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THE USE OF THE CONFOCAL MICROSCOPE IN THE STUDY OF ANCIENT HUMAN BONES

ABSTRACT: The authors demonstrate that ancient human bones exhibit natural autofluorescence. The red autofluorescence appears to be typical of ancient bones and therefore must be interpreted as a phenomenon related to diagenesis. They studied 4 samples of bone: 3 samples of ancient remains (Roman and medieval bones) and 1 sub-modern to make a comparison.
The laser scanning of the samples makes it possible to observe and record the images of the bone tissue relative to various planes below the surface of the histological specimen and to follow the three-dimensional disposition of the histological structures of the bones. The authors think that the confocal microscope could be utilised for histological and anthropological research on ancient bones.

KEY WORDS: Confocal microscope – Ancient bone – Fluorescence

INTRODUCTION

Histological specimens of ancient human bones are generally examined with a stereomicroscope at low magnifications, and with transmitted light optical microscopes which can employ polarized light, at higher magnifications. The scanning electron microscope is also useful for examining the detailed structures of ancient bones. All of these instruments, however, are limited to superficial examinations of the specimens: indeed, microscopes that gather reflections (of either visible light or electrons) can only examine the surfaces of the specimens, while those that employ transmitted light require sections thin enough so that light or electrons could cross them. With the confocal microscope one could, on the other hand, analyze not just the surface, but to a certain depth which can be considerable but varies depending upon the material in question. This new capability provides unexpected opportunities, and is here applied to the study of bones for the first time.

BACKGROUND

The confocal microscope exploits fluorescence, in other words the capacity of some materials, when irradiated with light of a given wavelength, to reemit the energy in the form of light waves of a longer wavelength. The microscope is already commonly used in the study of a number of biological structures, from diatoms to lymphocytes to cancerous cells. Some biological structures and certain macromolecules are fluorescent on their own (*primary fluorescence or autofluorescence*). For example, elastic fibers (brilliant blue fluorescence), wax-like substances and the riboflavins (various shades of yellow), vitamins (various shades of yellow, green and blue), and the porphyrins (strong red) (Radley, Grant 1951).

Materials that do not display primary fluorescence must be impregnated with fluorescent substances (*fluorochromes*) (*secondary fluorescence*) before they can be examined.

The confocal microscope employs a laser beam to examine the sample at depth, and the individual serial

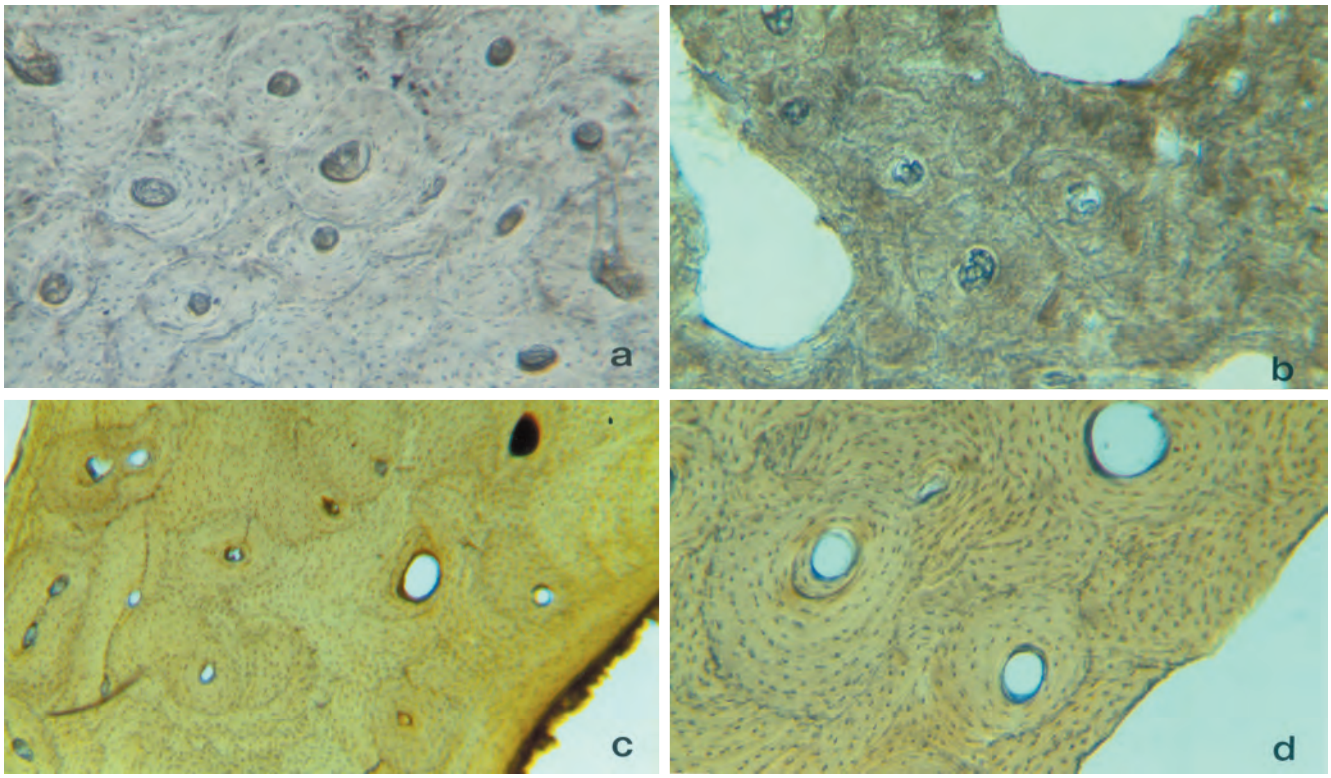

FIGURE 1. Transverse sections observed through a transmitted light optical microscope: a) sample #1 (sub-recent bone, femur, 100×); b) sample #2 (T51 Ind. B from Teramo Sant'Anna, femur, 100×); c) sample #3 (E113 from Herculaneum, fibula, 100×); d) sample #4 (E130 from Herculaneum, fibula, 100×).

images gathered can then be processed to obtain three-dimensional reconstructions. One can also make "virtual" cross sections of a specimen.

Until we undertook this study, it was not known if bone exhibited primary fluorescence; it turns out that the confocal microscope will be especially useful in providing information on the three-dimensional paths followed within a specimen by structures such as Havers channels or osteocytic lacunae.

MATERIALS AND METHODS

The samples examined here were pieces of non-decalcified human bone taken from the *linea aspera* of the femur, or the interosseous ridge of the fibula, of four individuals, (*Table 1*); one sample dates to sub-current time, one is from an archaic necropolis, and two are from Roman times.

The thin sections (*Figure 1*), which are 100µ and 150µ thick, were obtained using a microtome with a diamond-coated circular blade (Leica 1600). After washing for 10 minutes in alcohol of increasing concentrations (from 70% to 100%) and xylene, the sections were mounted on slides using Canada balsam. In preparing the specimens plain glass slides, which are generally not fluorescent, were used. The ancient bone specimens were studied with a confocal microscope consisting of a krypton/argon Fluoview laser generator (Olympus) mounted on an Olympus BX50 transmitted light microscope at the Laboratory of Anthropology of the Ministero per i Beni e le Attività Culturali's Laboratorio di Antropologia del Servizio Tecnico per le Ricerche Antropologiche e Paleopatologiche. The sample of modern bone was examined with an MRC 1024 BIORAD confocal microscope (with the same krypton/argon laser) mounted on a Zeiss optical microscope at the Laboratorio di Fisiologia Cellulare of the Università degli Studi "G. D'Annunzio" in Chieti. The images were processed using a program that runs under Windows version 3.11.

OBSERVATIONS

The confocal examination of the transversal sections of the femoral cortical of the recent sample of dry human bone showed, first of all, that the material has very little primary fluorescence (*Figure 2*). Regardless of the wavelength selected for the observation (we used both green and red light), the architecture of the treated bone from the recent skeleton revealed only a non-uniform, tenuous primary fluorescence; the only intensely fluorescent areas are the walls of the Havers channels and the osteocytic niches, and the canalicolae that, in life, hosted the cytoplasmic elongations of the osteocytes. This distribution is probably due to the presence of protein remains in the areas that exhibit primary fluorescence. In

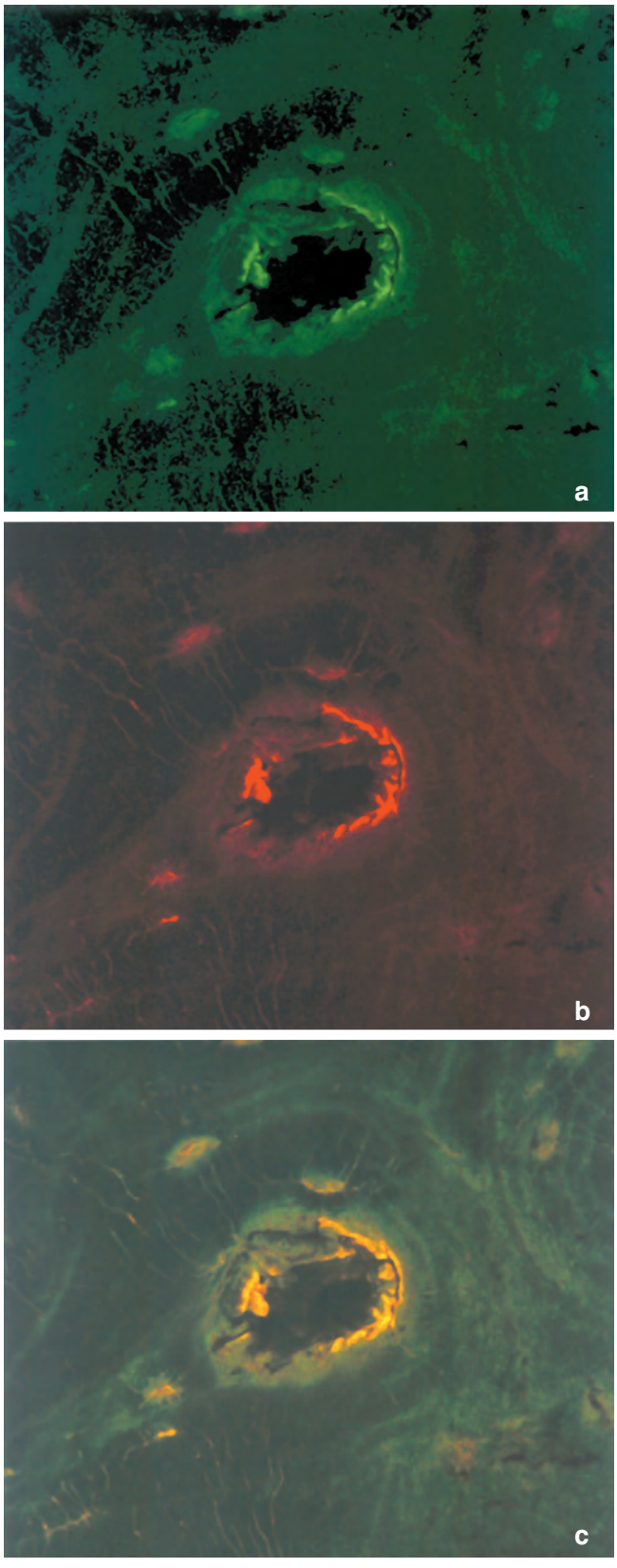

FIGURE 2. Confocal observations of sample #1 (sub-recent bone) at the green (a), and red (b) wavelengths, and combination of the two images (c). There is nearly no autofluorescence at the level of the architectural structure of the bone tissue, while the areas of intense emission are limited to the walls of the Havers channels, osteocytic niches, and canalicolae (proteinic remains, 100×).

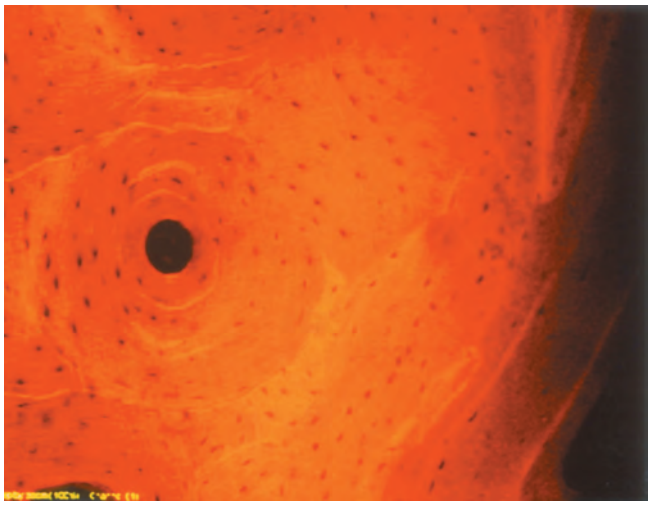

FIGURE 3. Transverse section of sample #4 (E130, fibula) observed with the confocal microscope: note the intense and diffuse autofluorescence (observed at the red wavelength, 63×).

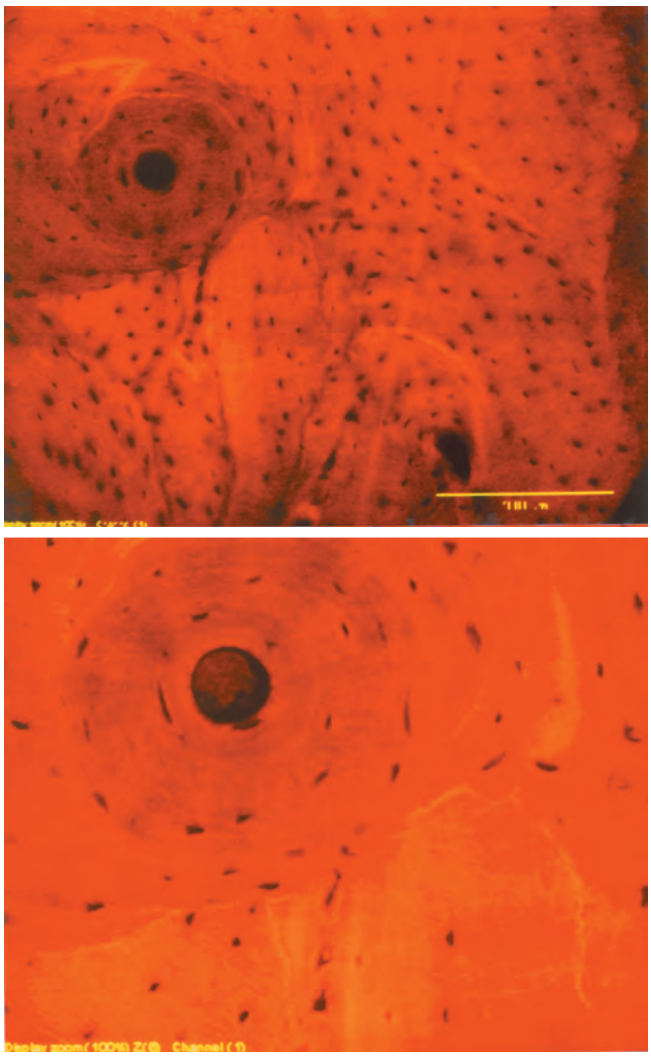

FIGURE 4. Transverse section of sample #3 (E113) seen with the confocal microscope at the red wavelength: 63×, note the intense autofluorescence and the clarity of all the structures typical of the histological architecture of a normal human bone: the cementation lines and the relationships between osseous breccia and active osteons are especially clear.

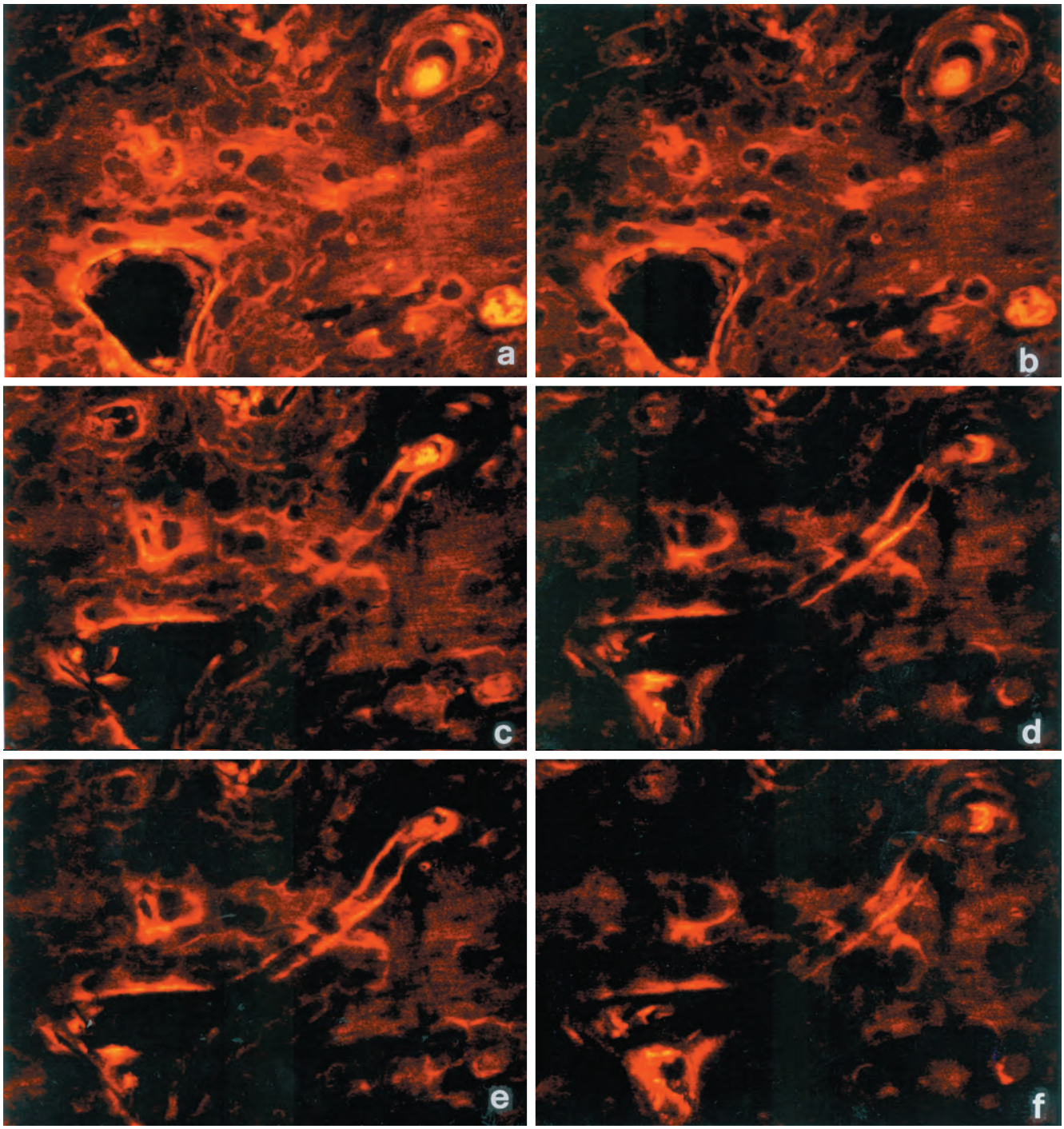

FIGURE 5. Transverse section of sample #3 (E113) seen with the confocal microscope (observed at the red wavelength; 63×): six images at differing depths (a–f) below the surface of the sample, obtained through laser scanning; one can follow the path of a Volkmann's channel.

fact, the fluorescence of the walls of the Havers channels corresponds to the areas in which the (prevalently proteinic) remains of the vascular system and blood accumulate in the bones of the dead. The primary fluorescence of the niches and canalicolae instead follows the distribution of the remains of the Rouget-Neumann sheath, which is again proteinic (Capasso, Di Tota 1993). These observations therefore would tend to indicate that recent dry human bone does not exhibit primary fluorescence; the phenomenon only occurs in the limited areas where proteinic remains related to the putrefaction of the soft parts of the bone structure accumulate.

To the contrary, all the samples of ancient bone from archaeological excavations exhibited intense red autofluorescence (*Figures 3*, *4*). Though this fluorescence is not uniformly distributed throughout the samples, the areas in which the proteinic remains accumulated are not areas of high-energy emission. The examination of the

ancient remains also showed how well the structural part of the bone samples was preserved: the structure of the active osteons is clearly visible, with the concentric disposition of the bony lamellae around the vascular (Havers) channels. One can also see the irregular shape of the interstitial lamellae that fill in the areas between the osteons (osseous breccia).

The primary fluorescence makes it possible to follow the paths of the cementation lines, in other words the non-fibrillose layers that delimit the osteons and the interstitial systems: they are pale orange, with respect to the more intense color of the surrounding structures. The many osteocytic lacunae which are also quite evident, appear as black, like the vascular canals.

The laser scanning of the samples makes it possible to observe and record the images of the bone tissue relative to various planes below the surface of the histological specimen. This in-depth scanning allows, for example, the study of the structure and morphology of the Volkmann canals, which connect adjacent Havers channels, and to follow their paths through the osseous breccia by joining a sequence of contiguous sections (*Figure 5*).

By registering a series of images obtained through in-depth laser scanning and processing them with a computer, one can obtain virtual transversal sections of the specimen, sections that reveal the bone structure throughout the entire thickness of the sample (100µ) along planes perpendicular to that of observation. For example, this technique was used to perform a transverse section of sample number 4, which revealed variations in the caliber of three Havers channels observed longitudinally. The image obtained (*Figure 6*) allows the study of the vascular channels and the variations in their morphologies along their lengths (for example increases or decreases in their cross sectional areas), and also to observe the connections there may be with perforating vascular channels (Volkmann's channels) or elongations of the osteocytic lacunae.

We were also able to follow the three-dimensional disposition of the histological structures in the fresh human bone studied with the confocal microscope, though the images obtained were not as richly detailed due to the low autofluorescence of the recent bone.

The greatest emission of fluorescent radiation in the ancient bony tissue was likely related to processes of refraction and reflection of the electromagnetic waves by minerals that were present in greater amounts in the ancient bone matrix, in other words, the appearance of structural or chemical alterations to the hydroxyapatite during diagenesis.

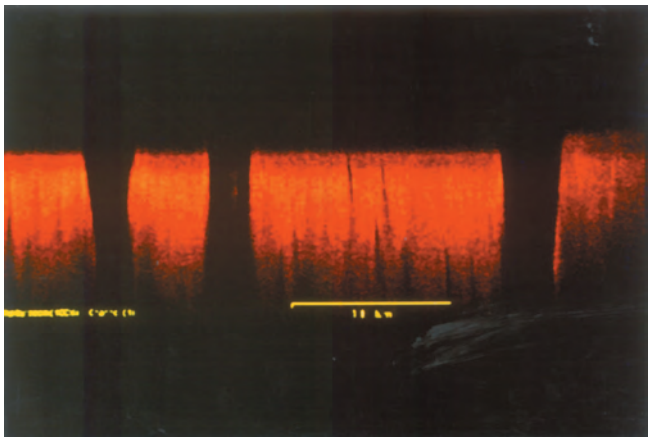

FIGURE 6. Virtual longitudinal section of sample #4 (E130) obtained through the elaboration and interpretation of a series of scans on the surface and at depth that cover the entire thickness of the sample (about 100µ). Note the three Havers canals with their longitudinal variations in width; the longitudinal series with less energy are less autofluorescent areas interspersed in the laminar structure of the osteons (observations at the red wavelength, 63×).

CONCLUSIONS

Dry recent human bone is only slightly autofluorescent, and the zones of fluorescent emission (concentrated along the walls of the vascular channels, osteocytic niches and canalicolae) are likely related to the presence of proteinic remains.

Ancient human bone, instead, exhibits natural autofluorescence. This red autofluorescence would appear to be typical of ancient bone, and therefore must be interpreted as a phenomenon related to diagenesis. At present we cannot say if the appearance of autofluorescence is related to a relative enrichment in mineral salts or to possible physical and/or chemical alterations to the hydroxyapatite.

New studies are necessary to determine this; in the meantime this study shows that autofluorescence is characteristic of ancient human bone, and this means the confocal microscope provides another means for anthropological and palaeopathological study. Indeed, unlike the microscopes normally used to study samples of

TABLE 1.

Samples	N°	Origin	Date	Sex	Age at death
Femur, sec.150µ	# 1	Chieti	Sub-modern	Male	Adult
T. 51 Ind. B Femur, sec.150µ	# 2	Teramo	VII–XI cent. A.D.	Male	Adult
E 113 Fibula, sec. 100µ	# 3	Herculaneum	79 A.D.	Female	30–35
E 130 Fibula, sec. 100µ	# 4	Herculaneum	79 A.D.	Female	20–25

bone tissue, the confocal microscope allows us to use laser scanning to collect information on the three-dimensional disposition of the various anatomical structures of the bone. One can therefore use a single histological specimen to study not just the appearance of the tissue along the surface of the section, but also to observe the tissue architecture of the underlying plains and view, albeit virtually, a longitudinal section of the sample.

If further studies of greater numbers of samples confirm that autofluorescence is typical of ancient human bone tissue, it will open the way to research on 1) the reasons of the fluorescence; 2) the chronology of its appearance; 3) its relationship with the characteristics of burial. This course of research, which is already underway in our laboratories, may also determine the relationship between appearance and intensity of autofluorescence and time elapsed from the moment of death.

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REFERENCES

CAPASSO L., 2001: *I fuggiaschi di Ercolano*, "L'Erma" di Bretschneider, Roma.

CAPASSO L., DI MUZIO M., DI TOTA G., SPOLETINI L., 1990: Gli inumati della necropoli medievale di Teramo Sant'Anna: studio antropologico preliminare. *Ricerche antropologiche a S. Maria Aprutiensis*. Pp. 12–16. Edifrafital S.p.A., S. Atto di Teramo.

CAPASSO L., DI TOTA G., 1993: Probable persistence of the remains of a Rouget-Neumann sheath into the canalicular system of ancient human bone. *Internat. J. of Osteoarcheology* 3: 101–105.

CHIARUGI G., BUCCIANTE L., 1972: *Istituzioni di anatomia dell'uomo*, vol. 1, tomo 2, Casa Editrice Vallardi, Milano.

MAZZI V., 1977: *Manuale di tecniche istologiche e istochimiche.* Piccin Editore, Padova.

PASQUINELLI F., 1981: *Diagnostica e tecniche di laboratorio*, vol. 2. Rosini Editrice s.r.l., Firenze.

RADLEY H., GRANT H., 1951: *Fluorescent analysis in U.V. light*. Chapman & Hall.

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