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REPLICATION TECHNIQUE FOR STUDYING MICROSTRUCTURE IN FOSSIL ENAMEL

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Abstract

The present paper describes a two stage impression technique using a silicone elastomer suitable for field replication studies; which requires high dimensional stability, defined resolution, and being capable of reproducing inaccessible details. A test object consisting of etched pearlite possessed fine detail [>1μm to <0.1μm] which was suitable for testing negative/ positive replica combinations. Coltene President light-body impression material was capable of resolving parallel side depressions of widths ≧0.2μm, and it possesses very good dimensional stability with time, allowing the production of positive casts to be deferred for several months. Low viscosity Spurr resin reproduced this detail, and flowed into inaccessible sites.

Although there was evidence of bulk contraction on curing, there was no significant shrinkage on flat [linear] surfaces or in vertical relief dimensions.

Replicas of fractured hominid teeth showed good surface detail, and reproduction of inaccessible three-dimensional features on enamel surfaces. Enamel prism shape was pattern 3.

Key Words: Hominid teeth, fossil enamel, microstructure, replication technique.

Introduction

The principal reason for the use of replication techniques in paleontology and anthropology are that the original specimens are too valuable to study directly in the SEM, with the further advantages that selected areas of large objects can be examined easily [Pfefferkorn and Boyde, 1974], and also inaccessible areas which cannot be visualised directly on the original can be observed. Complete bibliographies on earlier replication techniques are given in Pfefferkorn and Boyde [1974] and subsequently by Pameijer [1978] and Barnes [1978, 1979]. A replica is the production of a copy of the original produced by reproduction procedures. The first reproduction is a negative replica or impression, which may be replicated again giving a positive copy, which must be dimensionally accurate, and capable of resolving fine surface detail.

Grundy [1971] was the first to use silicone-based dental impression materials for the negative impression, and an epoxy resin to create the positive replica. Numerous authors, including Al-Hamadani and Crabb [1975], Barnes [1978, 1979], Shipman [1981], Scott [1982] and Rose [1983], have tested replica combinations of different dental impression materials and casting resins to assess their value in reproduction of surface detail in various applications. These studies, however, used only qualitative criteria in their assessment of the replication technique.

Quantitative assessments of resolution using physical tests have been employed by very few investigators. Schoen et al [1978] used a surface contact profilometry method on negative replicas of a serrated reference specimen with a periodicity of 100μm, and a peak height of 10μm, using dental impression materials. Kusy and Whitley [1985] employed non-contact profilometry techniques using laser scattering on negative and positive replicas of gratings with 1200 and 2400 lines/mm with nine impression materials and four epoxy resins, together with capillary flow tests. They concluded that addition-cure silicones, in particular Reprosil light, were capable of replicating detail of less than 1μm. Pameijer [1978], using replicas of metallographic scratched specimens, described a resolution of down to 0.17μm and Bromage [1985], using cleaned predentine, claimed a resolution of 0.14μm, although the collagen fibrils of these dimensions were not clearly resolved on the positive replica [his Fig. 2B].

There remains a need for an accurate impression technique suitable for field usage. Principal factors relevant to this application are impressions with high dimensional stability, which enable the preparation of the positive cast to be deferred if necessary until return to the resident institution; with defined resolution; capable of reproducing inaccessible details within undercuts; which can be used in hot climates. There is also a requirement for a simple resolution standard which can be used to assess the resolution of the positive replicas.

The purpose of the study was to: [1] test a 2-stage negative impression material for dimensional stability with time, and of the dimensional accuracy of the positive replica; and to construct a test specimen suitable for resolution tests which is widely available; and [2] present examples of dental tissue ultrastructure in replicas of fractured fossil hominid teeth from East Africa.

Materials, Methods, and Results

Replication Methodology

Replica techniques. Impression material [negative replica]. An addition-reaction polymethylvinylsiloxane silicone [Coltene President; Coltene SA, CH-9450 Altstatten, Suise] material was used in a two-stage impression technique.

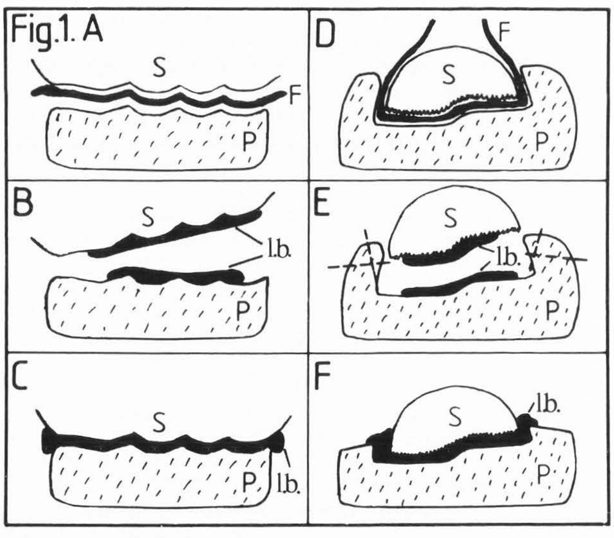

Figure 1 : Preparation of two-stage impression.
1A,D. Specimen [S] with foil [F] closely adapted to its surface, pressed into putty [P] base.
1B,E. Specimen and putty mould with thin layer of light-body impression [l.b.] injected onto both surfaces. Undercuts on putty impression trimmed [1E]
1C,F. Specimen bedded into putty mould under firm pressure.

The first stage used a putty elastomer [Coltene Putty], with a layer of aluminium foil of 50-100μm thickness closely adapted to the specimen to provide a spacer between the specimen and the putty base [Figs. 1A, D]. Equal quantities of putty base and catalyst are mixed by kneading, which hardens in approximately 6 min at 23°C. The foil provides a limited space for the second stage impression material, and also serves to prevent porous specimens becoming impregnated with monomer. The putty mould can be trimmed after hardening to eliminate undercuts [Fig. 1E]. Light-body base and catalyst material is mixed with a spatula on disposable pads [working time 3.5 min at 23°C], introduced into a syringe and a thin layer injected onto the surface of the specimen, and into the putty mould [Figs. 1B, E]. The specimen is bedded into the mould under firm pressure, forcing impression material into inaccessible regions with excess being extruded around the specimen margins [Figs. 1C, F]. President retarder is available to extend the working time, which is particularly useful in a hot climate. Specimens are released by flexing the putty mould. Relevant physical properties of Coltene light-body impression material are:- maximum extension to fracture 90%; tensile strength 2.26MPa; viscosity 44±4PaS

Casting resin [positive replica]. A low viscosity [ca 60PaS] vinyl cyclohexene dioxide epoxy resin of medium hardness composition [Spurr, 1969] was principally used. It was convenient to make up relatively large volumes [about 40ml] of the resin at one time, which could then be subdivided into aliquots of 5ml, for immediate use or stored frozen at -20°C until needed. Aliquots were thawed, and prewarmed together with the negative impressions at 50°C for 15 min. The negative impression was tilted, and resin was run into the impression using a syringe, avoiding entrapment of air bubbles. The resin was cured for 72 h at 50°C.

Earlier specimens, including the fossil replicas, were sputter coated with gold for approximately 2 min, divided into short bursts [Barnes, 1978], in a Polaron E5000 coater at 20mA and 1.2kV. Later specimens including Pearlite replicas were coated initially with ca 1.5nm carbon, in a 'cool' Polaron E5100 coater, followed by gold at 15mA and 1.00kV continuously until a thickness of >10-20nm was deposited.

Bromage [1985] reported that R. S. Epoxy Adhesive [R. S. Components Ltd, P.O. Box 99, Corby, England, NN16 9RS] which is a quick-setting domestic adhesive, was particularly suitable for replica studies, and this resin was also tested using the Coltene President impression material using his protocol.

Impressions of the Pearlite test object were also made using Reprosil putty and light-body impression material [Kusy and Whitley, 1985].

Dimensional stability tests. Impression materials [negative replica]. The manufacturers of Coltene President claim a dimensional stability of 0.13% shrinkage for putty and 0.14% for light-body.

Casting resins. [a] Spurr resin undergoes a volumetric contraction of 5% following curing [Causton, 1981], which suggests that a linear

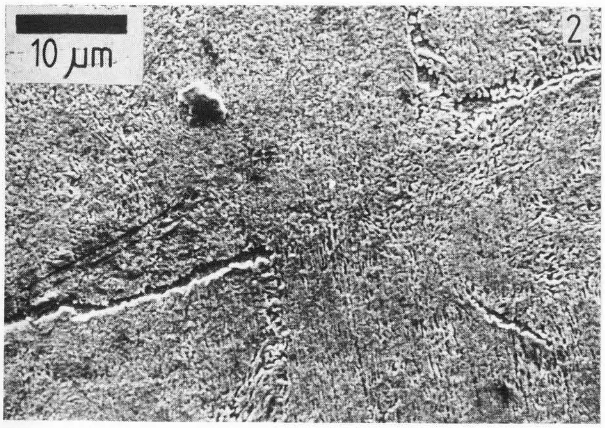

Figure 2 : Survey view of etched lamellar pearlite.

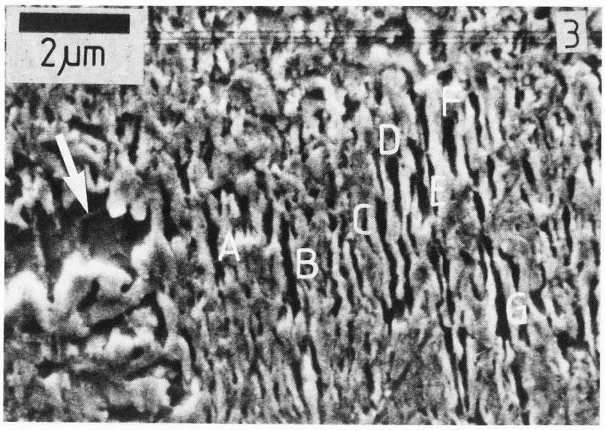

Figure 3 : Detail of central region of Fig. 2 of etched pearlite. Note L-shaped structure [arrow] which is ≦ than 0.1µm high in the floor of broad depression. The width of the labelled [A - G] etched depressions is ≦0.2µm.

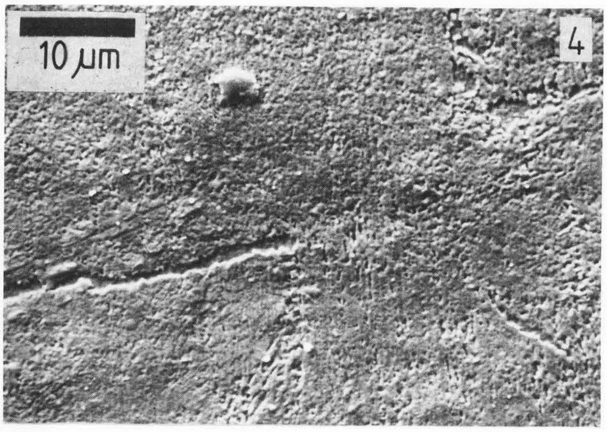

Figure 4 : Survey view, Spurr replica [President light-body impression]. Compare with Fig. 2.

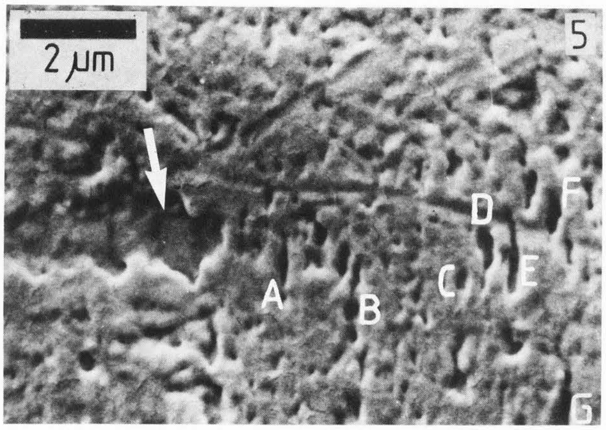

Figure 5 : Detail, Spurr replica. Compare with Fig. 3. Note L-shaped structure in broad depression has been replicated [arrow], together with other low relief details. The individual labelled [A-G] etched depressions have been replicated with varying completeness.

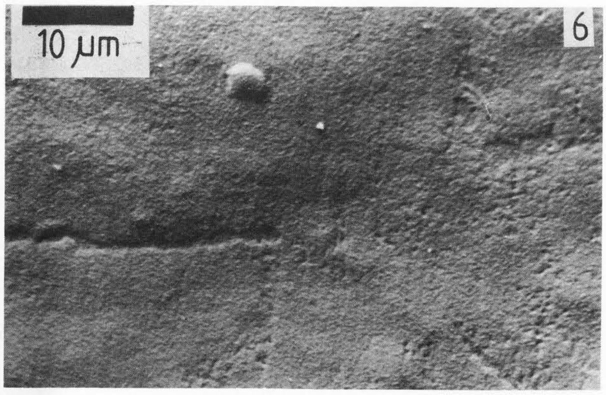

Figure 6 : Survey view, R. S. Epoxy replica [President light-body impression]. Compare with Fig. 2. General details of broad depressions have been replicated.

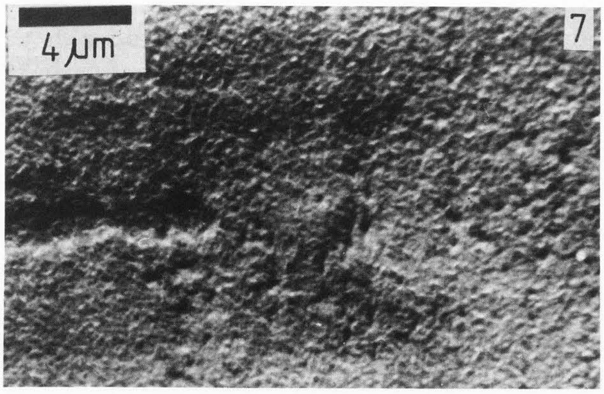

Figure 7 : Detail of R. S. Epoxy replica. Compare with Fig. 5. Note rounding of depression margins, granularity, and absence of detail.

contraction of 1.71% would be expected. Evidence of contraction was visible after 72 h curing at 50°C by the presence of a concavity on the upper surface of the replica. [b] R.S. Epoxy resin is reported by the manufacturers cited by Bromage [1985] to show less than 0.1% shrinkage.

Tests were made on the dimensional stability of the impressions at intervals between 1 h and 3 years after setting, and on the positive Spurr replicas before and after sputter coating with gold using an impression of an incident-light micrometer [Leitz] with a 1mm scale divided into 100 units of 10µm spacing, with 2µm wide engraved lines. Measurements were made using a Zeiss microscope using an incident light Epiplan x8 objective and a Filar vernier eyepiece micrometer. Measuring error was <0.2%. The impression showed no measurable contraction, even after 3 years. Uncoated Spurr replicas showed an average shrinkage of 0.6%, which increased to a maximum of 0.8% after coating with gold.

The tests of the two-stage impression [negative replica] and the positive replica showed negligible linear dimensional change, although there was the possibility of changes in vertical dimensions [relief]. This was checked using microscopical techniques and with a Planer Surfometer SF101. A calibration slide with a groove 2mm wide and 43.1µm deep was used as the test object. Impressions and Spurr replicas were made, and both positive and negative replicas were sectioned perpendicular to the surface. Measurements of the sections were made using the Zeiss microscope as before. Spurr replicas were measured on the profilometer. Optical measurements showed that the positive and negative replicas were accurate to within 2%, and profilometer values within 3%, which approaches the measuring error of the two systems.

Resolution Tests. Test specimen. Pearlite was chosen as a replication resolution standard, since it is widely available, contains fine structural detail of varying dimensions from >1µm to <0.1µm, and is physically tough.

Pearlite is a constituent of steel, consisting of alternate thin plates of ferrite [Fe] and cementite [Fe_3C], of widths down to <0.1µm. Specimens of silver steel, or patented wire, were polished, and then briefly etched for approximately 10 secs [or until the surface becomes grey] with 1% nitric acid in 95% methanol, which preferentially dissolves the ferrite phase. The specimens, which were washed in methanol, air dried, and sputter coated with about a 20 nm layer of gold to prevent oxidation, were relatively durable and could be replicated repeatedly.

The test specimens of pearlite, and positive replicas were viewed normal to the electron beam in the secondary electron mode at an accelerating voltage of 7.5kV in a Cambridge S600 SEM. The short etching procedure used produced an etch depth of 0.1-0.2µm, established by a modification of the method of Boyde et al [1978]. A representative field of etched pearlite is shown in Fig. 2 [x1,000] and Fig. 3 [x10,000]. Fine lamellae of cementite of widths varying from 1.0 to <0.1um remain proud of the surface, separated by parallel-sided depressions of a similar range of widths [Fig. 3]. Linear depressions ≤0.2µm are labelled [A - G]. Low relief features [arrow] are visible in the floor of the broad depression.

Positive Spurr replicas from Coltene light-body impression [on a putty base] of the same field are shown in Fig. 4 [x1,000] and Fig. 5 [10,000]. There is loss of fine detail at the highest magnification [Fig. 5] but the linear depressions A - G seen on the original specimen in Fig. 3 are represented, albeit incompletely. The low relief features [arrow] in the broad depression are visible on the replica.

Positive R.S. Epoxy replicas from a Coltene light-body impression are shown in Figs. 6 and 7. General aspects of the broad depressions are visible [Fig. 6, x1,000], but at higher magnifications [Fig. 7, x10,000] there is rounding of the shallow depression margins, and an absence of reproduction of the ≤0.2µm linear depressions.

Positive Spurr replicas from a Reprosil light-body impression [on a putty base] showed poor quantity of reproduction, similar to the appearance in Fig. 7.

Shelf life of impression materials and effects on resolution. The manufacturers claim a shelf life of 24 months for Coltene materials. Impressions of the Pearlite test specimen were taken using light-body impression material aged 4 months and 27 months, and positive Spurr replicas made. There was no appreciable reduction in resolution attained between the two impression materials of different ages.

Replicas of Hominid Dental Tissues

Negative impressions were made, using the two stage Coltene President impression technique, of intact enamel and fractured internal surfaces of Plio-Pleistocene hominid teeth from Koobi Fora, Kenya; and Olduvai Gorge, Tanzania. Positive Spurr replicas were subsequently cast on return to my home laboratory. Details of the specimens studied are given in Beynon and Wood [1986]. Specimens were initially selected using a dissecting microscope. It was essential to thoroughly clean the surfaces to be replicated using acetone to remove protective varnish coatings. In dentine there was usually total occlusion of dentinal tubules, but in a minority of specimens tubules were patent. An example of replication of fine detail [including undercuts] in the form of terminal branching of dentinal tubules is given in Fig. 8. Enamel is thick in *Australopithecus boisei* teeth [Beynon and Wood, 1985, 1986], and there is relatively little prism decussation in inner enamel [Fig. 9]. Evidence of a pattern 3 prism shape was obtained from direct replicas of upstanding enamel prisms [Fig. 10, stereo pair], which illustrates both the capacity of the primary impression material to penetrate into inaccessible sites, and of its subsequent successful reproduction by Spurr resin. The possibility that the enamel prisms illustrated in Fig. 10 were extracted during replication was excluded, by casting a second positive replica from the original negative impression three years later. This duplicate cast showed some slight loss of detail, but the

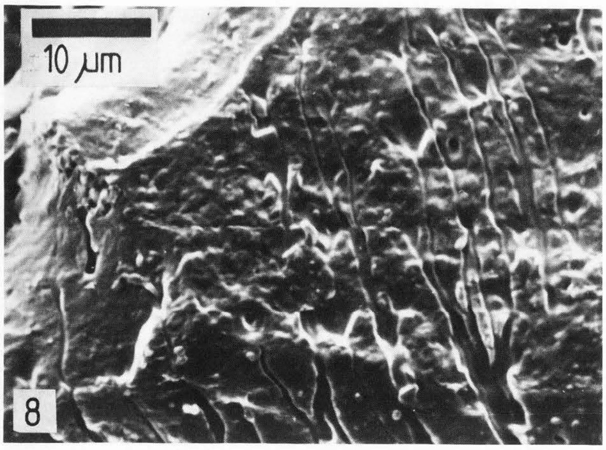

Figure 8 : KNM-ER 807. Early *Homo*. Right M^2. Outer dentine showing dentinal tubules with extensive terminal branching.

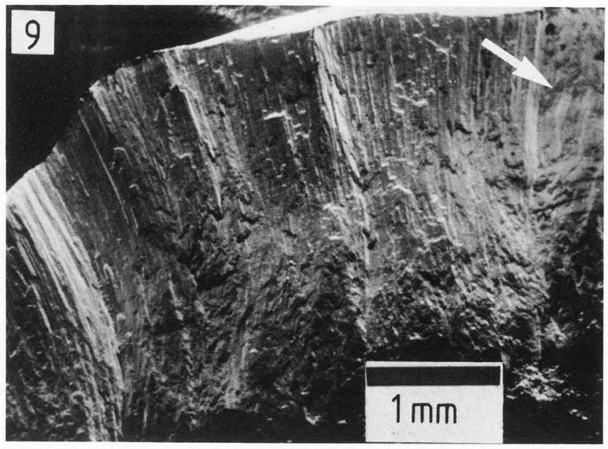

Figure 9 : KNM-ER 1479B. Robust australopithecine. Molar or premolar fragment. Survey view showing straight enamel prisms with relatively little prism decussation, and 'turret' [arrow] of upstanding enamel prisms.

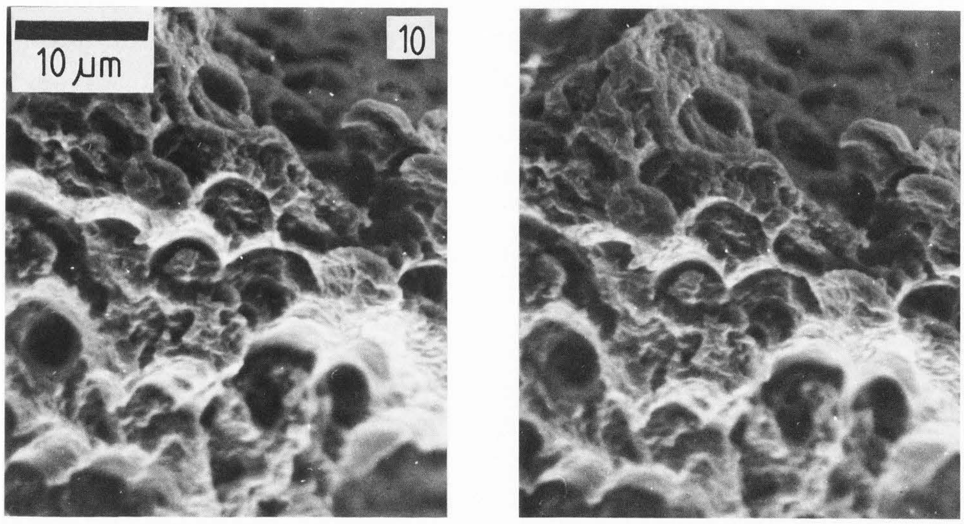

Figure 10 : KNM-ER 1479B. Stereo-pair of pattern 3 enamel prisms at the tip of the 'turret' in Figure 9.

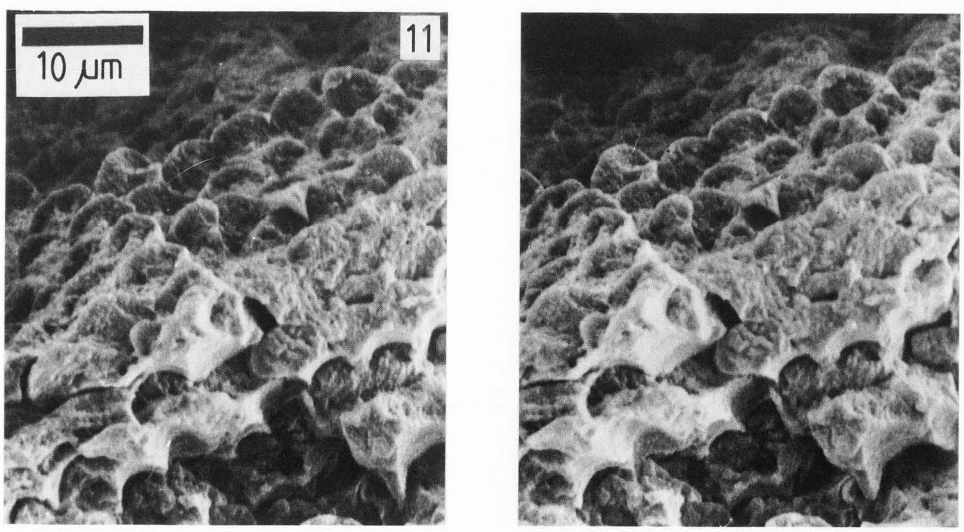

Figure 11 : KNM-ER 1479B. Stereo-pair. Duplicate cast [Jan 1986] made of original impression [Jan 1983] from which the replica illustrated in Figure 10 was prepared.

overall reproduction was relatively good [Fig. 11].

Discussion

The choice of impression material is potentially wide, but for the purposes outlined in this study the use of addition-cure elastomers is preferable to condensation-cure material, which includes Xantopren, and which has been used by several clinical authors [Kusy and Leinfelder, 1977; Scott, 1982; Rose, 1983]. These condensation-cure materials are potentially dimensionally unstable with time due to evaporation of condensation by-products, although McCabe and Storer [1980] noted a relatively low value of dimensional change with this product. They commented that greater accuracy can be achieved using a two-stage impression technique, with putty and light-bodied material. A practical advantage of the two-stage technique is that the primary putty impression is closely adapted to the specimen surface, and the second stage low viscosity pseudoplastic impression material is subjected to high shear forces [McCabe, pers. comm.], being forced into inaccessible areas which may not be replicated by a single stage impression technique. Kusy and Whitley [1985] draw attention to the importance of adequate flow of impression materials, particularly in areas of high relief, which characterise the fractured tooth surfaces present in this study. Their observation that Reprosil light was the best impression material was not confirmed, but this could be due to incompatibility with Spurr resin.

The finding of good dimensional stability of the Coltene President light body impression material over an extended period is believed to be useful in field replication applications, and the ability to make duplicate positive casts is noteworthy.

Spurr resin has been used previously in replica studies by Al-Hamadani and Crabb [1975]. It was chosen in the present study for its exceptionally low viscosity, and low surface tension. The silicone rubber impression material possesses high surface energy which greatly facilitates flow of the resin into inaccessible undercut areas [Figs. 9, 10, 11]. A potential penalty in the use of low viscosity resins is that they show greater shrinkage on curing and there was evidence of bulk shrinkage. The tests on dimensional stability using Spurr resin on both flat surfaces and those with vertical relief showed negligible dimensional change. Spurr resin contains toxic components and should be handled with care [Causton, 1981]. Curing of the positive replicas was carried out in a fume cupboard.

Etched pearlite appears to be a useful material for resolution tests, with structural detail in the range >1μm to <0.1μm and a low surface relief of 0.1-0.2μm. The etched surface consists of alternating upstanding and depressed lamellae, with a form approximating to a square wave, which is more demanding as a reproduction standard than serrated surfaces with a saw-tooth or sinusoidal form, since the flow of material into parallel-sided depressions is more difficult than into a V-shaped depression.

The linear resolution of the replication methodology described here is of the order of 0.2μm, and is capable of reproducing surface relief of <0.1μm. The epoxy adhesive described by Bromage [1985] was substantially worse in replicating the test specimen, although this may reflect incompatibilities between materials. Spurr resin is resistant to the electron beam [Spurr, 1969], although a minimum thickness of 10nm of gold was necessary to avoid surface damage, with a 20nm layer being relatively stable at the 7.5kV accelerating voltage used.

The method has been used on fractured surfaces of fossil teeth, in a field situation in Africa, and it may have use in other field replications. The fidelity of reproduction of undercut and inaccessible locations has been shown to be high. The laboratory dimensional stability tests indicate that direct measurements can be made from the positive replica.

Acknowledgements

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Discussion with Reviewers

A. Boyde: Barnes [1978, 1979 and thesis] studies of replication techniques looked at sharpness with which a knife edge was replicated. Is that not quantitative? And if not, can such valuable qualitative studies be dismissed as "only qualitative"?
Author: An ultramicrotome glass knife as used by Barnes is an exacting test object for comparative replication studies. A glass knife, or its replica however, do not lend themselves to numerical description or measurement, and no two glass knives are directly comparable. It is for these reasons that I do not consider this test system to be quantitative.

Reviewer II: No discussion is given to the question of the extent to which putty based addition cured silicone rubbers shrink less than putty based condensation polymerisation silicone rubbers. Indeed, assuming that the condensation cured silicone resins do shrink, - is this of relevance?

The author assumes the long term shrinkage of putty based condensation silicone rubber impressions, but we do not know that this occurs. Perhaps a control study, using both types of silicone rubber, should have been done.
Author: I have not investigated the extent of shrinkage of addition-cure as compared with condensation-cure putty primary impression material. The volumetric shrinkage of either cure-type of putty is much less than that of the equivalent fine body second stage impression material, owing to the presence of a much greater volume of inert filler particles in the putty material. Condensation cure polymers release volatile alcohols, which can be measured as weight-loss over a 24 hour period [McCabe, J. F., Wilson, H. J. 1978 Addition curing silicone rubber impression materials. Brit. Dent. J. **145**: 17-20]. The important feature of the two stage impression technique is that the first [putty] stage undergoes polymerisation [and any short term contraction which may occur] before the second impression is made. This layer of fine body material is thin [around 0.1mm] and the effects of shrinkage are thereby diminished.

There remains the possibility of long term shrinkage of condensation-cure materials, although I have not measured it; if any does occur then direct measurement on replicas would not be possible. I have used addition cure materials for the reasons outlined, which I have measured and shown to be stable over extended time periods.

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