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Comparative analysis of isolated cellular organelles by means of soft X-ray contact microscopy with laser-plasma source and transmission electron microscopy

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Summary

Soft X-ray contact microscopy (SXCM) is, at present, a useful tool for the examination at submicrometre resolution of biological systems maintained in their natural hydrated conditions. Among current X-ray-generating devices, laser-plasma sources are now easily available and, owing to their pulse nature, offer the opportunity to observe living biological samples before radiation damage occurs, even if the resolution achievable is not as high as with synchrotron-produced X-rays. To assess the potential of laser-plasma source SXCM in the study of cellular organelles, we applied it for the analysis of chloroplasts extracted from spinach leaves and mitochondria isolated from bovine heart and liver. X-ray radiation was generated by a nanosecond laser-plasma source, produced by a single shot excimer XeCl laser focused onto an yttrium target. The images obtained with SXCM were then compared with those produced by transmission electron microscopy observation of the same samples prepared with negative staining, a technique requiring no chemical fixation, in order to facilitate their interpretation and test the applicability of SXCM imaging.

Introduction

The possibility of examining the structure of cells in their normal living state by an X-ray microscope has been pursued by biologists and physicists for many years. Soft X-ray contact microscopy (SXCM), which utilizes a radiation corresponding to the wavelengths between 2.3 and 4.4 nm, known as the water window region, allows untreated living biological specimens to be examined at optimal contrast. The water window lies between the oxygen and carbon k absorption edges, where the absorption of carbon is 10 times that of oxygen (and hence water).

Several groups have developed microscopes working in the soft X-ray spectral region, using synchrotron or table-top (primarily laser-plasmas) X-ray sources. Current high-brightness synchrotron radiation sources, together with Fresnel Zone Plates microfocusing optics, allow high-resolution imaging of untreated biological samples (Kirz *et al.*, 1995; Attwood, 1999). However, synchrotron machines are not always readily accessible to investigators. Laser-plasma sources, by contrast, are more easily available and suited for the construction of more compact microscopes. Moreover, they have the advantage of generating very short X-ray pulses, thus reducing blurring resulting from brownian motion and the possibility of damage caused by radiation to biological structures. The main drawbacks of laser-plasma-based X-ray microscopy are poorer resolution and longer time needed to obtain images, as the contact technique usually employed requires chemical development and subsequent AFM imaging of the X-ray exposed resists.

Whatever the X-ray source, soft X-ray microscopy can be considered a useful complementary technique to light and electron microscopy. The theoretical resolution of light microscopy is limited by the wavelength of light to about 200 nm, whereas transmission electron microscopy (TEM) offers subnanometre resolution; however, the specimen preparation, required in both microscopies, makes it likely that artefacts will introduced. In particular, the standard preparation for TEM observation, which requires chemical fixation, dehydration,

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embedding, sectioning and staining to enhance contrast, makes the imaging of whole intact living cells impossible.

Until now, soft X-ray contact microscopy has been used almost exclusively for the observation of single cells, mainly unicellular organisms such as the green alga *Chlamydomonas dysosmos* and the cyanobacterium *Leptolyngbya* sp. VRUC 135 (Albertano *et al.*, 1997), *Saccharomyces cerevisiae* yeast cells (Batani *et al.*, 1998) or even cultured animal cells (Yada & Shinohara, 1994), but information about isolated cellular organelles is not available, except for a few images of chloroplasts (Ford *et al.*, 1994). Nonetheless, it would be desirable for life scientists to extend the application of this promising technique to the analysis of subcellular structures. To test this possibility, we decided to observe mitochondria isolated from bovine heart and liver and chloroplasts isolated from spinach leaves with a soft X-ray contact microscope. The images produced were then compared, as a reference, with those obtained with a TEM after negative staining, a method which, similarly to SXCM specimen preparation, does not require chemical fixation, embedding and sectioning of the samples, and allows intact whole cell organelles to be observed. Furthermore, negative staining is a well-established microscopic technique, which has been used for decades for ultrastructural studies, and has furnished part of the morphological information on which currently accepted models of molecular and cellular structures are based. For this reason, it can be a valuable aid for the correct interpretation of biological SXCM images, as preliminary studies have demonstrated (Limongi *et al.*, 2002). However, it has to be pointed out that negative staining TEM is only suitable for the observation of viruses, bacteria, subcellular structures and macromolecules (Haschemeyer & Myers, 1972). A remarkable advantage of this method is that it offers the opportunity to analyse the morphology of isolated entire organelles, so that it is possible to estimate both their shape and their average size. Moreover, in the examination of mitochondria, it allows us to distinguish the two typical conformations described by Hackenbrock (Muscatello *et al.*, 1972; Tzagoloff, 1982): the orthodox (well-developed cristae, most of the internal space occupied by the matrix) and the condensed conformation (increased intermembrane space and reduced volume of the matrix).

Another important issue in this investigation was that of sample preparation, which is crucial for obtaining clearly defined images; for this reason, a significant part of our work was dedicated to the optimization of methods to prepare specimens of isolated cell organelles for SXCM observation.

Materials and methods

Preparation of chloroplasts

Chloroplasts were isolated from fresh spinach leaves, according to the method of Whatley & Arnon (1963), by differential centrifugation with a Sorvall Super Speed RC5B centrifuge.

Leaves (50 g), washed with distilled water, were ground by hand in a precooled mortar in ice-cold extraction medium (100 mL 0.35 M NaCl, 10 mL 0.2 M Tris buffer, pH 8, and about 50 g cold sand) for *c.* 2 min. The homogenate was filtered through 6–7 layers of gauze and centrifuged twice at 200 ***g*** for 2 min. The green supernatant liquid was centrifuged for 7 min at 1000 ***g***. The chloroplast pellet, suspended in 0.35 M NaCl, was washed once at the same centrifugal force and resuspended in about 2 mL 0.35 M NaCl by a small clearance Potter homogenizer with 2–3 up-and-down strokes by hand. The chloroplast preparations were tested in order to assess their photosynthetic ability by means of the Hill reaction activity. Oxygen production was measured with an oxygraph equipped with a Clark electrode, by a method described by Mantai *et al.* (1970).

Preparation of mitochondria

Mitochondria were isolated from bovine heart and liver by differential centrifugation and prepared for observation either directly or after further purification on a sucrose density gradient. The organs were quickly removed from the animals and placed in ice. The heart (except connective and external fat) was minced, washed twice in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 0.2 mM K-EDTA, in order to remove any traces of blood, suspended in the same medium (1 : 4 w/v) and homogenized in a large and then in a normal clearance Potter homogenizer, with one up-and-down stroke, at high speed. The homogenate was then filtered. During these operations the pH, which tends to diminish, was maintained at 7.4 by adding KOH. The homogenate was centrifuged twice at 575 ***g*** for 5 min with a Sorvall Super Speed RC5B centrifuge and the supernatant fraction was recovered and centrifuged at 15 600 ***g*** for 5 min. The mitochondrial pellet was washed once at the same centrifugal force and suspended in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4) or in 0.148 M NaCl. To purify the organelles, the pellet in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4) was layered on discontinuous sucrose density gradient (0.8, 1, 1.2, 1.4 and 1.6 M sucrose) and centrifuged with a Spinco L2-65 B ultracentrifuge with SW 27 rotor at 110 000 ***g*** for 60 min. The mitochondria recovered from the 1.4 M sucrose layer were washed and resuspended with 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4) or 0.148 M NaCl.

The bovine liver was finely minced with scissors, washed in 0.25 M sucrose, 20 mM HEPES, 2 mM K-EGTA (pH 7.4), suspended in the same medium (1 : 4 w/v) and homogenized in a large clearance Potter homogenizer, with three up-and-down strokes, at low speed.

Mitochondria were separated by differential centrifugation and purified with the same procedure used for the heart, except for: HEPES buffer was substituted for TRIS buffer; the supernatant fraction was centrifuged at 10 000 ***g***; the discontinuous sucrose gradient was 1.2, 1.4 and 1.6 M; mitochondria were recovered from the 1.6 M sucrose layer. All these operations were accomplished at 0–4 °C.

The mitochondrial preparations were tested to verify their functional activity, by determining the oxidation rate and the control of respiration by phosphate acceptor. Mitochondrial oxygen consumption was measured with an oxygraph equipped with a Clark electrode, as described elsewhere (Amicarelli *et al.*, 2001). Only the preparations having an acceptor control ratio higher than 3.5 were used.

Protein determination

The protein content of mitochondria and chloroplasts was determined by the Biuret reaction, pretreating the samples with 0.5% deoxycholate, before adding trichloroacetic acid, using bovine serum albumin as a standard.

TEM analysis

Samples of mitochondria, isolated from bovine heart and liver, and of chloroplasts, isolated from spinach leaves, were examined after negative staining. The drop method was applied (Haschemeyer & Myers, 1972); this procedure requires less than 1 min to be accomplished. Each sample was appropriately diluted with the suspending medium (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, or 0.148 M NaCl for the mitochondria and 0.35 M NaCl for the chloroplasts), in order to obtain a final protein concentration of 1–2 mg mL^{-1} for the mitochondria and of 5–10 mg mL^{-1} for the chloroplasts.

A small droplet of each suspension was placed on a grid coated with a support film (Formvar). After 10 s the grid was washed with several droplets of staining solution, ammonium molybdate adjusted to pH 7.4 with ammonia, isotonic with the suspending medium (2% for the mitochondria and 4.7% for the chloroplasts). The last droplet was left for 10 s and then blotted with filter paper, leaving a thin film of staining solution, which solidifies rapidly on the sample into a smooth glossy film. The stain surrounds the organelles on the Formvar film as a result of surface tension interactions. The samples were examined with a JEOL JEM 100 C transmission electron microscope at 80 kV. Generally, negatively stained particles, when observed with the electron microscope, appear as light areas because of the low scattering power of the particle compared with the electron-dense surrounding stain. Moreover, the stain protects the biological material from the electron beam damage, so that it is not necessary to fix the sample.

SXCM

For SXCM observation the untreated biological sample is put on an X-ray-sensitive photoresist (in our case poly methylmethacrylate, PMMA, was used). Exposure to X-rays produces on the resist local changes in the chemical properties, proportional to the X-ray dose that reaches its surface. When a biological sample is present between the X-ray source and the resist, the X-ray absorption of the different zones of the sample gives an absorption profile that, after a chemical development, produces a three-dimensional image of the sample on the PMMA, with a 1 : 1 magnification; for further details see Albertano *et al.* (1997).

For our observations samples were exposed to X-rays for about 25 ns: this very short exposure time allows images to be obtained before the appearence of structural alterations, owing to radiation damage. The different photoresist profiles were observed with a Digital Nanoscope IIIa atomic force microscope (AFM). In the resulting magnified images the clearer areas corresponded to zones of higher X-ray absorption, and hence of higher carbon content, in the samples.

The resolution limit, experimentally determined, was about 70 nm.

The X-ray plasma-source

For our SXCM a laser-plasma driven by an excimer laser was employed as an X-ray source. This laser is a discharge pumped XeCl system emitting at UV radiation and its characteristics are described in Bollanti *et al.* (1990). The laser radiation is focused on a target placed in the middle of a vacuum chamber. The focused laser spot has a diameter of 20–30 μm and from this region soft X-rays are emitted. The duration of the X-ray pulse is 25 ns, corresponding to a laser intensity on the target of 2.5×10^{13} W cm^{-2} and to an X-ray source size of 30 μm diameter. In our experiments yttrium was used as a target. The Y plasma produces strong emission lines in the water window energy region (280–540 eV). The vacuum chamber was filled with He gas at atmospheric pressure.

Preparation of biological samples for SXCM and imaging

The time required for the preparation of one sample is about 10 min. This time is spent principally to optimize the distance between a thin X-ray-transparent window (Si_3N_4, 100 nm thick) and the photoresist on which the organelles in their suspending medium are put. This operation is aimed at immobilizing the biological samples in order to diminish the brownian movements and the diffraction and penumbral effects that may reduce image resolution. Moreover, it produces a thinning of the layers of suspending medium, which are always present at either side of the sample (i.e. between the sample and the Si_3N_4 window, on one side, and the resist, on the other side), and which are an important limiting factor for the obtainment of optimally resolved images, as they lower contrast by absorbing X-rays. To accomplish this operation, the specimens were observed, immediately before insertion into the chamber, by a light microscope in the environmental holder, and the liquid medium thickness was adjusted to 5–10 μm.

In our experiment the X-ray fluence ranged from 10 to 20 mJ cm^{-2}. After exposure, the resists were washed in a sodium hypochlorite solution (1 : 1 in water), to remove any traces

of organic matter and finally chemically developed using methylisobutylketone (MIBK) and isopropyl alcohol (IPA) 1 : 1 solution. The development time of the photoresist represents a critical parameter because it strongly influences the quality and the resolution of the images. In fact, if this time is too long, the developer begins to corrupt the lateral walls of the resist surface forming the three-dimensional image, and details of this, especially if very thin, may be altered or even lost. Unfortunately it is not possible to establish a general rule for photoresist development time. The best method is to proceed in sequential steps (approximately 15-s intervals) and to observe the photoresist by the AFM until the best image contrast is obtained.

Results and discussion

Analysis of chloroplasts

Chloroplasts were isolated in an extraction medium with a controlled osmolarity to prevent structural alterations. However, the suspensions obtained in each preparation comprised both intact and damaged organelles, as was observed by phase contrast light microscopy, where the intact chloroplasts appeared as shiny discs and the damaged chloroplasts appeared dark (data not shown).

With TEM observation of negatively stained samples, the intact chloroplasts appeared rather homogeneous, with no structural features visible inside them, probably owing to the presence of the envelope, which prevented the stain from penetrating (data not shown). By contrast, the damaged chloroplasts had lost the envelope membranes and part of their stroma content, so that the stain penetrated more easily into the organelles, thereby revealing the multilayer stacks of thylakoids (grana) which appeared as light disc-shaped structures with a mean diameter of 0.5 μm (Fig. 1A). At higher magnification, single thylakoids were also distinguishable, which were covered with small particles corresponding to protein complexes protruding from their membrane surface (Fig. 1C).

For the SXCM preparation of the samples, the optimal concentration of organelles on the resist, to obtain a monolayer, was estimated with an optical microscope. The images were obtained in the same exposure conditions: about 10 mJ cm^{-2}

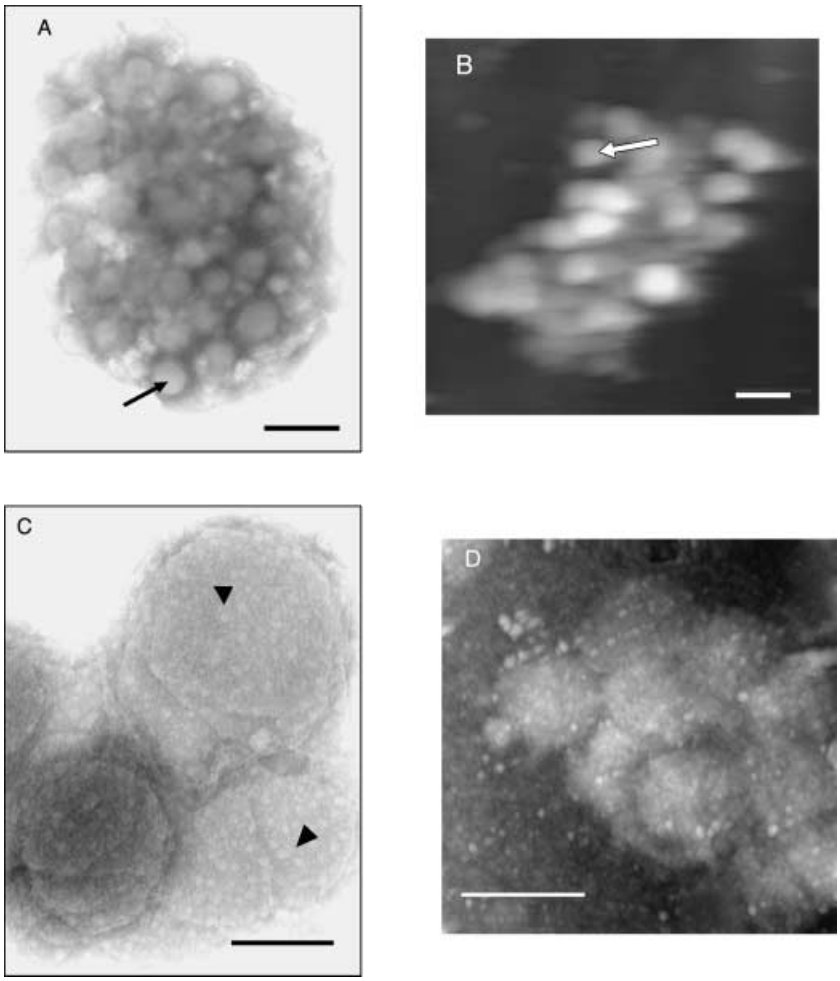


Fig. 1. Chloroplasts isolated from spinach leaves and observed by TEM (A,C), after negative staining with 4.7% ammonium molybdate, or by SXCM (B,D). In A and B the appearance of the internal membrane system is shown; the interconnected stacks of thylakoids (grana) are evident (arrows). C and D illustrate grana observed at higher magnification; in the TEM image single thylakoids can be discerned, with protein complexes on their membrane surfaces (arrowheads). Scale bars = A,B,D, 1 μm; **C**, 0.2 μm.

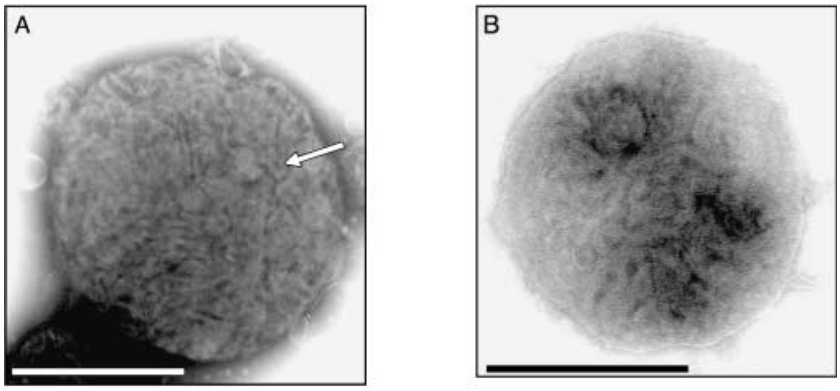


Fig. 2. TEM images of mitochondria isolated by differential centrifugation from bovine heart and suspended in 0.25 M sucrose (A) or 0.148 M NaCl (B) solution and negatively stained with 2% ammonium molybdate. The numerous cristae (arrow) are clearly evidenced by the surrounding electron-opaque stain, which penetrated into the organelles filling the intermembrane spaces. No differences are observed in the structure of the mitochondria suspended in either of the two media. Scale bars = 0.5 μm.

in water window Y target, 25 ns X-ray pulse. The time of development was about 2 min and the image relief range was 0–170 nm.

Also with SXCM two different populations were observed: whole chloroplasts and broken chloroplasts. The intact organelles showed a rather homogeneous brightness, owing to the presence of the envelope, which retained the stroma, and the internal membrane system; their high carbon content was responsible for the strong X-ray absorption in the water window region (data not shown). Damaged chloroplasts, which had lost their envelope, had a granular appearence as a consequence of the partial loss of their stroma: this caused a reduction of X-ray density around the granal stacks, making them more evident (Fig. 1B). These results are in accordance with those previously reported by Ford *et al.* (1994), who examined chloroplasts isolated from leaves of pea plants and suspended in a high-molarity sucrose medium. Moreover, as with negative staining TEM, thylakoids could be observed at higher magnification (Fig. 1D).

Analysis of mitochondria

Preliminary tests on bovine heart mitochondria isolated by differential centrifugation revealed that sucrose, the medium generally used to isolate these organelles, is not suitable for the preparation of biological samples to be observed with the SXCM: the images obtained were always blurred and the mitochondria were not easily distinguishable. This is presumably due to the high carbon content of sucrose molecules, which reduces the natural contrast in the water window of biological samples suspended in this medium. For this reason the mitochondria were resuspended in a saline medium (0.148 M NaCl solution), containing no organic substances and having the same osmolarity as 0.25 M sucrose. The mitochondria isolated by differential centifugation, resuspended in the two different media and negatively stained with 2% ammonium molybdate, had the same aspect when observed with the TEM (Fig. 2A,B). They appeared ellipsoidal (average major axis, 1.4 μm; average minor axis, 0.8 μm) or spherical (average diameter, 1 μm) and generally had a typical orthodox conformation. The outer membrane and the infoldings of the inner membrane were clearly identified as the intermembrane space was filled with the electron-dense stain. It was also possible to observe the numerous and narrow cristae, characteristic of the cardiac tissue mitochondria.

For SXCM observations, in order to obtain on the resist a monolayer of mitochondria sufficiently spaced to distinguish them clearly from each other, the mitochondrial suspensions were appropriately diluted. The optimal dilution rate was theoretically determined on the basis of the measured protein concentration of each suspension, knowing the average dimensions (found from TEM observations) and protein content (about 70% of total mass) per mitochondrion, and then tested by SXCM observation. With an average protein concentration of 15 mg mL^{-1}, a suitable monolayer of mitochondria (in a 250×250 μm^2 square window) was obtained with a dilution rate of 1 : 4. The images were obtained at the same exposure conditions described in the chloroplast analysis section.

By SXCM the organelles isolated by differential centrifugation and resuspended in isotonic NaCl solution showed zones with a high degree of X-ray absorption (Fig. 3), probably due to the abundant system of inner membranes.

A further purification of the mitochondrial suspensions prepared by differential centrifugation was also necessary, as they contained numerous vesicles and membrane fragments deriving from broken organelles, which co-sedimented with the mitochondria and made their SXCM imaging rather difficult. For this purpose, the pellets recovered from differential centrifugation were purified by a discontinuous sucrose gradient. The purified mitochondrial fraction was finally resuspended in 0.148 M NaCl. The appearance of such organelles, after negative staining, was identical to that observed in the mitochondria isolated by differential centrifugation (Fig. 4A; compare with Fig. 2A,B).

By SXCM the organelles purified as described above appeared better contrasted, with abundant well-defined clear zones, corresponding to accumulations of cristae membranes, and dark zones, corresponding to the intermembrane space and to the mitochondrial matrix (Fig. 4B–D). Despite this, the images of bovine heart mitochondria obtained with SXCM

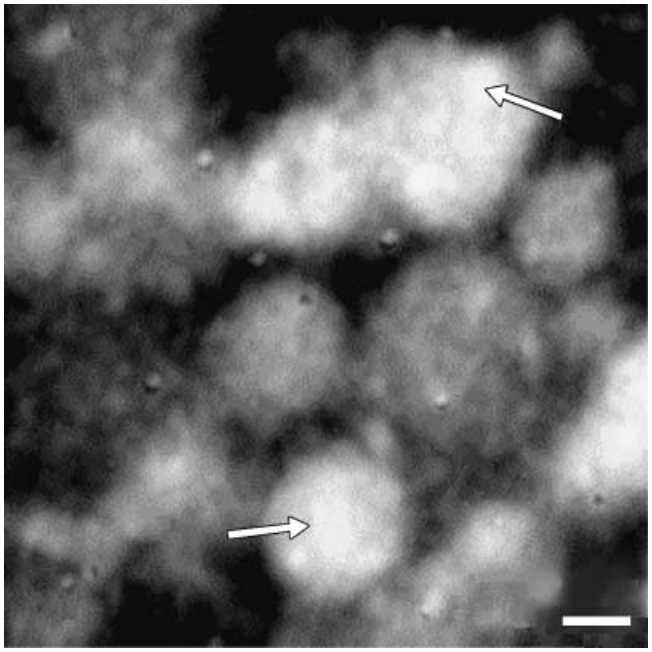


Fig. 3. SXCM image of mitochondria isolated by differential centrifugation from bovine heart and suspended in 0.148 M NaCl solution. High X-ray-absorbing areas are visible inside the organelles (arrows). Scale bar = 0.5 μm.

were not sufficiently resolved, probably as a consequence of the presence of a large number of densely packed cristae inside them. Therefore it was decided to examine mitochondria isolated from bovine liver. It is well known that these organelles are characterized as possessing fewer cristae than those present in heart cells (Tzagoloff, 1982), and this could render them more suitable for the study of the inner mitochondrial structure by means of SXCM.

Mitochondria isolated from bovine liver and resuspended in NaCl isotonic solution were observed, both with TEM and SXCM, only after purification in sucrose density gradient. When observed with TEM, after negative staining with 2% ammonium molybdate, they appeared mainly spherical, with an average diameter of 1.2 μm; most of them showed a condensed conformation, with a wide intermembrane space and large and deep infoldings of the inner membrane (Fig. 5A,C).

With SXCM bovine liver mitochondria had a similar appearence, with a large low-absorbing zone, probably corresponding to the wide intermembrane space observed by TEM (Fig. 5B,D). In each preparation, a small percentage of mitochondria were found damaged (Fig. 6), with disruption of the outer membrane; this is a normal and inevitable occurrence with the standard isolation and purification procedures.

Conclusions

We investigated the potential applications of SXCM to the morphological analysis of organelles isolated from living cells,

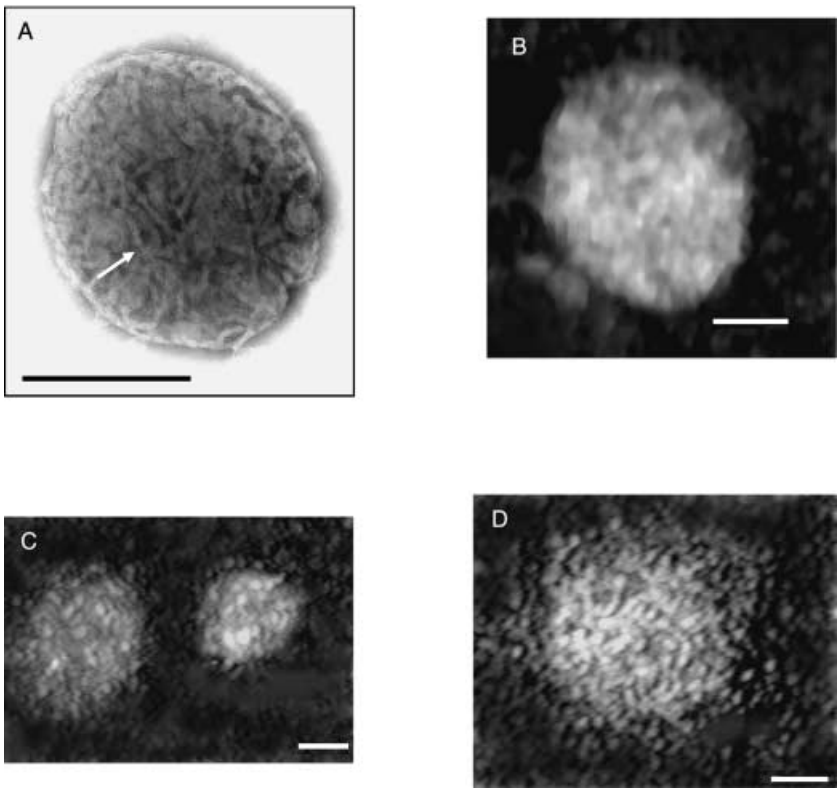
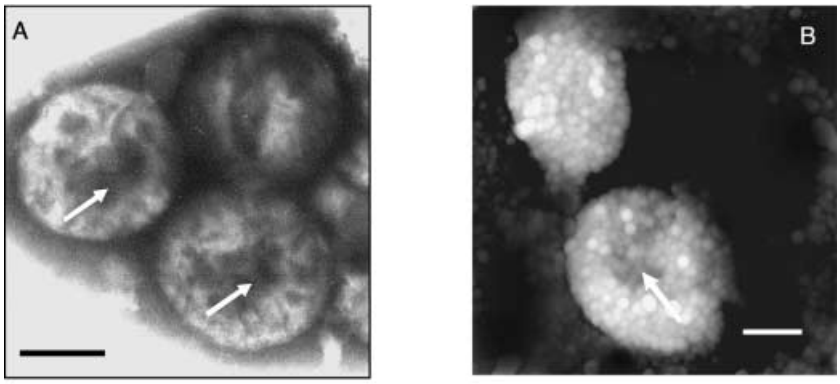


Fig. 4. Bovine heart mitochondria purified by sucrose density gradient centrifugation, suspended in 0.148 M NaCl solution and observed by TEM (A), after negative staining with 2% ammonium molybdate, or by SXCM (B,C,D). In the SXCM images distinct clear zones are visible inside the organelles; they correspond to densely packed cristae membranes, which are clearly distinguishable in the TEM image (arrow). Scale bars = 0.5 μm.



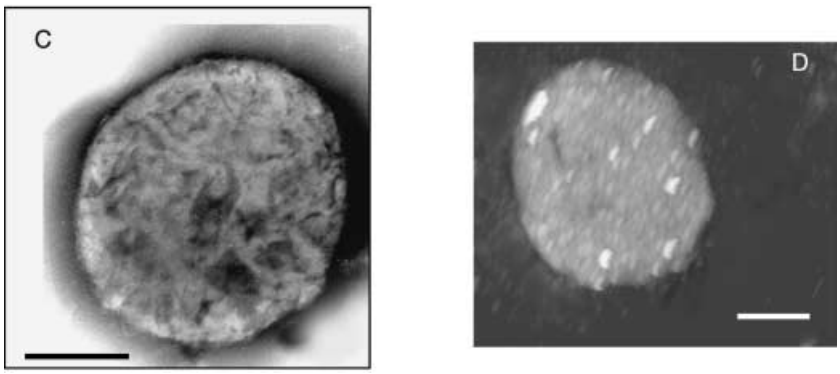


Fig. 5. Bovine liver mitochondria purified by sucrose density gradient centrifugation, suspended in 0.148 M NaCl solution and observed by TEM (A,C), after negative staining with 2% ammonium molybdate, or by SXCM (B,D). In the SXCM images some large low-absorbing zones are visible inside the organelles; they correspond to the wide intermembrane spaces that are characteristic of the condensed conformation and are more evident in the TEM images (arrows). Scale bars = 0.5 μm.

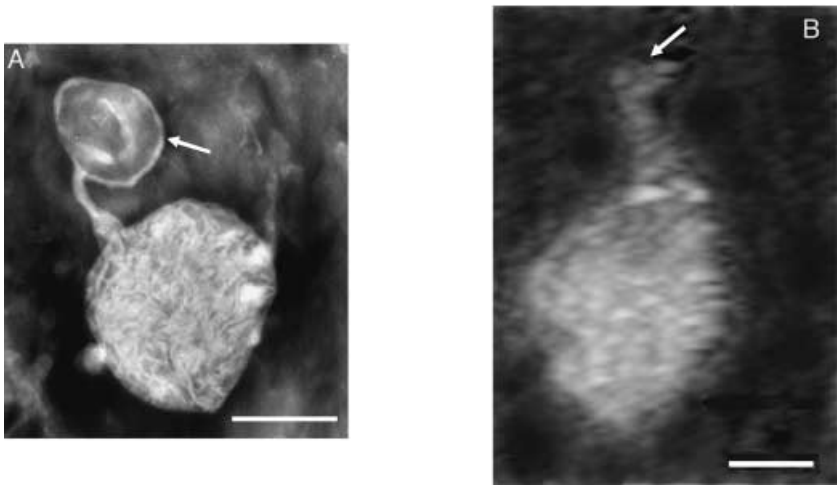


Fig. 6. Damaged mitochondria found in preparations of organelles isolated from bovine heart (A, TEM image; negative staining with 2% ammonium molybdate) and liver (B, SXCM image), purified by sucrose density gradient centrifugation and suspended in 0.148 M NaCl solution. A large vescicle outside the organelles (arrows) originated from the extrusion of part of the inner membrane, with its matrix content, owing to disruption of the outer membrane. Scale bars = 0.5 μm.

using the comparison with negative staining TEM of the same samples as an aid for the correct interpretation of the images obtained, and elaborated a suitable method for the preparation of these biological specimens for SXCM examination.

Chloroplasts isolated from spinach leaves and prepared with a conventional method, using SXCM observation, exhibited a sufficiently resolved structure only if they had lost their envelope and part of the stroma: in these organelles the spatial arrangement of the internal membrane system was evident, which retained its organization in stacks of thylakoids (grana). This is in accordance with the images obtained with TEM observation of chloroplasts prepared with the same procedure and negatively stained, although the TEM images had a better resolution, which allowed single thylakoids with their surface protein complexes to be seen.

Mitochondria isolated from bovine heart and liver, when observed using SXCM, did not show a clearly distinct internal structural organization, comparable with that visible in TEM images of negatively stained organelles. This result was to be

expected taking into account the actual limit of resolution of the SXCM apparatus, which is about 70 nm: this value, although lower than that achievable with light microscopy, is still too high to allow the fine details of mitochondria structure to be discerned. A further difficulty was encountered in the examination of mitochondria, owing to the strong soft X-ray absorption of the sucrose medium in which they had been suspended, according to conventional preparation procedures. This problem was overcome by using a different suspension medium, with a saline composition and totally devoid of organic substances.

From our results it can be concluded that laser-plasma source SXCM cannot yet be considered suitable for the ultrastructural analysis of subcellular components with a complex internal membrane organization. This technique, which was originally developed for submicrometre observation of living biological samples in their natural hydrated state, is still limited by the low power of resolution of current instruments, which restricts their use to the observation of whole cells and isolated organelles, but with a poor discrimination of their internal structures. Improvements in SXCM instrument performance, with significant enhancement of image quality, are expected from the use of newly developed, higher energy X-ray sources, such as third-generation synchrotron machines, and from the amelioration of microfocusing optics for transmission X-ray microscopy. Similar technical advances, in conjunction with optimized sample preparation procedures, such as those proposed here, could render X-ray contact microscopy more suitable for applications in the study of subcellular structures.

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