# Preliminary results of combined scanning near-field optical microscopy and atomic force microscopy applied to a model biological system: *Clostridium tyrobutyricum* spores

N. Andreeva[1], G. Ferrini[1], D. Bassi[2], F. Cappa[2], P.S. Cocconcelli[2], S. Prato[3], B. Troian[3] and F. Parmigiani[4]

[1] *Dipartimento di Matematica e Fisica, Università Cattolica, Brescia, Italy*
[2] *Istituto di Microbiologia, Università Cattolica, Piacenza, Italy*
[3] *A.P.E. Research, Area Science Park, Basovizza, Trieste, Italy*
[4] *Dipartimento di Fisica, Università degli Studi, Trieste, and Sincrotrone Trieste, Basovizza, Trieste, Italy*

(Received: October 3, 2008; accepted: March 13, 2009)

***ABSTRACT*** Innovative microscopies for the measurements of biological parameters can be helpful to reduce environmental impact, to prevent food contamination and poisoning and to assess the biocompatibility of nano-materials. In this frame, we applied scanning near-field optical microscopy (SNOM) and atomic force microscopy (AFM) to a model system constituted by *Clostridium tyrobutyricum* dormant spores and their vegetative cells. SNOM was used to acquire the information about optical properties of *C. tyrobutyricum* spores with sub-wavelength resolution. Comparing reflection and transmission SNOM images revealed the presence of structures inside the spores. A detailed topographic study of the spore's external layers was carried out with AFM. To obtain information about the morphology of spores and vegetative cells at the nano-scale level, we applied AFM in the lateral force mode, that allowed us to have a qualitative picture of the elastic and viscoelastic properties of the spore coat.

## 1. Introduction

In this work, the first results obtained by the combination of two scanning probe microscopy (SPM) techniques, atomic force microscopy (AFM) (Binning *et al*., 1986) and scanning near-field optical microscopy (SNOM) (Lewis *et al*., 1984; Pohl *et al*., 1984), applied to a simple biological system are discussed. Both techniques can be used with living cells without introducing sample modification due to its preparation.

AFM is used not only to obtain topographical images, but also to measure the local mechanical properties of the sample (adhesion, elasticity, friction). However, a force-microscopy approach is not suited for the investigation of processes taking place below the immediate surface. SNOM, on the contrary, allows us to obtain valuable information from the sample by evanescent light interaction, with a probing depth of several hundred nanometers and to achieve an optical lateral resolution substantially below 100 nm (Lange *et al*., 2001).

As a simple biological model for our investigation we chose *C. tyrobutyricum* spores. The spore is a dormant, non-reproductive form produced by some kinds of bacteria to ensure their survival under environmental stress. The main interest to spores is stimulated by their ability to remain dormant for extended time periods and exhibit a remarkable resistance to environmental

 

insults (i.e., heat, radiation, toxic chemicals, and pH extremes) that are lethal to vegetative cells. Spores can survive in deep-sea sediments and then spread to different Earth environments. *Clostridia* types are widely spread; in fact, some of them can be found in marine sediments from Atlantic and Pacific coasts (*C. oceanicum*) (Smith, 1970). *Clostridia* could also contaminate sea-food (*C. botulinum*) and be a danger to man. We have chosen *C. tyrobutyricum* as a model due to its non-pathogenicity and its importance as contaminant in the food industry (Cerf *et al*., 1967; López-Enríquez *et al*., 2007).

We present preliminary results on the use of AFM and SNOM techniques, that reveal the benefits gained by this combination for the investigation of biological samples. As a comparison we use images from scanning electron microscopy (SEM). Despite the popularity of SEM among microbiologists (Allen *et al*., 2000; Muller and Engel, 2001), based on the fact that this type of microscopy allows image acquisition with sub-nanometer resolution, it should be noticed that electron microscopy is an invasive method and could not be applied on living cells.

## 2. Materials and methods

### *2.1. Sample preparation*

*Clostridium tyrobutyricum* ATCC 25755 (T) was used in all the experiments. The reference strain was propagated in Reinforced Clostridial Medium broth (Oxoid, UK) with 50% (w/w) Sodium Lactate syrup (Merck, Germany) added to produce a final concentration of 2.8% (w/v) and incubated at 37°C in anaerobic bags equipped with Anaerocult A packs (Merck, Germany). Spore suspensions were obtained by adapting the biphasic culture from Cerf *et al*. (1967). A cellulose tubular membrane (CelluSeptT4, wall thickness 40 μm, dry cylinder diameter 47.7 mm, MWCO 12,000-14,000, Orange Scientific) filled with 150 ml of RCM broth was embedded in a bottle holding 400 ml of the same medium and was inoculated with 1.0% of a 24-h-grown *C. tyrobutyricum* culture. The culture was incubated in anaerobiosis at 37°C for at least four days and then maintained 15 days at room temperature. After this, the spores were harvested, cleaned from cellular debris, centrifuged (at 8,000 × g at 4°C for 10 minutes), washed three times with sterile water, and finally stored at 4°C in water until use. Spore purity was more than 90% inspected by phase-contrast microscopy. Only a few intact vegetative cells were visible.

For SNOM/AFM investigation we used *C. tyrobutyricum* spore water solution in concentration of $1.8 \times 10^7$ ml$^{-1}$. A drop of 5 μl spore solution was embedded on poly-L-lysine-coated glass and dried in air for 30 minutes at room temperature before SNOM/AFM analysis.

For the SEM study the protocol of sample preparation includes carbon coating. For this 5 μl of stock spore water solution ($1.8 \times 10^7$ ml$^{-1}$) was put on a positively charged nylon membrane (Roche Diagnostics GmbH Germany). Since spore water solution is negatively charged, it bounds on a positively charged nylon membrane at room temperature. Samples were then dehydrated stepwise in ethanol: in 75%, 85%, 95%, and finally 100% ethanol for 1 hour each at room temperature. Critical point drying was performed in a Baltec CPD030 dryer. The specimens were mounted on SEM discs and coated with carbon for electrical conductivity using a Jed4X (Scherrer and Gerhardt, 1972).

Finally, they were observed with a Philips XL30 ESEM scanning electron microscope (Palumbo *et al*., 2004). The specimens were analyzed under both low and high vacuum SEM

conditions. The analysis was performed under the following conditions: high vacuum $3x10^{-4}$ Pa, 7000 count rate by dead time 33%, dwell time 60 ms and low vacuum $1.2x10^{2}$ Pa.

### *2.2. AFM experiments*

Topography acquisition was carried out with AFM (APE Research, Italy) working in amplitude modulation (Giessibl, 2003), which is a non-contact mode technique (Mironov, 2004) suitable for dealing with fragile biological objects. In amplitude modulation the microscope cantilever is forced to oscillate at a certain frequency orthogonally to the sample surface. Due to the interaction between the tip and the sample, the tip oscillations are damped. Reconstruction of the surface topography is fulfilled by using the amplitude of the tip oscillations as a feedback to trace contours of constant amplitude by moving a piezoelectric actuator to modify the tip-sample distance. The AFM non-contact mode scanning allows us to reduce the mechanical influence of the tip on the surface and to avoid tip contamination while working with biological samples. For AFM topographic imaging we used non-contact silicon nitride cantilevers with spring constant of 40 N/m and curvature radius of 10 nm (NSC 15, MMasch, Lithuania).

It is well known that AFM is also a powerful tool for tribological measurements (Gallyamov, 1999). The friction distribution of the outer spore's multilayer surface was measured using the AFM in lateral force mode spectroscopy (NT-MDT, Russia). This mode of scanning allows us to discern the areas with different friction ratios and in addition to reveal features of the sample topography at the nano-scale level. The physical mechanism of the lateral force mode is based on the measurement of the torsion deformation (twist) of the cantilever during scanning. As far as friction is the force resisting the relative lateral (tangential) motion of solid surfaces, measuring the torsion deformation of the cantilever allows us to determine areas of sample topography with different friction ratios. In the case of a relatively flat surface the change of the twist angle as the cantilever moves from one area to another is related to the change of the local friction forces. Under conditions of highly corrugated topography, the lateral force mode allows us to underline topographic features of a sample at the nano-scale level.

For AFM scanning in a lateral force mode we used contact mode silicon nitride cantilevers with a spring constant of 0.15 N/m and curvature radius of 10 nm (CSC 17, MMasch, Lithuania).

### *2.3. SNOM experiments*

SNOM (APE Research, Italy) measurements were made in an illumination mode (Ohtsu and Kobayashi, 2004). In this mode the light coming from a diode laser (wavelength 635 nm) is coupled with a single mode optical fiber (LovaLite, France). The end of the fiber with sub-wavelength aperture is coated with an opaque aluminum film to enhance the contrast of the optical images. The near field component of the incident light is created on the sub-wavelength aperture at the end of the fiber. During scanning, the near field excites the sample that scatters light in the far field. Scattered light is collected both in reflection and transmission. The distance between the fiber and the sample is regulated with a shear-force feedback system. To control the tip-sample separation shear-force feedback uses the damping of lateral oscillations of the fiber caused by the interaction with the sample. This type of feedback allows acquisition of sample topography in addition to optical images in reflection and transmission, but with less resolution in comparison with AFM. Since the resolution of the SPM method is determined by the probe

curvature radius, optical fibers, the tip curvature radius is of the order of hundreds nanometers, while the AFM tip curvature radius usually is of the order of tens of nanometers.

Considering only near-field scattering at the sub-wavelength fiber aperture, there are several mechanisms of probe-sample interaction influencing SNOM images in reflection and transmission. The near-field dipole-dipole interaction between fiber and sample results in forwards (i.e. into the sample surface) and backwards (i.e. away from the sample surface) far-field light scattering. Backward far-field scattering is influenced only by the superficial layer of the sample that interacts with the near-field (typically 50-100 nm). Forward far-field scattering penetrates into the volume of a semitransparent sample and can be reflected, transmitted or absorbed by the internal sample features. Since SNOM light detectors collect all far-field contributions, it is clear that scattering from internal features of the sample could influence SNOM images both in reflection and transmission mode. We point out that this fact does not negate the possibility of obtaining SNOM optical images with sub-wavelength resolution.

## 3. Results and discussion

First we compare the AFM/SNOM images with SEM images, to discuss the structure of *C. tyrobutyricum* spores revealed by different techniques. According to images taken with SEM (Fig. 1), spores have an oval shape with average dimensions of 1.5 x 2.5 μm. With the SEM technique the image is originated by the electron reflection from the spore surface. The procedure of spore preparation for SEM studies, i.e. stepwise dehydration and carbon coating, allowed us to distinguish the spore's inners structures (Bassi *et al.,* 2009). These substructures can be correlated with spore morphology: exosporium, coat, cortex and core. Exosporium is the thin outer layer of the spore made of proteins, polysaccharides and lipids and consists of a multilayer structure. The coat is made up of highly cross-linked keratin and layers of spore-specific proteins and could take 50% of the spore's volume. The cortex mainly consists of cross-linked peptidoglycans and contains diaminopimelinic acid and dipicolinic acid. The core is the innermost spore structure, containing the components of the vegetative bacterial cell (the cell wall, cytoplasmic membrane, cytoplasm, nucleoid).

The AFM observation of spore topography gives some additional information with respect to SEM images. The average dimensions of oval shaped spores measured with AFM were 2.5 x 3.0 μm. The bigger sizes of the spores with respect to SEM analysis can be explained by the different preparation procedure. Since spores for AFM studies were dried in air, without stepwise dehydratation in ethanol, the presence of water in them influenced their volume, increasing their sizes with respect to dehydrated spores for SEM analysis.

As can be seen in Fig. 2, AFM topographic profiles reveal the presence of the exosporium, confirming the SEM analysis. Moreover, in the AFM images the difference in the volume enclosed by the exosporium compared to that enclosed by the spore structures inside the coat is evident. Both SEM and AFM images allow us to estimate the tendency of exosporium to form around one or both spore poles and extend up to one third of the spore length. But only an analysis of the height distribution made with AFM (Fig. 2b) demonstrates that the volume occupied by the exosporium is relatively small compared to the whole volume of the spore.

High-resolution AFM images (Fig. 2c) reveal that the surface of exosporium consists of

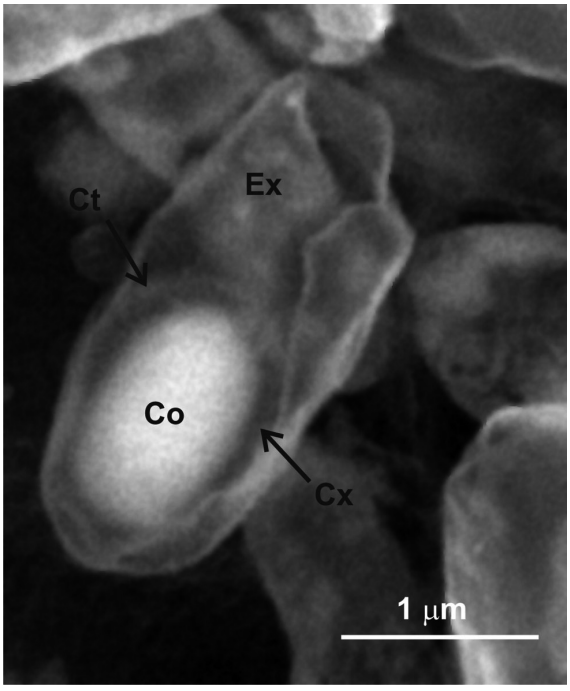


Fig. 1 - SEM image of a *C. tyrobutyricum* spore. The spore is surrounded by an amorphous exosporium (Ex), containing the light-stained core (Co) and the dark-stained cortex (Cx) which is separated from the exosporium by the light coat (Ct).

amorphous material. It is possible that the topography of exosporium is influenced by the drying process as a consequence of the spore's surface dehydration. In fact, the temperature of the sample drying process has an influence on surface topography. Increasing the temperature during the drying process led to decreasing the sizes of features of the spore topography at the nano-scale level. This is also an evidence of the amorphous structure of the exosporium outer layer.

Results of AFM scanning in the lateral force mode are shown in Fig. 3b. Their comparison with AFM topography (Fig. 3a) proves the possibility of being able to distinguish topographic features at the nano-scale level with this technique. In particular, note the presence of 100 nm thick fibers found on the substrate (Figs. 3b and 3c). The presence of flagellar structures was also described in other works devoted to AFM investigations of *Clostridium* (Plomp *et al*., 2007) and could represent a residue originated from the vegetative phase. Interestingly, the examination of images taken in the lateral force mode (Fig. 3b) puts in evidence the distribution of areas with different frictions on the spore surface. On some spores the area over the coat exhibits lower friction than the area on the spore's "tails", formed by the exosporium.

SNOM shear force topography images (Fig. 4a) do not give additional information to AFM topography. Moreover, as can be seen, by comparing AFM and SNOM topographic images (Figs. 2a and 4a), the resolution of SNOM topography is less than that obtained with AFM (Lange *et al*., 2001). However, as said above, the significance of SNOM measurements relies on the study of optical properties at sub-wavelength resolution. Indeed, our SNOM measurements of spores taken in reflection and transmission reveals regions with a different optical contrast that can be attributed to the spore's internal structures (Figs. 4b and 4c), according to the mechanisms outlined in Section 2.3. The contribution of internal structures is justified because the

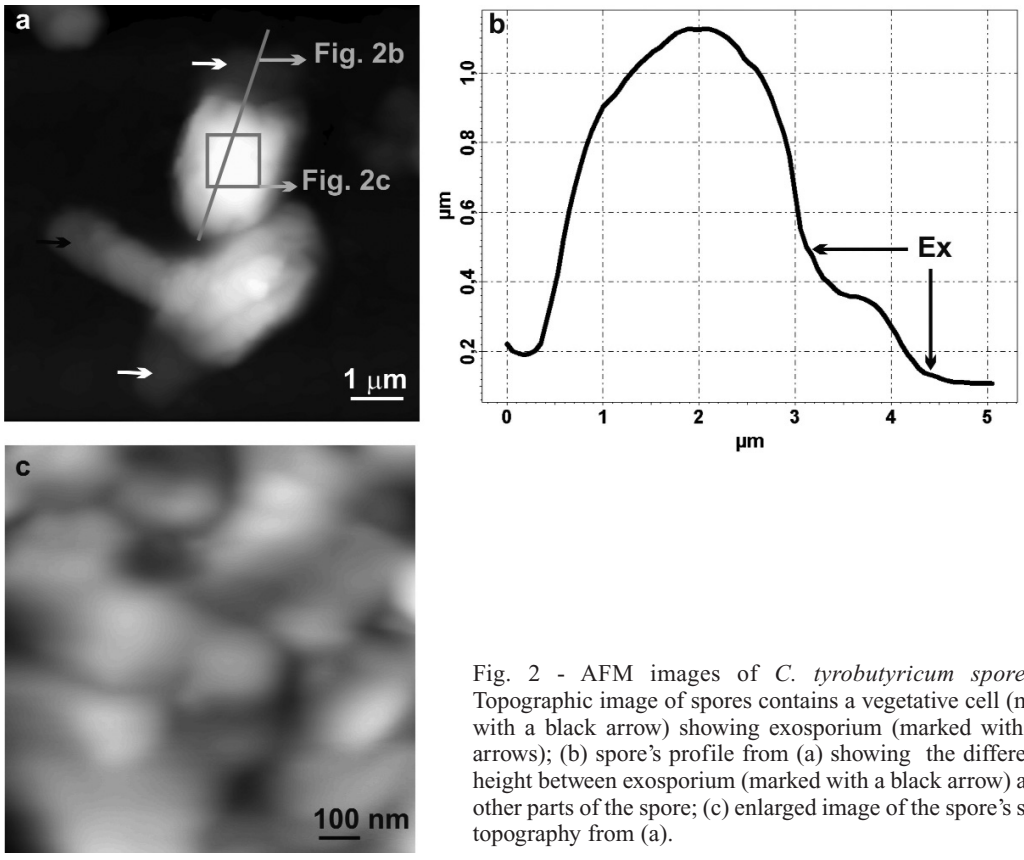


Fig. 2 - AFM images of *C. tyrobutyricum spores*. (a) Topographic image of spores contains a vegetative cell (marked with a black arrow) showing exosporium (marked with white arrows); (b) spore's profile from (a) showing the difference in height between exosporium (marked with a black arrow) and the other parts of the spore; (c) enlarged image of the spore's surface topography from (a).

composition of the spore's outer layer is believed to be essentially homogeneous and thus not capable of producing regions with a different optical contrast due to an evanescent field interaction at the surface.

The SNOM image in reflection (Fig. 4b) reveals two different structures inside the spore with good contrast, resolved as a dark area in the centre surrounded by light-stained structures on the spore's poles. On images in transmission the bright central zone of the spore is located in the center of the cell and surrounded by a dark area.

It is interesting to compare the inner structures of a spore obtained with SNOM and SEM. For this purpose the union of the SNOM image in reflection with that taken in transmission is compared with the SEM image in Fig. 5. As shown, it is possible to find a correspondence between the inner structures observed with SEM and those in the SNOM images by size analogy: core, cortex and exosporium. In the SNOM image (Fig. 5), the core is seen in transmission as a bright central zone of the spore. The cortex is revealed as a dark area between the core and the light-stained structures (associated with exosporium), observed in reflection from the poles of the spore.

As these preliminary studies demonstrate, the SNOM technique has a great potential for development, especially if used for optical measurements with different light sources. The investigation of the same sample using lasers with different wavelengths allows not only the

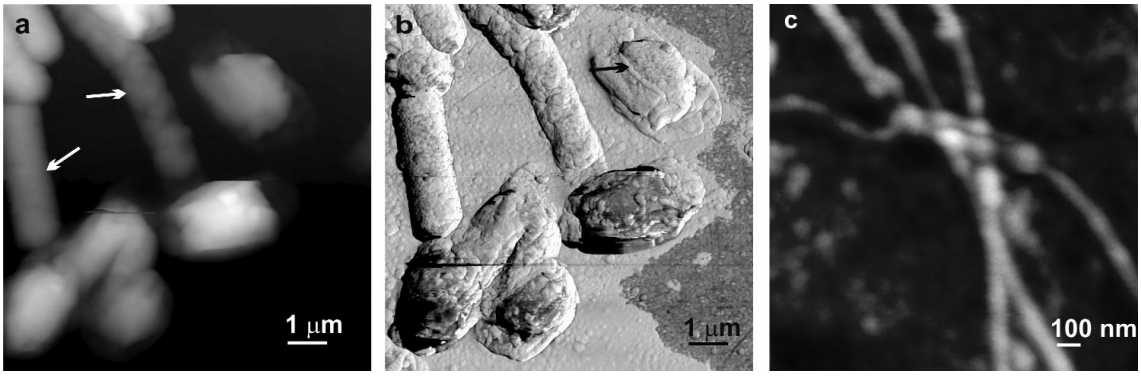


Fig. 3 - AFM images of *C. tyrobutyricum* spores: a) topographic image of spores contains a vegetative cell (marked with white arrows); b) AFM image of the same sample taken in lateral force mode showing the remnants of the bacterial flagellar structures; c) topographic image of flagella structures found in the sample solution.

resolution of structures but also the fulfillment of local spectroscopic measurements. Local spectroscopy combined with precise AFM topographic measurements will make it possible to determine the presence of biological markers inside biological objects and identify their position with subwavelength resolution (Cricenti *et al*., 2003).

## 4. Conclusions

Relying on the results of our study, we conclude that the combined use of the AFM/SNOM technique allows us not only to image biological samples under natural conditions but also to obtain information about their mechanical and local optical properties. Information about mechanical properties of the sample surface supplements the high resolution topography and could be used for further investigation of the interaction between the sample and different substrates. The possibility of obtaining local optical properties of biological objects (especially in transmission) gives information about their inner structures. It should be mentioned that data

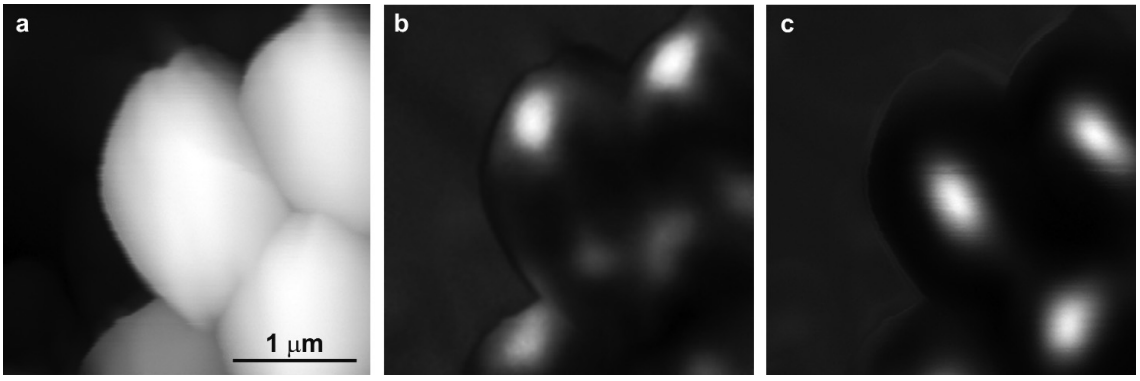


Fig. 4 - SNOM images of *C. tyrobutyricum* spores: a) topographic image of spores; b) image of spores taken in reflection; c) image of spores taken in transmission.

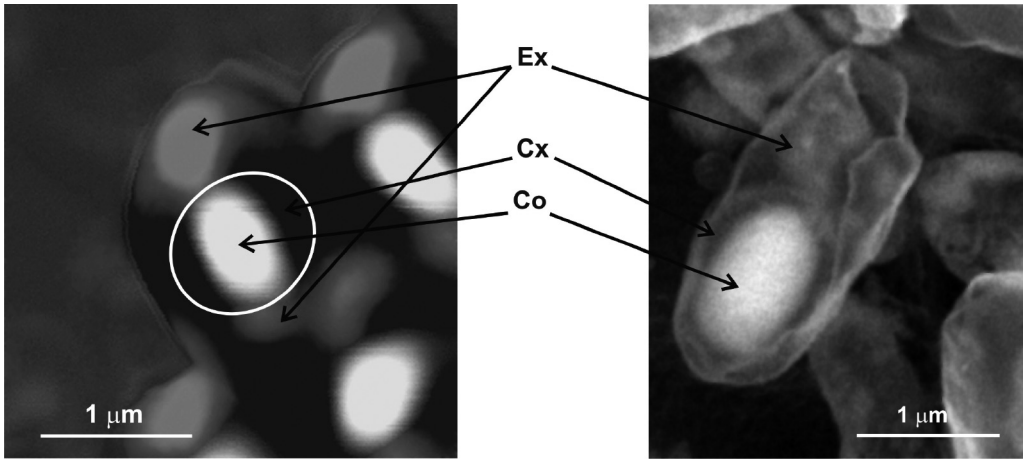


Fig. 5 - Comparison of SNOM optical measurements (left image, obtained taking the union of SNOM reflection and transmission images) with SEM (right image). According to our assumptions, it was possible to resolve the core (Co), cortex (Cx) and exosporium (Ex) inside a spore with SNOM.

acquired with the SNOM technique on the spore's inner structures correlates with that observed with conventional SEM methods. We suppose that further comparison of SNOM images obtained with different wavelengths will allow us to identify the presence of different biological markers, like the dipicolinic acid (which is the main marker for spore presence and identifies ungerminated spores).

The AFM/SNOM technique is of interest not only for obtaining versatile information about the object of study, but also for improving the resolution of SNOM topographical measurements in combination with AFM. So, the combination of atomic force and near-field optical microscopies is a promising approach for the investigation of biological objects in their natural environmental condition.

**Acknowledgments**. This research has been partially funded by the Fondazione Cariplo.

## REFERENCES

Allen T.D., Cronshaw J.M., Bagley S., Kiseleva E. and Goldberg M.W.; 2000: *The nuclear pore complex: mediator of translocation between nucleus and cytoplasm.* J. Cell. Sci., **113**, 3885-3886.

Bassi D., Cappa F. and Cocconcelli P.S; 2009: *A combination of a SEM technique and x-ray microanalysis for studying the spore germination process of Clostridium tyrobutyricum.* Research in Microbiology, 2009. In press.

Binning G., Quate C.F. and Gerber C.H.; 1986: *Atomic Force Microscope.* Phys. Rev. Lett., **56**, 930-933.

Cerf O., Bergère J.L. and Hermier J.; 1967: *Termorésistence des spores de Clostridium tyrobutyricum et Clostridium butyricum.* J. Dairy Res., **34**, 221-229.

Cricenti A., Generos R., Luce M., Perfetti P., Margaritondo G., Talley D., Sanghera J.S., Aggarwal I.D., Tolk N.H., Congiu-Castellano A., Rizzo M.A. and Piston D.W.; 2003: *Chemically resolved imaging of biological cells and thin films by infrared scanning near-field optical microscopy.* Biophys. J., **85**, 2705-2710.

Gallyamov M.O.; 1999: *Scanning probe microscopy of nucleic acids and thin organic films*. Ph.D. Thesis in Physics, Moscow State University, 227 pp.

Giessibl F.J.; 2003: *Advances in atomic force microscopy.* Rev. Mod. Phys., **75**, 949-983.

Lange F., Cambi A., Huijbens R., de Bakker B., Rensen W., Garcia-Parajo M., van Hulst N. and Figdor C.G.; 2001: *Cell biology beyond the diffraction limit: near-field scanning optical microscopy.* J. Cell. Sci., **114**, 4153-4160.

Lewis A., Isaacson M., Harootunian A. and Muray A.; 1984: *Developoment of a 500° spatial resolution light microscope: I. light is efficiently transmitted through 1/16 diameter apertures.* Ultramicroscopy, **13**, 227-231.

López-Enríquez L., Rodríguez-Lázaro D. and Hernández M.; 2007: *Quantitative detection of Clostridium tyrobutyricum in milk by real-time PCR*. Appl. Environ. Microbiol., **73**, 3747-3757.

Mironov V.L.; 2004: *Fundamentals of scanning probe microscopy.* The Russian Academy of Sciences. Institute of Physics of Microstructures, Nizhniy Novgorod, 97 pp.

Muller S.A. and Engel A.; 2001: *Structure and mass analysis by scanning transmission electron microscopy.* Micron, **32**, 21-31.

Ohtsu M. and Kobayashi K.; 2004: *Optical near fields. Introduction to classical and quantum theories of electromagnetic phenomena at the nanoscale.* Springer-Verlag, Berlin-Heidelberg, 205 pp.

Palumbo E., Favier C.F., Deghorain M., Cocconcelli P.S., Grangette G., Mercenier A., Vaughan E.E. and Hols P.; 2004: *Knockout of the alanine racemase gene in Lactobacillus plantarum results in septation defects and cell wall perforation.* FEMS Microbiol. Lett., **233**, 131–138.

Plomp M., McCaffery J.M., Cheong I., Huang X. and Bettegowda C.; 2007: *Spore coat architecture of Clostridium novyj NT spores.* J. Bacteriol., **189**, 6457-6468.

Pohl D.W., Denk W. and Lanz M.; 1984: *Optical stethoscopy: image recording with resolution $\lambda/20$*. Appl. Phys. Lett., **44**, 651-653.

Scherrer R. and Gerhardt P.; 1972: *Location of calcium within bacillus spores by electron probe X-ray microanalysis*. J. Bacteriol., **112**, 559-568.

Smith L.D.; 1970: *Clostridium oceanicum, sp. n. a sporeforming anaerobe isolated from marine sediments.* J. Bacteriol., **103**, 811-813.

*Corresponding author:* Gabriele Ferrini
Università Cattolica del Sacro Cuore
Dipartimento di Matematica e Fisica
Via dei Musei 41, 25121 Brescia (Italy)
phone: +39 030 2406710; fax: +39 030 2406742; e-mail: gabriele@dmf.unicatt.it