Scanning Electron Microscopy

Volume 1985 | Number 3 Article 38

7-16-1985

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SCANNING ELECTRON MICROSCOPY/1985/III (Pages 1259-1271)
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OPTICAL AND SCANNING ELECTRON MICROSCOPY IN THE SINGLE OSTEOCLAST RESORPTION ASSAY

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(Paper received March 28, 1985: manuscript received July 16, 1985)

Abstract

The present studies relate to the single or isolated osteoclastic resorption function assay which we introduced in 1983 to overcome objections to assays based upon measurements of calcium release from bones, in which it was never strictly controlled whether the mechanism involved the destruction of bone with the formation of classical Howship's lacunae. The method may prove to be quite popular in the near future and has already been adopted by other research groups. In previous work, we had utilised stereophotogrammetry of scanning electron micrographs to measure the depth, volume and other parameters of the individual lacunae. However, increasing experience with the method has suggested that we can await a wide range of biological variability in single cell function in any one experiment. We have therefore tested other methods from which data could be obtained more rapidly to permit a better statistical analysis, albeit with reduced accuracy, of each resorption complex.

The main aim of the studies reported here was to evaluate various methods of optical and scanning electron microscopy that can be used for the visualization of osteoclasts and their associated resorption lacunae generated in vitro in slabs of dentine and bone. Optical microscopy was found to be complementary to SEM, enabling vital microscopy of unstained and stained cells. In particular, oblique illumination LM and tandem scanning reflected LM (TSRLM) proved to be of paramount value for this purpose. Fixed coated specimens could be most rapidly scanned for resorption lacunae using darkfield reflected LM or TSRLM.

Key words: SEM - optical microscopy - bone resorption assay - dentine - osteoclasts - tandem scanning reflected light microscopy.

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Introduction

Scanning electron microscopy (SEM) has proved to be an excellent tool for the visualization and measurement of resorption lacunae made by osteoclasts cultured on test substrates (Boyde et al, 1983a, 1984; Ali et al, 1984a,b; Jones et al, 1984). However, it can only be used at the end of the culture period. The purpose of this paper is to summarise results of experimental attempts to utilise light optical microscopy (LM) in the study of calcified connective tissue resorption. There are two clear possible advantages of LM methods:

One is the opportunity for examining living material to monitor the resorption process in vivo or in vitro with a view, for example, to determining such simple natural historical facts as whether the action of one osteoclast is spasmodic and whether it is synchronised within itself (in the case that it generates more than one loculus) or synchronised with or influenced by other cells in the same culture.

A second possible advantage is the scanning of much larger surface areas per unit time to discover the incidence and size of resorption lacunae in wet specimens and those which are prepared as if they were to be examined in an SEM. The possibility of higher speed scanning in an LM arises from the avoidance of the need to pump down each and every specimen to SEM operating vacuum; and the fact that the LM image is constant and can be read at high speed. It is further much simpler to be sure that one has properly scanned an LM specimen, though this would not be true if automated scanning and focussing were available on the SEM being used.

This is not to lose sight of the handsome and complementary advantages of the SEM in this research area which we have recognised and recommended in the past (Boyde et al, 1983a,b, 1984). Thus it is possible to put many specimens in the SEM at once and to scan them all rapidly, though sacrificing the possibility of recording stereopairs. In our own SEM we can load say 20 to 30 6mm diameter stubs in one holder and view them at normal beam incidence obtaining very satisfactory CBSE, BSE-ET, or directional BSE images (Fig 1). The depth of focus of the SEM, using a small final aperture, is a very considerable advantage over the LM, saving the

requirement to have flat, and well-levelled specimens in order to get at least each field of view all in focus at once (Fig 2).

We wish to work with biological resorbable substrates which are, as far as possible, homogeneous in composition. Bone and cartilage are too inconsistent or "macroporous" (Figs. 3,4,5). Thick cementum can be used (Fig. 6), but dentine is available in much larger amounts of continuous uniform tissue. Both cementum and dentine can be sawn into slices 100μm thick which are just practical to handle. Warping on cutting due to the rehydration of dried material and dehydration for SEM specimen preparation is a problem in sections which are thinner than 100μm. Since it is already impossible to make a transmission image which shows the location of 2μm surface depressions in 100μm of dentine in water and phase contrast is not possible with such a thick specimen, it is practically more convenient to use 200μm slices. One thus abandons transmission LM (TLM) methods which depend upon contrasts originating from the dentine substrate per se, but can still use TLM methods using staining (amplitude) contrasts of cells. As regards the possibility of using dental enamel, this is a stiff, brittle material and can be sawn as sections at 100μm. It is, however, difficult to find uncracked starting material in adequate quantities and its use is peripheral to most resorption research because it does not contain a collagen-based organic matrix.

The optical transparency of the dentine tissue slice is therefore a problem in limiting the use of the usual vital microscopy methods of phase contrast and Nomarski optics. There is certainly no point in struggling to apply these methods to a fixed, stabilised tissue sample because visualisation of cells and even resorption lacunae can be simply achieved by staining methods. Thus the suitability of vital methods and post-experimental, post fixation methods are considered separately.

Materials and Methods

Osteoclasts

Osteoclasts were harvested as part of a mixed population of cells obtained by mincing long bones (cleaned of muscle, periosteum and cartilage) of young mammals and birds (late pregnancy or early post-natal rabbits, early post-natal rats and pre-hatch chicks: Boyde et al, 1984, Ali et al, 1984a,b, Jones et al, 1985).

Substrates

Substrates were obtained mainly from sperm whale mandibular teeth (the gift of Dr R. Gambell) though we have proven the use of human and rhinoceros teeth and do not doubt the suitability of most large mammalian teeth. The teeth are cut using a water cooled, high speed diamond saw to prepare 6mm or 12mm square rods, from which numerous identical transverse slices are prepared using a water cooled, slow-speed diamond saw (Buehler Isomet) under low cutting load conditions. The dimensions of the sections were chosen to fit two sizes of standard multi-well dishes. Slices of cortical bone from adult human femur were used for comparison with the tooth material.

Substrates were cut with an Isomet diamond saw and polished or finished using abrasive surfaces, slurries or ground glass plates and standard diamond polishing equipment.

Examination of specimens

Fixed specimens were examined unstained by SEM or tandem scanning reflected light microscopy (TSRLM) and following staining by LM and TSRLM. Vital microscopy of unstained cells was attempted using normal transmitted LM with brightfield and darkfield illumination, phase contrast LM, transmitted brightfield using an inverted LM, reflected light darkfield LM, and TSRLM. The live cells were also stained with neutral red, DASPMI (Horster et al, 1983), Bisbenzimid, and methylene blue, and examined by LM.

Results

Substrate surface finish

The Isomet diamond saw leaves a pattern of =<1μm deep, roughly 20μm major wavelength grooves which cannot be confused with either any natural internal structure of the dentine or any feature which results from experimental or handling procedures. The grooves do have the disadvantage of introducing some topography-dependent contrast into reflection LM and SE and BSE SEM images which detracts from the possibilities of automatic (grey-level) image recognition and analysis of resorption lacunae, and a uniform "optical quality" polish would be desirable.

We have therefore attempted to improve the starting-point of surface-finish in various ways, each of which presents different problems.

(a) Fine grade bonded abrasive surfaces produce grooves which are as deep as those generated by the diamond saw but which have the disadvantage of a random orientation. Occasional deep gouges result probably from the debonding of abrasive particles. The slices also become less plane-parallel.

(b) Abrasive slurries (e.g. alumina) may leave fine particles jammed in the entrance of dentine tubules which provide strong and distracting contrast in reflection LM methods, and could possibly interfere with the biological experiment.

(c) Grinding between plane parallel ground glass sheets improves the plane-parallelness of the slices but leaves occasional gouges in the dentine surface (Fig. 7) which are easily confused with less regular resorption lacunae, particularly when using reflection LM methods.

(d) Diamond polishing methods should not use oils, because these are difficult to remove from the dentine after polishing. Fine diamond particles are very strongly reflective and are difficult to remove even by vigorous ultrasonication techniques: this is easily monitored by tandem scanning reflected light microscopy.

The possibility of preparing much thinner dentine substrates is attractive, but it would have to be based on the cementation of the dentine to a glass slide before grinding and polishing the section to the required thickness and finish. The

problems here are that any polymer which may be considered as a glue may (a) contribute to optical LM contrasts, (b) debond in water and (c) contain residual amounts of toxic materials. Further practical research is needed in this area.

SEM procedures

Since we were interested in dynamic biological events in the course of an experiment involving many samples, we have chosen some rapid methods of sample preparation for the SEM which are satisfactory for the present requirements and may be of interest in other fields. They are designed to answer specific questions which are also addressed by all the LM methods discussed below.

1) Are healthy looking osteoclasts about?

The sample is fixed in 3% glutaraldehyde in 0.15m pH 7.2 sodium cacodylate buffer for 5 mins or much longer, depending upon the urgency. It is then dehydrated in 70%, 100% and 100% ethanol steps, changed to trichloro-trifluoro-ethane (Freon 113), air-dried and sputter coated for SEM (Fig 8).

2) Has resorption occurred yet?

Whether this question can be answered by LM of the live cultures depends upon the extent of resorption and the thickness of the substrate. For slices >200μm, resorption needs to be quite advanced before it can be spotted. The earliest resorption stages are therefore most easily spotted in the SEM specimen whether examined by SEM or reflected light.

The sample is either (a) treated with a water jet to wash off most of the cells, dehydrated and dried from Freon 113, or (b) treated with a 50% dilution of concentrated sodium hypochlorite for 10 mins, washed in distilled water, dehydrated and dried as above.

These procedures allow SEM examination of a sample within 15 mins. Procedure 1 can be followed by procedure 2(a) or 2(b) on the same sample, with the advantage that the prior location of all cells can be seen in the shadow image made by the first metal coating (Fig 9); matching images of prior cell distribution can be made by (transmission or) reflection LM (Boyde et al, 1984). Removing cells with a water jet has an added advantage in that cells which have very great adherence to the substratum, such as resorbing osteoclasts, are hardest to dislodge and may be uncovered and identified functionally by this means.

Similar preparations may be used for light microscopy, as will be discussed in later sections.

LM Methods

Vital Microscopy The main aims of this study were to attempt vital microscopy of resorption in vitro and to investigate the extent to which LM methods might displace SEM as the prime means of measuring resorptive activity at the termination of an experiment. Vital microscopy is easy with thin specimens, and routine filming or time lapse video is simple if the sample is thin enough to allow a good phase image. However, the starting specimen is a 200μm thick dentine slice in sterile tissue culture medium, with a mixture of bone and bone marrow derived cells, amongst which are the large, multinucleated osteoclasts. Clumps of other cell types have to be distinguished from possible osteoclasts on morphological grounds: thus the problem is not just as simple as finding the cells on the substrates.

1) Normal, transmitted LM with brightfield illumination gives contrasts from too many origins. Most biologists who are used to working with inverted LMs to monitor cell distributions on transparent substrates are content with either normal or phase contrast images. In our case, however, the inverted microscope is not particularly advantageous because the dentine test substrates would also have to be inverted, thus potentially squashing the cells against the bottom of the dish or culture chamber and in any event restricting the access of medium to the cells; or the cells would have to be disturbed each time they were observed. A dentine section in water is not perfectly transparent, and the optical information derived from variations in the distribution of the dentine tubules and degree of mineralisation of the intertubular dentine matrix often overwhelms the image of the cell on the surface. However, previously dried substrates become more transparent with time in the medium, and extensive resorption can be provisionally identified using an inverted specimen in an inverted LM.

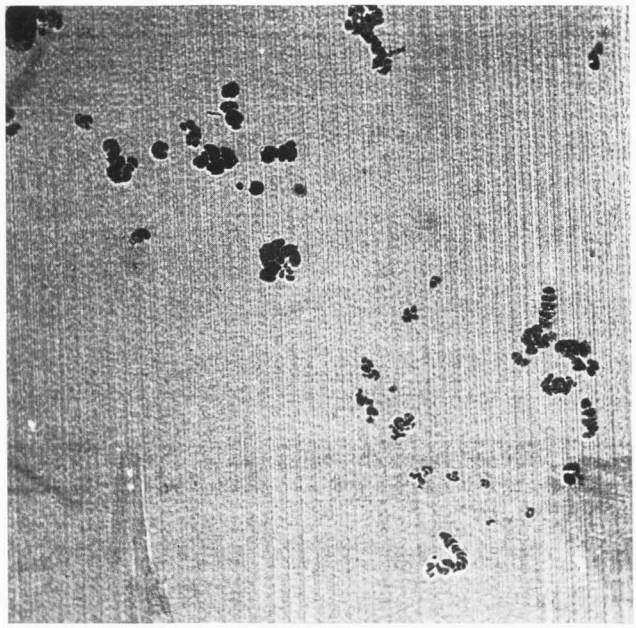

Figure 1: BSE image of resorption lacunae caused by rabbit osteoclasts in the diamond-sawn surface of a sperm whale dentine slice. FW (Fieldwidth) = 1130μm.

2) Interference LM: the measurement of the change of optical path length due to resorption can be monitored by ILM. However, the specimens are so large that the reference beam must be made to pass through a completely separate reference system as in the Mach-Sehnder interferometer. We have made

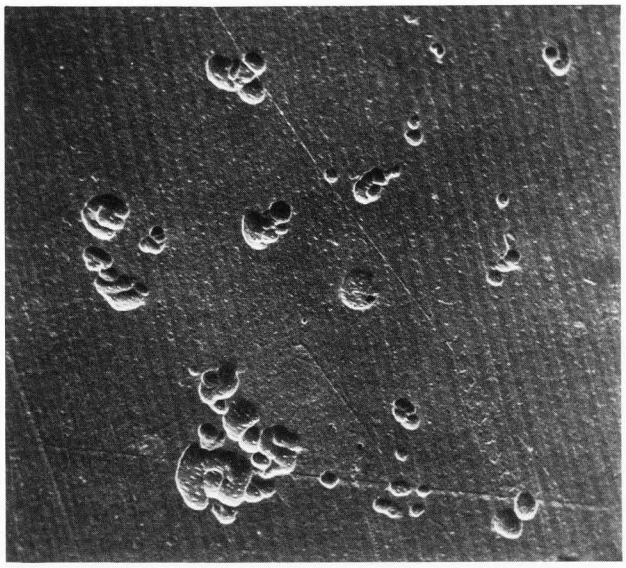

Figure2: SE image of rabbit osteoclast resorption lacunae in sperm whale dentine: 4 days in culture. FW = 450µm.

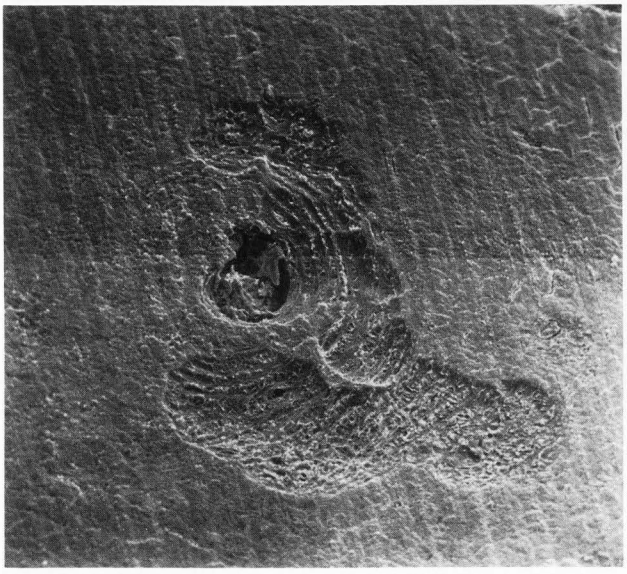

Figure 3: SE image resorption patch made by rabbit osteoclasts in human femur bone slice in 48 hour culture. Just to left of center is a Haversian canal - a space in the original bone structure. The presence of such spaces would make automatic image analysis very difficult. FW = 210µm.

trial observations with the Leitz **Horn** interferometer microscope, which show that, although this instrument is difficult to set up and tricky to use, one can make images from which the volume of a uniform composition substrate removed can be calculated. Each fringe represents one half wavelength of optical path difference, which for green light translates roughly into

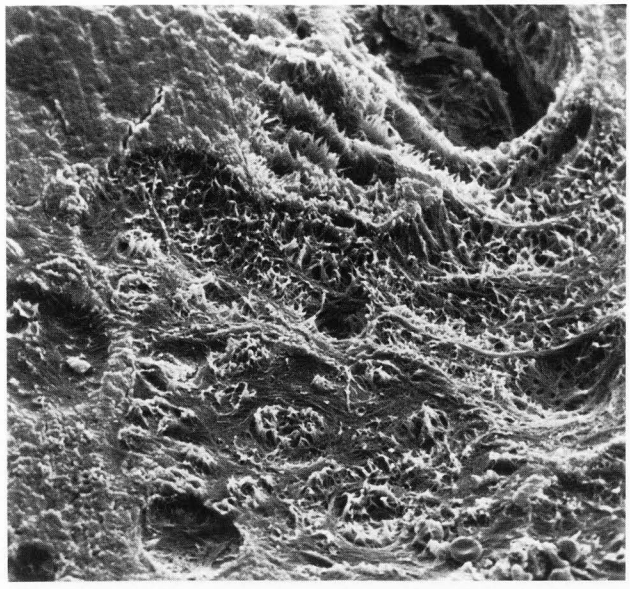

Figure 4: Higher magnification view of osteoclast lacunae in human femur. The different texture in the resorbed areas results from the structural features in the bone. For example, differently oriented lamellae, towards top center: Sharpey fibers, towards bottom center; osteocyte lacunae, of which there are 3 in this field. 48 hour culture. FW = 100µm.

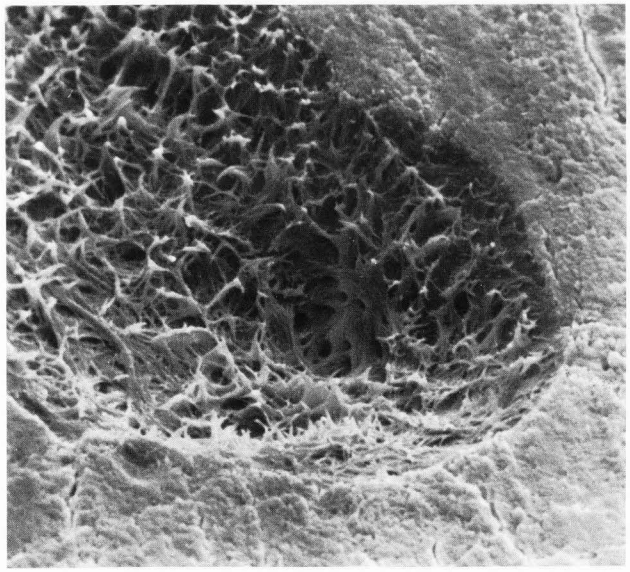

Figure 5: Higher magnification view of resorption lacunae made by rabbit osteoclasts in human femur bone, showing the presence of an original osteocyte lacuna at the center of the field of view. 48 hour culture. FW = 53µm.

1.5µm of sperm whale dentine. It is difficult to trace and count the fringes even in a photograph, and more research will be necessary before we can conclude that this will be a method suitable for routine experiments.

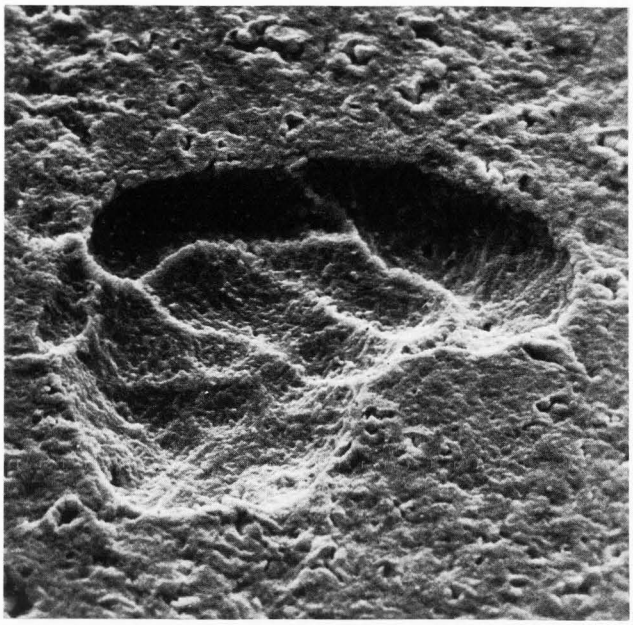

Figure 6: SE image of resorption lacuna in sperm whale cementum. An organic sample, in which both demineralised collagen and cells have been removed. FW = 55µm.

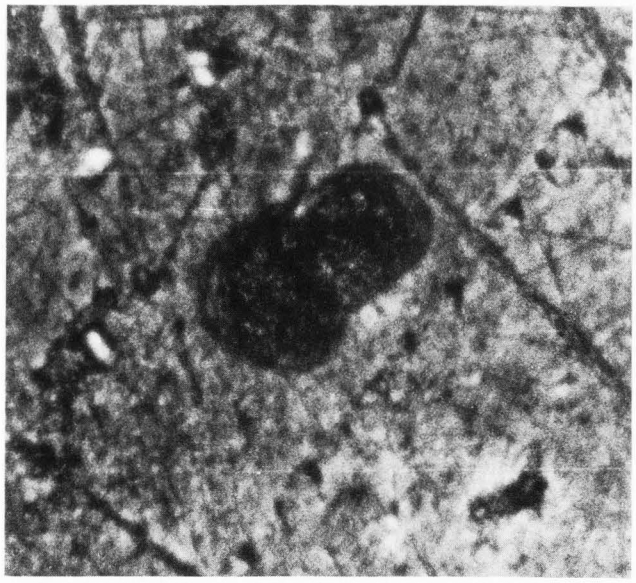

Figure 7: Transmitted brightfield light micrograph of sperm whale dentine surface prepared by grinding between glass plates. A resorption lacuna is at center. This method of preparing the surface prior to the experiment is unsatisfactory. Various gouges in the surface could be misinterpreted as resorption lacunae. FW = 175µm.

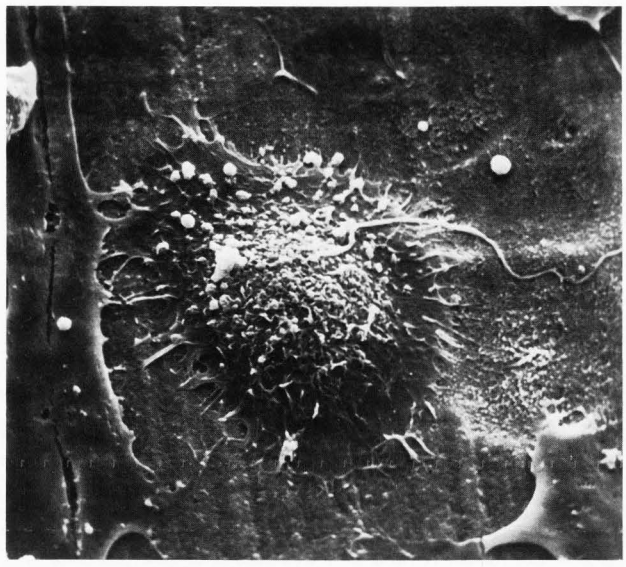

Figure 8: Rabbit osteoclast on sperm whale dentine prepared by GA fixation and air drying from Freon 113. FW = 43µm.

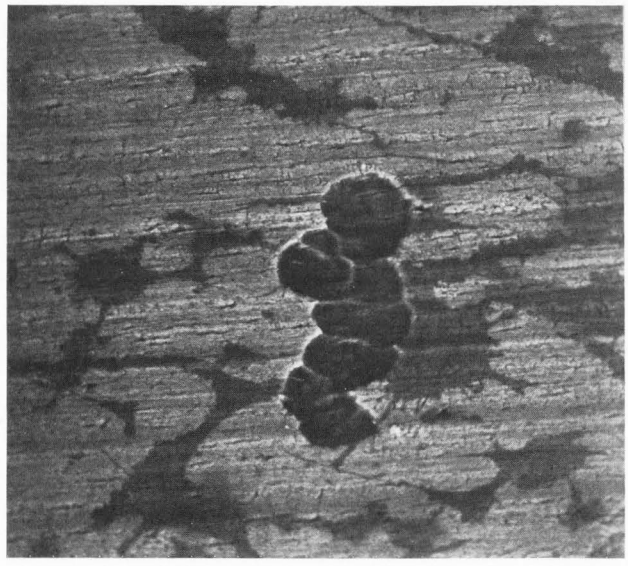

Figure 9: Sperm whale dentine resorbed by rabbit osteoclasts. First gold coated; then the cells were removed with a water jet and 5 mins treatment with an NaOCl solution, then lightly overcoated. This BSE image shows the "shadows" caused by the cells as density variations due to changes in the thickness of the gold coating FW = 185µm.

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3) Phase contrast (PCLM): The phase relationships necessary for phase contrast light microscopy to work are destroyed by specimens anything like as thick as the dentine slices which we have otherwise found so convenient for **post hoc** study using SEM methods. Thus attempts to use PCLM result in the formation of a less satisfactory, ordinary transmitted LM image.

4) Transmitted darkfield: using a central stop in the illuminating condenser made it possible to visualise cells on dentine substrates, but not with sufficient clarity to distinguish osteoclasts from incidental, large cell clumps.

5) Transmitted brightfield. In the case of large, advanced resorption lacunae, they could be detected if they were placed on the side of the dentine slice towards the objective. We were unable to demonstrate cells or resorption lacunae on dentine substrates when using an inverted LM, with the dentine specimen the right way up, i.e., having to look through the entire specimen thickness, with the specimen covered with tissue culture medium and a coverslip or Petri dish lid.

6) Reflected light-darkfield: using an inverted light microscope, we illuminated the specimen with an oblique beam at about 70 degrees to the optic axis on to the surface of the dentine slice (Fig. 10). This provided a reflection image of cells proud of the substrate surface. By colour filtering this light and simultaneously using a contrasting colour in transmitted illumination, we found that we could make images in which it was possible to see both cells and resorption lacunae, obviously best if the resorbing side was towards the objective.

7) Vital staining including fluorescent probe methods:

(a) Neutral red (Barnicot, 1947) Osteoclasts selectively accumulate neutral red. We used this substance in a one-part in 10,000 dilution in tissue culture medium (Fig. 11). Osteoclasts stained well within 5 mins (Fig. 12). We could then transfer the cells back to the medium without stain, when they could be studied for periods of hours, and restained if necessary. Osteoclasts visualised in this way have been found to survive for at least one week.

(b) DASPMI (Horster et al, 1983) is a fluorescent probe, excitation 455-499nm, recorded 520nm, which stains mitochondria. In practice it shows the distribution of cell mass rather nicely, with nuclei showing against the brighter cytoplasmic background.

(c) Bisbenzimid . We have previously described the use of the DNA fluorescent probe, bisbenzimid, in a combined LM-SEM study of osteoclasts in rat calvaria (Jones et al, 1983; Boyde and Jones, 1985). This dye was used as a 0.01 - 0.1% solution in tissue culture medium or saline. It can also be used to stain fixed preparations. Osteoclasts are distinguished by the clustering of nuclei and the shape of their nuclei and nucleoli.

(d) Methylene Blue. We used methylene blue as a vital stain for all cell types on the substrate surfaces. We found that it was actively taken up by all live cells, but more by the cells which did not stain with neutral red. Cells in cultures which were allowed to die, or caused to die by selectively and excessively illuminating one area of a specimen, did not take up the methylene blue. Methylene blue and neutral red could be used in combination to assess the relative numbers of cell types other than osteoclasts, and to visualise the numbers of vital cells. Preparations stained with either of these stains had faded at 24 hours post-staining but could be restained. Some specimens have been restained every day for 5 days, allowing their continued vitality to be monitored.

8) Tandem scanning reflected light microscopy (TSRLM):

We have used the new tandem scanning reflected light microscope (TSRLM - TSM Model 1983, Sluzba Vyzkumu, Praha, Czechoslovakia) in an attempt to visualise surfaces of calcified tissue substrates. The TSRLM provides an image produced only with light reflected from the layer upon which it is focussed (Petran et al, 1968; Boyde et al, 1983b, Boyde, 1985, Petran et al, 1985).

The TSRLM has provided us with a method in which we can visualise cells, whose nuclei appear as bright reflective features, and resorption lacunae, which appear darker than the surrounding unresorbed surface, in unfixed and unstained living specimens. The distribution of the cell bodies of osteoclasts is better demonstrated by ordinary reflected oblique illumination from the substrate side of the specimen. The distribution of internal features cannot be seen by normal reflection LM using the same specimens and objective lenses.

Methods for light microscopy of the fixed sample The LM methods which we found to be unsatisfactory for living specimens are equally useless with the fixed specimen. Thus normal bright and darkfield, transmitted and reflected light microscopy of the unstained specimen do not contribute: too little reflection occurs at the specimen surface. If the specimen is fixed, however, there is no objection to any of the usual staining and coating procedures used for LM and SEM respectively and by this we can profit greatly.

1) Staining for routine LM: Most usual light microscopic staining procedures can be employed to enhance the visualisation of cells on bone and dentine surfaces, if the stains do not interact directly with the substrate. This is not a problem for most reagents since the densely mineralised collagenous matrix is not very reactive: most normal histology is done with demineralised tissue when the reactive groups are uncovered.

Toluidine blue. After glutaraldehyde fixation, the samples are stained for 15 secs with a 0.1% solution in water, and examined in water: (or washed, dehydrated and mounted in balsam or DPX). All cell nuclei are clearly visible at this stain density (Fig. 13). Increasing the staining period to 2 mins or more makes it possible to see both cytoplasm and resorption lacunae more easily (Figs. 14 and 15). The staining within osteoclasts is sometimes so dense that it is no longer possible to count the osteoclast nuclei - which is a major objective of this investigation since we wish to be able to measure the volume of tissue resorbed as a function of the number of nuclear units in the osteoclast.

The fact that resorbed areas of the surface react with toluidine blue is probably due to the demineralisation of the dentine matrix caused by the osteoclasts (Fig. 16).

The most convenient means of studying the stained substrates is to use an inverted LM, with the specimen inverted: the sample surface then levels itself perpendicular to the optic axis and it is not necessary to tilt the section, if, as may be the case, it is wedge shaped.

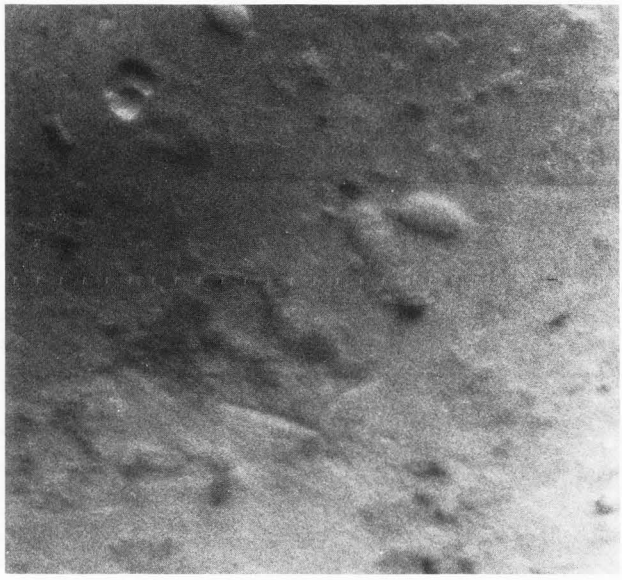


Figure 10: Sperm whale dentine with live cells on the surface photographed by oblique (darkfield) reflected light. Note that resorption lacunae could possibly be confused with cells standing proud of the surface if the direction of illumination were not taken into account. FW = 285µm.

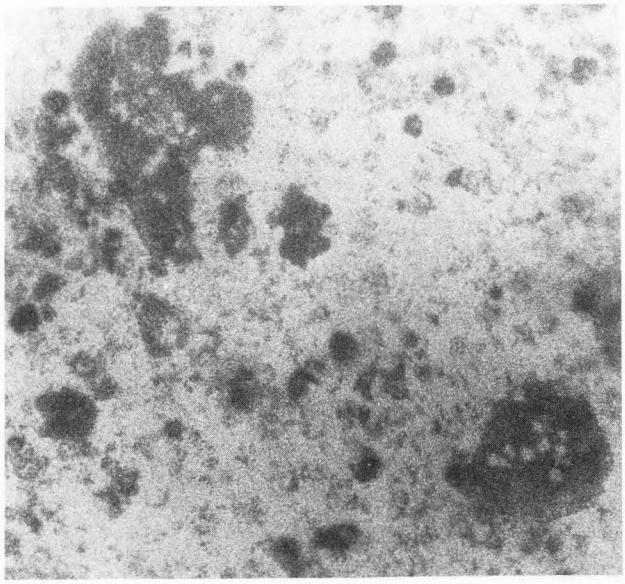


Figure 11: Rabbit osteoclasts on sperm whale dentine stained with the vital dye, neutral red. Spaces occupied by the nuclei appear pale. FW = 200µm.

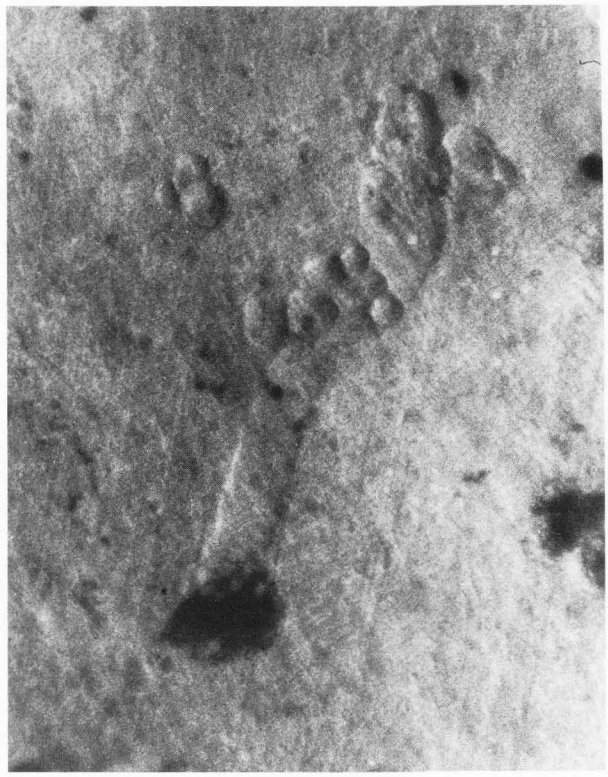


Figure 12: A single osteoclast, now stained with neutral red, had moved across the surface of this preparation in a 48 hour culture period, causing the long resorption track seen above and to its right. FW = 200µm.

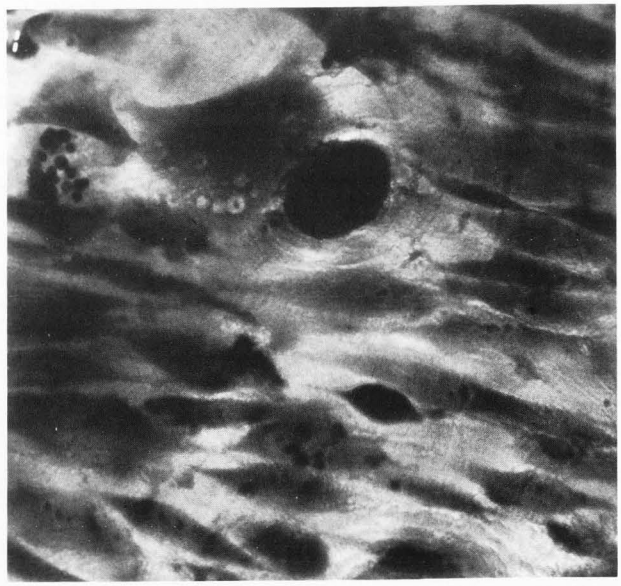


Figure 13: Toluidine blue stained cells on surface of human femur bone slice after 48 hours in culture. An osteoclast next to a Haversian canal can be identified at left. FW = 200µm.

Toluidine blue staining for LM thus constitutes an excellent method for monitoring the end result of the resorption process. Cells and Howship's lacunae can be seen and the latter measured for areas by projecting an image on to a digitizing pad and tracing, and for depth by through focussing with a high NA objective. Early resorption of dentine can be detected by the enhanced staining of the dentine tubules in the resorbed area.

2) DNA fluorescent staining: use of the bisbenzimid DNA stain also constitutes a convenient means of counting osteoclast nuclear number by fluorescence microscopy.

3) Simple silver staining techniques: reveal the location of resorption lacunae in the dentine test substrates (Fig.17). These methods will work with the cells still in situ, but are more selective and more practically useful if all the cells are first removed with a water jet. The best procedure is to "Water-Pik" (trade name) the unfixed sample with distilled water, since we found that actively resorbing osteoclasts were not removed by water jet or ultrasonication from glutaraldehyde fixed samples. Distilled water is used to remove chloride ions which would react with silver ions in the next step, which is to immerse the sample, resorbed side up, in a 0.1% solution of silver nitrate in distilled water for 3-10 mins under U-V illumination.

The specimen is washed extensively with distilled water at the end of this period. Excellent contrast is obtained between resorbed surfaces and the unaffected cut surfaces. However, within the resorbed areas, the staining intensity varies from yellow, to orange, or brown.

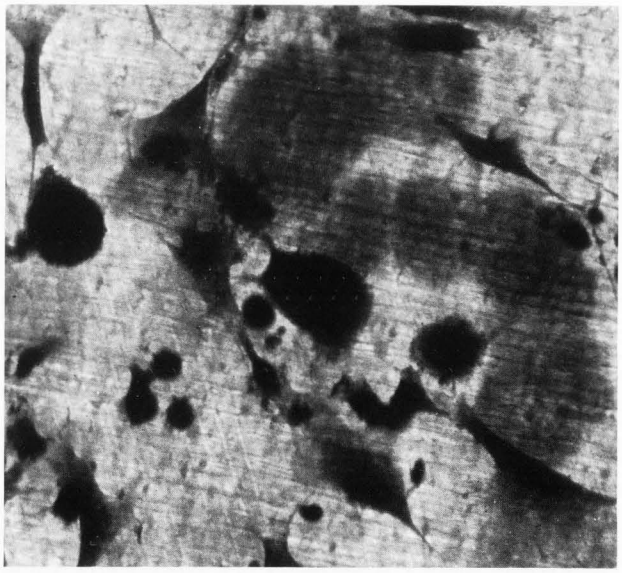


Figure 14: Toluidine blue stained preparation of rabbit cells, including osteoclasts, on sperm whale dentine. Osteoclasts in this overstained preparation are the much darker patches. The string of dark (grey) patches represent a snail track resorption lacuna caused in the 48 hour culture period. FW = 200µm.

These variations in staining intensity correlated with similar variations seen in toluidine blue stained samples when we removed the toluidine blue with ethanol, and then restained with silver as above.

The colour developed by the silver staining could be further intensified by immersing the sample (after extensive washing) in a 0.16% ammonium sulfide solution for a few seconds, followed by washing: this converts metallic Ag to black AgS and the colour reaction of the resorbed area from yellow, through brown-orange to very dark brown or black (Fig.18). We have been able to show that the darkest stained areas are those which show a significantly thick layer of demineralised collagenous matrix by SEM (Fig.19: Jones et al, 1985). These Ag or AgS staining methods therefore enable us to distinguish active resorbing sites in resorption lacunar patches from those which have been previously active.

Figure 15: Human femur slice on which rabbit osteoclasts had been cultured for 48 hours. Notice details of Haversian canals, and Volkmann's canals (running in the plane of this transverse section); also osteocyte lacunae - all tending to confuse the recognition of real osteoclastic lacunae and nuclei of other seeded cells on the section surface. The remaining osteoclasts are very densely stained. Such an image would be very hard to handle automatically. FW = 200µm.

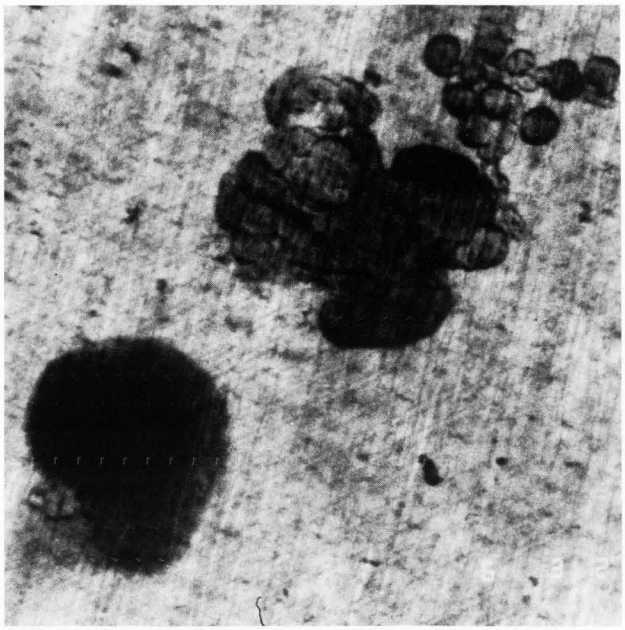


Figure 16: Higher magnification view of toluidine blue stained sperm whale dentine resorbed by rabbit osteoclasts showing the opening-up of dentine tubules at the base of the resorption lacunae. FW = 200µm.

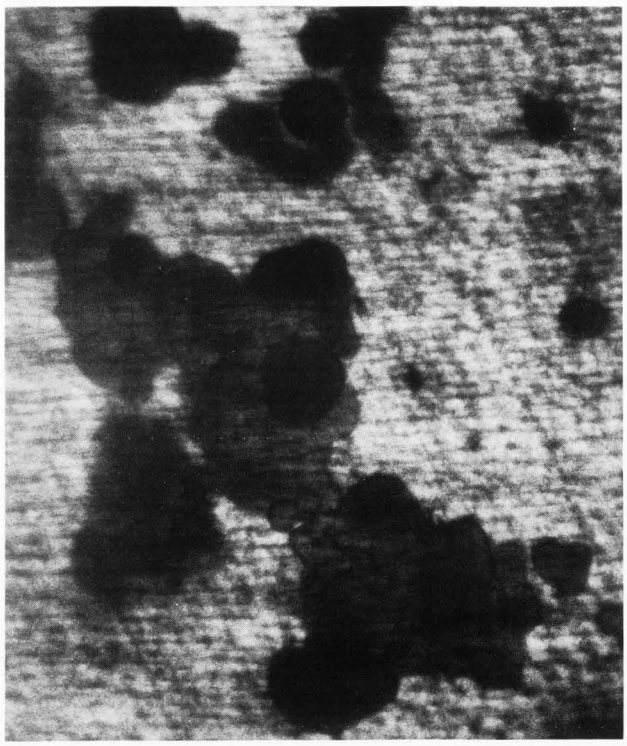


Figure 17: Silver (nitrate) stained surface of sperm whale dentine resorbed by chick osteoclasts. Note the variation in staining density of the different resorption bays. The cells were removed with a water jet. Culture period 3 days. FW = 200µm.

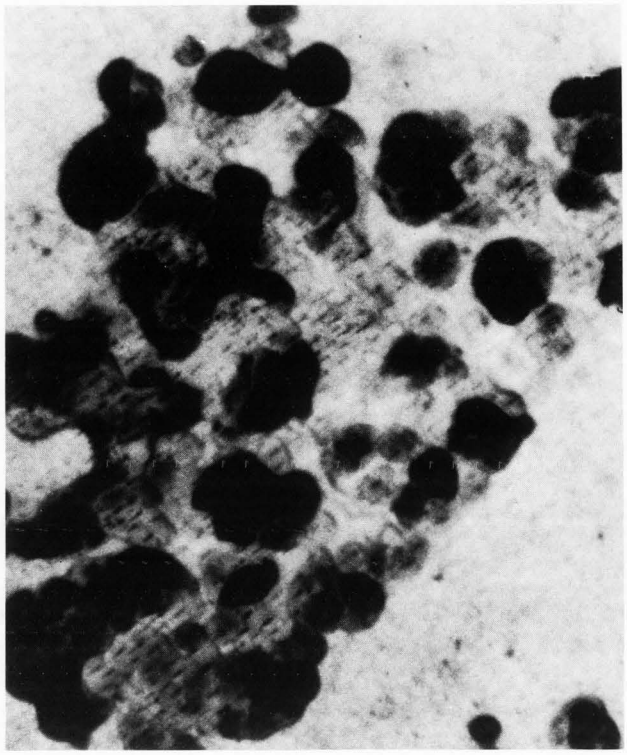


Figure 18: Resorption lacuna complex in sperm whale dentine caused by chick osteoclasts; stained with silver nitrate - the silver converted to its sulfide by ammonium sulfide. Note the great optical density of this reaction product. 4 days culture. FW = 200µm.

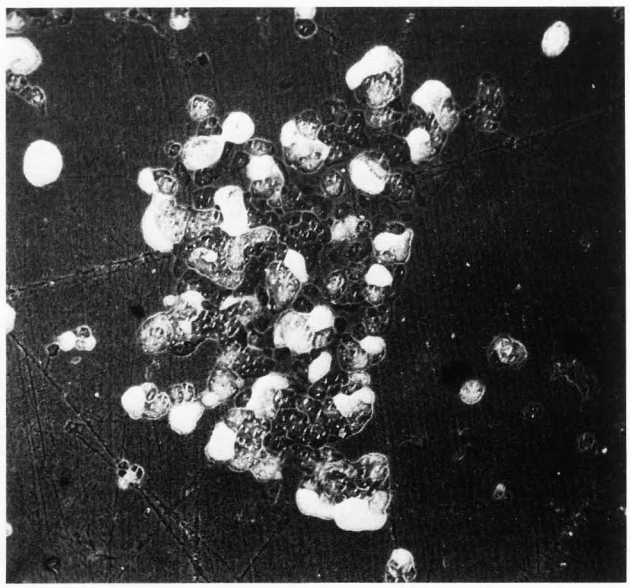


Figure 19: Silver (sulfide) stained surface of sperm whale dentine resorbed by chick osteoclasts. The most densely stained resorption bays appear bright in this BSE image. FW = 470µm.

4) The SEM specimen: Specimens prepared by CPD and metal coating as if for SEM are also excellent for reflected LM using the appropriate objectives calculated for no cover slip. The location of cells can be studied with simple epiillumination. If the specimen is coated with Au or Au:Pd with the cells in place, and these are then washed off with the Water-Pik or dissolved off in NaOCl, an excellent high contrast image can be obtained showing the prior location of the cells due to the strong reflection of light from the metal coating compared with the relatively very poor reflection from native dentine (Fig.20). This coating reflection contrast is so strong that it can also be imaged well in transmission LM if the coating side of the specimen is towards the objective. The contrast in this type of specimen preparation is ideal for image analysis of cell area and shape. However, the image does not provide contrast which recognises resorption lacunae from areas which were simply covered by cells including osteoclasts, and cell clumps on the substrate surface.

This "problem" can be turned to advantage as follows. As recorded above, fixed active osteoclasts (i.e., those in association with Ag staining areas by LM, fluffy demineralised collagen areas by SEM) cannot be removed by mechanical means. We can make preparations which show the area covered by cells by (1) CPD as for SEM (2) gold or other precious metal coating and (3) removal of the residual cells and disruption of their gold coating by hypochlorite treatment. Following such a sequence, the remaining holes in the gold coating represent the area covered by osteoclasts in intimate contact with the substrate.

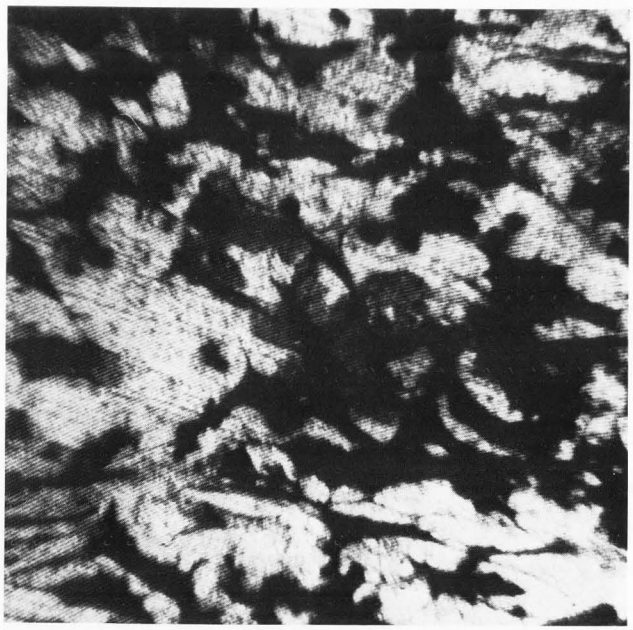


Figure 20: Tandem scanning reflected light micrograph of gold coated SEM specimen of partly resorbed sperm whale dentine from which the cells have been removed to cause the non-reflective, black patches. FW = 214µm.

The sample with a part - the part over the cells - of the first metal coating removed is not ideal for SEM as such, and we would normally give it an additional coating to prevent charging in the areas previously covered by cells. Particularly if the first coating is relatively thick, and the second thinner it is easily possible to recognise the cell shadowed areas in the BSE SEM image. It may also be possible to get sufficient reflective contrast difference to distinguish surface cell shadows from resorption bays, particularly as the contrast due to coating thickness difference is added to by the characteristic texture contrast of the resorbed surface.

Aluminium coating by evaporation gives even better reflective surfaces than Au or Au:Pd (which are nowadays most commonly used for SEM), and has several advantages for any contrasting mode dependent on reflection from the coating, i.e., whether this is observed by reflected or transmitted light.

5) TSRLM: This method gives excellent contrast between resorption lacunae and the surrounding unresorbed surface for all specimens which are observed water-wet, dry or under oil, and whether or not they are coated (Fig.21: see also Boyde, 1985, Figs.10 & 11). Naturally, the brightness of the image is improved by metal, especially by Al coating. Due to the comparatively very shallow depth of focus of the TSRLM the contrast due to resorption lacunae is always much greater than in normal bright or darkfield reflection microscopy and is usually sufficient to permit automatic image analysis. The reduced depth of field also makes it possible to measure the depths of the pits by through focussing (Figs.22 - 25), again also even in the uncoated specimen.

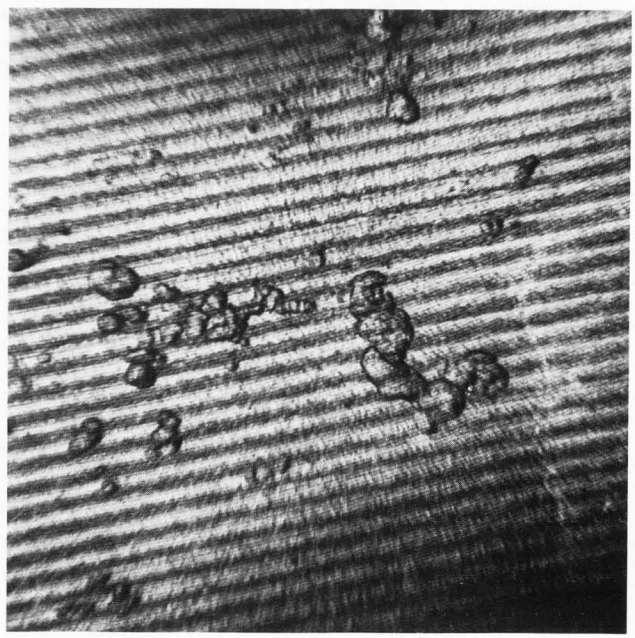
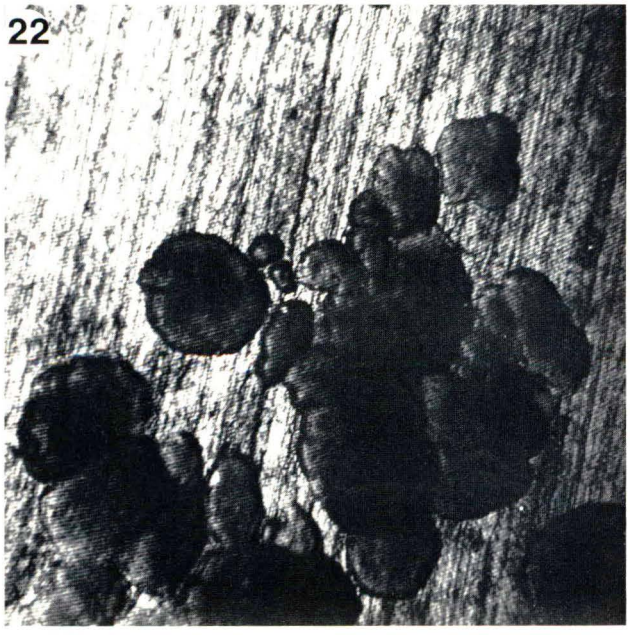
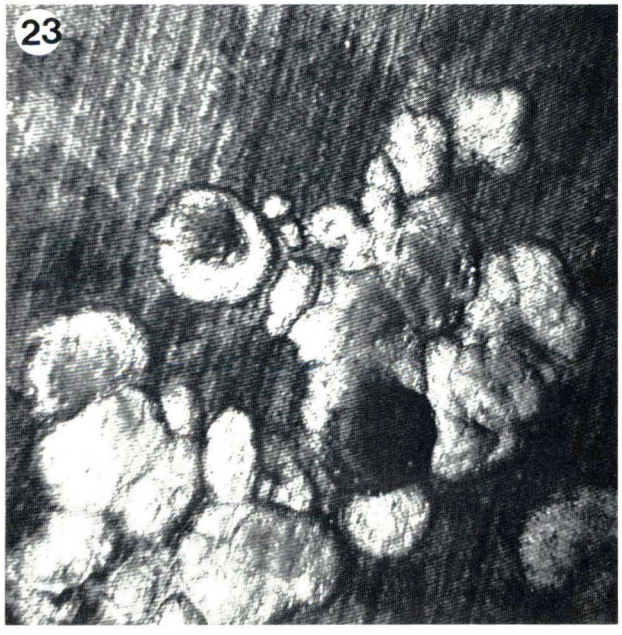
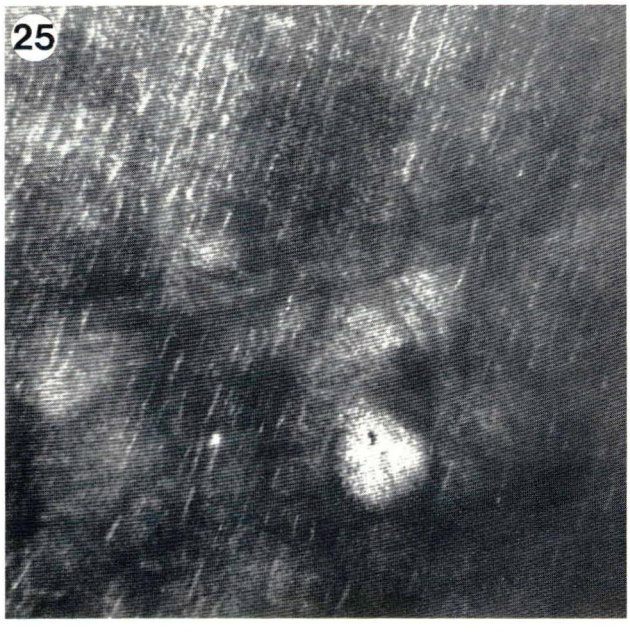
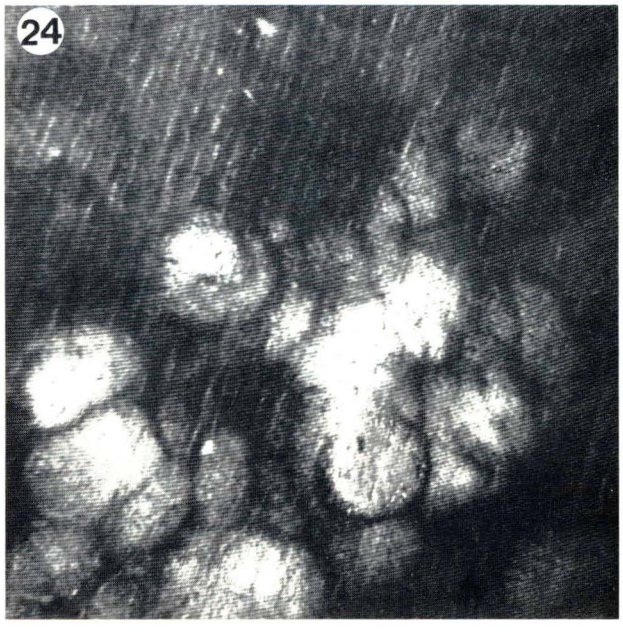


Figure 21: Low magnification TSRLM image (16X dry objective) of resorbed dentine test substrate showing high contrast for the resorption lacunae (made by chick osteoclasts). FW = 530µm.









Figures 22 to 25: Through focus sequence of TSRLM images of a resorption lacuna complex caused by chick osteoclasts in sperm whale dentine. The cells were removed and the sample prepared as for SEM examination.

Fig 22 is focussed at the surface of the dentine.
Fig 23 is focussed 3 microns deep.
Fig 24 is focussed 6 microns deep.
Fig 25 is focussed 9 microns deep, which is nearly at the bottom of the deepest bay.
Note the changes in contrast with focus. FW of each frame = 200µm.

Discussion and Conclusions

In previous studies of osteoclastic resorption lacunae (Boyde, 1968, Boyde et al, 1983a, 1984; Ali et al, 1984; Jones et al, 1985) the SEM has demonstrated great advantages. We have shown that it is possible to produce exact 3-D measurements of resorption pits by stereophotogrammetry of SEM stereopairs (Boyde et al, 1983a). However, if a particular problem requires a much larger biological sample size then measurement precision may have to take second place to an overview of the biological process.

The methodological studies reported here show that resorption lacunae and associated living cells can be imaged on dentine slabs without additional stains if one uses oblique illumination or the TSRLM. The latter method has the unique property of abstracting information from the focussed-on layer although one must take care lest the bright light may damage the live cells. Accepting the use of stains like neutral red, methylene blue and the fluorescent stain bisbenzimid, cells can be seen on the dentine slices, and the associated lacunae can be seen at the same time using oblique illumination LM. These methods will enable us to monitor the development of lacunae and the movement of the osteoclasts on a calcified collagenous substrate.

If the specimen can be examined after fixation, normal reflection LM with contrast enhancement due to careful selection of the coating technique and the time at which the coating is applied, allows far more rapid scanning of the samples. In this respect, darkfield RLM is better than brightfield RLM, but TSRLM has great advantages over any other LM method.

In conclusion, optical microscopy thus proves to be a valuable complement to SEM in the type of resorption assay studies which we wish to conduct. The advantage of using the relatively homogeneous dentine rather than compact bone for the resorption test substrate was confirmed using light microscopy.

Acknowledgements

These studies have been supported by grants from the Medical Research Council, the Science and Engineering Research Council, and the University of London Central Research Fund. Interference microscopes were kindly made available by Dr E Mendez. We are grateful for the technical assistance of Elaine Maconnachie and Roy Radcliffe.

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Discussion with Reviewers

Gary B. Schneider: The proposed techniques are being developed to investigate osteoclastic function. Is the smooth collagenous substrate provided by the dentine slices an appropriate model to study bone resorption?

Authors: The topography of the surface does not seem to affect osteoclastic resorption, but does make a difference to the ease with which we can identify and measure it. We have found that dentine and bone show very similar patterns of resorption in vitro. The great variation in mineralization in different osteonal regions of bone slices (see, for example, any BSE image of adult bone, or Boyde (1984) or Jones et al (1985)) means that this should be accounted for in any study involving measurement of plan area or volume; this problem is minimized by using dentine.

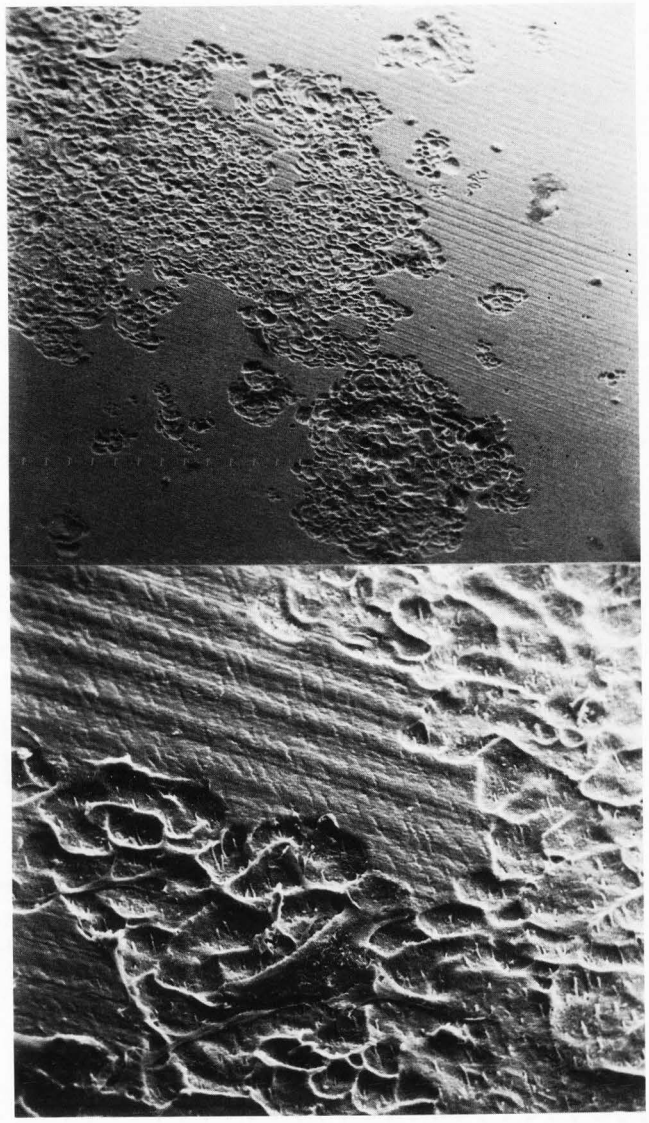
Mojmir Petran: The discussion of interference methods should include other devices (e.g. Microinterferometer by Linik, 1935) which are not affected in their function by the overcrowding of the image field.
Authors: Following your suggestion, we have now tried a Linik reflection micro-interferometer with no success.

Gary B. Schneider: What precautions do you take to avoid damaging cells by the bright light used in TSRLM?
Authors: The light in the TSRLM need not be particularly bright: we use short exposure periods and a low light level (image intensifier) TV camera.

E. Wisse: Osteoclasts have a true macrophage nature and their task seems to be the digestion of bone matrix. In your experiments you seem to combine materials and cells from various, sometimes pretty exotic origin. The question I want to ask you, is whether rat osteoclasts digest rat bone in a similar way as chicken osteoclasts digest whale or rhinoceros dentine.
Authors: The unique ability of osteoclasts is their facility to mobilize the mineral component of calcified tissues.. Yes, rat osteoclasts will mobilize rat bone as well as avariety of other biological and non-biological substrates (Jones et al, 1984).

Gary B. Schneider: Just because an osteoclast is firmly attached to the substrate, does this really provide an indication of functional activity? The cell may be firmly attached but not actively resorbing substrate at that given time.
Authors: Resorbing osteoclasts both in vitro and in vivo have long microvilli outside the clear zone of the resorptive site which are firmly attached to the substratum. In fixed tissue, in particular, this region and the ruffled border are very resistant to their attempted removal. Osteoclasts not overlying resorption lacunae are much easier to remove. Figures 26 and 27 show that the actively resorbing cells are also not removed by trypsinisation.

E. Wisse: Could you extend a little your recipe for harvesting osteoclasts? Suppose one would be interested in harvesting osteoclasts, it would possibly not be successful to just mince long bones, like you describe in Materials and Methods. Briefly, what else is needed to get the cells out?
Authors: We clean the bones of adherent tissue, including periosteum, cut away the epiphyses and mince the bone with a scalpel blade. (Marrow can be first picked out from lengthwise sliced bone if a purer population is required.) The resultant bone debris is mixed with the medium by rigorously sucking it up and down in a large-barrelled, smooth-ended pasteur pipette to release more osteoclasts. After a brief settling period (about 30 minutes), non-adherent cells are washed off and the substrate with attached osteoclasts is placed in fresh medium.



Figures 26 and 27: Sperm whale dentine resorbed by chick osteoclasts from a marrow fraction - 8 day culture. The specimen was trypsinized to remove the cells, which selectively leaves behind a reduced population of osteoclasts which are better attached to the surface. The remaining cells are those which are in an active phase of resorption.
Fieldwidths Fig. 26 = 1mm, Fig.27 = 200µm.

E. Wisse: Has anybody tried to develop an "artificial" thin layer, composed of collagen and hydroxyapatite, in order to circumvent the problem of making thin slices of matrix material, digestible by osteoclast? Such a layer could be designed to optimally fit the best microscopy method. It is not difficult to make thin layers of collagen (we use them to culture liver endothelial cells), and these might quite easily be saturated with calcium and phosphate.
Authors: We are currently developing and testing "artificial" layers for just this purpose in collaboration with N. Maroudas.