectors for fast, backscattered electrons in an SEM (Lebiedzik, 1979; Raski *et al.*, 1988). This method presents problems where slopes are too steep and/or there is physical" shadowing" (total obstruction of the signal arriving at one detector).

Substratum	n=	Depth:Area	Depth:Volume	Volume:Area
DENTINE sperm whale	115	0.5977	0.6678	0.9222
BONE human lamellar	120	0.5152	0.6845	0.8676

SEM Stereophotogrammetry

FIG. 5. Table of coefficients of correlation for depth:area, depth:volume and area:volume of pits made in dentine or bone by chick osteoclasts in a 24 h culture period. Measurements made by SEM stereophotogrammetry with Ross Instruments SFS3.

Profiles and Depths from Partial Knowledge of the Geometry in the SEM

Several methods have been proposed which provide limited three dimensional data, in particular line profiles and depths, more economically than can stereophotogrammetry. Generically, these methods require knowledge of angles of view and look for projections of shadows of straight edges (McAdams and Gasiecki, 1975) or electron beam induced "burn" marks on the sample surface (Hoover, 1971) or the eclipsing of one set of edges by another (Boyde, Jones and Reynolds, 1978).

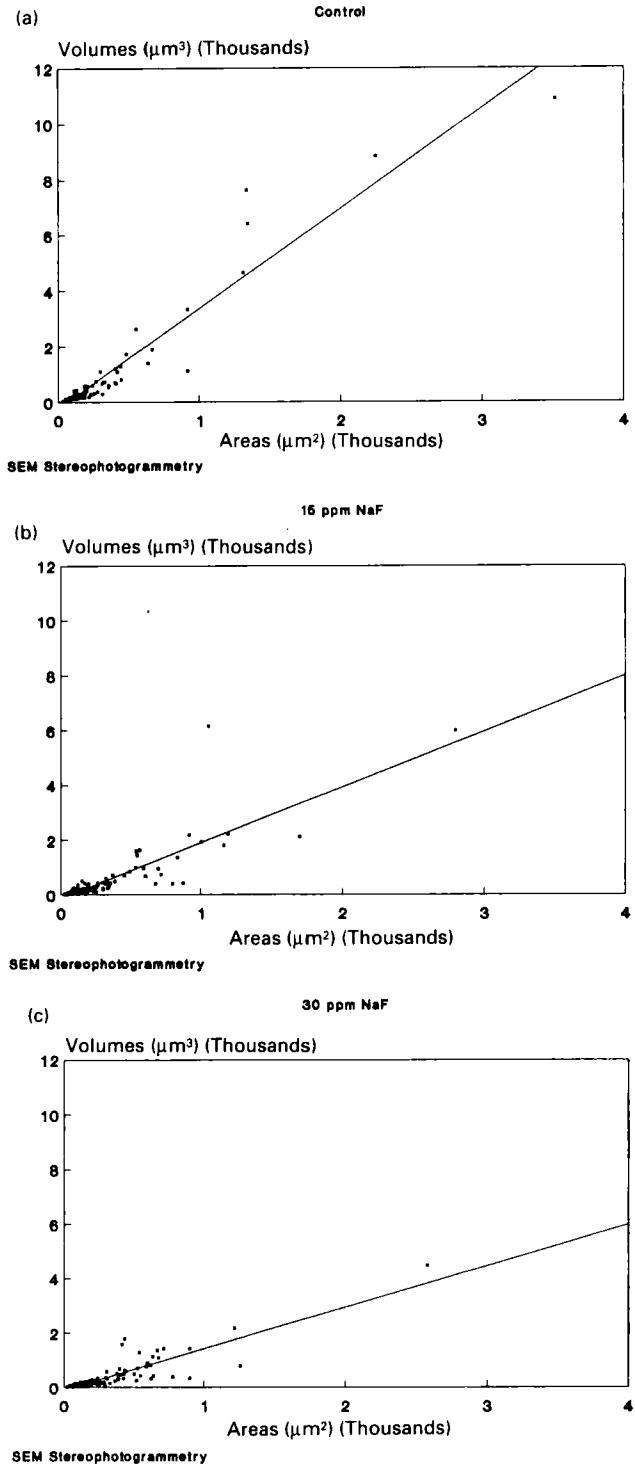


FIG. 6. Correlations between volume and area in the resorption of sperm whale dentine by chick osteoclasts: controls (a), experimental groups treated with 15 ppm NaF (b) and 30 ppm NaF (c). Measurements made by SEM stereophotogrammetry using Ross Instruments SFS3.

Light optical methods obviously include interferometry, measuring microscopes, "classical" close range stereophotogrammetry and the reflex microscope (Scott, 1987).

Sheet of Light Methods

The profile of a reflective surface can be recovered by illuminating it with a sheet of light (obtained from a slit aperture or by rapid scanning of a laser beam). The illuminated object is examined at a large angle or at right angles to the illuminating source, with the object scanned in the Z direction or rotated. Such systems have been designed for both microscopic and macroscopic applications (Moss *et al.*, 1989).

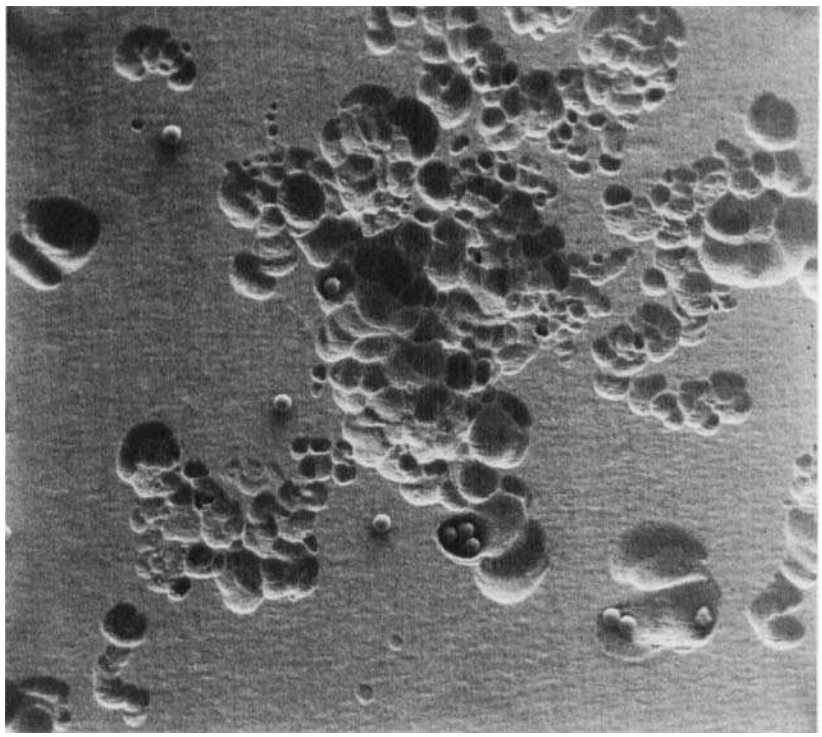


FIG. 7. Celestial spheres of 10 μm diameter (polystyrene latex spheres made in an orbiting spacecraft) as scale markers in resorption lacunae made by osteoclasts which differentiated in a pre-hatch chick long bone marrow culture. SEM image 10 kV.

Interference Fringe Methods

Determining the shift of interference fringes is a time honoured method of measuring step heights on nearly flat specimen surfaces in optical microscopy. It is limited in practice to measuring very small height differences of a few wavelengths and can not be applied to the problem of measuring resorption lacunae. However, it gives useful information about the flatness of polished surfaces, for example the step height at the edge between embedding plastic and bone, dentine or enamel in embedded hard tissue specimens which have been polished for backscattered electron imaging for density determination in an SEM (Figs. 8 and 9).

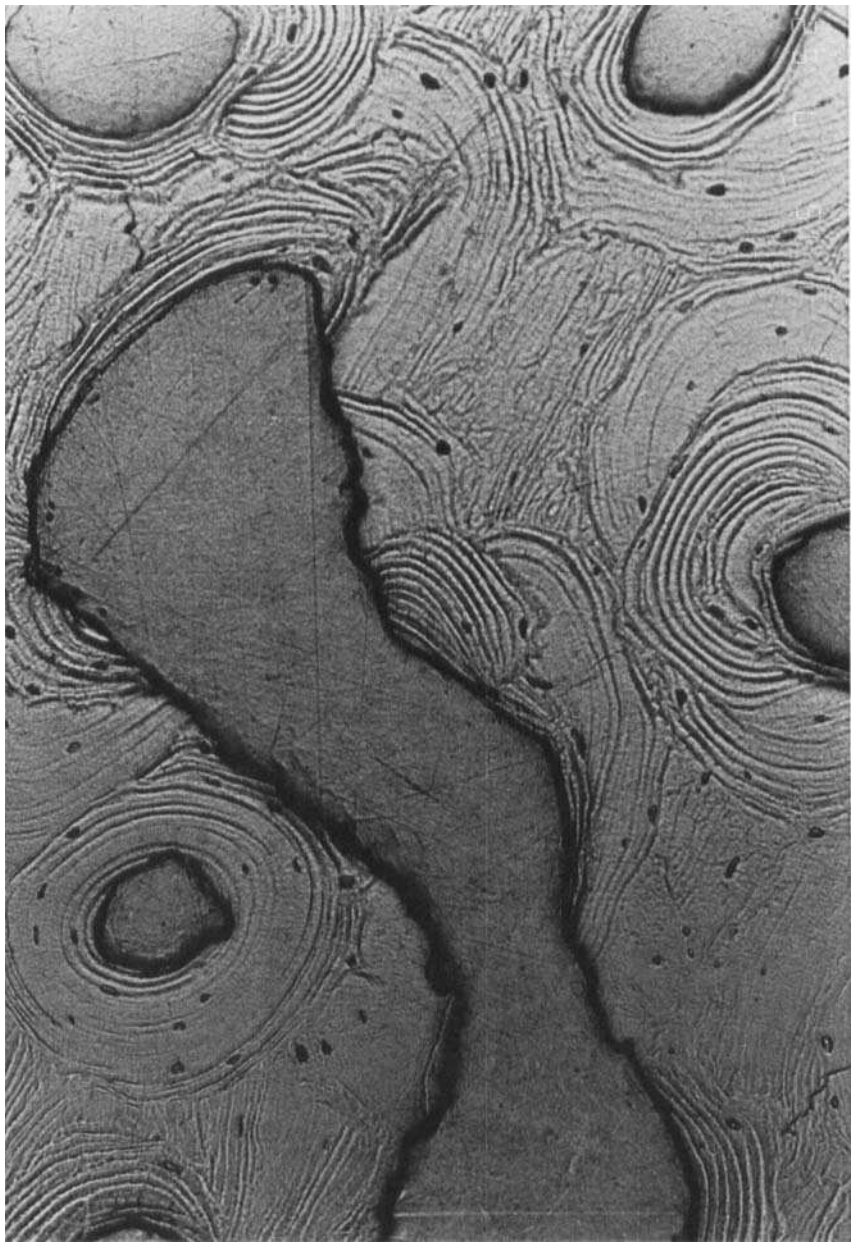


FIG. 8. Ordinary reflected light micrograph of the surface of a polished block of bone embedded in polymethyl methacrylate (PMMA). Fieldwidth 800 μm.

A Mach-Zehnder interference microscope has been used (by courtesy of the Blackett Laboratory, Imperial College London) in a trial determination of the volume of resorption lacunae (Figs. 10, 11 and 12). This is a transmission microscopic method, measuring the optical path difference with a laterally displaced reference beam. The reference beam does not pass through material of exactly the same nature as the sample beam, because of the local variability of

composition of the dentine or bone slices which are used. Nevertheless, with the more uniform dentine, it would provide the means for a dynamic three dimensional measurement of resorption in progress by live osteoclasts in tissue culture (Fig. 12), bearing in mind that the osteoclast itself contributes to the optical path difference and that the osteoclast moves with time.

FIG. 9. Interference fringe image of same field as Fig. 8 using sodium vapour lamp orange monochromatic light ($\lambda = 589$ nm).

Laser Interferometry and Related Methods

There are now many automated laser interferometric methods available for the characterisation of surfaces with nanometre precision. None of these provides a practical solution in the particular problem field of the hard tissue research laboratory. The result is an extraordinarily precise estimate of Z, with unacceptable precision in X and Y (Biegen and Smythe, 1988; Virdee, 1988).

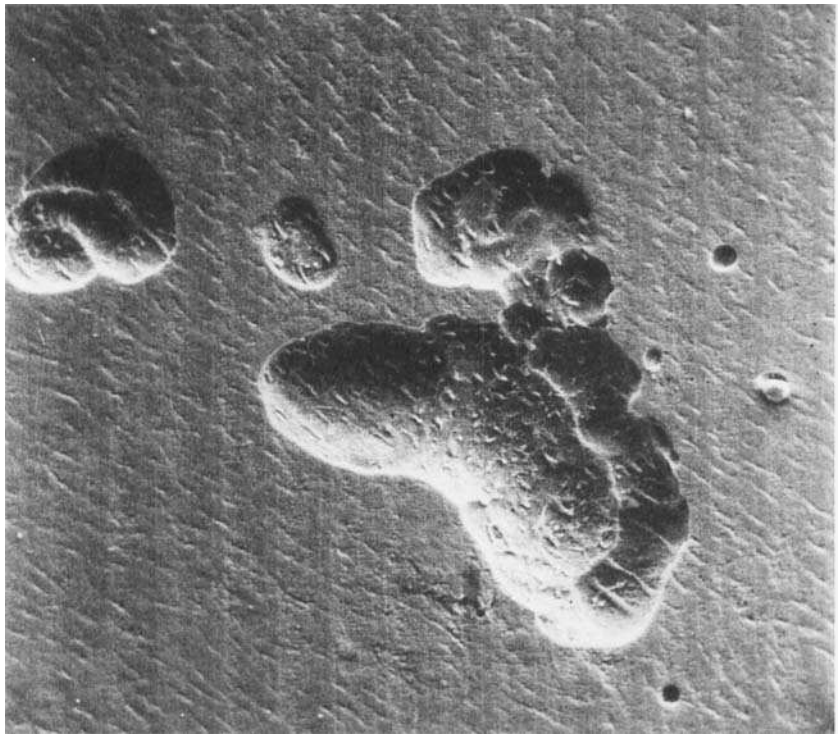


FIG. 10. Scanning electron micrograph of resorption pits made by chick osteoclast in sperm whale dentine. Fieldwidth 500 μm.

Scanned Tip Microscopies

Scanned tip microscopes, including scanning tunnelling microscopes and atomic force microscopes, are a major centre of current interest. Most use non-contacting probes with piezoelectric elements and feedback control to keep constant current or voltage conditions between a probe and a sample surface. They are capable of such extremely high resolutions that single atoms can be imaged. Working backwards, however, they have not been made into devices which are crude enough to measure features such as the osteoclastic resorption lacunae. They provide exceptional (sub-ångstrom) resolution in X, Y and Z, but cannot handle deep pits and steep slopes (*The Journal of Microscopy*, 152 (2 and 3), 1988, gives an extensive bibliography in this area).

Climpson (1987) made a scanning contact tip profiling system to image specimen surfaces at an appropriate scale. Such Talysurf™ and Talystep™ instruments may damage the sample surface, and again the probe cannot get into small pits and follow the steep slopes of small features. However, this is a practical solution to the mapping of larger and much flatter areas.

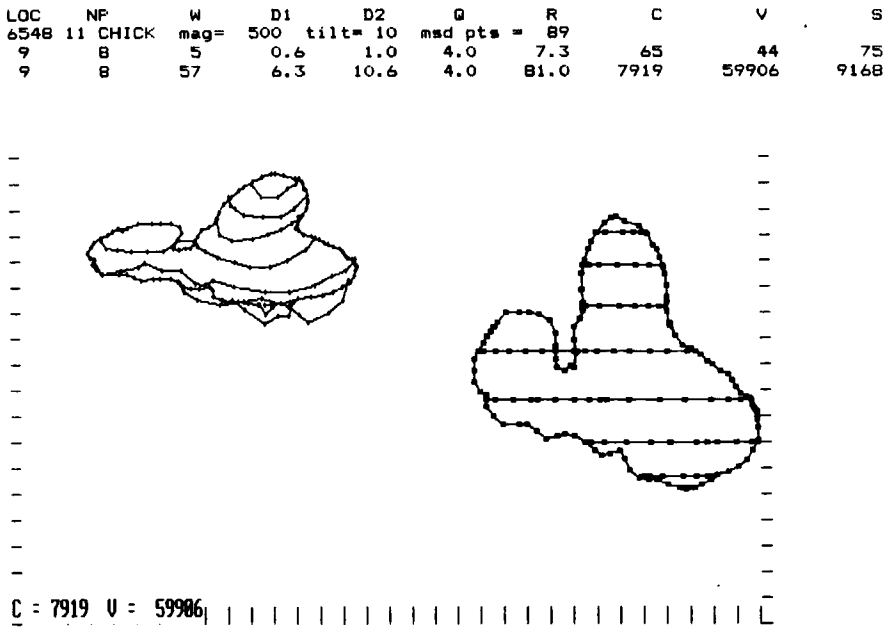


FIG. 11. Screen dump output (90° rotated, mirror inversion) from Ross Instruments SFS3 showing the lines along which measurements were taken to derive the volume etc. of the pit shown in Fig. 10 (pit maximum depth = 10·6 μm, area = 7919 μm^2, volume = 59 906 μm^3).

Conventional (non-confocal) Optical Microscopy

If scanned tips and interference fringes cannot enter the holes that are to be measured, why not use simple limit microscopy either (1) with a large depth of field or (2) with a minimal depth of field configuration?

Low aperture, long working distance, large depth of field, stereobinocular microscopes can be used to provide images for a stereophotogrammetric solution; one picture can be recorded through each channel of a stereobinocular microscope, or the specimen can be shifted under one objective, or tilted. In one current application in the authors' laboratories, an 80 mm macro lens is used with a 35 mm camera back, providing a roughly 1:1 magnification at the film. Normal and osteoporotic lumbar trabecular bone samples are tilted through a small angle. The depth of the specimen is known, and it suffices for the purpose of the experiment to calibrate by measuring the parallax front to back. The increment of this parallax is used to determine relative depth through the section to reconstruct the positions of trabeculae (Jayasinghe and Boyde, 1991).

Optical microscopes with high aperture, short working distance objectives have a depth of field so small that it is possible to determine the position of a feature in the surface by focussing, as in "depth measuring microscopes", bringing the point to be measured to a standard position within the optical system (for example, the DMM 2000 depth measuring microscope system of Unitron Inc.).† The resolution is limited by the mechanism used to move the sample in X and Y and Z (focus), and by the precision to which the focus can be set and read, the focus being a function of the depth of field of the objective lens. The latter will be too great to permit its utilisation in measuring sub-micrometre depth features, but simple focussing has been used to measure resorption pit depths (Fig. 13) and to estimate their volumes

†Unitron Inc., P.O. Box 169, Bohemia, NY 11716, USA.

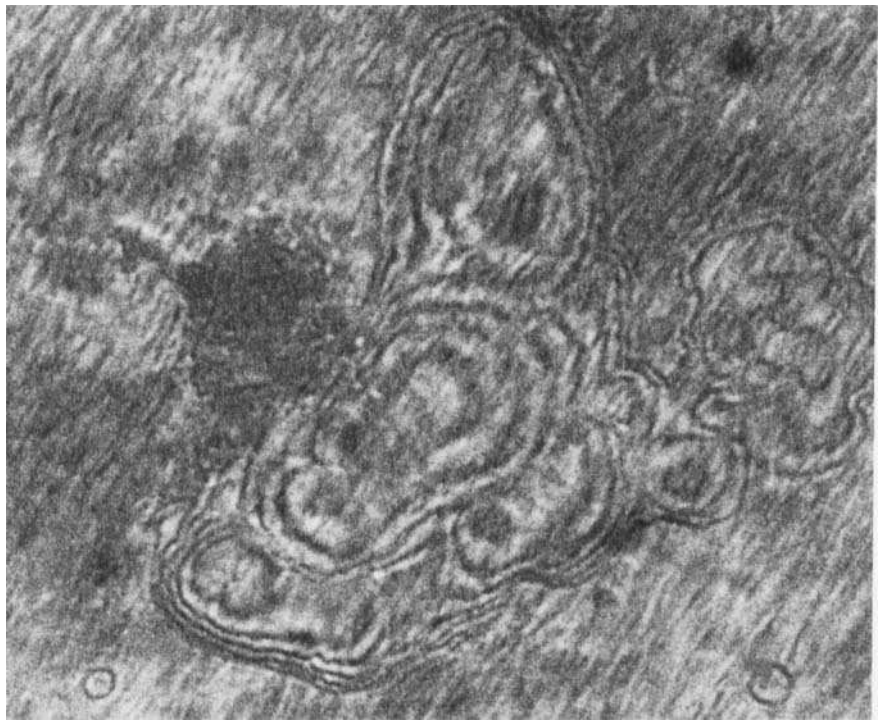


FIG. 12. The same pit as shown in Figs. 10 and 11 viewed in the Mach-Zehnder interferometer microscope using green light. Interference fringes show contours of equal optical path difference.

by a stereological grid overlay method (Walsh *et al.*, 1991). It would be possible to focus the microscope automatically by transferring the image to a video system and determining the focus as that stepper-motor focus control position at which the maximum high frequency information is obtained. Working with a relatively high magnification objective and regarding a point as a small field would provide means for determining the average level of a part of a surface in a specimen.

It must be noted that focussing or autofocussing to determine height in an SEM would not be a sensible procedure, whether this was done by mechanical movement of the specimen or by changing the final (focussing) lens excitation. SEMs have a large depth of field and this can not be reduced to the desired level by increasing the aperture (Fuchs *et al.*, 1990). Furthermore, electromagnetic lenses exhibit hysteresis. For both reasons, it would not be possible to determine best focus to one micrometre, and there is a practical interest in determining heights/depths to a small fraction of a micrometre. Nevertheless, work claiming to be able to use this as an approach to measurement of resorption pits has been published (Shimizu *et al.*, 1989a and b, 1990).

Confocal Microscopy

The most important improvement in possibilities comes from the utilisation of the confocal principle. The illumination in the intermediate image plane of the objective is apertured so that the aperture(s) (real or virtual) is (are) imaged in the focal plane in the specimen to illuminate, efficiently and brightly, only a corresponding point (or points). The imaging system only examines the same point,

by collecting the signal via a "confocal" aperture in the intermediate image plane of the objective on the image forming side of the microscope. Thus only a small volume element in the plane of the specimen surface (or within a translucent object) is imaged. There will be a sharp response peaking to a maximum at focus for an object being translated through this one point in space and the signal intensity can be used as a measure of focus.

Turning this principle into an image forming system requires scanning, for example by scanning the specimen mechanically past the stationary probe, scanning the objective lens, or by scanning the optical probe with galvanometer mirrors or rotating polygonal mirrors or acousto-optic deflection devices. It is also possible to scan the physical apertures in illumination and detection. In the latter case, it is possible to have a large number of illumination and detection apertures operating simultaneously. The best known of these Nipkow disc-based devices is called the Tandem Scanning Microscope (TSM) (Boyde, 1985 and 1990; Boyde, Jones *et al.*, 1984). The confocal laser scanning microscopes using a single scanning beam of light have become very popular and were soon recognised for their potential in the precise measurement of surfaces (Hamilton and Wilson, 1982; Sheppard and Hamilton, 1983; Sheppard *et al.*, 1983; Cox and Sheppard, 1983). However, because only one point is illuminated and imaged at any one moment, their frame speeds are generally too slow for a large amount of practical survey work. Recent commercial instruments using acousto-optic deflectors on the line axis have been able to achieve video rate performance and these instruments also perform well in the practical measurement field (Boyde, Maconnachie *et al.*, 1991a and b; Jones, Boyde *et al.*, 1991).

The TSMs can be used in two different ways to characterise three dimensional surface morphology. With white light, the fact that even the very best microscope objectives have a significant longitudinal chromatic abberation can be exploited. A mirror object changes its colour with focus as the response of the microscope changes for different wavelengths along the optic axis. If the object is not flat, features in the object surface at the same distance from the objective lens appear in the same colour; in other words the image provides colour-coded contour maps (Boyde, 1985, 1990; Boyde, Xiao *et al.*, 1990). There are problems of field curvature, but the field curvature will be constant for a given lens. Depth can then be measured as the colour of the pixel in the image, assuming that the nature of the material at each pixel does not differ to affect the colour of the reflected light. With a colour video camera, the co-ordinates in the colour triangle could be measured.

The second and more obvious approach to depth measurement with the TSM (or any other confocal microscope) is to use either monochromatic light or an objective with minimal longitudinal chromatic aberration, transferring the image to a digital frame store. Using a constant intensity light source and a video camera in which the gain and black level can be set to fixed values, the focus is changed in very small increments, grabbing images in sequence and determining the focus level at which each pixel reaches its brightest value (the height of the specimen surface for that pixel) (Boyde, Dillon *et al.*, 1990). This procedure works well if the slope is not so great that no light is reflected back into the objective lens. It is therefore necessary to use a high aperture lens. The maximum available is a numerical aperture, NA = 1·4 for an oil immersion lens. However, biological objects tend to be transparent anyway and may become remarkably transparent when immersed in a high index medium. It may therefore be necessary to apply a reflective coating. Dry biological objects are effective reflectors and it is possible to work with the maximum numerical aperture of 0·95.

In the Noran TSM and Noran 8502 image analysing computer combination, the specimen may be focussed in increments down to 0·02 μm, but normally at 0·125 μm or 0·25 μm (Boyde, Dillon *et al.*, 1990). The focussing is done under computer control using piezoelectric elements with eddy current sensors to remove hysteresis effects. The frame grab period can be considerably less than one second. The processing time at each focussing level is one second. This means that a surface having topographic relief of 10 μm can be mapped in less than one minute (Fig. 14).

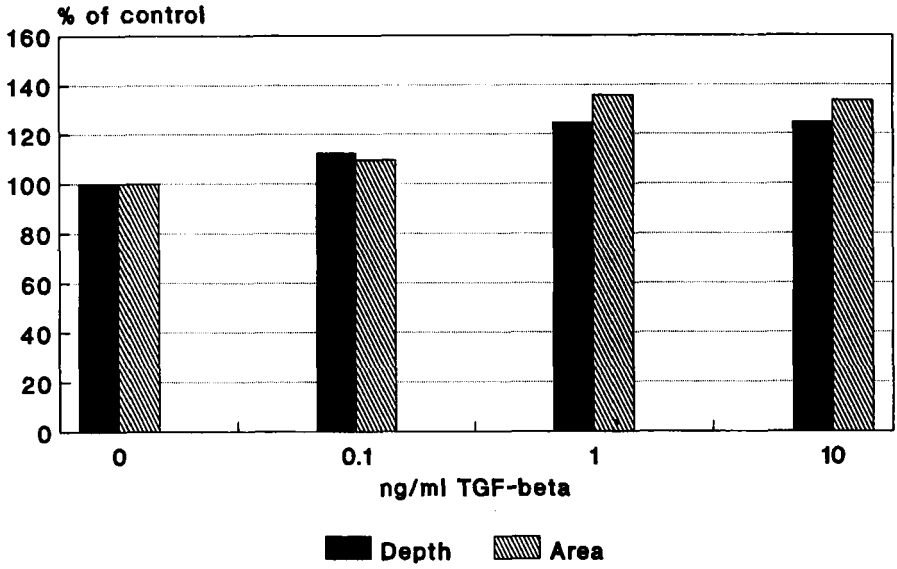


FIG. 13. The mean values of the depths and areas of pits made by rat osteoclasts in dentine in the presence of 10 per cent serum and 0·1, 1 or 10 ng ml^{-1} TGF-β, shown as a percentage of control values. Measurements made by ordinary reflection light microscopy (Bausch and Lomb, 50/0·45 objective).

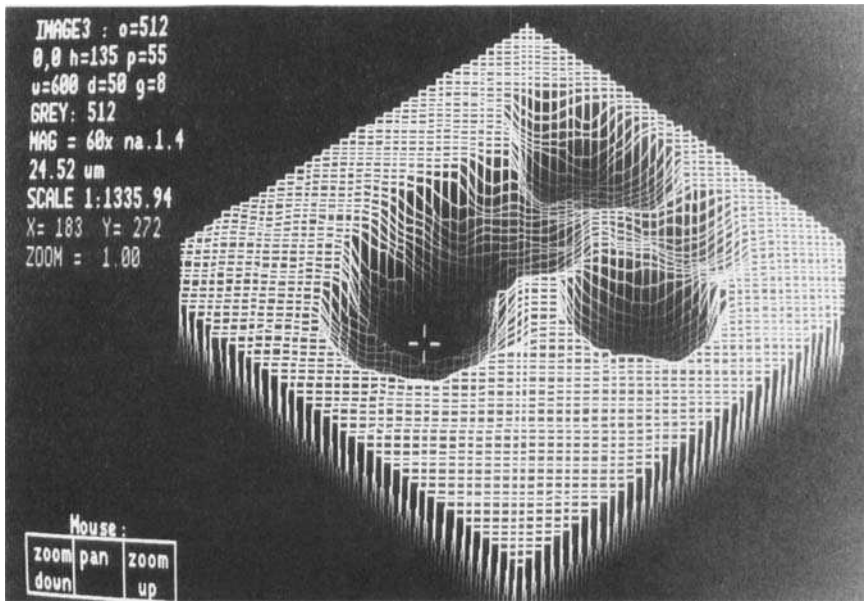


FIG. 14. Reconstruction of the surface of a slice of sperm whale dentine with a resorption pit bottom measuring 24·5 μm deep to the arbitrary, top reference plane. Data acquired by through focussing, determining the focus value at which the greatest signal intensity was returned, using a Noran TSM interfaced to a Noran 8502 image analysing computer. Pit was made by osteoclast which differentiated from pre-hatch chick long bone marrow in 19 day culture on sperm whale dentine slice.

Real specimens may have some surface slopes which are so steep that they do not reflect light back into the objective. To fill in this missing information, values of the pixels in these undefined regions are averaged with those of the neighbouring defined points, effectively putting straight slopes on missing data segments in the image.

Volumes, areas and any other required data are derived from the map of the specimen surface. Again, the most frequent application concerns the measurement of pits made by osteoclasts (Fig. 15). In order to determine the level of the surface surrounding the pit, a binary image is made by dilating the binary image of the pit (created by stating that the pit is the area having more than a certain depth) to a constant and safe distance and width outside the pit feature, taking the average of the surface in this domain to subtract from the values which are found in the pit proper. The volume of the pit is then the sum total of all the soundings within the pit multiplied by the area per pixel (Boyde, Dillon *et al.*, 1990; Boyde, Jones and Maconnachie, 1990; Ford *et al.*, 1991).

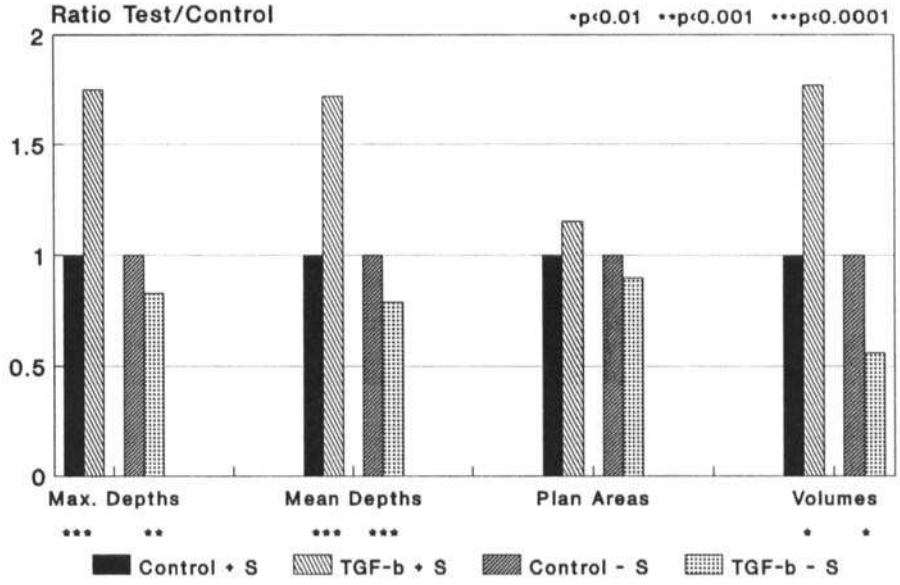


FIG. 15. Maximum and mean depths (μm), plan areas (μm^2) and volumes (μm^3) of pits made in medium with or without added 1 ng ml^{-1} TGF-β in the presence (+S) or absence (−S) of 10% heat-inactivated foetal calf serum. Mean values for the TGF-β pits are given as a percentage of control values. Measurements made by automatic confocal imaging and analysis (Noran TSM interfaced to Noran 8502 computer).

With a video rate laser scanning microscope (1LM11 Lasertec Corporation, Japan), the sample is focussed by stepper motor control. As with the Noran system, the operator finds focus levels just above the surface and just below the deepest part of the pit, and touches a button which activates the acquisition of a similar extended focus or range image in which the recorded values are the depth of the sample surface. At present, this software is so organised that the operator can choose a single line in the line direction of the video display monitor along which the profile is to be reconstructed. The width and the depth of the pit in the profile are obtained by moving cursors interactively on the display screen (Fig. 11), after which the data may be read out automatically into arrays, upon which appropriate statistical manipulations can be conducted (Boyde, Maconnachie *et al.*, 1991a; Jones, Boyde *et al.*, 1991). Since only the width and mean-maximum depth are to be obtained, it is satisfactory to ignore the undefined data points which persist in the trace of the steep edges of the pits (Figs. 16 and 17).

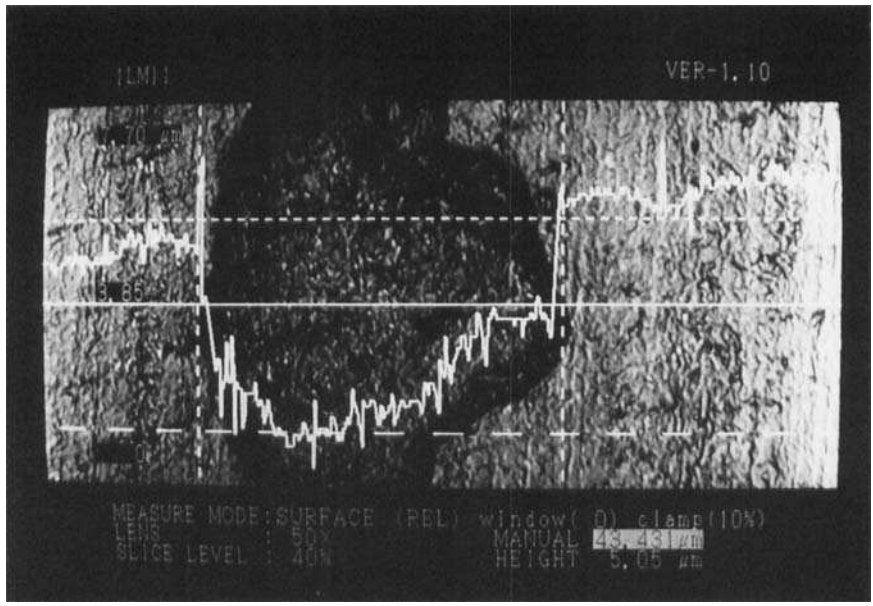


FIG. 16. Photograph of the display monitor of the Lasertec 1LM11 video rate confocal laser scanning microscope showing the measurement of the width and depth of a resorption lacuna along the continuous white line crossing the centre of the image. The short-dashed horizontal line has been moved interactively to a level half way between the surrounding surface profile to each side of the pit; the matching long-dashed horizontal line has been moved to cut through the mean level of the profile in the depth of the pit. The two vertical dashed cursor lines mark the edge of the pit.

Chick Osteoclasts 24 h on Human Lamellar Bone: TS or LS

n=		+ fringe	- fringe	fringe
100	*ts depth	3.54(0.22)	4.07(0.20)	0.53
100	ls depth	3.83(0.18)	4.20(0.18)	0.37
200	*pooled depths	3.68(0.14)	4.14(0.13)	0.46
100	ts width	22.54(1.51)	22.23(1.36)	
100	ls width	23.11(1.16)	23.07(1.08)	
200	pooled widths	22.83(0.95)	22.65(0.87)	
100	*ts w/d	7.59(0.52)	5.78(0.30)	
100	ls w/d	6.73(0.41)	5.86(0.25)	
200	*pooled w/d	7.16(0.33)	5.82(0.20)	

Means(S.E.M.)um

FIG. 17. Table of measurements made with the Lasertec 1LM11 confocal microscope of the depths and widths of pits made in human compact bone cut longitudinally (ls) or transversely (ts), such surfaces presenting collagen mostly perpendicular and more parallel to the plane of the section surface respectively. The specimens were measured before and after removing the demineralised collagen fringe. The results are important in demonstrating that there is no meaningful difference in the behaviour of chick osteoclasts resorbing bone as a function of the microscopic orientation of the mineral and collagen components of the bone matrix, and that the mean fringe depth can be measured by this method.

Measurements of width and depth give a good index of the volume of the pit, but do not short cut the measurement of volume as such, because the pits do not have a sufficiently regular shape. Since, however, the orientation of the pits on dentine is random and the widths are measured in a random direction with respect to themselves (but not to the instrument), it is likely that a good indication of biological trends might be obtained from the mean values of large numbers of widths and depths (Fig. 18).

The immediate future for osteoclastic resorption lacuna surveying would seem to lie with the development of even more sophisticated software for the control of the acquisition and analysis of confocal LM images, rather than with a development of stereophotogrammetry. However, even with the existing state of development, confocal LM approaches are much less tedious than operating stereophotogrammetric equipment. The data throughput is higher and photographic images (with all the associated real and time costs and the later archiving problems) can be avoided.

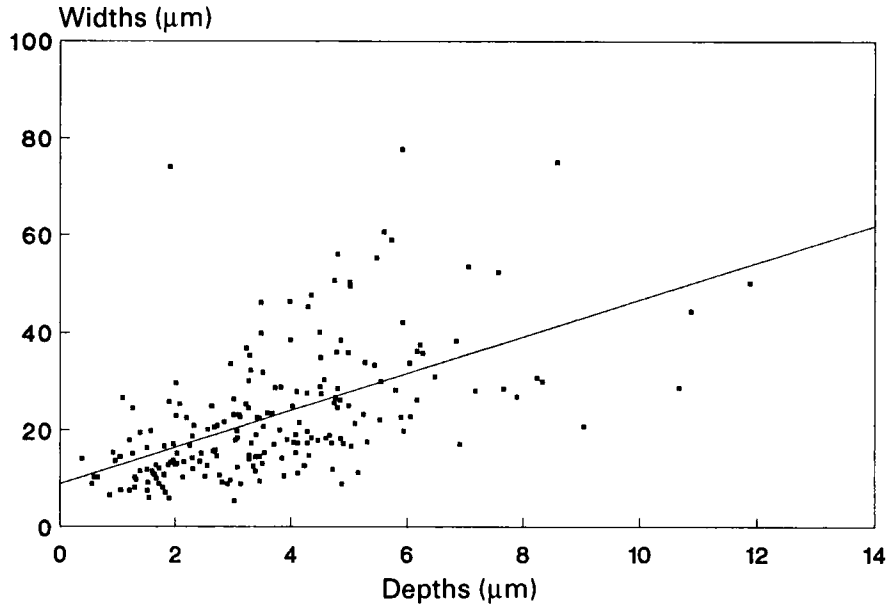


FIG. 18. Scatterplot of depths (μm) against widths (μm) of resorption pits made by chick osteoclasts in slices of human compact bone cut in the transverse and longitudinal axis. The changing relationship between the areas and depths due to the lateral movement of the osteoclast during resorption pits is evident. Measurements made with Lasertec 1LM11 confocal microscope.

A further advantage of the use of confocal LM in surveying hard tissue resorption lacunae is that they can be examined and measured whilst wet. In the SEM case (Fig. 19), the specimen has to be dry at the surface. In the process of resorbing hard tissues, osteoclasts first dissolve the mineral component out of the mainly collagenous matrix to allow proteolysis to occur. During active resorption, therefore, there is a layer of demineralised collagen or "fringe" lining the resorption pit. The thickness and the volume of this fringe layer can be determined by measuring pits before and after removal of the fringe. In the SEM case, drying collapse in this fringe layer can be minimised, but not prevented, by freeze drying (or less well by critical point drying). For confocal imaging, the surface can be made more reflective whilst still wet with a thin layer of silver deposited by immersing the sample in a dilute silver nitrate solution for a few minutes before beginning the observations. Kept wet throughout, pits made by rat osteoclasts in sperm whale dentine increased in volume by a factor of 1·187 when the "fringe" was removed ($N=10$, standard deviation=0·066).

Dentine collagen resorption in vitro

15 pits measured by SEM stereophotogrammetry

Pit	+Fringe	-Fringe	Fringe
Depth	2.58 (0.19)	3.08 (0.27)	0.52 (0.10)
Area	499.3 (86.6)	532.4 (171)	33.1 (7.7)
Volume	654 (132)	835 (171)	180.5 (44)

48h culture: deer antler osteoclasts
Means μm (S.E.M.)

Specimens critical point dried
Fringe removed by peroxide

+Fringe/-Fringe %

Depth ▪ 17%
Area ▪ 6.2%
Volume ▪ 22%

FIG. 19. Table showing the mean values of the depths (μm), areas (μm²) and volumes (μm³) of pits made by osteoclasts, isolated from growing fallow deer antlers, in sperm whale dentine, before and after removal of the demineralised collagen fringe using hydrogen peroxide. Measurements made by SEM stereophotogrammetry and Ross Instruments SFS3.

PRECISION, RELIABILITY AND RESOLUTION IN MEASUREMENT

SEM Stereophotogrammetry

From theory,

$$Z = p/(2\sin(\alpha/2))$$

where Z=height difference perpendicular to imaginary central photography; p=measured parallax, which can be measured to a precision of 3 μm at photoscale; α=tilt angle difference, in this case 20° set to precision $\delta\alpha$ of 0·05°, but with the same setting error on every occasion. With $\alpha = 20°$, $Z = 2{\cdot}8794 \times p$, so that for a parallax (setting repeat within range) of ±3 μm there is a measuring precision of ±8·382 μm at photograph scale. This figure is divided by the magnification to reach object scale, giving a value of ±0·0084 μm at 1000×. If $\alpha = 20{\cdot}05°$, then $Z = 2{\cdot}8723 \times p$; subtracting values of Z, 2·8794−2·8723=0·0071. The uncertainty from α would therefore be ±0·71 per cent. A BASIC programme for incrementing all relevant parameters to estimate an error region was published by Boyde (1974b).

For area determination non-stereoscopically in the SEM case (Kanehisa and Heersche, 1988), the pixel resolution must be considered. (It has been found necessary to use SEM magnifications of 625×, 1250×, 2500× and 6250× as appropriate to the size of the pit in this stereophotogrammetric work. The 100 mm record monitor resolution resolved 1000 lines; at 1000×, one pixel represents 0·1 mm.) A circular pit of diameter 20 μm imaged at 2500× would be 400 pixels wide and there would be an uncertainty of 0·5 per cent in width and 1 per cent in

area due to the finite pixel dimension. (In practice, the situation would be far worse, because users ignore both the changes of magnification with the field of view (Howell, 1978a and b) and the need to calibrate the magnification for one working distance reached by focussing mechanically, not by changing lens excitation.)

Regarding depth resolution in the non-stereoscopic case, Fuchs *et al.* (1990) give the best case as the depth of field being equal to 10 per cent of the fieldwidth and the usual case as 100 per cent of fieldwidth, corresponding to Z resolutions of 10 μm and 100 μm respectively at 1000×, and the use of 200 μm and 50 μm apertures respectively in the Cambridge S410 SEM. In short, focussing can not be used to measure pit depths in an SEM.

Conventional (non-confocal) Light Microscopy

From theory, Abbe's formula for the lateral resolution, d, of a light microscope is $d=1{\cdot}22\ \lambda/(\mathrm{NAc}+\mathrm{NAo})$, where λ=wavelength of light, NAc=numerical aperture of condensor and NAo=numerical aperture of objective. For a reflecting microscope illuminated though the objective via a beam splitter, then NAc=NAo. For a 50× objective used for pit measurement in a conventional, long working distance (Bausch and Lomb) reflecting microscope, the NA=0·45 and, with $\lambda=0{\cdot}5$ μm, $d=\pm 0{\cdot}68$ μm. Thus, for a pit of 10 μm radius, there would be an uncertainty of 7 per cent in the measurement of width and 14·3 per cent in the calculation of area, irrespective of magnification and any further uncertainties introduced by problems due to finite pixel dimensions in image digitising systems. For the very best, very short working distance dry objectives, NA=0·95 and, for $\lambda=0{\cdot}5$ μm, $d=\pm 0{\cdot}32$ μm, halving those possible errors.

For depth resolution, h, Francon (1961) gives $2h=\lambda/(4n\times\sin^2(u/3))$, where n=refractive index of immersion medium and u is the angle of the half cone of light that enters the objective (NA is the product $n\times\sin u$). With the same 0·45 NA objective, therefore, using $\lambda=0{\cdot}5$ μm, $n=1$, $u=13{\cdot}4°$, gives $h=\pm 1{\cdot}168$ μm. Thus reading by eye and with a perfect mechanical stage, a pit depth could not be measured to better than $\pm 1{\cdot}2$ μm. Pits of 20 μm diameter typically have depths ranging from 1 μm to 6 μm; clearly measurement by focussing with such a lens would be hopeless for shallower pits. However, increasing the NA of the objective has a dramatic effect. For NA=0·75 (as used by Walsh *et al.*, 1991), $\lambda=0{\cdot}5$ μm, $h=\pm 0{\cdot}369$ μm. For NA=0·95, $\lambda=0{\cdot}5$ μm, $h=\pm 0{\cdot}182$ μm.

Confocal Microscopy

From theory and practice, both the lateral and the vertical (axial) resolution are improved by a factor of 1·4 in the confocal case (Brakenhoff *et al.*, 1985); according to this criterion, d will reduce to $\pm 0{\cdot}23$ μm and h to $\pm 0{\cdot}13$ μm for a 0·95 NA objective. A more important factor, however, is that the integrated intensity (power) falls dramatically in out of focus confocal images. Sheppard and Wilson (1978, see Fig. 1) show that integrated intensity falls by 2 per cent for a defocus of 0·097 L for NA=1; in other words, a measurable change would be found for a change in focus of 0·048 μm for $\lambda=0{\cdot}5$ μm. Hamilton and Wilson (1982) made a practical demonstration of a vertical resolution of better than 0·1 μm with an NA=0·5 objective and $\lambda=0{\cdot}633$ μm. With a method which involves the sensitive measurement of the intensity, as in the UCL automated method with the Noran TSM and 8502 image analyser, a reproducibility of $<0{\cdot}05$ μm can be found using white light and a 0·95 NA objective.

Returning to the example of a pit of width 20 μm and depth 3 μm and trying to calculate the volume as $2/3\ \pi\times\mathrm{radius}^2\times\mathrm{depth}$ from these measurements alone (which would require a most unnaturally regular pit shape), a ($\pm 2\times 0{\cdot}05$ μm) $\pm 3{\cdot}33$ per cent uncertainty in depth compounded with a ($\pm 0{\cdot}23$ μm) $\pm 2{\cdot}3$ per cent uncertainty in radius (area 4·6 per cent) combines to give a mean theoretical volume uncertainty of $\pm 7{\cdot}9$ per cent (in this case, for the volume of 628·5 μm^3, the range is 580 to 680 μm^3).

Experimentally, repeating the entire process of measuring one pit with a mean

volume of 383 μm^3 with the UCL automated TSM procedure (Boyde, Dillon *et al.*, 1990) 30 times gave a standard deviation of 28 μm^3 or 7·4 per cent by volume. In this procedure, it should be noted, very large numbers of soundings are taken within the pit and within the surrounding unmolested surface, so that the possible theoretical errors must even out around the mean value; the observer can introduce no bias.

Magnification

The authors used the same magnification standard for all the microscopes used in their work. This was a silicon standard with a repeating grid pattern of 10, 100 and 1000 μm squares as in a chequer board.

What Should be Measured in a Resorption Assay?

If measuring volume is expensive, then what should be assessed in a resorption assay? A few of the alternative approaches which have been used in the literature are presented here.

(1) Measure depth and width precisely by CSLM.
(2) Measure depth at one place, area by planimetry and calculate volume (to an implied precision of 0·1 μm^3 or 1 part in 106) (Kanehisa and Heersche, 1988).
(3) Measure the depth of a few pits, the areas of all pits and estimate volume per slice assuming that the mean depth was representative (Chambers *et al.*, 1984).
(4) Measure area and assume that this relates directly to volume by citing a previously determined correlation (Hunter *et al.*, 1991).
(5) Measure area per slice, sometimes without real values being given and express the results as a percentage of control values (Zaidi *et al.*, 1987; Sato and Grasser, 1990; Evely *et al.*, 1991).
(6) Measure the area resorbed and the number of pits per random field of view.
(7) Count the number of pits per osteoclast.
(8) The number of pits made by each osteoclast is obviously a useful index of activity but, intuitively, a measure of the size of these pits seems necessary. To this end, Murrills *et al.* (1990) counted the number of pits in single "foci" (a focus being defined as a clutch of pits in a 400 μm by 400 μm field, possibly made by one osteoclast, but possibly not) as well as measuring the numbers of foci per slice, pit areas and the pit/osteoclast ratio for the slice.
(9) Count the number of pits per (small) slice, but make many replicates (Arnett and Dempster, 1987).

Possibly Erroneous Biological Assumptions in the Resorption Assay

In an *in vitro* bone cell resorption assay, assumptions are made concerning the cells that do the job, their cellular and nutritional environments, the substratum provided, as well as the measurement of the work accomplished. Some of these assumptions are listed briefly below.

(1) The number of cells in each aliquot is identical.
(2) All cells are equal, in size and activation state and cells will start, cycle and stop resorbing at the same time, irrespective of nuclear number and other factors.
(3) All substrates will receive equal and uniform seeding distribution of cell types.
(4) Settling and attachment efficiency is equal for all cells (taking into account size, clumping/time/temperature/washing vigour).
(5) Temperature and pH, and the concentration and influence of other cell types are all equal throughout the experiment (neighbours/osteoclasts/osteoblasts/immune cells/precursor cells).

(6) The relationships between area, depth, volume and shape are constant throughout the formation of the pit.
(7) Area:depth:volume:shape are constant and identical in experiment and control and in different experiments.
(8) Substrate resorbability (possibly related to composition and orientation of collagen fibres or crystals) is uniform across each and every substrate.
(9) All substrates are of equal size.
(10) All experiments have identical time span even within one clutch.
(11) All pits can be resolved by the microscopic technique (note surface roughness and minimum size dropout).
(12) The scanning search procedure is 100 per cent efficient.
(13) Search thoroughness is uniform across the entire clutch of samples (note time of day and boredom).
(14) Samples with no resorption pits are not omitted if slices are being compared.
(15) The observer is unbiassed, has no preconception of the result, and does not know which sample is being measured.

The inherent biological variability necessitates the measurement of several specimens and as many pits as possible for a meaningful result to be acquired. This calls for efficiency of measurement as well as accuracy.

ACKNOWLEDGEMENTS

The authors are grateful to P. Howell for his help with the development of the SEM based stereoscopic methods. They thank the Science and Engineering Research Council (SERC) for financial support in developing both SEM and TSM-based methods and the Medical Research Council (MRC) for the grant for the first TSM. Project support has been given by Research into Ageing and the Wellcome Trust. Lasertec UK Ltd. provided generous support through loans of their 1LM11. Thanks are also due to R. Radcliffe and M. Arora for technical assistance. This paper is dedicated to the memory of the authors' dear colleague, Elaine Maconnachie, who did so much to help them over so many years.

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Résumé

Les auteurs examinent les systèmes de mesures tri-dimensionelles d'objets microscopiques tels que, en particulier, les cavités creusées dans des substrats expérimentaux, déposés sur des surfaces plates, par des cellules isolées résorbant les os (ostéoclastes). On peut recourir à de telles mesures pour évaluer le travail effectué par ces cellules. Il semble que dans ces mesures, on tende à remplacer la stéréophotogrammétrie au microscope électronique à balayage par la microscopie à lumière confocale qui possède une très faible profondeur de champ et emporte la préférence.

Zusammenfassung

Die Autoren diskutieren Systeme zur dreidimensionalen Ausmessung von Mikroskopabbildungen, insbesondere der Vertiefungen, die durch isolierte, Knochen resorbierende Zellen (Osteoklaster) in Testsubstraten mit ebenen Oberflächen verursacht wurden. Solche Messungen können zur Prüfung der von diesen Zellen geleisteten Arbeit genutzt werden. Die Mikroskopie mit konfokalem Licht und einer sehr geringen Gesichtsfeldtiefe scheint die Stereophotogrammetrie mittels Rasterelektronenmikroskop als der bevorzugten Methode für solche Messungen zu ersetzen.

DISCUSSION

Chairman (Mr. Newton): Thank you very much indeed, Professor Boyde. Could I ask the first question please? How can you check the accuracy of these systems when you are working at sub-micrometre levels? How can you guarantee these accuracies? Do you have calibration objects?

Professor Boyde: We have calibration objects made for the semi-conductor industry which provide the calibration in *X*, *Y* and *Z*. However, we are not so much concerned about the absolute calibration of the system. We use exactly the same system with exactly the same set of built in errors to make all the measurements in an experimental and a control group of observations; if the numbers were out by say one per cent, it would be not be a worry. However, if the error were ten per cent one way in one specimen and ten per cent the other way in another specimen, then it would be important.

Chairman: In fact it is relative rather than absolute accuracy that is critical.

Mr. Naru: You showed an extremely interesting example of the comparison between a pit with the demineralised collagen fringe, measured again after dissolving the demineralised collagen. How did you ensure that the true orientation was recovered for the second measurement run?

Professor Boyde: The slice of dentine was glued to a glass microscope slide; the mechanical stage on the microscope used is such that one can just click the slide

back in again and it comes back to the same spot. Thus it was only necessary to handle the specimen attached to this glass slide during the peroxide treatment to remove the collagen fringe. There are enough small features in the surrounding unaltered surface to permit shifting the second map image by a few pixels to get the exact overlap before subtraction.

Professor Dowman: You mentioned in your presentation that you used some real time photogrammetric methods. They then seemed to be surpassed by the new techniques that you have used since. What happened to those techniques? Were they not useful to you?

Professor Boyde: We could not obtain the resources to develop the method. I was very fortunate in getting one of the first SEMs in the country but have not been able to raise the money since to get another one. I am sure that we could develop real time measurement to an extremely fine system with a good modern SEM.

Chairman: Could I ask how the cost of a confocal microscope compares with an SEM?

Professor Boyde: The first SEM that we bought from the Cambridge Instrument Co. in 1966 cost £16 850. The first confocal microscope that we purchased in 1983 from Czechoslovakia with a grant from the Medical Research Council cost £15 000. In 1988, our department bought a BioRad Lasersharp MRC 500 confocal microscope system for £68 000. Zeiss and Leitz confocal systems currently sell for just less than £100 000, about the same as a good digital SEM. The European agent of the American manufacturer of the TSM with which we work is currently asking about £35 000.

Professor Cooper: I have a non-photogrammetric question. It occurred to me, while we have been sitting here listening to your fascinating presentation, we have all lost a significant amount of bone through the activities of the osteoclasts. Your ingenious work has led to some comparative resorption rates of these cells. I wonder whether any modification of their activity is either desirable or possible. Is it necessary that they are there? Are they entirely bad for us or good for us?

Professor Boyde: The activities of osteoclasts are a finely integrated part of a system for the continuous repair of the skeleton by the replacement of little bits at a time. We replace most of our entire skeletons in about fifteen years.

Professor Cooper: Not sixteen?

Professor Boyde: That would be about the same as the cycle time for me coming to talk to this Society! Modification of osteoclastic activity is certainly indicated in disease states where it gets out of proportion to the rate of replacement by new bone tissue. A great deal of research is currently directed towards investigating natural control mechanisms and pharmacological agents which can be used to influence the resorption: formation balance.

Professor Cooper: Would you be looking for slower acting osteoclasts or faster acting ones?

Professor Boyde: The most important thing is to keep the balance between the removal of effete tissue and its replacement. We do not have any idea how osteoclasts judge that one piece should go and another piece should stay. Nor do we know how the balance is kept between taking away and putting it back. At the menopause, many women lose a relatively greater proportion of bone tissue. If it was put back in proportion, it would not matter.

Professor Cooper: Perhaps a (chicken) osteoclast implant might be proposed in the future?

Professor Boyde: There are factors produced by bone resorption that stimulate new bone formation in that locality. One way to control bone formation would be to control its resorption.

Chairman: Well, we've been really close up in Professor Boyde's talk tonight and now we must really close up and get off home. In finishing, I would like to thank him for his fascinating talk this evening in which we have heard about some extremely interesting developments in the field of microscope photogrammetry. I hope that the Technical Committee chairman will make a note to ask Professor Boyde back to talk to the Society in 2007 and so maintain the 16 year cycle. Would you please show your appreciation in the usual way?