MD-239 : Synchrotron radiation micro-CT for the investigation of bone quality
ESRF Long Term Proposal
Starting : 2006/II to 2009/I (6x18 shifts)

Purpose of the Long Term Proposal:
The purpose of this Long Term Proposal is to explore bone quality using the unique 3D quantitative imaging capabilities of SR micro-CT for the investigation of the tissular and cellular properties of bone, and to gather an international research community in the field of bone around this tool. The following sub-projects are studied :

1. Quantification of micro-cracks after biomechanical stimulation
2. Quantification of ultra structure system
3. Investigation of bone quality in mice models
4. Bimodal investigation of bone quality by SR micro-CT and acoustic microscopy
5. Quantification of bone micro-vascularisation in rat model
6. Analysis of bone formation in scaffolds
7. Bone mineralization in normal and diseased bone

The following teams are involved in the LTP :

1. Inserm U630, CNRS 5220 (CREATIS), Lyon, DR. F. Peyrin (coordinator)
2. Inserm E366, Laboratoire de Biologie du Tissu Osseux (LBTO), Saint Etienne, Dr L Vico, Pr. MH Lafage-Proust ,
3. CNRS UMR 7623, Laboratoire d'Imagerie Paramétrique (LIP), Paris, Dr. P. Laugier, Dr. F. Padilla
4. CNRS UMR 7052, Laboratoire de Biomécanique et Biomatériaux Ostéo-Articulaires, Paris, Dr. V. Bousson, Pr. JD Larédo, Dr.R C. Bergot
5. Magnetic Resonance Science Center, San Fransisco, USA, Pr. S. Majumbar,
6. Istituto Nazionale per la Ricerca sul Cancro (Unige/IST), Genova, Italy, Pr. R. Cancedda
7. Q-BAM Group, Dept. of Orthopedics, University of Halle-Wittenberg, Germany, Dr. K. Raum,

Experiments:

Exp.	Dates	Shifts	Participants	Projects
MD-239	06/09/2006	3	1,2	7
MD-239	20-24/11/2006	9	1,2,5	1
MD-239	25-28/04/2007	12	1,2,3,6,7	4,5,6
MD-239	2-3/05/2007	3	1,2,3	2
MD-239	2;4-6/07/2007	9	1,2,4	1
MD-239	12-17/11/2007	12	1,2,6,7	5,6

Total allocated: 48

Related experiments:

Exp.	Dates	Shifts	Participants	Projects
MD-218	1-3/12/2006	9	1,5	7
MD-293	12-14/12/2007	6	1,3	1

Scheduled in 2008 :

Exp.	Dates	Shifts	Participants	Projects
MD-239	2-4/05/2008	9	1,2,3	1,4,5
MD-239	30;2-3/07/2008	9	1,2,3	1,3,6
MD-239	9-10/07/2008	6	1,2,7	1,3,6

Total scheduled: 24

Description of work

Experiments were performed on beam line ID19 where we used the synchrotron radiation (SR) micro-CT setups. The beam-time allocated in the Long Term Project was shared between the sub-projects. At the present stage, data have already been collected on the different topics but the sub-projects are still in progress. In addition the simultaneous development of appropriate image processing methods is required to perform data analysis. We present preliminary results on the different topics.

1. Quantification of micro-cracks after biomechanical stimulation (partners 1,2)

The goal of this work is to investigate how fatigue-damage micro-cracks form and where they propagate within bone. The resolution of commercialized micro-CT is currently too limited for such a study and micro-cracks have so far never been investigated by a 3D method.

The first experiment was conducted in Nov 2006 . The preparation of the samples was done by the LBTO (Saint-Etienne). Human femoral heads were extracted during arthroplasty. Cylindar trabecular bone samples (diameter 10mm) were then extracted and divided in a Control group and a Loaded group. The Loaded group samples were maintained viable in a controlled perfusion culture-loading chamber over three weeks and underwent a cyclic compressive strain until a change in the Young modulus. After stimulation, all samples were then embedded in methylmetacrylate (PMMA) and cut as small parallelepipeds (5 x 5 x10 mm^3).

Micro-cracks are described as plane defects with a thickness close to the micrometer level, thus the choice of the spatial resolution is an issue : it should be as high as possible to observe the cracks and as small as possible to have a large field of view (FOV). SR micro-CT images were then acquired at two spatial resolutions: 1.4 and 5 µm. At 1.4 µm, since the FOV was 2.8 mm x 2.8 mm x 1.7 mm, the standard acquisition provides “local tomography” data. We tried to acquire data in “stitching mode”, which consists in recording several scans of the sample with different positions of the rotation axis, in the aim of merging the different projections before reconstruction to increase the FOV. Ten samples were acquired in this mode (i.e two scans were recorded by samples). The same samples were also acquired in SR micro-CT at a spatial resolution of 5 µm to get the entire sample. However, the reconstruction at 1.4 µm revealed strong artifacts observed at the periphery of the images due to distortions in the optic. It was thus difficult to merge the different projections and generate artifact free images at this scale.

A second experiment was conducted in July 2007. The same type of samples (trabecular human femoral heads) was used. Three different modes of fatigue were tested by changing the frequency and the duration of the stimulation (groups 1, 5 and 10). All samples were then

embedded in PMMA and a small parallelepiped 5mmx5mmx10mm was extracted. SR micro-CT images of the samples were acquired at two spatial resolutions: 1.4 and 5 µm. At 1.4 µm, two Regions of Interest (ROI) were acquired at different height in each sample since the FOV was smaller than the total sample. The reconstruction provided: 8 images in the C group, 5 in group 1, 8 in group 5 and 10 in group 10. The images allowed the visual observation of cracks. Figure 1 illustrates a 3D rendering of one sample, one tomographic slice at 1.4µm, and a zoom showing how the cracks are seen on the images.

The processing of the images is under progress. However, due to the small size of the cracks and different sources of noise in the image (photon noise, ring artifacts...), their automatic detection is challenging. An adapted denoising technique was developed to improve the visibility of the micro-crack (Larrue A, Rattner A, Laroche N, Vico L, Peyrin F. Feasibility of micro-crack detection in human trabecular bone images from 3D synchrotron microtomography. Conf Proc IEEE Eng Med Biol Soc. 2007;2007:3918-21). Due to the high resolution used, a large data set has to be managed (300 GB). Volumes of interest (512x512x1024) including cracks were manually extracted. These volumes are currently being automatically processed in order to segment the cracks and the lacunae. We expect to be able to measure different quantitative parameters such as the number, the volume, the thickness, length, and orientation of cracks.

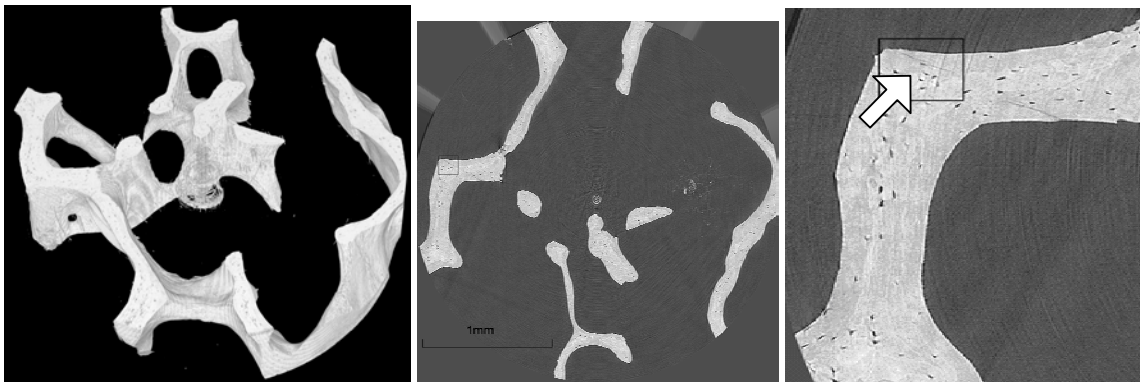

Figure 1, from right to left: 3D display, SR microCT slice and zoom showing a micro-crack (arrow)

Further experiments will be performed to see in which respect the imaging acquisition itself does not induce cracks, and additional data will be acquire to document the effect of mechanical constraint on the cracks.

2. Quantification of ultra structure system (partners 1,2)

The aim of this study is to evaluate the feasibility of visualizing the canalicular system from SR µCT at submicronic spatial resolution (voxel size: 0.28µm).
We used iliac crest biopsy samples embedded in PMMA from human osteoporotic patients (LBTO). To fit the field of view, small cylinder samples (diameter: 500µm, height: 1mm) were extracted from cortical region using a high precision drilling machine.
SR micro-CT images at 0.28µm were acquired in different conditions (energy: 13 and 18keV, exposure time : from 0.7 to 0.1s per projection, 2000 projections).
After reconstruction, we observed sample motion during the scan due to a high absorbed dose on the sample. When using an exposure time of 0.1 s the motion was negligible, but the signal to noise ratio (SNR) was quite low. Some features of the lacuno-canalicular system are visible

but due to the noise level their detection remains is difficult (Peter Z., Bleuet P., Lafage-Proust M.H., Peyrin F., Feasibility of Three-Dimensional Imaging of the Lacuno-Canalicular Network from Synchrotron Radiation Micro-CT, 17th IBDW (International Bone Densitometry Workshop), Kyoto, Japon, Nov. 2006).
Work in progress concerns the processing of the data. Further experiments will be necessary to study the best mode of preparation of the samples to avoid motion during the scan.

3. Investigation of bone quality in mice models (partners 7,1)

Recent work in mouse models give strong evidence that bone structure and strength are regulated by an identifiable set of genes. Two studies related to the effect of gene on the bone phenotype were conducted.

a) Effect of Leptin on bone phenotype *(partner 7,1)*
The obese gene with its product leptin is raising increasing interest but its effects on bone formation, bone remodeling and on the bone phenotype itself is still conflicting.
The aim of this study was to further elucidate the bone pathology in wild type (wt) and leptin deficient (ob/ob) mice, using 200-MHz scanning acoustic microscopy (SAM) and SR micro-CT.

The samples were prepared in the Q-BAM Group. 23 ob/ob mice and 32 wt mice were sacrificed at ages of 3, 6, 9, and 12 months. From each mouse both femurs were extracted, fixed in 70 % ethanol, dehydrated in alcohol and embedded in a modified PMMA. Transverse sections in anterior-posterior direction were cut using a diamond saw. In vitro Scanning Acoustic Microscopy (SAM) measurements at 200 MHz were performed on transverse sections (Fig. 2, left).

Afterwards collateral femora were attached face-to-face, and a cross section was prepared and scanned similarly to the aforementioned procedure. A subset of samples from each group was imaged by SR-microCT (Fig. 2, right). The spatial resolution was set to 2.8 µm, and the energy to 20.5 keV.

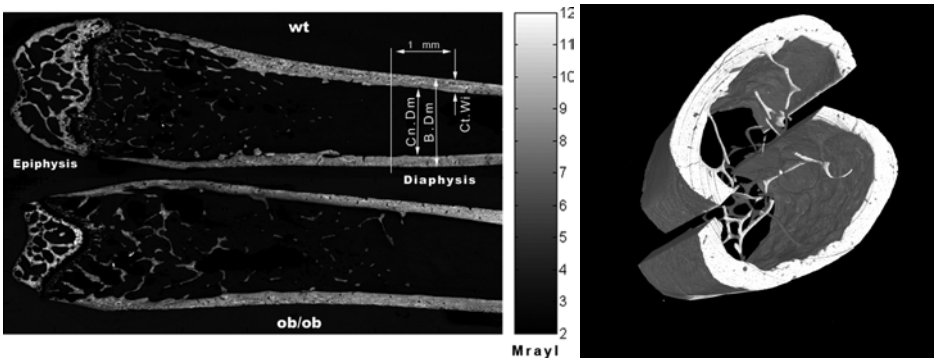

Figure 2 : 200-MHz acoustic impedance images of wt and ob/ob femur samples (left). The indicated 1-mm region has also been imaged by SR-µCT (right).

Data analysis is in progress. From SAM, morphology parameters (cortical thickness, cortical index, cavity density, porosity, etc.) and acoustic impedance parameters will be extracted. From SR-microCT, the tissue degree of mineralization (DMB) and micro-structure parameters will also be extracted. The site-matched correlation between SAM and SR-microCT will allow

evaluating the kinetics of tissue mineralization and tissue stiffening during maturation in normal and Leptin deficient mice.

In the next experiment, additional images will have to be acquired to complement the different groups and achieve publication level.

b) Effect of IFG-I on fetal bone *(partner 5,1)*

A protocol was designed to investigate the role of Insulin like growth factor-I (IGF-I) which is supposed to be critical during the accelerated periods of bone formation and resorption during early skeletal development.
Samples were prepared at UCSF (San Francisco, USA). Mice heterozygous for the IGF-I gene were bred and the females sacrificed at gestation days 14, 16 and 18. Each fetus were genotyped to identify IGF-I deficient (IGF-I -/-) and wildtype (IGF +/+) animals. The lumbar spine and tibia were excised from 3 IGF-I -/- and 3 IGF +/+ animals for each time point. Six groups were constituted corresponding to day 14,16 and 18, and knock-out (KO)or wild type (WT). The samples were fixed in 70% ethanol for storage.

Micro-CT experiments were performed at a spatial resolution of 0.7µm (detector size : 2048x1024). Due to the small size of the samples, the energy was set to 10 Kev. We performed a total of 85 scans, corresponding to approximately 6 samples per groups. Due to the very high radiation dose level, sample deformation could be observed during some scans, thus some of them had to be repeated two to three times. The projections were pre-processed for motion correction using a customized program developed at ESRF and reconstructed.

Typically the final image size varied from 1400x1400x1024 to 2048x2048x1024 (4GBytes). The data were processed by both teams in order to extract quantitative parameters of bone structure and mineralization in the different groups. The day 14 images were removed from the quantitative analysis since visually no bone was apparent on the images at this stage. The day 16-KO images were also removed for the same reason. Regions of Interest (ROI) were manually extracted in the remaining spine and tibia images and quantitative parameters were extracted.

The results have to be discussed and interpreted with the biologists and a publication will be prepared.

4 . Bimodal investigation of bone quality by SR micro-CT and SAM (partners 3,7,2,1)

The goal of the project was to combine evaluation at the same spatial resolution level SR micro-CT and Scanning acoustic microscopy (SAM). SAM in the very high frequency range (0.2-2 GHz) provides images with a spatial resolution (1-10 µm) comparable to that of light microscopy and can map the spatial distribution of acoustic impedance (Z), a parameter related to bone tissue density and elastic stiffness. The combination of the degree of mineralization of bone tissue (DMB) provided by SR micro-CT and Z using SAM leads to the quantitative assessment of tissue intrinsic elastic stiffness.

Three studies described below are in progress.

a) Evaluation of elastic and material properties of trabecular bone induced by adaptive mechanical remodeling *(partner 1,2,3)*

Experiments were carried out on 24 bovine trabecular bone samples provided by the LBTO. The samples were separated in 3 groups of 4 samples each. Specimens of C (control) group were immediately fixed after extraction and embedded in methylmetacrylate, NL (no loaded) and L (loaded) groups were maintained viable in a controlled perfusion culture-loading chamber over three weeks. Specimens of L groups underwent a cyclic compressive strain mimicking human jump, whereas NL samples were left free of loading.
The LTP allowed to acquire SR micro-CT images in the following conditions : voxel size 2.8 µm, energy of 20.5 Kev, 1500 projections. The samples were also explored with 400 MHz SAM (4 µm spatial resolution)

After tomographic reconstruction, a cross section surface in each 3D micro-CT images corresponding to that scanned with SAM was selected using different steps (Figure 3). The gray level values representing the linear attenuation coefficients were converted into a DMB (g/cm3), and the mean DMB of the superficial slice was calculated. Fig 1 illustrates a SAM and DMB slices.

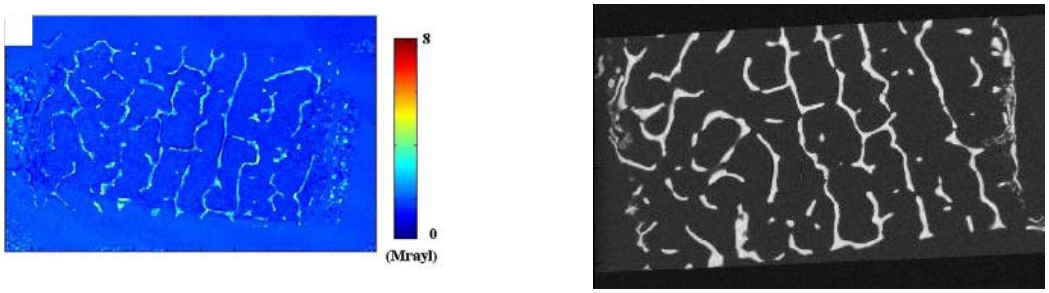

Figure3 : SAM (200 MHz) and SR µCT slice of a sternum trabecular sample(10 mm x 5 mm)

The acoustic impedance Z appeared to respond significantly to mechanical loading, while no differences were observed in the DMB. The data are still being processed.

b) Evaluation of elastic stiffness of cortical bone tissue using high- resolution SAM combined with SR-µCT *(partner 1,2,3)*

This study aimed at mapping elastic stiffness of bone tissue from SAM and SR-µCT data and comparing Young's modulus obtained by SAM (Ea) to local Young's modulus directly evaluated using the nanoindentation technique (En).

SR micro-CT images were acquired on seven cortical bovine bone samples provided by the LBTO. The conditions were the following: voxel size 5.1 µm, energy of 28 Kev, 1500 projections. The samples were also explored with 200 MHz SAM (4 µm spatial resolution)

After reconstruction, 200 MHz acoustic impedance maps and site-matched SR-µCT maps of tissue DMB of bovine cortical bone cross sections were analyzed using a customized image analysis software (partner 3). Preliminary results obtained from one bone sample indicated a strong correlation between Z and En (R^2=0.90) whereas the correlation between Z and DMB

was only moderate (R^2=0.26). The data are being processed and will be complemented by additional data.

c) **Evaluation of elastic stiffness of cortical bone tissue using high- resolution SAM combined with SR-µCT** *(partner 1,2,3,7)*

In vitro Scanning Acoustic Microscopy (SAM) measurements at 50 MHz were performed by the Q-BAM groups on one excised human femur. Then samples were further divided into posterior, medial, anterior and lateral sections. Cylindrically shaped samples with a diameter of 4.4 mm (length 5 mm) and the orientation of the long axis parallel to the radial axis of the femur shaft were drilled in order to be imaged at the ESRF.

The LTP allowed acquiring SR micro-CT images on seven cortical bone samples in the following conditions: voxel size 10 µm, energy of 26 keV, 1400 projections.

Site-matched correlation between DMB extracted from SR micro-CT and elastic coefficients extracted from SAM will be analyzed. It is expected to characterize the anatomical site dependent variations of mineralization, mesostructure and bone matrix elasticity. This study should allow to define relevant parameters and measurement sites for in-vitro measurements, and to develop a multi-parametric predictions model for assessing structural and elastic properties at the organ level.

Publication/ Communications related to the current investigations:

Rupin F, Saïed A, David V, Raum K, Peyrin F., Vico L, Laugier P. High Resolution Acoustic Microscopy: a New Method to Investigate Remodelling Process of Trabecular Bone. 2007 IEEE International Ultrasonics Symposium Proceedings, New York October 28th-31st, 2007 (in press).

Rupin F, Saïed A, Raum K, Vico L, Laugier P. 400 MHz acoustic microscopy of trabecular bone remodeling. World Congress on Ultrasonics (WCU), Vienna, Austria, April 9-11, 2007.

Rupin F, Saïed A, David V, Raum K, Vico L, Laugier P. Etude du remodelage osseux par microscopie acoustique haute résolution. 10ème JAPSUS (Journées Acoustique Physique, Sous-Marine et Ultra-Sonore), PARIS, France, 23-25 Mai 2007.

Rupin F, Saïed A, David V, Raum K, Vico L, Laugier P, Assessment of bone remodeling using high resolution acoustic microscopy. 2nd European Symposium On Ultrasonic Characterization Of Bone, ESUCB 2007, July 19-20, Halle, Germany.

5. Quantification of bone micro-vascularisation in rat model *(partner 1,2)*

The role of bone vascularisation in response to physiological or pathological stimuli that regulate bone remodeling is not fully elucidated. Recent data obtained from in vitro studies suggested an intense cross-talk between bone and vessel cells and that unloading-induced bone loss might be associated with local hypoxia. However the investigation of micro vascularisation by imaging techniques is still challenging. In previous works we showed the unique possibility with SR micro-CT to examine simultaneously the vascular network and the bone phase after injection of contrast product (Fei J., Peyrin F., Malaval L., Vico L., Lafage-Proust M.H., Bone vascularisation Imaging: Comparison of Histology, Conventional and Synchrotron Radiation

Micro Computerised Tomography in the Adult Rat Long Bones, 28th ASBMR, Philadelphia, USA, Sept 2006). However, depending on the quality of the vasculature opacification, the identification of the vascular and trabecular network may be challenging.

In this study we propose to use SR micro-CT to investigate the modifications in the vascular and bone network involved by Intermittent Parathormone (PTH) which is the only osteoporosis treatment able to significantly stimulate osteoblastic formation and increase bone mass.

The samples were prepared by the LBTO. Twenty-four young adult male Wistar rats were divided in two groups : PTH and Control (C). Rats were subcutaneously administered PTH (Preotact, Nycomed, Denmark) at a dose of 100 µg /kg/day and control rats were given a VEH (0.9% sterile saline) 5 days per week for 4 weeks. After the experimental protocol, all rats were double labeled with tetracycline by intraperitoneal injection at 5 and 1 day before sacrifice, respectively. After sacrifice, Barium sulfate was injected into the abdominal aorta to opacify the bone vascular network. The right femur were dissected free from the animal, fixed and embedded in PMMA. Samples were fashioned into 4 mm2 blocks for imaging.

SR experiments were performed in Nov 2007. SR micro-CT images were acquired with a spatial resolution of 2.8 µm. The theoretical FOV should have been 5.6mm, but due to a problem in the location of the scintillator on the optic, the actual FOV was reduced. In order to avoid local tomography, we acquired data in "half acquisition" mode with an off-centered rotation axis. This mode requires a specific handling of the projection data which was not available in the ESRF reconstruction algorithm and caused some delay in the reconstruction. However the software was recently operational and the data are now being processed.

Future work requires the automatic segmentation the vessel network from the bone network and from the background. Quantitative parameters will then be extracted and a statistical analysis will be performed to study the influence of the PTH. Figure4 illustrates a 3D rendering of the vessel network (red) on the bone network (white).

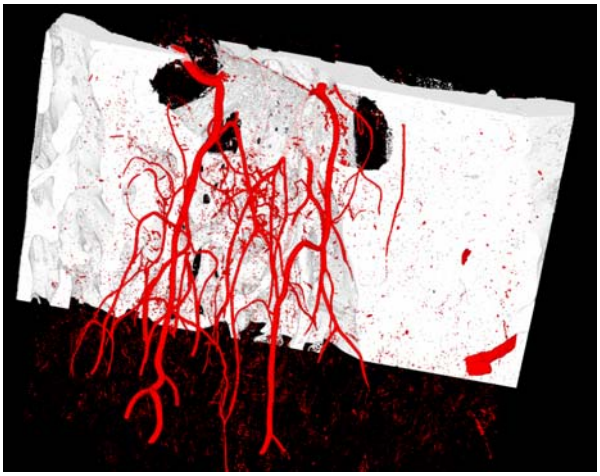

Figure 4 : illustration of the vessel network (red) and the bone network (white) in a vertebral rat sample obtained by SR micro-CT

6. Analysis of bone formation in scaffolds *(partner 1,6)*

The purpose of this study is to evaluate bone formation in different scaffolds elaborated by the Unige/IST, Genova, Italy, (Pr. R. Cancedda). In previous work it was shown that SR Micro-CT

produced bulk 3D information at micro-resolution, and SR microdiffraction provided useful information on interfaces to the atomic scale (Cancedda R., et. al., Biomaterials, 2007, 28(15):2505-2524). However, the pre-bone fibrous structures can not be resolved by neither microCT nor microdiffraction. In this study, we proposed to use holotomography to investigate whether it is adequate to analyze quantitatively this fibrous matrix.

We studied Skelite scaffolds based on silicon-stabilized tricalcium phosphate, which were shown to interact with the body's bone cells and can be replaced by natural living bone (Mastrogiacomo M., et al., Biomaterials. 2007, 28(7):1376-1384). Bone formation was activated by different mechanism, either in vivo (by implantation in immunodeficient mice with Bone Marrow Stromal Cells (BMSC)) or by an in vitro system developed at Unige/IST, Genova. The aim of this in vitro system is to mimic the physiological bone microenvironment by cultivating bone forming cells and bone resorption cells together on porous scaffold.

Data were acquired during the experiments in April and Nov 2007. Different types of scaffolds were analyzed: small Skelite disks (cylinders Φ9 mm x 1.2 mm) and in vivo implanted scaffolds (4x4x4 mm^3). Images were acquired at a spatial resolution of 5µm. Note that holotomography is more demanding in beam-time since several scans of the same sample have to be acquired at different distances. The first experiment allowed acquiring 9 empty disks (absorption CT), one disk with cells (holotomography) and one implanted scaffolds (holotomography). The second experiment allowed acquiring 10 empty disks, one disk with cells and 6 in vivo implanted scaffold. All acquisitions were performed in holotomography.

The reconstruction in holotomography is less straightforward than in absorption tomography since it requires a first phase retrieval step. The first phase retrieval algorithms developed at ESRF (P. Cloetens, R. Barrett, J. Baruchel, J.-P. Guigay, and M. Schlenker, "Phase objects in synchrotron radiation hard x-ray imaging," J. Phys. D.: Appl. Phys. 29, 133–146, 1996) were more adapted to samples with a weak absorption which is not the case for bone scaffolds. New methodological developments done on phase retrieval algorithms recently allowed to extend the method to hard samples (M. Langer, P. Cloetens, J.P. Guigay F. Peyrin, Quantitative comparison of direct phase retrieval algorithms in in-line phase tomography, submitted Med Phys, 2007). The application to bone scaffolds seems very promising since the soft tissue can effectively be resolved with this technique (see Figure 5).

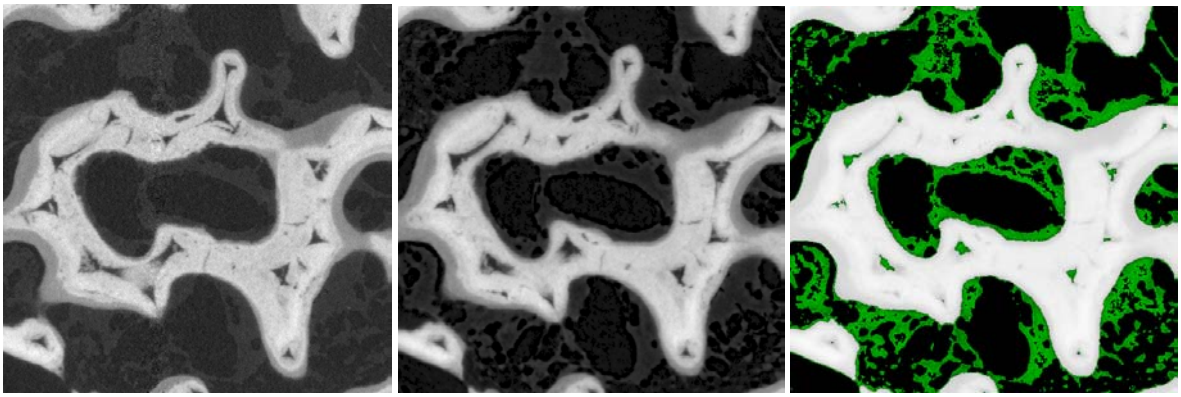

Figure 5 : illustration of the reconstruction of a bone scaffold (after in vivo implantation). from left to right : absorption image, holotomographic reconstruction, segmentation of the soft tissue in green from holotomographic reconstruction.

The next beam-time allocation should allow the imaging of the same samples already acquired in Nov. 2007 after cell culture or in vivo implantation and thus, the quantification of bone growth.

Conclusion

The unique capabilities of SR micro-CT as a quantitative and high resolution imaging technique are exploited to investigate bone quality. The first experiments have already provided valuable data which are being processed. Data processing is an issue due to large volume of data generated for each experiment. In addition, the analysis of some structures which are at the limit of resolution of the image is challenging and requires the development of specific image processing algorithms. The next experiments will allow complementing the existing data and improving some technical points.