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Applications of X-ray synchrotron microtomography for non-destructive 3D studies of paleontological specimens

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ABSTRACT Paleontologists are quite recent newcomers among the users of X-ray synchrotron imaging techniques at the European Synchrotron Radiation Facility (ESRF). Studies of the external morphological characteristics of a fossil organism are not sufficient to extract all the information for a paleontological study. Nowadays observations of internal structures become increasingly important, but these observations should be non-destructive in order to preserve the important specimens. Conventional microtomography allows performing part of these investigations. Nevertheless, the best microtomographic images are obtained using third-generation synchrotrons producing hard X-rays, such as the ESRF. Firstly, monochromatisation avoids beam hardening that is frequently strong for paleontological samples. Secondly, the high beam intensity available at synchrotron radiation sources allows rapid data acquisition at very high spatial resolutions, resulting in precise mapping of the internal structures of the sample. Thirdly, high coherence leads to additional imaging possibilities: phase contrast radiography, phase contrast microtomography and holotomography. These methods greatly improve the image contrast and therefore allow studying fossils that cannot be investigated by conventional microtomography due to a high degree of mineralisation or low absorption contrast. Thanks to these different properties and imaging techniques, a synchrotron radiation source and the ESRF in particular appears as an almost ideal investigation tool for paleontology.

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1 Introduction

Paleontologists aim to obtain the maximum knowledge about the fossils they study. It is therefore increasingly important to have access to internal or histological structures of the samples. Numerous studies have been performed using destructive methods, mainly by sawing fossils, for microscopy investigations. This kind of approach provides very important data, for example about teeth structure and development [1–3]. In some cases (i.e. for a majority of microfossils) these techniques are the only way to have access to the principal features of the fossils. These techniques have the advantage of being quite cheap and very efficient, but they present at least two major drawbacks. The first one is that they do not give access to the three-dimensional structure. The second one, even more important, is that they are destructive. Fossils represent a non-renewable resource. Except for some groups of fossils that are well represented and that can be studied by destructive techniques, many of extinct species are known only through a very limited number of samples (sometimes by only one specimen). Thus, it is inconceivable to destroy or even to damage rare fossils in order to study their internal or histological structures. In particular, this aspect is of critical importance for hominid fossils.

Before studying important specimens, fossil owners need to be guaranteed that the investigation technique is totally non-destructive. X-ray imaging techniques are therefore well adapted because they are non-invasive.

X-ray projection radiography has been the first X-ray technique used historically; unfortunately it is often insufficient to give meaningful data on a thick or dense specimen (poor absorption contrast) and the lack of the third dimension largely limits the exploitation of data.

The third dimension is accessible in images produced by computed tomography (CT) scanners, which were developed for medical purposes. CT is now a reference tool for three-dimensional non-destructive studies, in particular for paleoanthropologists [4–7]. On tomographic slices and volumes, the full visualisation of the sample is possible and therefore it is perfectly suitable for quantitative measurements [8] (2D and 3D morphometry, virtual reconstruction using fragments or damaged fossils, endocranial extractions and so on).

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Nevertheless, medical scanners can provide only very limited spatial resolutions. Even in the best case, it is difficult to observe structures smaller than 0.5 mm. They cannot be used when precise measurements are needed, for example of the dental enamel thickness [6, 9, 10] or when details on the micron scale or samples of submillimetric sizes have to be studied.

Laboratory tomographs, not necessarily devoted to medical applications, have evolved over the last few years and can reach high spatial resolutions. They can reveal very small details (sometimes down to the micrometre scale) and are nowadays the benchmark for a wide variety of applications including paleontology [11, 12]. However, despite the high quality of the data that can be obtained by some of these machines, investigation of numerous fossils remains difficult. Limitations arise from the polychromatic X-ray source spectrum of these machines and from the intrinsic nature of the fossils (highly mineralised samples, which often exhibit very low absorption contrast).

Using a third-generation synchrotron source optimised for hard X-rays, such as the European Synchrotron Radiation Facility (ESRF), instead of conventional sources, changes dramatically the situation for investigations of fossils. Indeed, X-ray beams used for X-ray synchrotron microtomography (SR-μCT) at the ESRF present three main properties that enhance significantly the data quality and the imaging possibilities: the monochromaticity, the high beam intensity and the partial coherence.

Through several examples taken in different fields of paleontology, we present here a rapid overview of what can be done at the ESRF thanks to these beam properties.

2 Monochromaticity and beam hardening effect

The first important property of the X-ray beams used at the ESRF for microtomography is the monochromaticity.

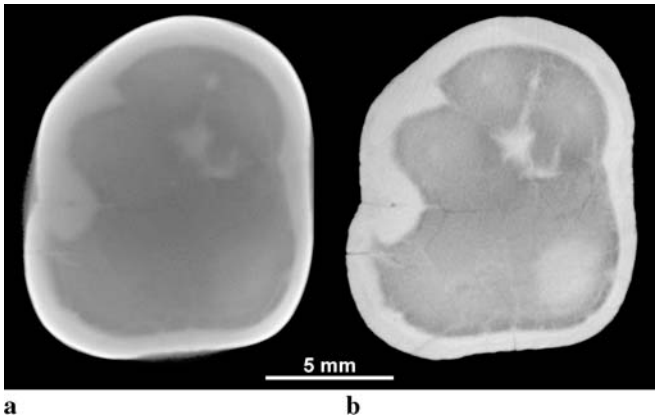

FIGURE 1 Comparison for the same microtomographic slice between a scan performed (**a**) with an industrial microtomograph using a polychromatic X-ray source (voxel size 20 μm) and (**b**) with a monochromatic X-ray beam at an energy of 50 keV (voxel size 30 μm) on the ID19 beamline of the ESRF. The sample is a second lower molar of the middle Miocene Thai hominoid primate *Khoratpithecus chiangmuanensis*

When using conventional sources the beams are generally polychromatic, and the reconstructed slices present in many cases the so-called 'beam hardening' artefact. This artefact is due to the differential absorption of the X-ray spectrum by the sample [6, 13, 14]. In fact, because the low energies are more absorbed by the sample than the high ones, the harder part of the X-ray spectrum becomes predominant in the transmitted beam. This effect leads to a misleading recovery of the linear absorption coefficients on the reconstructed slices, mainly appearing as brightening of the sample borders. Beam hardening is related to the density of the material. Hence, as fossils are generally highly mineralised, this artefact is often strong (see for example [11] or [12]). Dental enamel thickness measurements [6] constitute an example of these misleading results. Figure 1 presents, for a second lower molar of the Thai middle Miocene hominoid *Khoratpithecus chiangmuanensis* [15], a comparison between two scans, the first using a polychromatic beam from a conven-

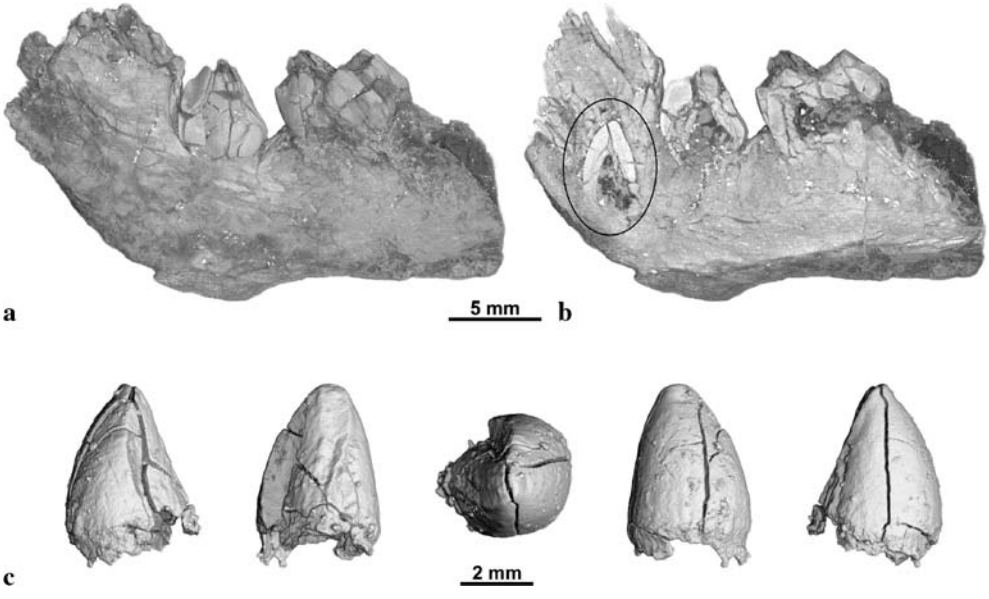

FIGURE 2 3D extraction of an unerupted third premolar germ from a mandible of an undetermined late Eocene primate from Thailand. (**a**) 3D rendering of the external morphology of the sample. (**b**) Virtual vertical slice through the mandible showing the presence of an unerupted dental germ. (**c**) 3D rendering of the tooth after segmentation. Data were collected on the ID19 beamline with a voxel size of 15 μm. Thanks to monochromaticity, no beam hardening effect is present

tional X-ray source and the second using a monochromatised synchrotron beam (beamline ID19 of the ESRF). This comparison clearly shows that beam hardening can substantially degrade results of microtomographic investigations of fossil samples.

A monochromatic intense beam leads to reconstructed slices free of beam hardening effects. Data processing is easier as absorption information can be related more accurately to the local densities in the sample. Results can be exploited for quantitative studies with a very good precision and reproducibility [16]. It also greatly facilitates the segmentation of the 3D images of the sample (Fig. 2) and the extraction of relevant parameters.

Hence, a monochromatic beam in addition to a nearly parallel beam geometry [14] produces images of the internal structures in mineralised fossils that are of much better quality than those obtained using conventional sources.

3 High beam intensity and spatial resolution

The second major asset of a synchrotron radiation source is the very high X-ray beam intensity, which is orders

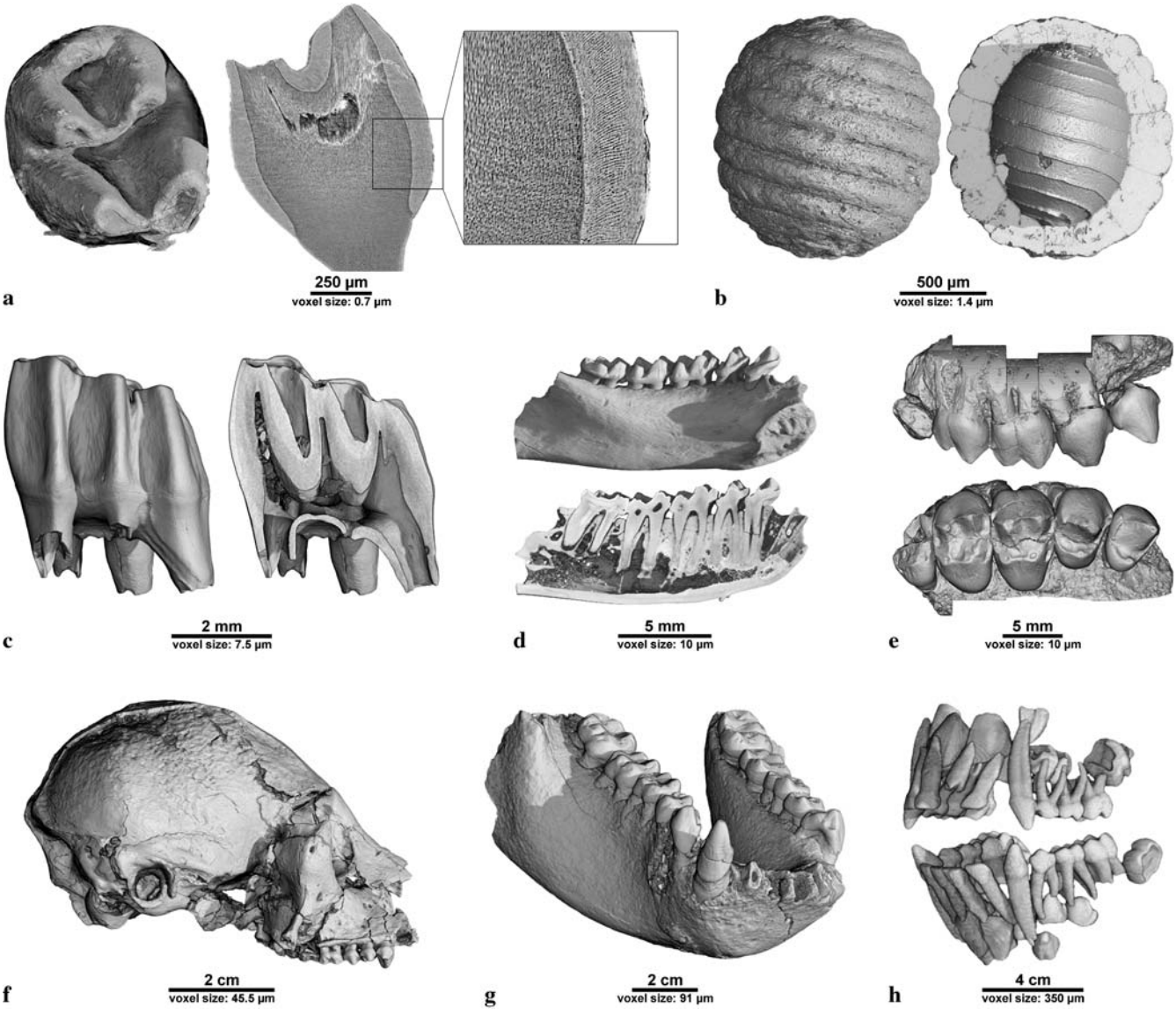

FIGURE 3 Examples of applications of X-ray synchrotron microtomography on paleontological and biological samples illustrating the wide range of spatial resolutions that can be used at the ESRF. Resolutions decrease from the first toward the last sample. (**a**) Molar of a modern murinae rodent (beamline ID19). With such a high resolution it is possible to observe the enamel and dentin microstructure non-destructively. (**b**) Cretaceous charophyte algae *Peckisphaera gigantea* (beamline ID19). On this sample, we virtually reconstructed the internal cast showing the morphology of the non-preserved oospore. (**c**) External morphology and virtual slice in a molar of the middle Miocene rodent from Spain *Ruscinomys sp*. (beamline BM5). (**d**) External morphology and virtual slice in a mandible of the late Eocene Omomyiform primate from France *Microchoerus sp*. (beamline ID19). (**e**) Upper teeth of the late Eocene anthropoid from Thailand *Siamopithecus eocaenus* (beamline ID19). Data were collected in local microtomography, the maximum width of this fossil being about 20 mm. (**f**) Skull of the early Miocene platyrrhine primate from Argentina *Homunculus patagonicus* (beamline ID17). (**g**) Mandible of the late Miocene hominoid primate from Thailand *Khoratpithecus piriyai* (beamline ID17). Original data were acquired with a voxel size of 45.5 µm but the 3D processing was done on the complete volume after a binning by a factor of two. (**h**) Virtual pulling out of the lacteal and definitive teeth of a modern skull of a young chimpanzee *Pan troglodytes* (beamline ID17). Data were collected with a linear germanium detector

of magnitude higher than that produced by X-ray tubes. This high flux allows using monochromators, still maintaining a sufficient flux for imaging. The monochromatic beams are produced using either crystals (mainly silicon) or multilayer coated mirrors. Quasi-monochromatic conventional sources are also available, but their flux is too low to perform X-ray imaging of fossils with a good picture quality, or in a reasonable time.

Even when very high resolution detectors (pixel size < 1 micron) are used, the intense monochromatic beam permits exposure times within a fraction of a second for a single projection. At the ESRF, the resolution range, expressed as pixel size, is currently from 350 μm (on the medical beamline ID17) down to 280 nm (on the 'long imaging' beamline ID19) with various detectors of intermediate resolutions. This wide range of resolutions is important for paleontologists as they study a great variety of samples with a very wide range of sizes (to summarise, from bacteria to dinosaurs). Through complementary beamlines, the ESRF is able to cover the major part of this range. Several kinds of paleontological samples have already been imaged at the ESRF and clearly demonstrate the possibilities of SR-μCT for non-destructive studies of fossils (Fig. 3) [15–20].

4 Partial beam coherence and phase contrast imaging

SR-μCT using a third-generation synchrotron optimised for hard X-rays leads to the best possible results that can be obtained on fossils in absorption mode. Nevertheless, many fossils display strong chemical modifications due to diagenesis. The fossilisation process results, for numerous paleontological samples, in very weak absorption contrast. Third-generation synchrotrons, through the high coherence of the X-ray beams, bring new imaging techniques that can solve this problem for a large number of fossils [16].

The transverse coherence of the X-ray beam is increased when the distance from the synchrotron source increases (about 145 m for the ID19 beamline) and when the spatial extent of this source decreases. In this case, a 'long' propagation distance (typically 0.5 m) between the sample and the detector allows detecting X-ray interference patterns associated with sample details that are indistinguishable in absorption mode (i.e. using incoherent sources as in the conventional scanners). Different imaging techniques based on this propagation effect exist: phase contrast microradiography, phase contrast SR-μCT and holotomography [14, 21–26]. Each of these techniques allows novel paleontological applications. Some examples showing briefly these new possibilities are presented hereafter.

4.1 *Phase contrast microradiography*

Propagation-based phase contrast on microradiographs leads to efficient edge detection that superimposes with the absorption contrast. All the interfaces in the sample that correspond to abrupt phase changes appear clearly. Compared with a microradiograph in the absorption mode, the result appears sharper and very small details can be revealed [23, 27–29]. One of the most spectacular applications of this technique in paleontology is the investigation of inclusions in opaque amber. Amber is a fossil tree resin that sometimes embeds organisms (flowers, insects, spiders and so on). Most of the time, it is quite transparent and inclusions can be directly seen with optical systems (binocular stereomicroscopes or microscopes) after polishing the surface. But some amber blocks are totally opaque and then cannot be studied by optical methods. For this kind of sample, it is even impossible to know if there is any inclusion. Absorption microradiography can show some inclusions when organisms exhibit sufficient absorption contrast, but most of the embedded organisms remain invisible with this technique. Using phase contrast microradiography to image these opaque amber blocks reveals the details of the inclusions, even using a medium-resolution detector. Figure 4 shows phase contrast building up when increasing the distance between the sample and the detector (pixel size of 7.5 μm) on a late Albian (Cretaceous) hymenopterous insect from France. This investigation technique is likely to become a reference tool for the study of inclusions in opaque amber. After a precise localisation of the organisms, the most interesting ones can be imaged in three dimensions by SR-μCT with a moderate phase contrast (as described below). Figure 5 presents such a three-dimensional extraction of a Cretaceous beetle (Coleoptera: Dermestidae, origin: France). It was virtually extracted from resin and separated from the bubbles, the cracks and the impurities by 3D segmentation. All the details of the cuticle are visible. The animal can be studied as if it was not embedded in resin. This method is especially interesting for the study of the oldest Mesozoic ambers, in which the majority of pieces of resin are opaque. It can also be applied on transparent amber pieces, in order to achieve 3D reconstructions of important specimens.

4.2 *Phase contrast microtomography*

Computed tomography slices are reconstructed from projections acquired over 180°. If phase contrast microradiographs are used instead of absorption ones, it leads to phase contrast SR-μCT [24, 30]. This technique yields 3D data with edge detection superimposed on absorption contrast. The amount of edge enhancement depends on the distance sample/detector, on the X-ray energy, on the resolution of the detector and on the phase properties of the sample itself.

This imaging method can bring impressive results on fossils that present very weak absorption contrast [16]. Moreover, it reveals features that usually can be investigated only on microscopic slides. One of the most impressive applications of phase contrast SR-μCT concerns fossil teeth exhibiting no absorption contrast, due to diagenesis. Figure 6 shows a comparison between absorption and phase contrast scans of a molar of an early Oligocene eosimiid primate from Pakistan. Edge detection allows distinguishing the position of the enamel–dentin junction despite the fact that there is no observable absorption contrast due to the mineralisation pattern of this tooth. Studies of enamel thicknesses and distributions are particularly important for paleoanthropologists [5, 6, 10, 16, 31–34]. Numerous fossil teeth display this kind of mineralisation and cannot be investigated by classical, absorption-based, microtomography. Using phase contrast allows revealing the enamel–dentin junction, and hence studying these fossils non-destructively with a very good accuracy.

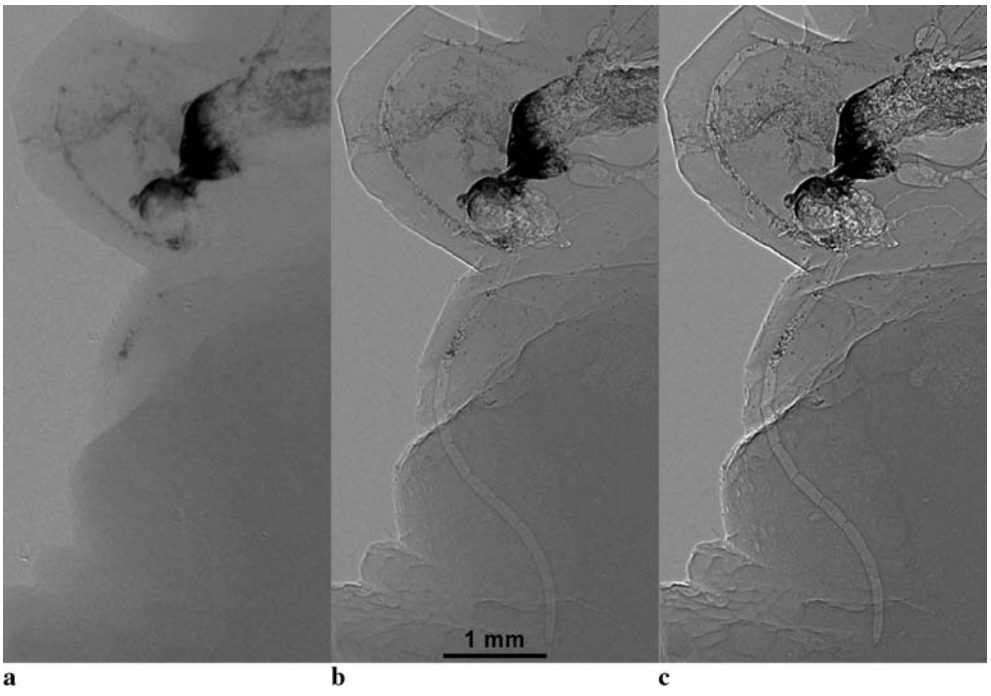

FIGURE 4 Phase contrast microradiography on a Cretaceous hymenopterous insect from France embedded in opaque amber. The pixel size is 7.5 microns. The images were acquired on the beamline ID19 at an energy of 20 keV. (**a**) Distance sample/detector 10 mm (absorption microradiograph), (**b**) 500 mm, (**c**) 990 mm. Phase contrast reveals a lot of anatomical features that remain invisible on the absorption microradiograph

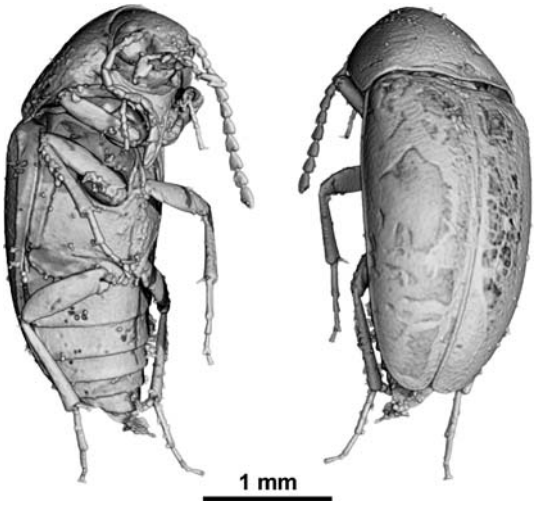

FIGURE 5 3D rendering after segmentation of a Cretaceous beetle from France embedded in opaque amber. The scan was performed on the beamline ID19 with an energy of 20 keV and a voxel size of 7.5 μm. The sample/detector distance was 150 mm in order to obtain a moderate phase contrast contribution

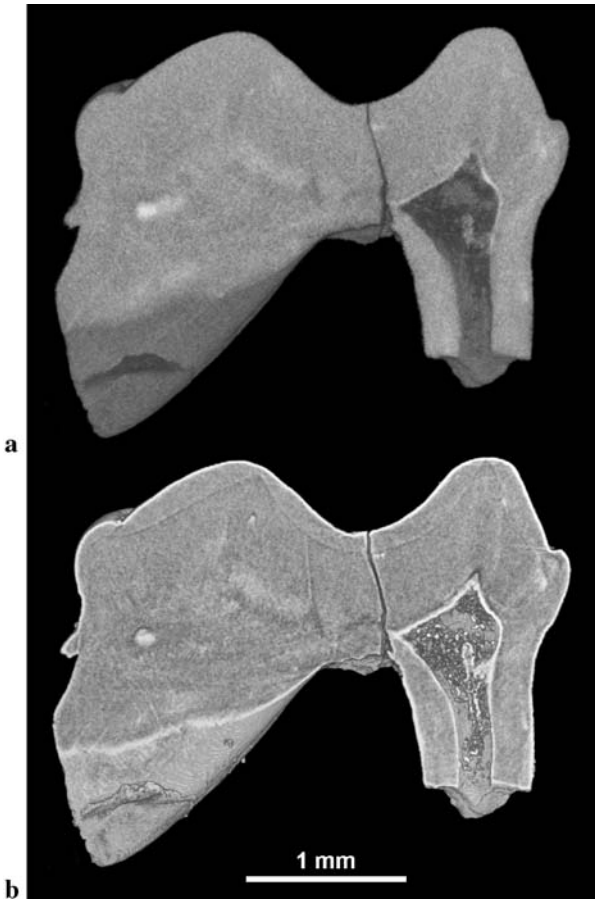

FIGURE 6 Comparison between a scan in absorption mode (**a**) and another with phase contrast in edge detection mode (**b**) on a molar of an early Oligocene eosimiid primate from Pakistan. Phase contrast enhances greatly the visibility of the internal structures and materialises the enamel–dentin junction that is invisible on the absorption scan. These scans were performed on ID19 with a voxel size of 6.7 μm at an energy of 40 keV. The sample/detector distance was 15 and 500 mm, respectively

Dental development and microstructure are other aspects that are important for paleontologists, since they can bring information about life history, ageing, growth, dentition process, diet, mechanical adaptations and so on [35–46]. Until now, the only way to study them was to cut teeth in order to make microscope slides or preparations for scanning electron microscopy. Evidently, this destructive technique cannot be applied on important fossils. On some of them, phase contrast SR-μCT can bring a good non-destructive alternative for these investigations. Indeed, even with medium spatial resolutions, this technique succeeds in some cases in enhancing growth features that can then be studied on virtual slices. Figure 7 shows the impact of an increasing phase contrast ef-

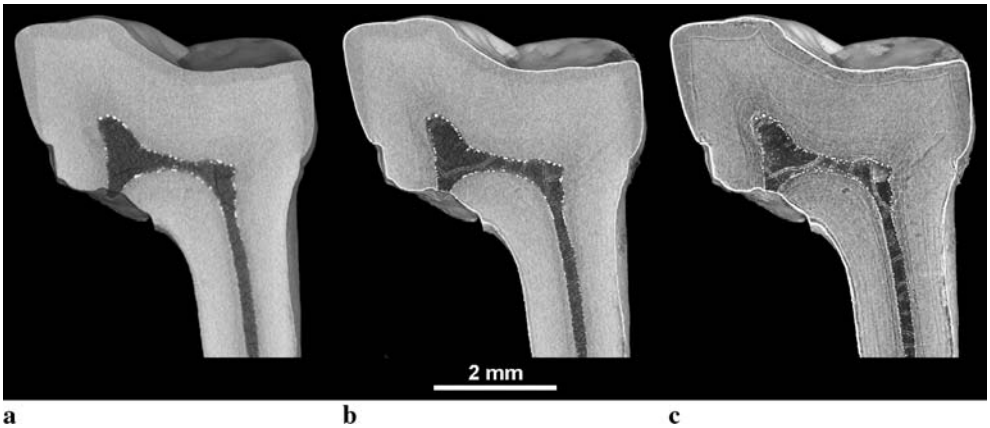

FIGURE 7 Increase of the propagation phase contrast effect on virtual microtomographic vertical slices through a molar of a latest middle Eocene undetermined primate from Myanmar. (**a**) Sample/detector distance 10 mm (absorption), (**b**) 400 mm, (**c**) 990 mm. The scans were performed on the ID19 beamline with a voxel size of 6.7 μm at an energy of 50 keV. In the last picture, phase contrast clearly reveals the enamel–dentin junction and a lot of large-scale developmental and microstructural features in the dentin

fect on a molar of a small undetermined latest middle Eocene primate from Myanmar. With quite strong phase contrast (sample/detector distance $= 0.99$ m), SR-μCT reveals various microstructural and developmental features in the dentin of this sample. In this case, it is possible to investigate the general pattern on a whole tooth, even if the most interesting features require far more precise data.

For the studies of enamel microstructure and development, the size of the investigated structures is generally a few micrometres (enamel rods or daily growth features). Thanks to the very high resolution that can be achieved and the possibility to perform local microtomography on bulk samples, histological features normally accessible only by microscopes can sometimes be non-destructively investigated by SR-μCT [16, 47]. Figure 8 presents a small part of a molar cusp of the late Eocene anthropoid primate from Thailand *Siamopithecus eocaenus* [48]. On this sample, it is possible to see with a good precision the enamel microstructure and some very fine developmental features.

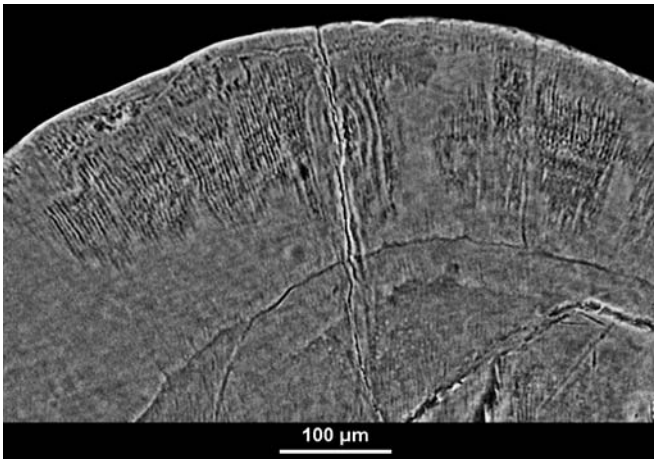

FIGURE 8 High-resolution virtual slice through the top of a cusp on a molar of the late Eocene anthropoid primate from Thailand *Siamopithecus eocaenus*. This scan was performed on ID19 with a voxel size of 0.7 μm in local phase contrast microtomography with an energy of 30 keV. The sample/detector distance was 250 mm resulting in a quite strong phase contrast effect. In this picture, the enamel microstructure is clearly visible down to the rod level, as some fine incremental marks (Retzius lines). Enamel is on the *upper part* of the image

Phase contrast SR-μCT is able to reveal many features of fossils that cannot be investigated with conventional microtomography and thus it enlarges extraordinarily the panel of paleontological samples that can be studied non-destructively.

Moreover, the beam coherence allows another imaging technique that can bring potentially even more impressive results on fossils in the future: quantitative phase tomography or holotomography [25, 49].

4.3 *Holotomography*

Phase contrast is due to differences in material properties in the sample. The optical phase corresponds to the projection of the decrement of the real part of the refractive index, which is in its turn determined by the electron or mass density. In edge detection mode, only the interfaces corresponding to changes of the density appear. By taking several scans at several distances, it is possible to reconstruct for each angular position a quantitative phase projection using a phase retrieval method [25]. Then, from the complete set of retrieved phase projections, a tomographic reconstruction can be performed using the standard filtered back-projection method. The result is a 3D quantitative phase map of the sample whose grey levels are directly linked to the electron density. This technique can reveal density differences in the sample that are invisible on absorption scans. Holotomography is particularly well adapted to phase objects, which are not often encountered in fossils, or to details showing very low absorption contrast. Nevertheless, a preliminary test was successful on a Tertiary charophyte from southern France (*Chara costulata*) still embedded in mineral matrix. Figure 9 presents a comparison between a slice in absorption mode and the result of the holotomographic reconstruction. In this case, this technique enhances many features that are hardly distinguishable on the absorption scan. Most of these details can be observed with phase contrast microtomography, but, as this method enhances only the interfaces, it is impossible to separate the different parts according to their grey levels, whereas this is easily done on the holotomographic reconstruction. With this

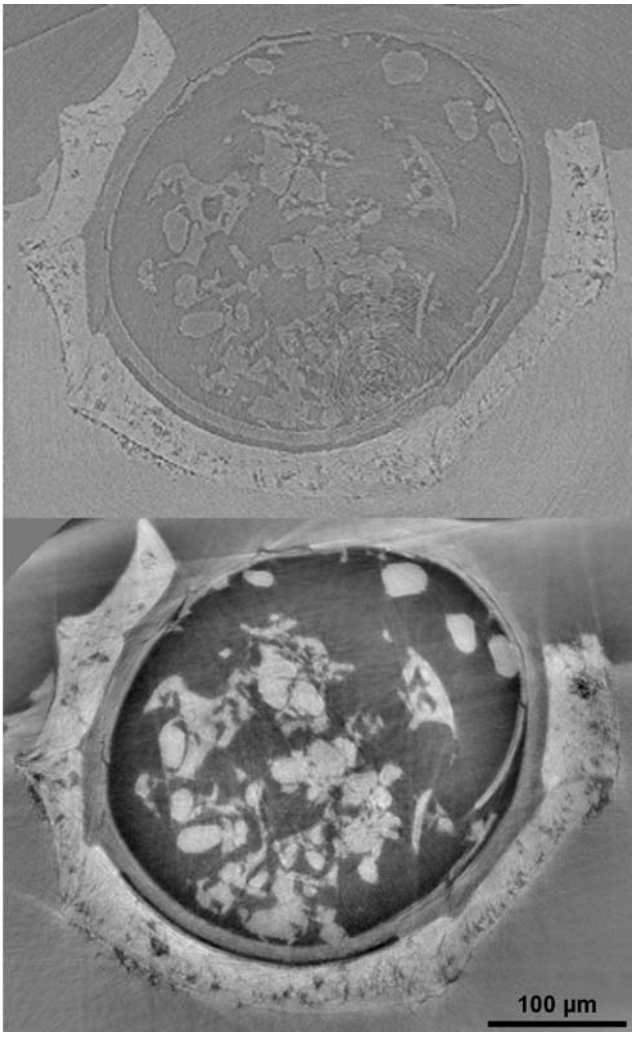

FIGURE 9 Slices in a tertiary charophyte *Chara costulata* in absorption mode (**a**) and after a holotomographic reconstruction (**b**). Scans were acquired on ID19 with a voxel size of 0.7 µm at an energy of 23.1 keV. Phase retrieval was performed from four scans with sample/detector distances of 10, 138, 177 and 244 mm, respectively. Holotomography enhances a lot of anatomical features that are only poorly visible on the absorption scan. The different parts of the sample present different grey levels which allow us to distinguish them clearly. The two slices presented here are not oriented in exactly in the same way, since the sample was removed from its holder between the absorption scan and the holotomography

kind of data, segmentation can be performed in order to virtually extract in 3D the different structures. This procedure can bring significant additional information for paleontological studies of the fossil that cannot be obtained using simple absorption or even phase contrast scans.

Presently, holotomography is not yet adapted to study fossils, except for particular samples such as the one presented here. But the expected evolution of this technique should lead to a very promising tool for samples mixing strong absorption and low contrast.

5 Conclusion

Thanks to the potentialities offered by the monochromaticity, the high flux and the coherence properties, a third-generation synchrotron source, like the ESRF, appears as an invaluable investigation tool for paleontologists. It allows imaging the internal structures of a large variety of fossils with a resolution and precision not obtainable with other non-destructive methods. Moreover, thanks to newly explored roads such as holotomography on dense objects, the future appears even brighter for fossils presenting very low absorption contrast. The fact that it is now possible to explore the internal features of a fossil without damaging it should stimulate the paleontological community to use synchrotron radiation for studying fossils of outstanding scientific importance, as was already the case of the fossils attributed to the oldest known hominid, *Sahelanthropus tchadensis* [7, 17]. The ESRF, by opening up to the paleontological community, could contribute to a better knowledge of the life history on our planet, and of our far origins.

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