PROPOSAL FOR A X-RAY MICROSCOPY BEAMLINE IN THE SPANISH SYNCHROTRON ALBA.

I. Abstract

In the area of Bio-Medicine there is an enormous need for a technique that could provide three-dimensional information of whole cells with a resolution in the range of electron microscopy, but with the time scale similar to light microscopy. A very attractive possibility is offered by X-ray microscopy in the soft energy range, that uses the native contrast mechanism (water window) of biological material in an aqueous environment. The goal of this proposal is to create a resource that exploits the unique properties of x-rays to image cells and tissues. The microscope is based on a simple full field transmission design to be installed in a bending magnet.

The proposal is organized as a complete Program that would take into account the definition of experimental systems suitable to develop adequate sample preparation techniques, as well as new, improved approaches for labelling and chemical detection of cellular components in the subcellular native environment. Also, the development of data acquisition procedures for tomographic reconstructions and time series of dynamic processes are challenging aspects that should run together with the beamline design and construction. The proposal mobilizes the expertise from specialists in light and electron microscopy, cell biologists, structural biologists and chemists, together with physicists and engineers. This project is a concerted effort to set up not only an instrument but rather a challenging proposal towards the development of a new approach in Structural and Functional analysis in Biology.

II. Introduction.

Determination of the structure and the topological relationship of the cellular components, as well as their dynamics, are prime objectives in modern Cell Biology. They depend upon the development of new experimental approaches that would allow the analysis of the biological material "*in toto*" under physiological conditions. Confocal microscopy is well suited for these approaches, and it is presently a very successful alternative in these studies. The main strengths of confocal microscopy are the possibility to get three-dimensional representations of the living cells, as well as the possibility to visualize molecular markers with sufficient temporal resolution at a higher resolution than that of conventional light microscopy, both in lateral and vertical dimensions. These characteristics make possible the spatial and temporal reconstruction of markers in near physiological conditions, and the last decade has witnessed the powerful combination of molecular biology tools (as the engineered fluorescent proteins), the use of a variety of light microscopy tools, and the digitalisation of image acquisition and storage.

The main limit of confocal microscopy is the spatial resolution on the boundary up to 200 nm for fluorescence imaging (and much worse for transmitted light imaging). It is not evident whether light based microscopy might attain near molecular resolution ever, as although techniques have been developed to overcome the diffraction limit in resolution, these approaches have been very limited in their application and their extension to complex heterogeneous materials are doubtful (Gustafsson, 2000;Klar, 2001). An obvious alternative is the use of shorter wavelength radiation, and electron microscopy (EM) is an excellent example of that approach. EM has been extremely successful to reveal the subcellular organization at an impressive resolution level, up to the point that our present knowledge of the cellular organization is based mainly in EM data. Nevertheless, EM has several important limitations: The sample has to be introduced in the vacuum required by the electron beam, the thickness of the samples is limited to very thin layers (up to a few tens of a micron), excluding the observation of whole cells. Another additional complication is that high resolution transmission EM images are two-dimensional projections and, thus, complex superposition limits the

proper three-dimensional interpretation. Furthermore, it is not possible to get temporal series of living samples.

Recent advances, including the combined use of high voltage microscopes, together with cryogenic procedures for sample preparation and data acquisition, and the development of tomographic methods, have allowed to extend the use of EM into the subcellular level, showing the possibility to visualize the complex organization of cellular machines, organelles and cytoskeletal structures, as well as their topological relations within the subcellular environment. The resolution limit for this type of microscopic analysis is around 3-4 nm (Walz et al., 1997;Grimm et al., 1998;Baumeister et al., 1999;Baumeister and Steven, 2000;Bohm et al., 2000;Nicastro et al., 2000;Medalia et al., 2002;Grunewald et al., 2003) , thus approaching the 0.4-2 nm resolution boundary characteristic of the EM reconstructions of isolated macromolecular aggregates using cryo-EM and computer reconstruction procedures (Henderson and Unwin, 1975;Penczek et al., 1992;Henderson, 1995;Frank, 1996;Baker et al., 1999;Grimes et al., 1999;van Heel et al., 2000).

In spite of the dramatic advances of cryo-EM, the limits imposed by the sample thickness that can be imaged using electrons (around 1 micrometer in standard microscopes, and up to 2-3 micrometers in the higher voltage microscopes), and the impossibility to get information from living specimens without complex sample preparation procedures, impose a clear constrain in the application of these approaches for subcellular analysis at molecular resolution.

There is an enormous pressure for a technique that could provide three-dimensional information of whole cells with a resolution in the range of EM, but with the time scale similar to light microscopy. A very attractive possibility to avoid the limitations of either confocal or electron microscopy is X-ray microscopy, that uses the native contrast mechanism (water window) of biological material in an aqueous environment. High flux of tunable X-rays produced in synchrotron facilities in the soft energy range (< 700 eV) can be used under different setups: Full field transmission X-ray microscopy –TXM-, scanning transmission X-ray microscopy –STXM- fluorescence and luminescence X-ray microscopy (Schmahl, 1980;Kirz et al., 1990).

The photon energy that presents a more interesting profile in Biology is the range 250 eV up to 1800 eV. In particular, the region between the K shell absorption edges of carbon (284 eV) and oxygen (543 eV) is very attractive as these photons readily penetrate in aqueous samples. In this energy range (the so called "water window"), biological material absorbs around one order of magnitude higher than water, thus producing images with a high intrinsic contrast. The advantage of X-rays over other charged particles (as electrons) is their higher penetration and the corresponding decrease in radiation damage. Also important is the possibility to visualize samples at atmospheric pressure (and even in aqueous environment), thus opening the possibility to plan structural analysis of the biological samples under fully functional conditions.

Although biological X-ray microscopy is in the early steps of development, there are different experimental setups already implemented for X-ray microscopes that show special interest in biological applications. In particular, full field TXMs using zone plate optics and CCD recorders, as the setup in the ALS (Advanced Light Source, Lawrence Berkeley National Laboratory, USA), have proven to yield resolutions exceeding by almost an order of magnitude that currently attained by light microscopy (Meyer-Ilse et al., 2001;Larabell and Le Gros, 2003;Larabell and Le Gros, 2004). These instruments are fast, thus allowing tomography and three-dimensional reconstruction, as well as time acquisition series. They offer high spatial resolution combined with penetration around 10 micrometers in biological samples (one order of magnitude thicker than the maximum penetration of electron beams in EM). Although not specially suited for the purpose, as chemical analysis requires higher energies, they are also suitable for elemental information retrieval opening the possibility for labelling of specific structural components. The typical geometry of sample holders in these microscopes allows the design and easy use of complex chambers to control, change and monitor the conditions of the sample during the data acquisition. The drawbacks of these instruments are their relative inefficiency that implies the use of relative high doses.

We strongly believe that soft X-ray microscopy has the unique power to provide structural and chemical information in fully hydrated biological specimens with thickness above 10 micrometers at a resolution better than 30 nm. The setting of a beamline with those imaging capabilities, represents a challenge for the years to come in

a relatively new field, that would also open new venues for correlative studies using light and electron microscopy.

We are aware that the successful application of X-ray microscopy to Biological systems relay not only in the technical description of the Microscope, but rather in the coordination of a complete Program that would take into account the definition of experimental systems suitable to develop adequate sample preparation techniques, as well as new, improved approaches for labelling and chemical detection of cellular components in the subcellular native environment. Also, the development of data acquisition procedures for tomographic reconstructions and time series of dynamic processes are challenging aspects that should run together with the beamline design and construction. The proposal mobilizes the expertise from specialists in light and electron microscopy, cell biologists, structural biologists and chemists. Their input will also be required by the engineers when it comes to devise sample holders as these will not only have to comply with the physicists requirements to achieve optimal signal to ratio results, but also with the requirements imposed by the specific samples to be observed. This project is a concerted effort to set up not only an instrument but rather a challenging proposal towards the development of a new approach in Structural and Functional analysis in Biology.

III. Beamline description.

1.- General considerations.

It is aimed to build a microscope easy to operate that should allow to record many images per day (~ 100) . For this reason a large deal of simplicity has been incorporated in the design. An existing instrument that has performed very well in this sense is the one installed at beamline 6.1.2 of the ALS which has been taken as guideline.
Another design issue has been to provide the x ray microscope with two ancillary visible light microscopes, located at its vicinity, in order to examine the samples immediately before mounting them into the microscope and also when they are already mounted. This second microscope should be used to precisely select the areas that it will be imaged by the x-ray microscope.

What follows is a succinct description of the main components and characteristics of the planned instrument. It has been assumed that a detailed and accurate design should be done in due time by the selected beamline scientist. Figure 1 schematizes the main optical components.

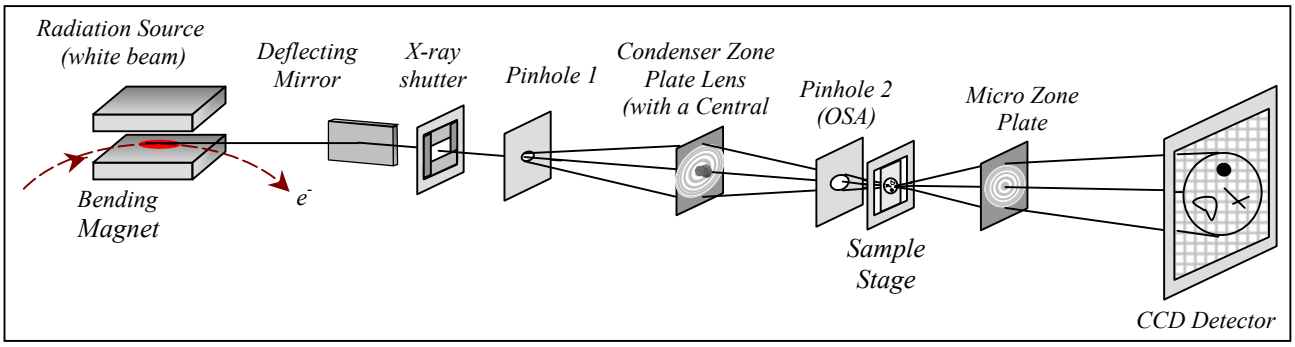


Figure 1: Schematic layout of the main components of the beamline.

2.- Mirror

Bending magnet radiation from the storage ring will be reflected at an angle of incidence about 3 degrees by a flat cooled mirror Ni or Au coated in order to suppress high energy photons. The mirror will be located as close as possible to the storage ring wall, after a set of primary slits which will define the beam dimensions. The mirror will be enclosed in a UHV chamber ion pumped.

It is envisaged to have a mirror with two strips: Ni and Au for operation at the water window energy range or at higher energies (1 keV maximum) respectively. As no high energy photons will be in the reflected beam, the microscope will not need to be in a lead shielded hutch which simplifies its operation.

3.- Acoustic Delay Line

To protect the vacuum of the storage ring from accidents due to rupture of one of the thin windows installed in the microscope, an acoustic delay line (not shown in Fig. 1) should be installed after the window . It should be designed to slow down a vacuum leak in order to allow the fast valve in the front end which separates the machine and beam line vacuum , to be closed in due time.

4.- Full Field Microscope

4.1. General description

The microscope is an x-ray analogue of a simple optical microscope. Figure 2 schematizes its components. The x-ray beam from the mirror is defined with an aperture that matches the condenser zone plate which focuses the beam onto the sample stage. The condenser zone plate (CZP) is used to illuminate the sample with an intense and uniform beam and it is also used as a monochromator. The wavelength selection is achieved by using the fact that for a given zone plate, its focal length is proportional to $1/\lambda$. By varying the distance between the CZP and the pinhole located just before the sample (order selecting aperture), it is possible to monochromatize the incident radiation with a band pass $\Delta\lambda/\lambda$ around 10^{-2}.

The transmitted x-rays from the sample are collected by the objective zone plate (OZP) optically matched with the CZP. The OZP is used in a very asymmetrical configuration to magnify the image onto a relatively distant CCD detector or image plate. The distance from the CCD to the OZP determines the magnification of the system and the outer zone of the OZP the resolution of the image.

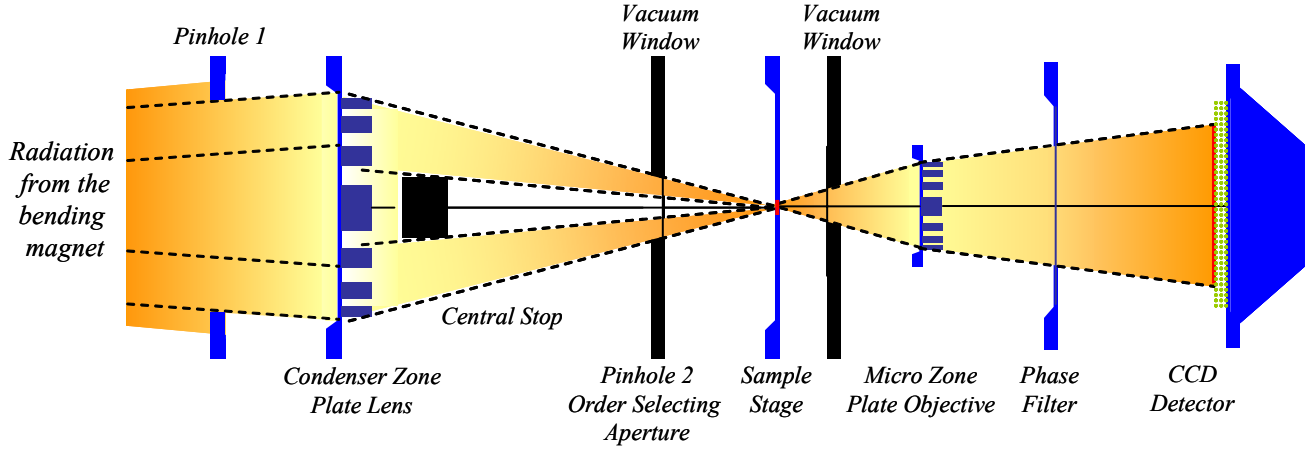


Figure 2: Scheme of principal optical elements for a Full-Field Transmission X-ray Microscope

4.2 Condenser Zone Plate Assembly

Pinhole 1 defines the beam that impinges the CZP which focuses the source to a spot of typical dimensions around 2-5 μm. As these are smaller in some cases than the sample size, it is necessary to have the possibility of scanning the CZP perpendicularly to the incoming beam . This implies the additional complication of also scanning pinhole 2 in order to be able to uniformly illuminate a relatively large sample. This design issue has to be carefully studied in the detailed design of the instrument.

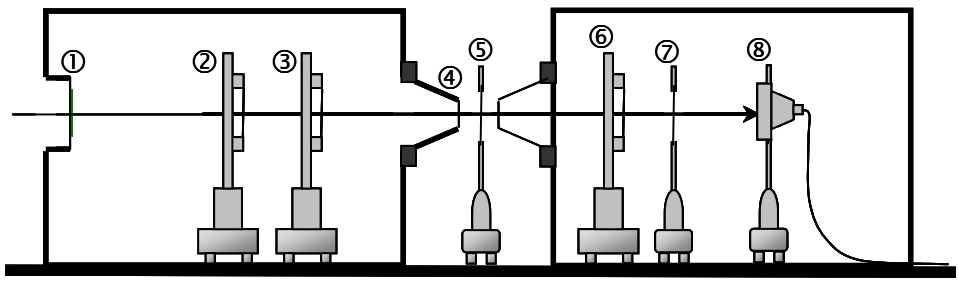
After the CZP a central stop has to be installed to prevent direct illumination to reach the sample in order to reduce the background.

Pinhole 1 and the CZP have to be mounted on a precise z stage allowing translations parallel to the direction of the incoming beam, in order to change the wavelength. It is aimed to span the range 100-1000 eV or 12-1.2 nm . To give an idea on the required z travel, if the minimum CZP sample distance is designed to be 30 mm which would correspond to the longer wavelength (12 nm) then to focus 1.2 nm radiation the distance has to be 300 mm

The CZP and associated mechanical translations will be installed in a high vacuum system which will have thin Si_3N windows along the optical path.

Figure 3 schematizes the different translation stages required for the alignments.

The whole vacuum chamber has to have the possibility of being removed from the beam path and accurately repositioned back on the optical axis in order to be able to mount an optical microscope to visualize the sample and define the sample area that will be x-ray imaged.



① X-ray shutter and filter
② Pinhole I (x-y-z translational stage)
③ Condenser zone plate lens (x-y-z transitional stage)
④ Pinhole 2 (OSA)
⑤ Sample stage
⑥ Micro zone plate objective lens
⑦ Phase filter
⑧ CCD detector

Figure 3: Schematics of the different positioning stages of the optical elements.

4.3 Objective Zone Plate Assembly

The OZP has to be mounted on a high precision x-y-z stage and the phase ring located after the OZP has also to be mounted in an independent x-y-z stage for precise alignment.

As the x-ray wavelength or microscope magnification are changed, both the OZP and phase ring have to be adjusted at the proper distance from the sample.

The whole unit has to be in a moderate vacuum housing.

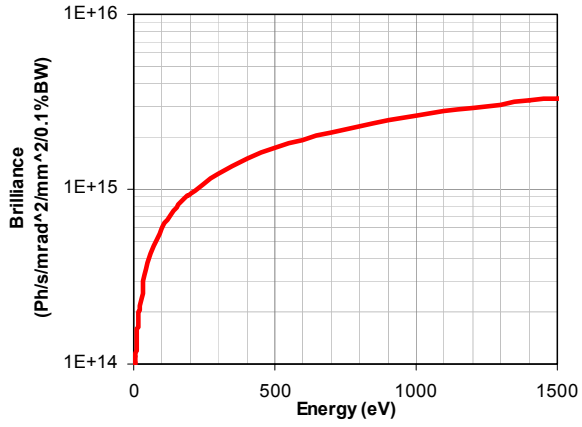
4.4 CCD detector

It is planed to buy a commercial CCD detector well suited for soft x rays that would be installed in the vacuum enclosure and mounted in a z travel stage of ~ 300 mm span.

Typical parameters could be 1000x1000 pixels and ~ 20 μm pixel size.

4.5 Expected flux

Let us estimate the expected photon flux available for the microscope . The spectral brilliance of a bending magnet source at ALBA is depicted in the figure below.



Spectral brilliance of a bending magnet source evaluated for a current of 250 mA in the ring.

At the water window energy range (300-500 eV) the brilliance is around 10^{15} photons/(s.mm^2.mrad^2.0.1%BW). The dimensions of the photon source are determined by those of the electron packets in the storage ring. Approximately they are (FWHM) $\Sigma x = 117$ μm and $\Sigma y = 59$ μm in the horizontal and vertical directions respectively. The

angular width of the coherent photon beam from the above 'point' source can be *estimated* with the diffraction limit condition $\Sigma \cdot \Sigma' = \lambda/2\pi$ which gives (for λ = 4.1 nm which corresponds to hν= 300 eV) $\Sigma x' = 5.6$ μrad and $\Sigma y' = 11$ μrad . Let us suppose that the condenser zone plate is located at L= 20 m from the source; then the coherently illuminated area will be $L\Sigma x' L\Sigma y' = 2.5\ 10^{-2}$ mm ^{2} . By multipliying the brilliance by ($L\ \Sigma x' L\ \Sigma y'$) ($\Sigma' x\ \Sigma' y$) one gets ~ 10^9 photons/s.0.1%BW. As the band pass of the condenser is 10^{-2} rather than 10^{-3}, we obtain ~ 10^{10} ph/s as estimation of the coherent flux illuminating the zone plate. This is a rather stringent evaluation since it is based on a high degree of coherence in the incoming beam. In practice, the degree of coherence can be relaxed somewhat and still obtain reasonable diffraction contrast from quasi-monochromatic incoherent sources that are relatively close each other (Born and Wolf, 1980). Taking into account these considerations, the above number can be increased by almost a factor 10.

IV. Experimental systems.

The building of the beamline must be coordinated with the definition of experimental systems with sufficient intrinsic interest but, also, best suited for a systematic study of the experimental conditions required for the different imaging modes by X-ray microscopy of biological samples. The systems that we propose to explore are key in present Cell Biology: Cell signalling, organelle movement, cell division, and interaction of cells with the environment.

The reason for choosing cell division as a model process to develop a X-ray microscopy beamline applied to biology are fourfold. Firstly, cell division is essential for life itself. From the proliferation of unicellular organisms to mammal development the partition of one cell to generate two daughters is a fundamental process. Secondly, cell division requires a dramatic reorganisation of the whole three-dimensional structure of the cell. As the cell prepares itself for division, the interphase cytoskeleton is disassembled and the cell division machinery, the mitotic -or meiotic- spindle is organised. Dissociation of the Golgi, and nuclear envelop, condensation of chromatin by may orders of magnitude, bipolar attachment of sister chromatids or homologues, chromosome

movements and congression into a metaphase plate, pole-ward segregation of each of the two copies of the genetic material, cleavage of the mother cell into two daughters and reorganisation of the interphase state in these cells are, in gross descriptive terms, the stages of the cell division process. This intricate and highly dynamic re-shaping of almost every cellular compartment offers an excellent model for microscopy as it was shown when it attracted the attention of the first high-quality microscopy studies in cell biology, over a hundred years ago. The third reason that justifies the choice of cell division as one of the biological processes to be used as a model for the development of a X-ray miocroscope is the wealth of structural detail. From the overall shape of the spindle, which can easily be observed by conventional optical microscopy, to the fine structure of the centrioles, that can only be resolved by high-resolution EM (Lange and Gull, 1996) the dividing cell provides a whole range of three-dimensional structures that are excellent targets to validate new microscopy techniques. Finally, cell division has a well-established relevance in biomedicine. Errors in the segregation of the genetic material result in aneuplodies that lead to lethality or disease, and uncontrolled cell division is an essential requirement for cancer progression. Neoplastic transformation is almost always associated with profound alterations of the cell division machinery and cell karyotype. Thus, in most cancers, supernumerary centrosomes that have varying amounts of pericentriolar material are often found. These strongly correlate with multipolar cell division spindles that organise distorted metaphase plates and drive unequal chromosome segregations. Genomic instability is also a hallmark of cancer. It may affect chromosome number and integrity and is suspected to provide a source of variability that may be used by cancer cells to evolve towards more malignant states. Getting cell division back under control, or simply arresting division in cancer cells is a prime objective of current basic and applied research, and one that can benefit from new high-resolution, less invasive microscopy techniques.

The basic principles that govern cell division, as well as many of the actual proteins that provide essential functions required for this process, are highly conserved from lower eukaryotes to mammals. Therefore, studies carried out in any well-characterised model system are bound to have a significant impact on our understanding of cell division in higher organisms, including humans. The most widely-used model systems in which to study cell division are fungi, yeast, nematodes and flies (Gonzalez et al., 1994;Oakley, 1999;Bowerman, 2001;Jaspersen and Winey, 2004). Frogs, too, are common in cell

cycle research, but mainly for biochemical studies. From an ultra-structural point of view, cell division in nematodes (C. elegans) and flies (D. melanogaster) may provide the closest resemblance to cell division in mammals. Sophisticated protocols are already available to prepare these cells for microscopy studies (Feiguin et al., 1998;Grill et al., 2003). Moreover, these two systems are amenable to genetic manipulation, thus providing a source of wild-type as well as mutant cells in which cell division proceed in the absence of a given protein (Gonzalez *et al.*, 1994;Bowerman, 2001). A detailed characterisation of the phenotypes brought about under these conditions is crucial to understand the functions provided by the missing proteins. Until now, these studies have been carried out by light and EM microscopy. It is expected that X-ray microscopy will generate data to fill the important gap that exists between these two approaches.

Drosophila is easy and inexpensive. More importantly, there are a number of groups in Spain, and particularly in the metropolitan area where ALBA will be installed that carry out state-of-the-art research in this organism. There is, however a clear need to set a small team of biologists who should take responsibility for developing the new protocols that will be needed. An essential aspect of developing a new tool is to have a comparative study of performance. Thus, the team of biologists should identify the specific questions that fit best to this project. These should be over structural issues where neither confocal, nor electron microscopy have been able to provide the required data.

Other fundamental aspect for the successful application of microscopic techniques in Cell Biology is the possibility to combine structural determination with chemical detection of different structural components. We would like to emphasize the importance of the development of new and improved procedures for labelling specific cell components specially suited for X-ray microscopy. The development of molecular labels would represent a main effort in our proposal. The incorporation of immuno-fluorescence and immuno-gold plus silver enhancement procedures developed initially for light and electron microscopy would be progressively adapted to the requirements of X-ray microscopy environment. Previous studies point to the use of transition metals such us Vanadium for the design of new probes for biological x-ray microscopy, so we will investigate the possibility to create or find new biological labelings that could provide enough x-ray contrast ((Morone et al., 2004).

We will develop new approaches for *in situ* tagging by exploring new methods to bring reagents outside the cells (aimed to investigate the physiology of the apoplastic space, where changes in composition and pH - by using pH-dependent fluorophores - have been observed in response to stress (López-Millán, 2000) and inside the cells (aimed to investigate the physiology of the symplastic space, where reagents may accede through plasmodesmata – frequencies of plasmodesmata are commonly greater than 1 million per square millimetre -, aquaporins, plasma membrane channels, specific transporters, and others). These approaches will be carried out in cell cultures and in intact tissues, together with the development of local chemical reactions to attach metals to specific cell components. Metals are widespread in cells, not only metallic micronutrients (such as Fe, Mn, Cu and Zn) but also pollutant heavy metals (Larbi, 2002), and references therein). Among pollutant heavy metals, lead (Pb) is a good candidate for plant cells, since Pb is taken up by roots, is internally mobilized and plants cope with high Pb concentrations without showing symptoms of toxicity or poisoning (Larbi, 2002;Marmiroli, 2004). Different processes can be investigated in plant physiology and soil sciences using Pb as a marker, since Pb is able to bind, through Pb-O bindings, to metal-binding proteins, ligno-cellulosic matrices, oxalate and parietinic acids, plant polysaccharides, mucilage (an organic polymer present on the root surface), and to soil salicylate, and humic and fulvic acids ((Marmiroli, 2004), and references therein). Within metal-binding proteins, expression of engineered proteins able to bind different metals or components with differential contrast will be explored. Other related applications will also be attempted by collaborating groups, including the study of the distribution of certain metallic micronutrients in plants that, as a consequence of abiotic stress situations (Morales et al., 1998, and unpublished results from experiments carried out at DCI, LURE, Orsay, France) or mutations (Ellis, 2003), have altered their metallic homeostasis and hiper-accumulate the metal, as well as in model systems. Areas of interest of these techniques are plant (stress) physiology and biotechnological applications related to Biotechnology of phyto-remediation processes.

The use of cell-penetrating peptides (CPPs) have emerged these last years as an efficient way to translocate plasma cell membranes. The versatility of peptide chemistry offers a nice opportunity to combine CPPs with probe molecules specifically designed for its

use in X-ray microscopy. One possibility is to use Cys residues as anchors between the CPP and gold colloidal nanoparticles.

The measure of X-ray excited visible light luminescence is another very promising approach for the visualization of specific intracellular entities. On the one hand, it is well known that in a scanning configuration, microscope resolution is determined by the spot size of the exciting radiation rather than the wavelength of the visible luminescence. On the other hand, however, the absorption of a single photon of the most commonly used 2-5 nm X-rays is able to destroy the conjugated aromatic rings present on the conventional fluorescent dies used in visible light microscopy. Here again, combined used of CPPs and robust luminescent lanthanide complexes could provide the basis for a new class of X-ray excitable molecular probes especially suitable for X-ray microscopy of living cells.

V. Sample preparation methods.

A key aspect for the successful application of any microscopy is the way the samples are prepared for visualization. The cumbersome procedures involved in sample preparation for EM, until the implementation of the cryo-techniques, have limited the extension of the use of certain applications in spite of their clear potential expectations. On the other hand, the straightforward sample preparation for confocal microscopy is instrumental for the extended use of these methods.

In principle, biological samples can be imaged directly under the X-ray beam, even inside the aqueous environment. This property must be explored in combination of optimised data acquisition methods, specially in the context of studies of dynamic processes, where the time resolution can be an unique bonus for this technique. The design of liquid sample holders will be instrumental in those imaging modes involving possibilities to modify the experimental micro environment of the sample (buffer conditions, temperature, change of chemicals, etc).

Nevertheless, it is progressively evident that for many interesting applications some sample preparation must be required. In spite of the high intrinsic signal to noise ratio of

images taken in the water window, getting to high spatial resolution will demand extended radiation of the sample that would eventually degrade its molecular structure. Also, the possibility to get X-ray tomograms at 20-40 nm resolution will demand the generation of at least a hundred of projection images. In these cases, a main avenue to explore is fixation of the sample by low temperature procedures (Weiss et al., 2000). Ample experience on freezing biological samples of variable thickness has been already obtained in cryo EM, where three-dimensional reconstruction is based usually on data obtained by cryo-EM on fast frozen samples within a layer of vitrified water. Also, electron tomography is based on the production of well preserved frozen section of cells. We plan to benefit from these experiences and further extend the analysis of fast freezing procedures by incorporating high pressure freezing, as well as exploring the use of cryo-protectants for thick sample preservation. Also, the improvement of the use of cryo-stages under the X-ray microscope will be very important for the direct examination of frozen cells that were not exposed to chemical fixatives or contrast enhancement agents and so, avoiding artefacts induced by these methodologies. The production of cryo-sections will be explored, specially for high resolution studies.

VI. Tomographic procedures

Recovery of three-dimensional information is a major step forward for the full exploitation of microscopy techniques. This is specially important in those microscopies (as EM and X-ray) where the direct images given by the microscope are, to a good approximation, two-dimensional projections difficult to interprete due to the complex overlapping of the three-dimensional data.

We propose to explore image acquisition protocols and data combination procedures to optimise the characteristic features of X-ray absorbence images. This will lead to complementary studies in the mechanism of image formation, image alignment and classification, segmentation procedures, etc (Fernandez et al., 2002).

Ample experience exists in the field of three-dimensional electron microscopy (3DEM) that can be of direct application in X-ray tomography, and Spain counts with well recognized groups in the area of 3DEM, covering all the way from method

developments to applications in key biological systems. It is the existence of these groups with strong scientific contacts in the EU, US and Japan that assures that Tomographic applications at ALBA will be of leading edge quality, coordinated with the most advanced experiences in this field worldwide.

The incorporation of realistic image formation models is a subject that, quite obviously, has to be tailored to the specific requirements of X-ray tomography, including the possibility to introduce depth dependent Point Spread Functions (under the assumption of linearity), which could be a way to increase the size of the specimens under study. It is clear, also, that ample experience in postprocessing, such as denoising, segmentation and quality assessment exists in 3DEM as well as in some application of confocal microscopy. Again, an specific tuning to X-ray microscopy can and should be performed working from the experience obtained in these other microscopies.

An important line of work will be related to the exploitation of the possibilities of three-dimensional tomography based on the penetration power of X-rays. The sample thickness that can be reconstructed by X-rays (10 micrometers) is about one order of magnitude higher than that usable in electron tomography. This new application field of EM will be able to deliver subcellular structure at 4 nm resolution but limited to small areas of the cell. In order to get together this high resolution, albeit partial information, we require an integrating framework that will ideally be deliver by X-ray microscopy: The 0,5- 1 micrometer slices reconstructed at high resolution by electron tomography will be docked into the 10 micrometer areas reconstructed by X-ray microscopy at moderate resolution. This approach is basically similar to that one successfully used in the docking of atomic resolution protein structures mapped into the lower resolution volumes obtained by cryo-EM. In this way, we can ideally devise an extended use of microscopies (Cryo-EM, Electron tomography, X-rays, Optical), covering different domains of progressive size (macromolecular, subcellular, cellular and multicellular) at progressive resolution levels (0,3 nm, 3 nm, 30 nm, 300 nm). Basically, this proposal would imply the possibility to combine different microscopies to study from the molecules to the cell.

Although the interface of X-ray microscopy and EM is an evident area of work in this proposal, it is also clear that the interaction with photon microscopies must be a subject

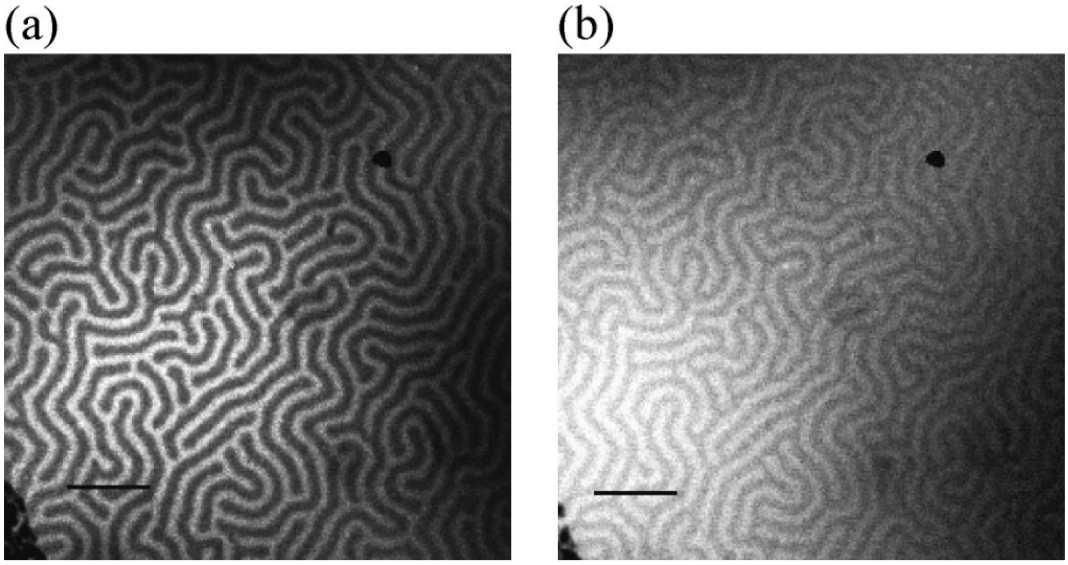
to explore. An interesting point underlying the development of multimodal microscopy interfaces is that there is a technological opportunity to incorporate into X-ray microscopy many new approximations under development in the field of image processing for electron and photon microscopy (Deerinck et al., 1994;Martone et al., 2003). This type of technological synergy has been very fruitful in the past and we look forward for these developments in near future.

VII. Other applications: Magnetism.

We are well aware that the beamline can be used for other applications with some modifications, something that is highly desirable due to the short number of beamlines availables in the Spanish synchrotron during its first operational period. As an example, applications on the Magnetic-imaging mode has been also proposed**.**

The combination of a transmission x-ray microscope (TXM) with x-ray magnetic circular dichroism XMCD as magnetic contrast mechanism allows us to image element-specific magnetic domains. In full transmission geometry (i.e. with the incoming beam perpendicular to the sample surface), element-specific studies of systems consisting of only few monolayers of magnetic material with out-of-plane magnetization can be easily performed with spatial resolution sufficient to distinguish features sizes down to 25 nm [fisher01]. By tilting the systems at an axis orthogonal to the photon propagation direction in-plane magnetized systems can also be addressed.

In order to switch to the magnetic imaging mode (magnetic transmission x-ray microscopy M-TXM), illumination of the sample with circularly polarized x rays is essential. From a bending magnet, linearly or elliptically polarized illumination can be obtained from in-plane or out-of- plane electron orbit radiation, respectively. Circularly polarized x-ray light is hence achieved by viewing the off-orbit contribution of the radiation from the bending magnet emitted about 2-3 mm above or below the orbital plane. Estimates of the degree of circular polarization taking into account the source parameters and the emission characteristics give values of about 60%, which is sufficient to achieve good contrast. Figure below shows an example from (Fisher et al., 2001).



Magnetic x ray transmission images of a multilayer sample 75 times 0.4 nm Gd /0.4 nm Fe, taken at the (a) Fe L_3 absorption edge and (b) L_2 absorption edge. The bar corresponds to 1 μm. The field of view of the images is about 40 μm^2 and the recording time is only of a few seconds. The images were recorded at remanence but they can also be taken on a field of several kOe. The white and black strips are the Fe domains with perpendicular magnetization out of plane or in plane. The color inversion of (b) relative to (a) arises from the different signs of the dichroic signals at both absorption edges. The resolution of the microscope is 25 nm. Although the images are from a relatively thick sample, domains of ultrathin Co films of only 3nm may also be imaged in a similar way.

The major advantage of M-TXM with respect to other microscopy techniques incorporating magnetic sensitivity is the possibility to record the images in varying external magnetic fields, because it is a photon-in photon-out approach at variance with electron based techniques. This important feature allows to collect information on the magnetization dependent evolution of magnetic domains within a complete hysteresis loop. Moreover, sub-nanosecond time-resolved magnetization dynamic studies can be performed by using the inherent pulsed time structure of the synchrotron radiation Alba source in single-bunch mode with a pump-and-probe scheme (Stoll et al., 2004).

VIII. Laboratory requirements.

Building of the Beamline must take into account the construction of a laboratory space fulfilling the requirements for cell culture, standard biochemical techniques, cold room, and light microscopy. This laboratory will be instrumental for the preparation and preliminary characterization of the samples to be imaged in the X-ray microscope. It is envisaged that a such a small laboratory run by an experienced postdoc with the help of a qualified technician should be able to carry out these functions.

The main features of this laboratory will be:

Standard biochemical techniques:

- Cold (dark) room (4 ºC), Fridges (-20 and –80 ºC)
- Liquid Nitrogen (N_2)
- Precision balances (chemical products)
- De-ionized and ultra-pure (MilliQ) water.
- pH-meters, Bortex, Agitators, other minor biochemistry lab equipment (glass and plastic lab material, etc).
- Centrifuge and ultracentrifuge (for organelle isolation)
- Spectrophotometer (to measure biochemical parameters, follow enzymatic activities, etc).

Microscopy:- Availability of a variety of sample holders (in shape and capacity), able to hold plant and animal tissues, liquid cell cultures and organelle isolations. These holders should have the possibility of changes some variables like pressure, temperature, light, humidity, etc.

- Microtome , and cryo- ultra microtome to cut slices of plant or animal tissues in the range of μicrometers and beyond, both at room temperature and frozen samples.
- Freezing systems, including atmospheric pressure and High-pressure freezing machines.
- Light Microscopes (conventional and fluorescence microscopes).

Cell culture facility.

Although many computer activities of the Program can be performed in remote mode, and the Beam design can be done in such a way as to maximize the remote handling of many of the operations involved in data adquisition and data validation, it is clear that a

minimal computer system must be accessible in the Beamline. For this reason, a computer room must be incorporated into the Beamline area to host the data processing required for *in situ* measurements. This computer room will be mainly required for assessing the quality of the data and the Tomographic reconstructions obtained from them. Since this is an emerging area, substantial "tuning" of existing developments as well as completely new procedures are expected to be required, and an on-line testing on them will be a need.

IX. Accompanying Program.

The biological programs coordinated in this proposal requires the mobilization of specific resources, both human and materials. Testing the different cell systems, development of new labelling procedures and the implementation of advanced tomographic methods will demand specific grants to cover their costs as well as the personnel involved. In principle, these grants should be handled as Strategic Actions, following the model of the Genomic and Nantechnology recent Programs launched by the MEC. Furthermore, it is critical to set up a training program for students, both at pre- and post-doctoral level, that will be the basis for the scientist team when the beamline will be available in five years time. These students will be trained in Synchrotron sources where the X-ray microscopy is presently under development and, to this end, a preferential Program will be developed to launch a project with the ALS at Berkeley both for training purposes, as well as for technological and biological applications.

X. References

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