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Tandem Scanning Reflected Light Microscopy: Applications in Clinical Dental Research

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TANDEM SCANNING REFLECTED LIGHT MICROSCOPY: APPLICATIONS IN CLINICAL
DENTAL RESEARCH

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Abstract

The Tandem Scanning Reflected Light Micro-
scope (TSRLM) enables the investigation of micro-
scopic structures both at and deep to the surface
of intact objects. The present paper reviews
studies undertaken to determine whether the TSRLM
would be usable and useful in the investigation of
natural and restorative dental materials in vitro
and in vivo.

It was found that the TSRLM could be used to
study normal and diseased dental tissues and the
new materials which are used to replace lost
substance. More importantly, it could be used to
characterize the interface between tooth and
optically translucent materials in bulk samples,
giving high resolution information from not only a
shallow depth of field, but at planes below cut
surfaces. This makes it possible to study
interfacial regions in three dimensions without
the risk of delamination that must accompany the
preparation of a microscopic section.

The use of fluorescent markers enables more
information to be derived from the tooth/adhesive
interface. Studies to date indicate the need for
the development of adhesion promoting agents which
incorporate a fluorescent radical in their
molecular structure.

Preliminary work using the instrument for
observation of cutting interactions between a high
speed bur and a tooth indicates some useful
potential in the study of cavity preparation
techniques and tissue failure mechanisms. Recent
developments of the TSRLM for three dimensional
imaging in other dental applications are outlined.
This microscope is an important advance in the
microscopic assessment of adaptation of
biomaterials to hard tissues.

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Introduction

The principle of the Tandem Scanning
Reflected Light Microscope [6,17] (TSRLM) and the
design of recent instruments [16] has been
described in the literature. The illuminating
light (LS) is intercepted by a rotating aperture
disc (N) which lies in the intermediate focal
plane of a standard light microscope objective
(OB: Fig. 1). This device provides an array of
separate scanning beams which light up
corresponding patches in the plane of focus more
intensely than out of focus layers. Reflected
light from these patches is imaged on to a
matching array of apertures on the opposite side
of the same aperture disc, and which are scanning
in the focal plane of the eyepiece (E). An
arrangement of mirrors converts the central
symmetry of the disc into congruency, so that the
array of apertures which chop the illuminating
beam is identical with the array on the
observation side. Thus both illumination and
"detection" are scanned in tandem, giving rise to
the name Tandem Scanning Reflected Light
Microscope (TSRLM). Reflected light from out of
focus layers in the specimen or from optical
surfaces in the microscope is intercepted by the
solid portions of the aperture disc or by light
traps in the microscope head. The only light
which can participate in image formation is that
stemming from the focussed on plane in the sample.
Contrast and brightness are high only for the in
focus layer. Although the TSRLM looks like, and
is used like, a conventional light microscope in
that the user looks through the eyepiece at a
steady image, the difference is immediately
noticed in use. On fine focussing, the image
changes rapidly: features are only seen in focus
because of the effective elimination of blur or
halo.

The TSRLM allows one to look into the
structure of a bulk semi-transparent specimen, up
to a certain depth below the surface, from the
outside. This is not possible with conventional
microscopes. It was conceived and designed as a
method of in vivo microscopy. There is no need to
prepare by fixation, embedding and sectioning: if
kept in a physiological environment specimens can
be maintained for as long as is usual under such
experimental conditions, giving images similar in
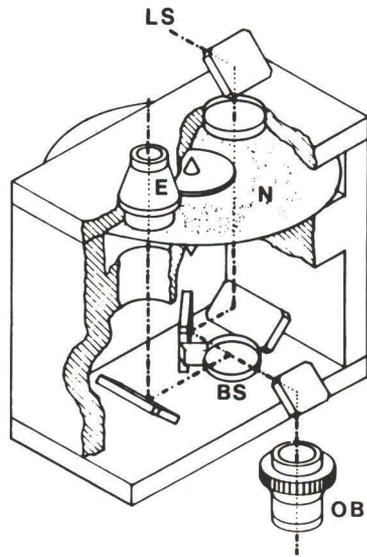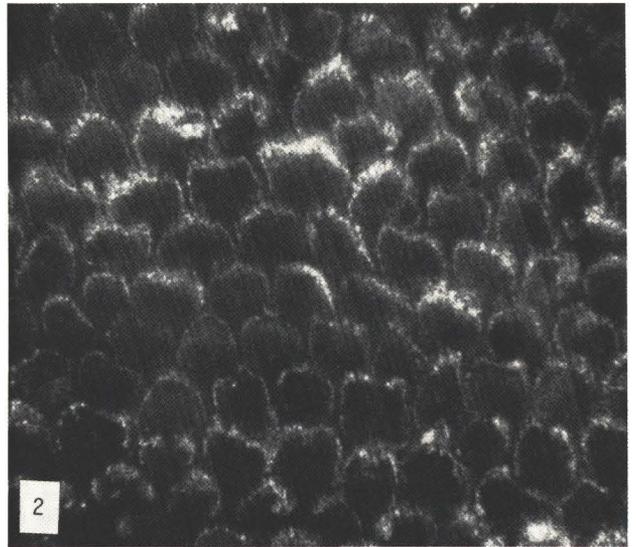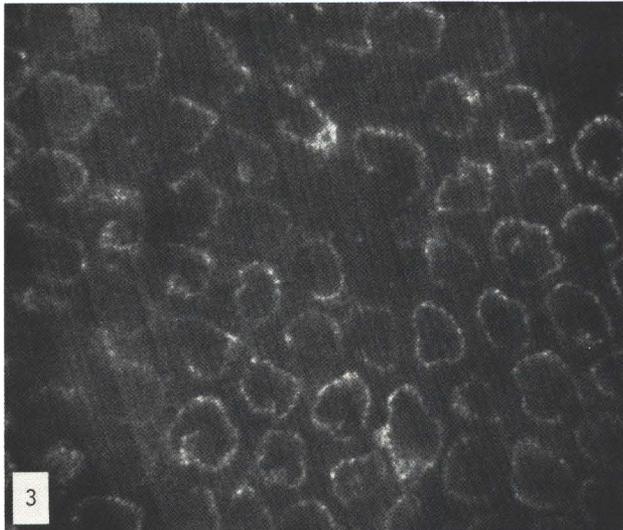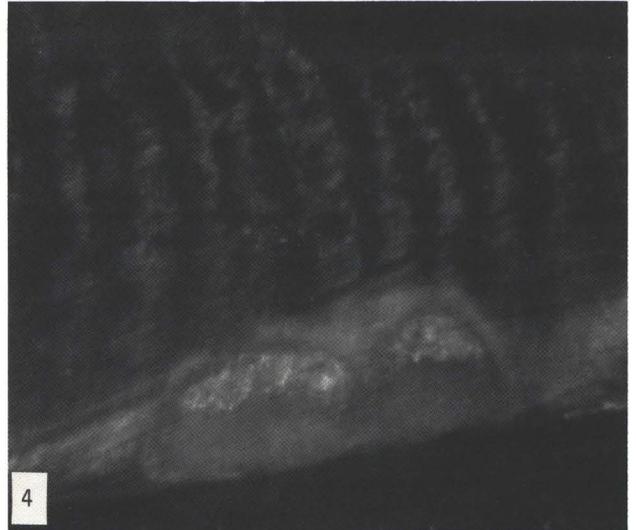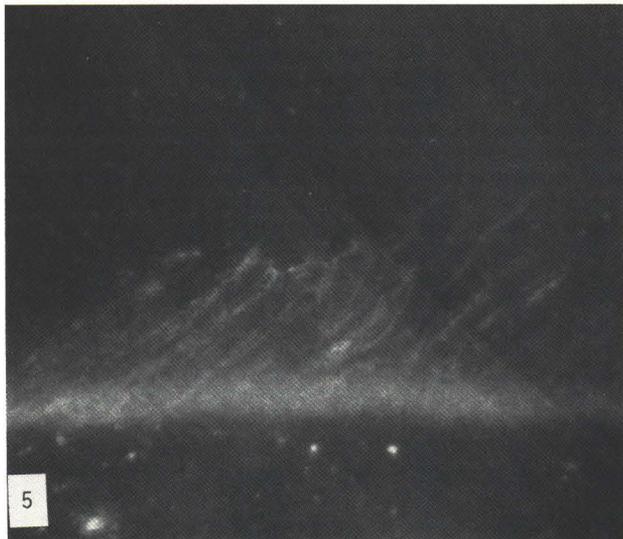
Fig. 1 Diagram of the TSRLM. Light (LS) enters top, reflected by a mirror to pass a field lens placed close to the 100 mm 1% transmissive aperture disc (N), with 17,600 roughly 30 μm holes in a pseudo-hexagonal array on Archimedean spirals. Light passing the disc is reflected twice before passing a beam splitter (BS), and is then reflected downwards to enter the 160 mm tube length RMS objective (OB). Light reflected in the specimen (or fluorescent light) passes back through the same lens, off the same final mirror, to be reflected by the beam splitter, and then suffers one more reflection before reaching the observation side of the disc. Light only reaches the instantaneously lit patches in the focussed on plane, from apertures matching one for one those on the eyepiece side (E); and only light from that plane can return through the disc. Other light hits solid portions of the disc. The last optical component is a Ramsden type eyepiece used to observe the image in the scanning disc. For further details see, for example, Petran et al. [17].

Fig. 2 Human enamel; specimen from Jewbury mediaeval cemetery, York, Microscope focussed 4 μm below wear facet, showing regular enamel prism packing. 100/1.3 oil. Fieldwidth 54 μm.

Fig. 3 Pleistocene Hyaena enamel, showing irregular prism packing pattern, 4 μm below surface. 55/0.95 oil. Fieldwidth 85 μm.

Fig. 4 Longitudinal section of human enamel with a 30% thickness artificial carious lesion. EDJ at the top of the picture. 10/0.75 oil. Fieldwidth 1 mm.

Figs. 5 & 6 Longitudinal section of a composite/eroded enamel margin, the resin tags can be seen to correspond with the underlying enamel. Fig. 5: restoration surface. Fig. 6: eroded enamel surface, (0.2N HCl applied for 150 seconds), 30 μm focus difference between figures. 90/1.1 water. Fieldheight 70 μm.

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character to those seen with phase contrast microscopy. Preparation can be reduced to removing surplus external layers to expose the layer of interest if it does not lie within the visible range from the external surface. In the usual operating mode, contrast arises from reflection at boundaries. The method also works well in fluorescence, giving images of sufficient intensity to be viewed comfortably with the naked eye and which can be recorded on normal (i.e., not very high speed) photographic emulsions.

Enamel is the tissue least likely to change in life or after death, and it may be perfectly preserved in fossils. The TSRLM has been used to study non-invasively the enamel structure in many living and fossil mammalian species [5, 10]. Details of enamel structure that can be resolved by the TSRLM include the shape of prism that it contains, allowing clear differentiation between prism shape and packing as shown in figures 2 and 3. The relative paths of the prisms, the circadian incremental growth features called cross-striations, and longer period growth lines at larger intervals may also be seen. The changes occurring in enamel caries can also be imaged [18] and the ability of the microscope to make thin optical sections of thick, easily prepared, slices

of tooth allows rapid examination of multiple specimens. If a thick (1.2 mm) section is made, both sides of the sample can be examined by focussing through the surface to a predetermined depth, avoiding the problems of delicate ground sections. This technique has been employed in the three dimensional reconstruction of carious lesions [2] (as seen in Fig. 4), for correlation with the changes in appearance of bite-wing radiographs when the X-ray beam angle is changed.

Dentine may also be studied to advantage in the TSRLM. The TSRLM can provide a high contrast image of a cut surface while imaging the internal structure close by [12]. The plugging of dentine tubule openings caused by smearing on cutting has been observed and the removal, by various means, of this "smeared layer" determined [18]. If necessary, specimens can be kept in near normal (although not vital) conditions and the placement of a dental restoration followed from beginning to end in the same sample [18].

A major problem with adhesive dental materials is the provision of an adequate cavity seal at the cervical margin of restorations, when these are finished onto dentine [7]. The structure, hydration and organic component of dentine presents a wide variety of options for different adhesive systems [1], these having been pursued by numerous manufacturers looking for a long-lasting bond between their chosen composite resin and the tooth. Unfortunately, if only a weak bond is produced, the contraction stresses set up in the polymerising restoration may lead to rupture at the adhesive/tooth interface. Conversely, if the bond is too strong, then unacceptable stresses will be transferred to the tooth [9, 11].

The TSRLM enables the distribution of bonding agents to be examined within the tooth and also in the overlying composite restoration. Early studies using the TSRLM [19] have examined an adhesive system which relies on the mineral component in dentine for bonding [8] Scotchbond^{TM}, 3M Ltd. St. Paul, MN, whilst more recently another system, which is said to attach to the collagenous component by reaction with glutaraldehyde, has been investigated [14] (**GLUMA**^{TM}, Bayer Dental Ltd. D-5090, Leverkusen, Germany).

Fluorescent markers have been used extensively for studying microleakage around restorations [13] and also for visualising contraction gaps [7]. Using the TSRLM, the penetration of bonding agent or resin monomer into tooth tissue can be studied by either the different refractive index and associated reflectance, or the fluorescent properties of the bonding agent, which can be manipulated by adding suitable fluorophores. Most of the normal "flare" or "halo" seen with conventional epifluorescence microscopy is lost in confocal scanning microscopy so that a much sharper image is produced. The TSRLM is also useful for studying the distribution and the packing of the filler particles in the resin phase of the composite restorative material [19].

Materials and Methods

Studies were originally conducted using human teeth, mostly sound third molars. These were
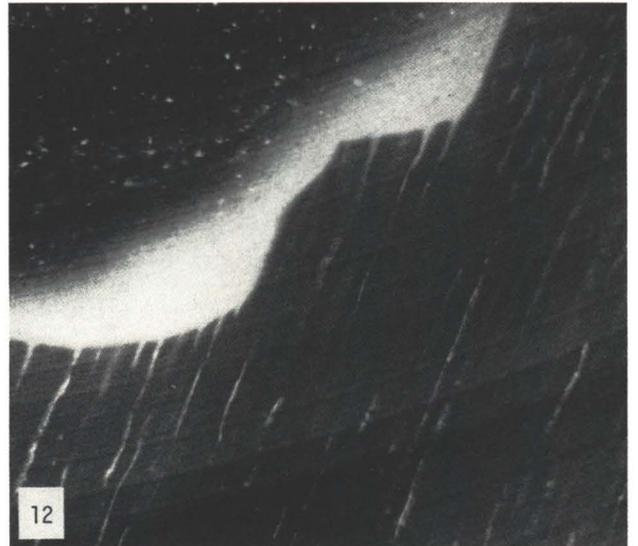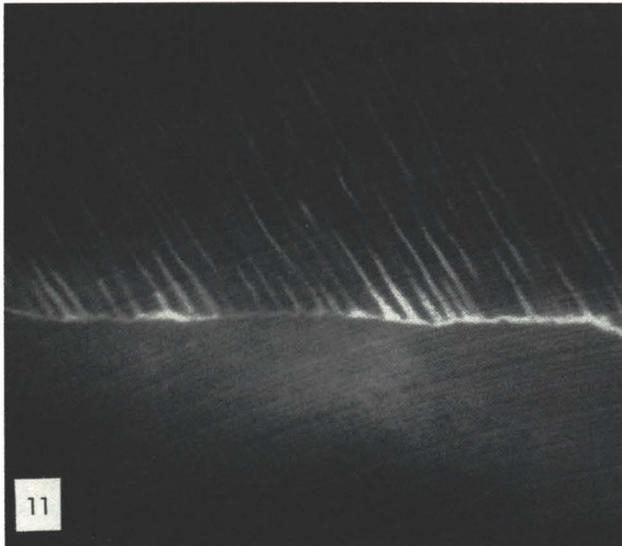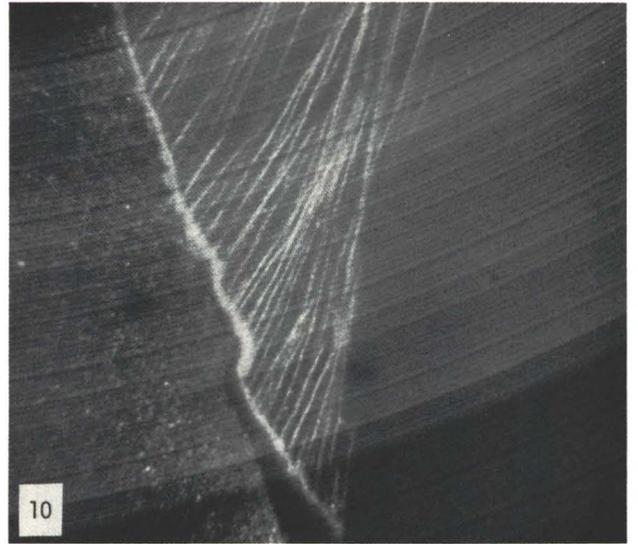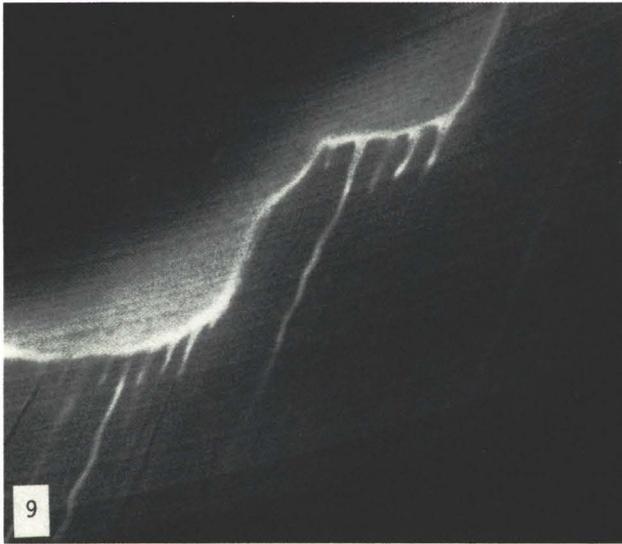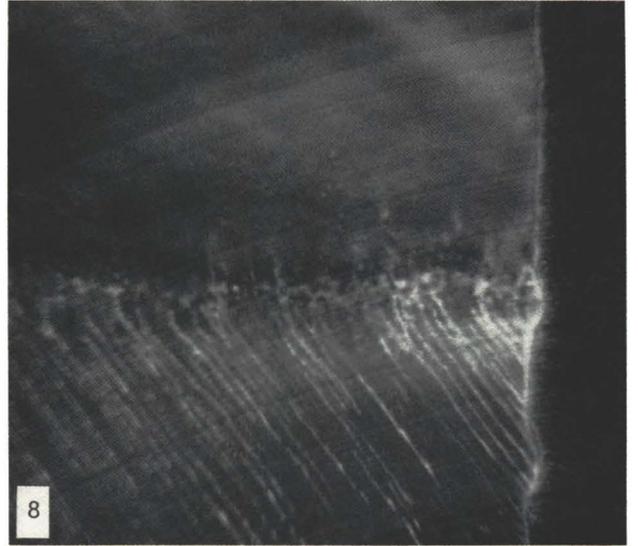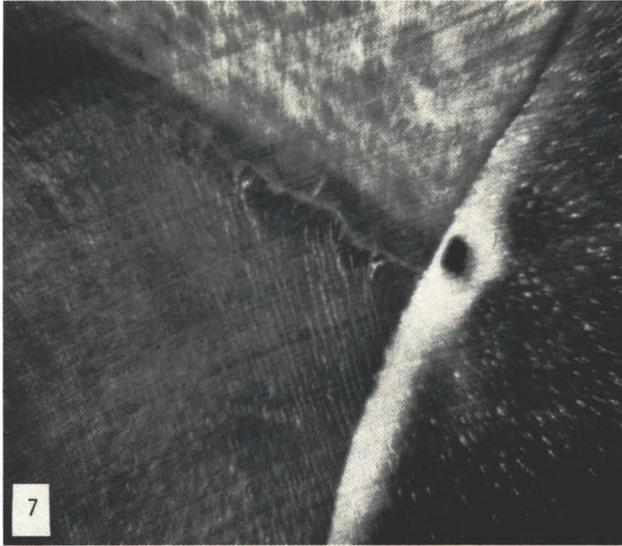
Fig. 7 Longitudinal section of human third molar: enamel top, dentine left, restoration right. Cervical cavity filled with **GLUMA**, **Enamelbond** and **Lumifor** composite resin. Brilliant sulphaflavine label in **GLUMA**, rhodamine B in **Enamelbond** resin. [NB: This regime was used for figures 7-14]. 532 nm blue filter showing fluorescence (mainly from **GLUMA**) and some information from dentine structure. The EDJ is faintly "picked-out" as a thin line by the fluorescent **GLUMA**. 20/0.80 glycerine. Fieldwidth 500 μm.

Fig. 8 Similar field to Fig. 7, but the sample is a bovine tooth: enamel top left, dentine bottom left, restoration right. The **GLUMA** is present as a thin film on the surface of the dentine, in contrast to Fig. 7, where pooling has occurred. The EDJ has been more diffusely penetrated by the fluorescent **GLUMA**, indicating considerable morphological differences between the two substrates in this region. 20/0.80 glycerine. Fieldwidth 500 μm.

Fig. 9 A cervical cavity cut in dentine (bottom right) with sharp internal line angles. A 405 nm filter (violet) enables fluorescence from **GLUMA** labelled with brilliant sulphaflavine to be seen. The bonding agent has penetrated dentine tubules presenting end-on, but to only a limited extent where they are nearly parallel to the cut surface. The diffuse "glow" above the bonding agent is from the **Enamelbond** labelled with rhodamine B, see Fig. 12. 40/1.3 glycerine. Fieldheight 220 μm.

Fig. 10 The surface of a **GLUMA** bonded restoration following removal of the dentine, originally to the right of the picture, with NaOCl (5 minutes) and 0.2N HCl (5 minutes). The "fronds" of bonding agent are supported in an aqueous environment whilst under examination. Delamination is visible between the bonding agent and the neighbouring composite. 40/0.75 water. Fieldheight 220 μm.

Fig. 11 A thin film of **GLUMA** between dentine (top) and composite restoration. Notice the widening of the dentine tubules following conditioning with EDTA. 405 nm (violet) filter. 40/1.3 glycerine. Fieldheight 220 μm.

Fig. 12 Same field as Fig. 9, with (green) 546 nm excitation filter for rhodamine B label incorporated into **Enamelbond** resin. The pooling of material is apparent in the line angles of the cavity. 40/1.3 glycerine. Fieldheight 220 μm.

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stored in 70% ethanol until re-equilibration with water just before use. Several experimental findings indicated the need to use fresh teeth with vital pulps and dentine containing live odontoblastic (cell) processes: the penetration of certain bonding agents into dentine is influenced by the exact nature of the tubule contents. For this reason, the practice of using only fresh teeth directly from the extraction clinic has been adopted; all operative procedures being completed within 30 mins to 1h after extraction. In order to verify that the in vitro techniques employed were realistic as in vivo models, cavities were cut and filled using identical techniques prior to extraction. The supply of human teeth was supplemented by bovine molars and incisors, obtained fresh from a slaughterhouse and stored for not more than 12 to 18h at 0 to 4°C before use.

In both the present studies [18, 19] using human teeth, cervical cavities approximately 4 mm wide and 3 mm high were cut with a 557 tungsten carbide fissure bur, running wet in an ultra-high speed handpiece. Cavities placed mesially and distally were extended below the enamel-cement and enamel-dentine junctions (i.e., finished on to dentine and so imitating a large proximal cavity, with both sharp and rounded internal line angles). In samples which were to be restored using a relatively weak bonding agent, a technique was devised which reduced the disruption produced by sectioning after restoration. In this situation, teeth were cut longitudinally using a diamond saw at slow speed under water. The section surfaces were ground flat on wet, 600 grit carborundum abrasive paper, and then carefully coated with a thin layer of rapid setting epoxy resin extended to the cavity margin, before cementing to a ground glass microscope slide. The restoration could then be placed as normal and the section gently removed from the slide, after soaking in water for 1h. This technique was employed with **Scotchbond**[TM] [19]. However, if the adhesive bond to dentine was known to be sufficiently strong to withstand the trauma of sectioning, then the tooth and restoration were cut after placement: this was found to be essential for systems which required an intact pulp to determine the penetration of bonding agent, such as the glutaraldehyde containing adhesives.

Fluorescent labelling

The visualisation of the distribution of the bonding agent can be greatly enhanced by the addition of fluorescent labels. Earlier studies used acriflavine hydrochloride added to the alcoholic component in **Scotchbond** [19]. This gives a yellow fluorescence when excited with blue light. More recent work has used brilliant sulphaflavine, which produces a yellow fluorescence with a 405 nm band pass filter (violet), which gives little structural information in the image. Alternatively, a 532 nm short-pass filter (blue) will give both fluorescence and structural features. Brilliant sulphaflavine can be used in one part of a bonding system whilst another component can be counter-labelled with rhodamine B: this may be excited with the appropriate 546 nm (green) filter to give an orange/red fluorescence with no interfering fluorescence from the brilliant sulphaflavine. It is thus possible to visualise separate components of the same system, such as the glutaraldehyde and resin components in the **GLUMA** technique.

Placement technique: Scotchbond

37% phosphoric acid gel was applied to the enamel for 30 seconds and then washed with water for 30 seconds trying to avoid contact with the dentine. Other than the addition of the fluorescent label, the **Scotchbond** was placed in accordance with the manufacturer's suggested instructions. Two coats were used and air dried between for the chemically activated system, or illuminated with blue light for the light activated system. The cavities were filled incrementally with a posterior composite resin restoration (**P30**[TM], 3M Ltd.). Dentine surfaces were covered with the material, which was cured first, and then the enamel margins.
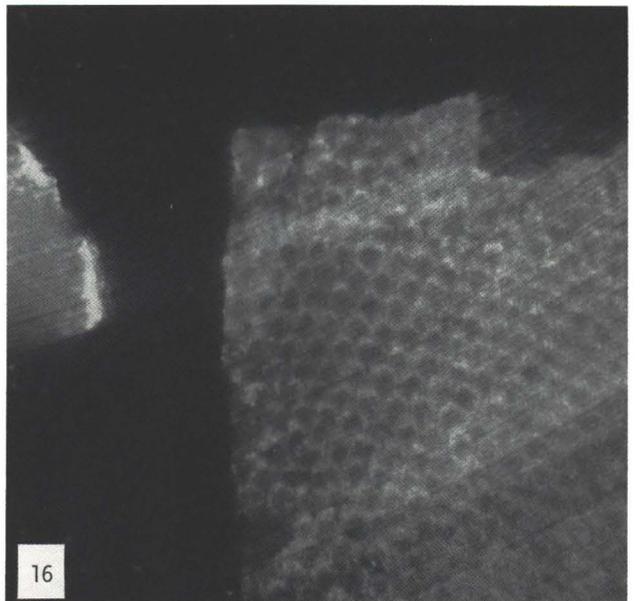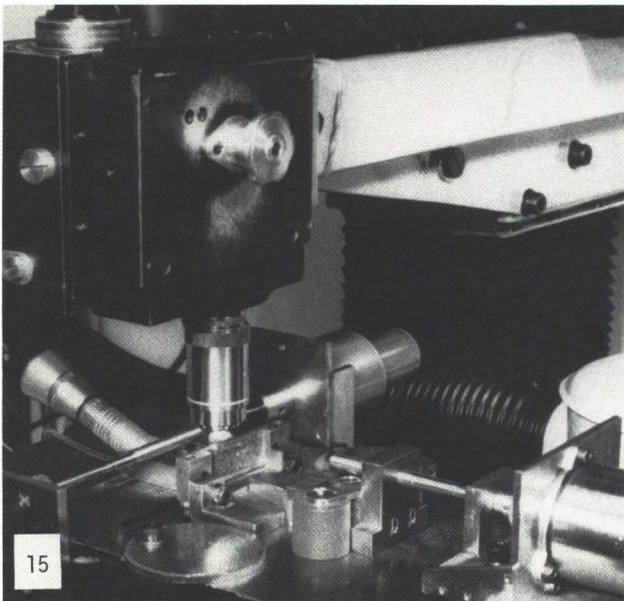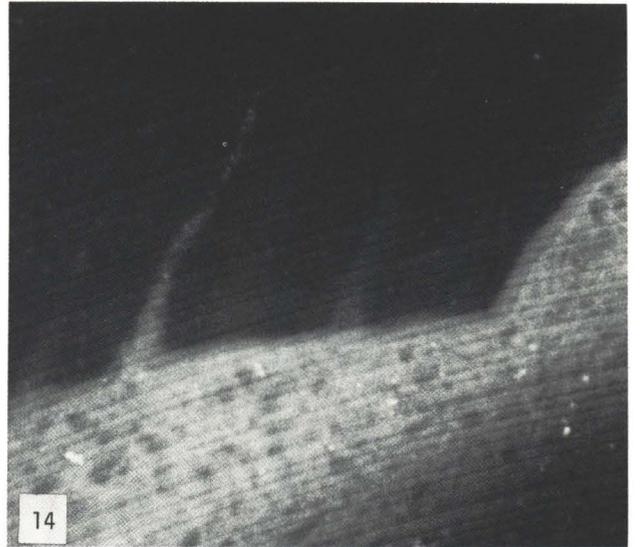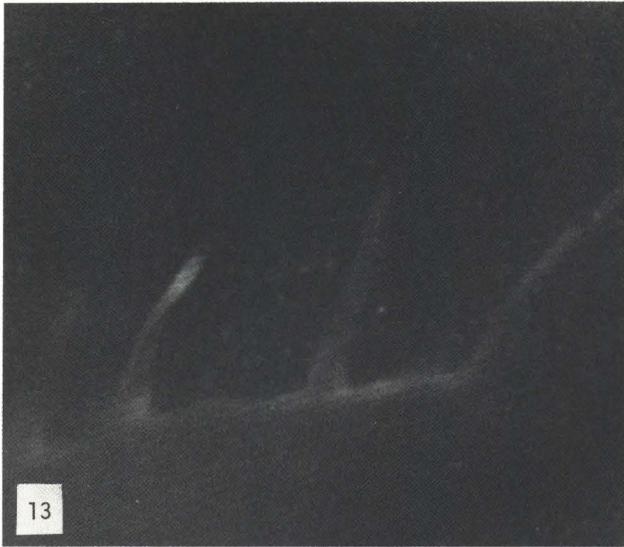
Fig. 13 A high magnification view of **GLUMA** distribution in relation to widened dentine (top) tubules. The Y-shaped dentine walls have been coated with the fluorescent material, leaving openings for the unfilled resin to penetrate. 405 nm (violet) filter. 100/1.3 glycerine, 15X eyepiece. Fieldheight 45 μm.

Fig. 14 Same field as Fig. 13 with fluorescence from **Enamelbond** resin alone. Notice the "backfilling" of this resin into the spaces within the **GLUMA**, as seen in Fig. 13, and the contrast with the filler particles in the composite restoration. 546 nm (green) filter. 100/1.3 glycerine, 15X eyepiece. Fieldheight 45 μm.

Fig. 15 The head of the TSRLM with the tooth cutting stage beneath. The eyepiece can be seen at the top left of the picture, with the fine focussing control visible on the side of the microscope. A standard microscope objective is focussed into a tooth which is held on the stage, positioned by the servo-motor seen on the right. The shank of a dental handpiece is held beneath the microscope head, to the left of the picture.

Fig. 16 A view after machining enamel with a 1mm diameter tungsten carbide bur; the tip of one blade can be seen end-on, including regions of cracking within the tooth. 50/1.0 water. Fieldwidth 180 μm.

Figs. 17 & 18 A stereopair showing the distribution of an elastomeric experimental bonding agent within dentine (right) and the neighbouring composite. Bonding agent labelled with brilliant sulphaflavine, with appropriate excitation filters. Fieldwidth 80 μm.

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Placement technique: GLUMA

The smeared layer was removed by treatment for 20 seconds with 0.5M EDTA pH 7.4. After washing, drying and coating with the **GLUMA** for 60 seconds followed by the light-cured **Enamelbond** resin, light-cured **Lumifor** composite was placed (both Trademarks, Bayer Dental). Compressed air was used to spread the two components of the bonding agent, which were labelled with brilliant sulphaflavine and rhodamine B, respectively. Some of the teeth had the pulp removed prior to preparation in order to see if this influenced the penetration of bonding agent.

Examination in the TSRLM

All specimens were kept in distilled water prior to examination. Normally, glycerine immersion objectives giving original magnifications ranging from x10-x100 were used, in conjunction with either a x10 or x15 eyepiece. In order to verify that the bonding agent (rather than free fluorescent label) was being examined, the dentine and enamel were removed with NaOCl and 0.2M HCl to allow visualisation of the bonding agent alone. This corroded specimen was then maintained in an aqueous environment and examined using water immersion objectives.

Results and Discussion

Bonding agent studies

Enamel etching produces a surface which is replicated by the resin giving rise to the finger like "tags" seen with the TSRLM [18]. Contrast between the bonding agent, or unfilled resin, and

tooth can normally be seen with an intact specimen. As with the SEM, it is possible to make the tags more obvious by removing the surrounding enamel. Fig. 5 shows some free floating resin tags, supported in water, as observed with a water immersion objective. The eroded surface of the enamel can be seen in Fig. 6 where the TSRLM has been focussed down by 30 μm. In studies where this has been undertaken with the TSRLM [18], the maximum tag lengths observed have been long (up to 70 μm), probably because there is little disruption of the specimen due to handling in air or vacuum. The more conventional tags were not often apparent with longitudinal sections because, generally, the enamel prisms were not etched end on: the maximal penetration of acid in this direction will produce the greatest resin tag length [3, 18, 19].

Dentine "conditioning" The phosphonate ester type of bonding agent (**Scotchbond**) has been shown to give good bond strengths when the mineral content of the tooth is high [8]. In order to achieve this, mineralizing solutions have been advocated [8], as well as the avoidance of any procedure which may lead to demineralization of the cavity surface. The manufacturers suggest that the smear layer be maintained intact. When enamel is etched with phosphoric acid it can be difficult to control the "run-off" of etchant when washing. Even when a protective lining is placed this may still leave dentine uncovered, especially close to the enamel/dentine junction, so producing a potential opened pathway (via the widened dentine tubules) to the pulp near the cavity margin. We have found [19] that the **Scotchbond** adapted well to the walls of the acid treated dentine tubules but ruptured from the bulk of the bonding agent in the restoration. This suggested that the remaining normally mineralized dentine in the tubules reacted with the **Scotchbond**, but the severely demineralized surface, low in calcium, failed to react satisfactorily. Contraction of the composite resin fractures the bonding agent.

In contrast, the glutaraldehyde/hydroxyethyl-methacrylate system (**GLUMA**) requires efficient removal of the smeared layer and mineralized dentine, in order to expose free collagen for attachment [14]. This is achieved by the use of EDTA, which will remove peritubular dentine as well as the smeared layer. The penetration of **GLUMA** was found to depend on a number of different factors.

With the pulp removed, and no tubule contents, it was observed that the material could extend to the pulp chamber. In specimens which were maintained intact and in as near physiological conditions as possible prior to restoration, the maximum penetration (along dentine tubules) was 300 μm. The glutaraldehyde could damage, by fixation, the pulpal tissues immediately beneath a deep cavity especially if a protective lining were inadequately placed. When restorations were placed in vital teeth, prior to extraction, penetration of **GLUMA** was comparable with the in vitro studies. It was found that the material could spread along discontinuities in the tooth structure, such as the enamel dentine junction (EDJ), for up to 500 μm in human teeth (Fig. 7) and even more (up to 1 mm)

in bovine samples (Fig. 8). This could have important implications in interpreting bond testing data between different adhesives, where dissimilar mechanisms of attachment are employed. One substrate and the sampling position within the tooth may favour a certain adhesive more than another.

Cavity shape was also a significant determinant of **GLUMA** penetration. If sharp internal line angles were employed, the material tended to be concentrated and this lead to greater interdigitation within the dentine (Fig. 9). The conditioning agent would also have been similarly concentrated and so it is not altogether surprising to find that these conditions favoured an easier pathway for the **GLUMA.** When the dentine tubules were within 10° of parallel to the surface, the penetration of the bonding agent was limited to 10-20 µm (Fig. 9), indicating the need for dentine tubular opening in order to achieve a broad area and volume within the tooth for bonding. However, a limited penetration may be enough to enable a sufficiently strong bond to be made to the dentine, with infiltration of hydrophilic and hydrophobic groups as advocated by some workers [15]. The true extent of the spread of the **GLUMA** was confirmed following removal of the tooth substance, when the "fronds" of bonding agent could be seen supported by the aqueous environment, during examination with water immersion objectives (Fig. 10).

The distribution of bonding agent

Work undertaken with chemically cured and light activated **Scotchbond** has shown that the pattern of distribution of the two materials within the overlying composite restoration is very different [19]. If the restoration was placed in one increment using the chemically activated system, then the bonding agent could be found distributed throughout, with possible poor consequences for the strength of the final product in clinical use. In this situation filler loads in the composite restoration could be substantially decreased by the incorporated resin. Bonding agent was most easily localized by applying a thin primary layer of composite, so restricting the dispersion of the fluid phase. The use of thin incremental layers may also reduce the stress built up within the restoration and tooth [9, 11]. The light-activated system allows better control of the bonding agent but can produce "lakes" of material in sharp internal line angles, due to difficulty in dispersing the material with an air syringe. If the phosphonate-ester type of bonding agent is prone to hydrolysis, or the resin component is dimensionally unstable, then a fairly thick layer of light-cured material would jeopardize the bond between composite and tooth. The more thorough mixing of the chemically activated **Scotchbond** with the resinous component of the restoration may help to safeguard against this eventuality, although the achievement of a strong bond with the dentine may be slower with the chemically activated material because it is not polymerized with blue light.

When examined at low magnifications, the **GLUMA** presented as a thin film between the adjacent tooth and the overlying unfilled resin and composite (Fig. 11). At high magnifications (1000X) it was apparent that this film was closely adapted to the walls of the etched and widened dentine tubules near the cavity surface, giving a Y appearance (Figs. 12 & 13). This was readily observed using the brilliant sulphaflavine labelled material excited with the appropriately filtered light. Where the unfilled resin had been counter-labelled with rhodamine B, the contribution of this component to the bond could be assessed in the same sample under examination. In cases where a Y formation had developed with the **GLUMA**, a backfilling with the resin was seen (Fig. 14). Alternatively, the resin could be seen apparently on its own, without evidence of the more volatile glutaraldehyde component.

As expected from the previous work with light-cured **Scotchbond** the light-cured **Enamelbond** resin with **GLUMA** exhibited pooling, and some difficulty was experienced in reducing the film thickness (Fig. 12). Even though the material was light-cured, there was still considerable mixing of unpolymerised and oxygen inhibited material with the adjacent composite resin (Fig. 14): incorporation of the rhodamine B into the unfilled **GLUMA** resin takes much longer than the time available during placement and polymerisation of the restoration. The label is 'fixed' in position on polymerisation. After removal of the tooth substance using sodium hypochlorite and hydrochloric acid, delamination between the composite restoration and the bonding agent was observed (Fig. 10). This may indicate a weak interface with the overlying composite.

Real time cutting and fracturing experiments

The TSRLM is also being used to observe tooth tissue structure whilst being cut by a bur. A stage has been made to hold a high speed handpiece with its bur so that the bur can be seen end on, as well as enamel prisms in the wall of a cavity (Fig. 15). The cutting interaction is recorded on videotape using a conventional TV camera, and analysed on play-back. Unfortunately, these results can only really be appreciated by seeing the live video recording, so that no form of illustration (including a still photograph taken after cutting: Fig. 16) will adequately portray the mechanisms involved.

Preliminary results have shown a "wave" or "domino" type of failure in sheets of enamel prisms machined with their long axes nearly parallel to the blades of a tungsten carbide bur. Cutting with a diamond bur is very much more indiscriminate, whilst with both, cracks and lines of failure are seen ahead of the advancing, machined edge.

These studies have raised the question of how much faster could the TSRLM be used in real-time recording? At present, the disc rotation speed is only 100 rpm: there is little doubt that it could go ten time faster, and with a stronger constructional material for the disc, at least another hundred times faster. Problems would arise from the brightness of illumination, in ensuring the balance of the disc at high speeds, and in finding a good high-speed recording medium. Even without these modifications, the microscope could still give useful information in experiments such as the controlled rupture of the bond between adhesive restorations and tooth substance by

observing the same field before and after failure.

Stereoscopic imaging

The TSRLM images a very thin layer below the surface of the block or thick slice of translucent material which is under investigation. To obtain three-dimensional reconstructions, images are recorded at regular focus intervals, printed on to photographic plates and made into a stack. Stereopair images can be made by contact printing with inclined axes of illumination through such a stack [6]. The information in such prints can, however, be contained in two original images corresponding to two directions of view through the original object, made directly by focussing during photographing [4]. The success of this new stereo imaging method depends upon the properties of the TSRLM, because only those features which lie in the plane of focus at any one instance contribute any information to the recorded image. Other confocal scanning optical microscopes (with a scanned specimen and a single beam) have the same property of depth discrimination, but do not give a real-time image. The TSRLM also works with white light, so that color effects are retained.

Another new means for acquiring stereo images in the TSRLM will be described in detail elsewhere (Boyde, Lampers and Dillon, in preparation). A Tracor Northern 8500 computer image analysis system has recently been coupled to the TSRLM at University College London. Utilising the frame store and image manipulation facilities, anaglyph (red - green) stereo images can now be presented with any chosen, synthesized tilt angle difference after only one through focussing sequence. Although this procedure loses the real colour present with a direct photographic method, it has the advantage that there is an immediate result upon which to judge whether to make a further analysis within a given specimen volume. It does, however, have the integral advantage that compensation for the residual errors in the manufacture of the aperture disc in the TSRLM can be accommodated using the frame store procedure. Thus an out of focus image - usually recorded with the objective positioned about 15 μm above the focal level for the sample surface - can be subtracted from each incoming image, which effectively compensates for both the irregularities in the disc and in the intensity of illumination, and, at least partly, for the local variations in the responses of the channel plate image intensifier and video camera. Figs. 17 and 18 are a stereopair, produced by the above technique, of an experimental bonding agent which penetrates through the undisturbed dentine smear layer, whilst also mixing with the overlying composite resin.

Conclusion

It is predicted that the TSRLM will prove useful in many aspects of biomaterials research, where there is a need to look at the internal structure of a translucent (dental) material which must remain as a bulk specimen. It may also be important in providing the means of making stereoscopic images at high magnification. The TSRLM is impressive in its original role as a vital microscope. The present studies indicate that it may also be useful in deriving new information from prepared specimens. Although thick or uncontrasted specimens can be studied, more can be seen if the specimen is specially prepared. The areas which most need to be explored and exploited are the use of the TSRLM in the fluorescent mode, producing 3-D images of intact tooth/restoration interfaces and with the use of reflective stains.

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Discussion with Reviewers

K.S. Lester: Could you spell out the original findings for bonded restorations that have so far been made with the TSRLM?

Authors: This is the first method by which significant areas of the bond region can be examined wet and in a natural, undisturbed condition. It is a very good method of assessing the relative extent of good and bad adaptation, although we have not illustrated this point here. Our results have shown that the bonding agents are distributed much more widely both within the tooth and the restoration than had previously been realised: they are difficult to control.

J.D. Eick: Is it possible that the fluorescence is giving some false interpretive results with some microstructures?

Authors: It is clearly much more difficult to distinguish fluorescent from reflective features in the black and white copies (our figures) of the original coloured images. We believe that this problem would be the main, if not the only, route to confusion. If one uses narrower bandpass filters for excitation and sharper short wavelength cut-off for observation one can improve the distinction of fluorescent from reflective features, but at the same time one loses the useful microstructural information conveyed by the latter.

B. Causton: Can the authors say how the diffusion characteristics of a multi-component adhesion promoter can be related to the diffusion rate of the fluorescent marker dye?

Authors: No. The question presumably means, How can you be sure or are you sure that the movement of the fluorescent marker dyes during the setting process reflects the migration of components normally found in the adhesion promoter? We eagerly await the development by polymer scientists (like yourself!) of adhesion promoters which incorporate a suitable fluorescent radical bound to an essential component of the system. We will

then no longer have to be concerned that the fluorescent marker may exhibit different diffusion behaviour than the other components in the system. Empirically, we have found that brilliant sulphaflavine is more readily soluble in the **GLUMA** than in water, and acriflavine hydrochloride is more soluble in the alcoholic component of **Scotchbond:** this would suggest that both these labels would be unlikely to partition into the tissue fluid in dentine. In the case of the **GLUMA** bond, corrosion studies have indicated that the extent of resin "tags" and of fluorescent label are more or less the same. The glutaraldehyde may well penetrate ahead of the resin proper.

K.S. Lester: You make the point that the instrument was originally introduced for in vivo work, and then discuss its application to appropriately prepared specimens. What are the limitations in dental research for its original in vivo mode?

Authors: The TSRLM was invented to satisfy the requirement to have a microscope which could be used to examine tissues in as near a lifelike or the living condition as possible. We believe that it will rarely be convenient to conduct studies similar to those described here on live patients! (who may object to many things, including fluorescent teeth). The most important factor is certainly that the movement generated by the heartbeat thwarts any attempt to work at a useful, high magnification.

K.S. Lester: Given the usefulness of the instrument and the increasing ease and versatility of stereo imaging, what are for you the most interesting areas to be examined by this technique in the clinical subject area of bonded tooth coloured restorations?

Authors: We would like to examine the bond region en face and stereo will be the only way to present and analyse such a wealth of information. We look forward to contributing to the evaluation of potential new materials before they reach the stage of clinical trials. Most important will be to provide information correlating the bond microstructure with mechanical testing data.

B.Causton: Do you think that fluorescence in the TSRLM may be strong enough to be quantified sufficiently well to allow diffusion coefficients to be determined?

Authors: We would be sure that the fluorescence in the TSRLM would be sufficiently strong for this purpose, given the availability of a TV rate imaging (and recording) system with a linear response and no auto-compensation.

G.J. Brakenhoff: With respect to stereoscopic imaging, while the TSRLM technique may provide white light and real time images, other confocal techniques (such as our on-axis technique) provide better resolution plus direct data storage in the computer system without the intermediary of a TV chain.

Authors: We realise that unitary beam, on-axis scanning holds the edge in resolution in X(Y) and Z, but it should be pointed out that the TSRLM already shows a dramatic improvement in resolution

in the Z direction compared with conventional optical microscopy. The TSRLM is so useful to us because this is achieved in a microscope which is otherwise like, and is used like, a conventional microscope. Furthermore, we expect the predicted marginal improvement in X(Y) resolution to be demonstrated in newly manufactured TSRLMs in the near future.

Stereo imaging will be important to us. Our methods enable us to do this at the limits of resolution previously accepted for conventional light microscopy - a great step forward over what was possible in conventional (half aperture) stereo imaging. However, to obtain the information that we require from investigations such as those which we consider here requires the rapid scanning of large volumes (large X,Y, and Z) of tissue by a trained human eye-brain complex. We take leave to doubt that this will be possible using a monochrome, on-axis confocal scanning light microscope. One has to be able to find what it is that one is going to analyse.

G.J. Brakenhoff: For completeness, could you reference earlier work on stereoscopic imaging in other confocal microscopes?

Authors: Here is a partial list mentioning one publication from each lab. (of which we are aware) previously working with confocal stereo image synthesis from (XY and) Z scans:-

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