FACT AND ARTEFACT IN CONFOCAL MICROSCOPY

T.F. Watson

Department of Conservative Dentistry
United Medical and Dental Schools
of Guy’s and St Thomas’s Hospitals
Guy’s Dental School, London Bridge
London SE1 9RT, United Kingdom


Abstract—High-resolution confocal microscopic images may be made of either the surface of a sample or beneath the surface. These images can be likened to optical tomograms, giving thin (> 0.35 μm) slices up to 200 μm below the surface of a transparent tissue: With microscopes running under normal conditions, the optical section thickness will be > 1 μm and the effective penetration into enamel and dentin a maximum of 100 μm. For maximum resolution, high-quality, high-numerical-aperture objectives should be used. Refractive index matching of the lens immersion media and the substrate will avoid distortions of images in the optical axis. Such errors could occur when imaging a considerable distance (> 40 μm) into a cell containing water, with an oil immersion objective above the cover slip. Care should be taken in the interpretation of computerized z axis reconstructions made from serial optical sections: Their validity should be checked with equivalent views made with the sample oriented in the same direction as the reconstruction. The use of fluorescent dyes in microscopy is a very powerful investigative technique. It is important that the dyes used not be labile and that they be well-fixed to the materials being examined, or the images may indicate the dye distribution rather than the material to which it is “attached”. Multiple labeling experiments must have crossover control experiments to verify the distribution of the individual dyes. Valuable information can often be gained by combining information from both reflection and fluorescence images. Two-photon laser excitation of dyes gives the potential for greater depth penetration and improved resolution.

Key words: Confocal, scanning, optical, fluorescence, microscopy.

Confocal microscopy has been quite slow to be applied in dental research, even though the principle behind this technique was discovered in 1955 (Minsky, 1961, 1988) and the first functional confocal microscope in a dental school was delivered in late 1983 (Boye et al., 1985; Watson and Boyde, 1985).

Review articles have been written about the potential applications of the technique in dentistry (Watson and Boyde, 1991; Watson, 1994), but little has been written about potential problem areas and how they may be avoided or overcome. The aim of this paper is not to describe the many applications of which confocal microscopes are capable, or to enter into lengthy discussions of the science behind their operating principles, but to highlight some of the practical aspects of their use in dental research. Examples will be drawn mainly from the work that has been undertaken over the last 13 years, looking at adhesive restoration/tooth interfaces. The reader who specifically wants to find out more about the technology and its associated techniques is recommended to consult the original references cited in Pawley (1995).

OUTLINE OF THE TECHNIQUE

The expression “confocal” derives from the use of an aperture in the conjugate focal plane of an objective lens in both the illumination and imaging pathways of a microscope: The area surrounding the aperture rejects stray light returning from areas which are not in the focal plane of the lens (Fig. 1). For a useful image to be made, some form of scanning device is required. Expressed simply, this type of scanning optical microscopy enables high-resolution images to be made of samples, often below the surfaces of translucent materials, with minimum requirements for specimen preparation.

High-resolution confocal microscopic images may be made of either the surface of a sample or beneath the surface. For subsurface images, an immersion medium is used to couple the objective lens optically with the sample, to produce reflection or fluorescence images of structures within semi-transparent materials such as cells and dental hard tissues. These images can be likened to optical tomograms, giving thin (> 0.35 μm) slices up to 200 μm below the surface of a transparent tissue. Such figures are the best that can be obtained, with optical systems specially configured. With microscopes running under normal conditions, the optical section thickness will be > 1 μm, and the effective penetration into enamel and dentin a maximum of 100 μm. The technique generates significant improvements in resolution, lying somewhere between that of conventional light microscopy and TEM/SEM (Watson and Boyde, 1991).
RESOLUTION AND CONTRAST

Factors affecting the resolution of optical microscopes (the ability to discriminate between two point sources of light) have been discussed at great length in text-books of microscopy (Inoue, 1986). Resolution is a feature inextricably linked with image contrast. By the confocal principle, both resolution and image contrast can be improved when compared with conventional light microscopy. However, other basic microscopy principles can have a profound effect on the quality of results achieved. For instance, it is imperative that the optical components be well-aligned and clean. Vibration is one of the biggest problems confronting resolution. Purchasing an expensive confocal microscope is a waste of money without putting it on an anti-vibration table. It has been said that 'you don't know you have a problem with vibration until you get rid of it!'

The theoretical resolution of a confocal system is primarily a function of the numerical aperture (NA, light-gathering ability) of the optical system and the wavelength (\( \lambda \)) of the light. Reducing \( \lambda \) to increase resolution is not always easy because of the problems with handling ultraviolet light and the special optical components this may require in the microscope. The importance of high-NA, high-quality objectives should not be underestimated for successful confocal imaging. The best results are obtained with a lens with as high an NA as possible, bearing in mind the refractive index of the mounting media used for our samples (oil, 1.5; glycerine, 1.4; water, 1.3). For specimens of enamel and dentin, immersion oil can work very effectively as a mounting medium. However, if left in place for a long time, it will tend to 'clear' the sample as structures such as dentin tubules disappear from view once the oil soaks into the sample. Glycerine is a
very useful material for examining hydrated dental samples, because following microscopic examination, it can be washed off easily so that experiments can continue. It should be noted that this hygroscopic fluid can have a profound dehydrating effect on water-sensitive materials such as glass-ionomer cements, but this effect can be used to advantage for dehydration stress studies of these materials (Watson et al., 1996). Water immersion objectives do not always produce good images of dentin structure, when compared with oil immersion (Fig. 2).

Problems arise if structures which contain a lot of water are imaged deeply below the surface by means of oil immersion objectives. Refractive index matching of the lens immersion media and the substrate is therefore of great importance, to avoid distortions of images in the optical axis, as the lens is focused progressively deeper into the sample. An example where such errors would be inevitable would be in the imaging of a considerable distance (> 40 μm) into a cell containing water, with an oil immersion objective above the cover glass (Visser et al., 1992).

It should also be remembered that there will always be less resolution in the optical axis of the microscope, and so great care should therefore be taken in the interpretation of computerized z axis reconstructions made from serial optical sections (Fig. 3). Wherever possible, equivalent views should be made with the sample oriented in the same direction as the reconstruction, to confirm the validity of the latter. For example, potential problems would be indicated by spherical objects appearing elongated in the optical axis of the microscope.

Reflection images or images derived from light back-scattered from the sample are a very powerful means of determining the integrity of a sample. The best images are obtained with microscopes illuminated incoherently (by white light), such as the tandem scanning microscopes (TSMs) rather than the confocal scanning laser microscopes (CSLMs). Laser light produces 'speckle', and this tends to interfere with image quality and can make image interpretation difficult. Notwithstanding, such a surface view is useful when used in conjunction with fluorescence images (Fig. 4).

One of the most difficult problems in confocal microscopy is the imaging of small reflective objects which are associated with a substrate of low inherent contrast. Toothpaste slurries on dentin illustrate the problem well: The slurry is highly reflective on the dentin surface. Focusing deeper into the sample (even when a water immersion objective is used) will give very misleading information about the depth penetration of the particles, because of massive diffraction effects (Fig. 5). Scattering of light from planes which are out of focus would be exacerbated by the use of an oil immersion objective in this situation.

**SURFACE RECONSTRUCTION AND MEASUREMENT**

A major application of confocal microscopes is the imaging and measuring of surfaces, being widely used in the electronics industry where integrated circuits are evaluated. Surface profiles can be generated from extended-focus computer images derived from multiple image planes, without the need for the specimen to be touched. These have the advantage of generating many useful measurements, such as the roughness parameter (R_α), over the complete area in view (Radford et al., 1997). The extent of further 3-D computerized reconstructions that can be made from these multi-dimensional data is limited only by the power of the computer (White, 1995). Virtually all confocal microscopes
are sold with some form of image processing equipment, a natural corollary of the digitized images that are produced. Storage of these images is getting easier and cheaper as computer technology advances. Video rate microscopy will require very large data transfer and image storage capacities (Watson, 1994).

As for all imaging with confocal microscopy, the higher the NA of the objective lens, the better the image quality (Fig. 6). For all samples, a dry lens (maximum NA, 0.95) will generally be effective. However, oil immersion objectives should offer advantages because of their improved light-gathering ability (NA < 1.4). The oil contamination of a sample may be unacceptable, and if the substrate under examination is translucent (e.g., resin composite, dentin, enamel), subsurface structures will confuse the image (Watson and Boyde, 1991). Some form of reflective surface

Fig. 4—Montage showing four images of a composite (C), primer (P), adhesive (A), and dentin tubules (T) interface. Top left: combined three-color image: P = yellow/green, A = red, T & C = blue (scarcely visible, but appearing pale blue in the air-inhibited zone at the adhesive/composite junction). While this image is attractive, it loses valuable information when compared with the individual images that produce it. Top right: reflection image showing tubules (T) and reflective surface of composite (C). Laser speckle is evident in this image. Bottom left: fluorescence image of primer (P), labeled with lucifer yellow. Fine structures, highlighted with open arrows, are less apparent in the combined view. 488-nm line Ar ion laser, 520-nm bandpass filter. Bottom right: fluorescence image of adhesive (A) labeled with rhodamine B. The presence of part of this layer is masked in the combined image. 514-nm line Ar ion laser, 600-nm longpass filter. (Imaged with Noran Odyssey, x60 OI/1.4 NA, fieldwidth 80 μm.)
Fig. 5—Reflection image of a calcium carbonate toothpaste slurry on dentin (maximum particle size, 10 μm). The circular image results from the insertion of an aperture in the microscope to improve contrast. Top: surface view of slurry. Bottom: focused nominally 8 μm below the surface. The reflective particles have a profound scattering effect, as shown by the light scattered laterally around this apertured image; a diffraction ring can also be seen (arrow). (Imaged with Noran TSM, x40 WI/0.75 NA; fieldwidth, 100 μm [photos courtesy of Dr. Mark Ide].)

(e.g., gold sputter-coating, as for SEM) will then be necessary to improve light reflection and prevent its absorption by the sample (Fig. 7).

Monochromatic light should be used to reduce the effects of lens chromatic aberration (Fig. 8), although with full-spectrum light sources, this can be used to give a color-coded image of the sample (Browne et al., 1992). Color changes can also be useful for helping to position ‘flat’ specimens at 90° to the optical axis of the microscope (Watson and Cook, 1995).

**FLUORESCENCE MICROSCOPY**

The use of fluorescent dyes in microscopy is a very powerful investigative technique. This has become particularly useful in confocal microscopy, especially with laser scanning microscopes which have tended to rely heavily on fluorescence techniques. Laser illumination has the advantage of high potential light intensities with the laser beam, but also the concurrent disadvantage of rapid photo-bleaching of dyes. The techniques involved are discussed at length in Pawley (1995).

The improved resolution and image contrast can indicate the distribution of sample components. An example of this could be the interface of a fluorescent-labeled adhesive within tooth tissue, where extended focus images can dramatically illustrate the penetration of these materials (Fig. 9). It is important that dyes used should not be labile and be well-fixed to the materials being examined. For instance, fluorescein is highly soluble in water; therefore, images of this dye may indicate its distribution rather than the material to which it is ‘attached’. A control experiment for determining the distribution of resin-based materials is to remove the mineralized tissue at the interface by etching. A reflection image is then made of the free-floating tags of material by means of a water immersion objective (Watson and Boyde, 1987). The tags should be the same length as the tags in the fluorescence image: If they are shorter, there is a strong possibility of dye partition from the labeled material. Dyes which have a great chemical affinity for the material under examination will be advantageous and may give other information if they are also sensitive to local environmental factors such as pH (Fig. 10) (Bhawalkar et al., 1996). Overloading of materials with dye can lead to ‘quenching’ (an anomalous reduction in fluorescence intensity), which can lead to misleading results (Fig. 11). In such conditions, the dye molecules may be too close to one another. Photons emitted from a dye molecule, following interaction with the excitation illumination, are absorbed by closely neighboring molecules, rather than emitted as fluorescent light. Material properties should also be checked to ensure that the dye is not changing the characteristics of the substance under investigation.
Multiple labeling experiments can produce attractive images, but it is important that control experiments be conducted to verify the distribution of the individual dyes. Such controls would be the use of dye R in one component and dye F in another in one sample, and then reversing the order of the dyes within the control sample.

The spectral excitation and emission characteristics of dye molecules may not be optimally separated by the dichroic mirrors, filters, and laser lines available in the confocal microscope in use. 'Cross talk' or mixing of the images of fluorescent dyes is always a phenomenon to be aware of in multiple labeling experiments. It can be recognized by making equivalent samples but only with single-labeled components. Similarly, 'reflection breakthrough' may occur with fluorescent samples which have strongly reflective objects in the same field of view (Fig. 12). The filters may be unable to stop all of the reflected light in these situations and can lead to misinterpretation of results. The strength of the reflection signal should be independently assessed from the fluorescence signal if there is any possibility of strong backscattering of light by the specimen.

Shadowing effects occur in confocal imaging, especially where 'dense' materials are examined. While light may penetrate a transparent material relatively easily, it will be attenuated, or even stopped, by superficial structures (Fig. 3). This will be apparent on through-focusing, as shown by a reduction in light intensity for the deeper layers. However, prior knowledge of the structure would indicate that an image should be produced from these planes of focus. Vertical (x-z) computer reconstructions derived from a stack of image planes should be treated with circumspection when these images are made of materials that are highly light-absorbing and

Fig. 7—Profile view (LHS) and extended focus reconstruction from which it is derived (RHS). The white line shows the position of the profile trace on the image. The vertical distance between the arrowheads is 18 μm. Hydrofluoric-acid-etched porcelain, sputter-coated with gold, imaged with x100 OI/1.4 NA. 546-nm illumination, CCD camera, Noran TSMDP software: MISIS Image. Fieldwidth, 50 μm.

Fig. 8—Diagram (after Boyde and Jones, 1995) showing color coding for depth in a confocal microscope illuminated with white light such as a TSM. R = red, G = green, B = blue. Such chromatic aberration will be a feature of nearly all lens/microscope systems and will affect the detection of light returning from the sample to the microscope. If fluorescent dyes of widely separate wavelengths are used in multiple labeling specimens, then the images obtained may be derived from different focal planes in the same sample, unless this problem is corrected for.
scattering (i.e., most dental biomaterials).

Greater depth penetration and resolution are obviously going to be of benefit, and new imaging techniques such as 2-photon laser excitation of dyes have this potential (Denk et al., 1990). The use of long-wavelength excitation removes the problems of photo bleaching of dye molecules. However, the laws governing the transmission of light still remain. With the longer excitation (near-infrared) wavelengths used in 2-photon imaging, there will be greater sample penetration by the exciting light, but it is still a requirement that the emitted fluorescent light be able to pass back through the sample. Therefore, opaque or light-scattering structures will still produce shadowing effects in the image.

FOURTH DIMENSION: TIME

Microscopic images will record the appearance of a structure which may or may not be changing. Classic microscopic techniques have tended to look at 'fixed' tissues, but more recently, the imaging of dynamic changes within objects has gained in popularity. Applications such as the mapping of intra-cellular ionic flux (e.g., calcium ratio imaging) have become practical with the advent of suitable fluorescent dyes (see relevant sections in Pawley, 1995). Capturing such fast-moving events presupposes a detection system which is sufficiently fast and sensitive to record the events as they happen. Even when working at video-rate (25 frames/s), many dynamic events will still be undetectable: 'Absence of proof is not proof of absence'. Two-photon imaging techniques offer significant advantages in high-speed imaging (Denk et al., 1990).

Many conventional confocal microscopes are incapable of producing full-frame images at video-rate and so will rely on 'line scans' to pick up rapid changes in the image.

Fig. 9—Single optical section (top) and extended focus (bottom) images of adhesive penetration into dentin. 20 slices, reconstruction 20 μm thick. Notice the lateral tubules of the dentin penetrated with adhesive but the voids in some of the main tubules. An acid erosion experiment would confirm or deny the presence of resin in the tubules, rather than dissociated dye. Adhesive resin labeled with APSS dye (4-[N-(2-hydroxyethyl-amino)styril]-N-methylpyridinium tetraphenylborate) (courtesy of Dr. P.C. Cheng and Dr. P.N. Prasad, SUNY, Buffalo, NY, USA). Imaged with Olympus Fluoview, 488-nm line Ar ion laser, 520 bandpass filter. x60 OI/1.4 NA. Fieldwidth, 130 μm.
Fig. 10—A resin-modified glass-ionomer adhesive, labeled with APSS which has been dosed with the liquid component of the cement. This dye has a great affinity for HEMA. Particulate distribution can be seen close to the dentin interface, with the glass particles displaced from the cavity surface by a swelling of the matrix component of the system: the ‘absorption layer’. The dye is showing fluorescence around the glass particles: This may be related to its fluorescence being strongest in an alkaline-localized environment. The dye was originally developed for 2-photon imaging, and when these samples are imaged with this technique, high-resolution images can be produced up to 80 μm below the surfaces of these dense materials. When imaged with a conventional confocal microscope, as here, the maximum useful penetration will be only about 10 to 20 μm. Imaged with Olympus Fluoview, 488-nm line Ar ion laser, combined reflection/fluorescence image. x60 Oil/1.4 NA. Fieldwidth, 75 μm.

within a sample: These may miss the area where changes are occurring. If there is an experimental requirement for high-speed imaging, then selection of a microscope with a high scanning rate is important (Watson, 1994).

Video imaging and storage onto video cassette tape are reasonably straightforward to arrange. However, subsequent analysis of the images and any associated data is inconvenient, if not difficult. Personal computer hardware now has sufficient capacity and reliability to allow for data transfer of digital images (512 x 512 x 8 bit at 25 frames/s) straight to hard disc (audio-visual specification). This allows for convenient editing of images and excellent opportunities for their combination with other experimental data captured concurrently (e.g., images of interfacial fracture in a shear bond test and equivalent load/displacement conditions)

(Watson, 1994).

While video-rate imaging offers many exciting possibilities, time lapse studies, of which all confocal microscopes are capable, will also generate interesting information regarding changes within materials over time. The major advantage of confocal imaging will be that the samples can be kept in near-normal conditions. However, as mentioned earlier, the microscope operating conditions must be stable, with no drift in the microscope stage or illumination intensity (Watson et al., 1996).

CONCLUSIONS

As with all new techniques, the inexperienced investigator should be aware of some of the artefacts inherent within the system. However, the widespread availability of confocal microscopes should give ample opportunity for dental researchers to capitalize on this technology.

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REFERENCES


