

## **ABSTRACTS**

### **Anatomical and functional medical imaging exploration on small laboratory animals.**

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The Animage Mice Imaging Facility proposes to biologists fast and robust methods dedicated to anatomical and functional medical imaging exploration on small laboratory animals (mice, rats and guinea-pigs).

To achieve this aim, we developed a multimodality platform of imaging systems with a 7T  $\mu$ -MRI, a high resolution ultrasound system, an X-ray  $\mu$ -CT and isotopic imaging systems such as autoradiography, beta micro-probe and a soon available  $\mu$ -PET.

We have had for one year the X-ray  $\mu$ -CT SkyScan 1076 In-vivo.

Some developments allowing an optimal conditioning of the animals during acquisitions were carried out : gas anaesthesia, respirator, insulating micro-bubble. This imaging modality allowed us both bone tissue and soft tissue relevant investigations within the framework of specific biological problems on :

- the consequence of a genetic modification,
  - Phenotype osteopetrosis diagnosis on KI mutant mice by 3D bone thickness measurements
  - Follow up of bone reconstruction on mutant mice lacking or over expressing thyroid hormone receptor
- the knowledge of physiopathological mechanisms,
  - Study of a lung injury model in the mouse
  - Study of atherosclerotic plaque calcification at the carotidian level
  - Morphometric study of the lumbar vertebrae

Study of a leaver endocrine tumor model.

**Evaluation of different in vivo models for bone cancer-related pain.**

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For many patients, pain is the first sign of cancer and 30 - 50 % of all cancer patients will experience moderate to severe pain, this number augments to 75 - 95% when patients with metastatic or advanced cancer are considered. Yet it has been reported that 45% of cancer patients have inadequate or under-managed pain control. Therefore, to significantly increase patients' quality of life, there is a need for the development of drugs that effectively treat bone cancer pain. The development and hence a well targeted treatment for bone cancer pain are still largely unknown but osteolysis (bone breakdown) seems to play an important role. Osteoclasts induce an acidic pH and release growth factors during bone resorption, thus stimulating sensory neurons. Other mechanisms involve the release of pronociceptive agents from the tumor itself and from inflammatory cells.

Several animal models of cancer pain were developed to study the basic neurobiological mechanisms of cancer pain.

We evaluated 3 mouse models for bone cancer pain using classical histology and micro-CT technology in order to determine the precise location of the tumor and the degree of bone destruction. In vivo testing, to evaluate the development of hyperalgesia and allodynia in the affected hindlimb, included different nociceptive tests such as cold-plate (thermal nociception), palpation induced lifting behavior (movement-evoked pain), von Frey assay and pin prick (mechanical nociception). Alterations in the pain measurements correlated with the degree of bone destruction although some variation is present between animals. On the other hand subcutaneous tumors did not induce any signs of increased pain sensation.

The increased pain sensation in the bone cancer models could be modulated by morphine treatment.

The used models and the multidisciplinary approach of this study yield new insights on the development of bone cancer pain and allows the evaluation of potential effects of analgesics.

## **In-vivo detection of lung tumors in mice by high resolution X-ray microtomography**

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In-vivo high resolution X-ray microtomography (micro-CT) has become a promising technique for imaging the inner anatomy of small laboratory animals. Visualization by micro-CT is fully non-invasive. Up to now, the major application of micro-CT in biomedical research was imaging bones and other calcified tissues.

A new interesting challenge for X-ray micro-CT is visualization of soft tissues. In small laboratory animals, there is an urgent need for a high spatial resolution, non-invasive visualization of lungs. Up to now, detection of these tumors has required sacrifice of the mice for histological examination and/or sectioning and microscopic observation, which are both destructive and time-consuming procedures.

Therefore, the feasibility of applying high-resolution microtomography (micro-CT) for the detection of lung tumors was investigated in living mice at an early and more advanced stage of tumor development. The chest area of anesthetized mice was scanned by X-ray micro-CT (Skyscan 1076, Aartselaar, Belgium). A healthy aged-matched group of mice was used as a control for two groups of diseased mice. Both in mice with a minor and heavy tumor load, micro-CT proved to be a fast and non-invasive imaging device for the detection of lung tumors. After validation of the CT data by histological sectioning, it was shown that the majority of the tumors could be distinguished in the reconstructed virtual slices obtained by micro-CT. The data from micro-CT were also confirmed by visual inspection of the inflated and excised lungs post mortem.

These results clearly indicate that in-vivo micro-CT opens broad perspectives for imaging tumor development and its progression in a non-invasive way. Micro-CT also allows for longitudinal evaluation of the treatment of lung cancer by drugs.

## Different Methods of Thresholding on Bone Tissue Measurement by Skyscan 1072 Computed Tomography.

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There is a large variability of results on bone morphology and topologic parameters related to the segmentation and thresholding methods.

We described in this work two methods of thresholding (global and local) focused on interface between bone and surrounding media. The first thresholding determination is based on the Tb.Th maximum. The second thresholding determination is based on the analysis of the grey level gradient between bone and the surrounding media.

We acquired on skyscan 1072 one femur of a rat female with following acquisition parameters:

75 kV, 100 $\mu$ A, 5600 ms, Al filter, pixel size: 11.1  $\mu$ m.

On one cross section, with a minimum of grey level (left) fixed, the maximum grey level (right) is positioned with large variation and the threshold criteria is the Tb.Th maximum.

Maximum level of segmentation	0.500	0.437	0.396	0.350	0.300	0.280	0.220
Threshold (Tb.Th max.)	235	232	230	226	220	215	195
Tb.Th ( $\mu$ m)	138.4	138.6	138.7	138.7	138.8	138.6	138.7
BV/TV (%)	13.16	13.2	13.2	13.2	13.2	13.12	13.18

If Tb.Th maximum is used as a criteria for the thresholding, there is no variation of bone parameters depending of the maximum grey level value of the segmentation.

On the whole data set, in addition to Tb.Th another criteria must be used a TbPF>0.

We compared average bone parameters obtained with global threshold adapted for each section and with global threshold selected on one cross section for the whole date set.

Threshold	Adjusted	160	215
Tb.Th ( $\mu$ m)	63.17	59.13	82.22
Variation (%)		-6.4%	+30.2%
TbPF (/mm)	0.26	1.27	-4.82

There is a large discrepancy between bone parameters according to the global threshold used. The global threshold depends on the position of cross section in bone. Thus an adjusted threshold is recommended but time consuming.

For the local thresholding, in theory, the bone trabecular quantity are underestimate if interface gradient is not over the low cut off gradient factor. Only the average gradient are available and not the interface gradient.

	Gradient Low Cut Off	Gradient High Cut Off	Low Cut-Off	High Cut-Off	Average gradient	Tb.Th ( $\mu\text{m}$ )
Skyscan with Smoothing	0.93	0.99	84	118	20	52.3

The modification of gradients cut off factors did not improve the Tb.Th measurements.

Conclusion:

The development of a plug in for a thresholding on a selection of cross section data set is necessary due to bone heterogeneity.

For the second method, a matrix of gradients is probably necessary to improve the local thresholding method to identify the interface gradient instead of the average gradient.

## **Steroid-induced changes in distal femur cortical bone of the BalbC mouse**

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The BalbC mouse is used at GSK in a reproducible mechanistic model of atopic asthma. To model the side effects of asthma treatment with steroid compounds on bone we have used micro-CT, dynamic and static histomorphometry of the distal femur of this mouse strain. In addition to an initial study indicating steroid-induced trabecular bone thinning (McLaughlin et al., 2002), we have found in subsequent work that cortical bone changes in this region of the femur provide a more reproducible measurement of steroid-induced bone thinning, which correlates with reduced bone formation rate. There is also a good correlation between the cortical thickness measured by micro-CT, and measurements made by histomorphometry or pQCT analysis. Results of our latest studies will be presented, and the possible relationships with bone strength will be discussed.

### Reference

F McLaughlin, J Mackintosh, B.P.Hayes, A McLaren, I.J. Uings, P Salmon, J Humphreys, S.N.Farrow (2002) Glucocorticoid induced osteopenia in the mouse as assessed by histomorphometry, micro CT and biochemical markers. *Bone* **30**: 924-930.

**Bone loss is accompanied by an increase in trabecular thickness in aging, ovx and tibolone treated rats: an in-vivo micro-CT study.**

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In the present study, we have followed the changes in trabecular bone structure in the tibiae of living aged female rats due to aging (sham), ovariectomy (OVX) and OVX treated with an anti-osteoporosis drug (tibolone: N.V. Organon). The three groups consisted of 5 rats each. The tibia of each rat was scanned at three time points, using the Skyscan 1076 in-vivo micro-CT system. The rats were scanned at week 0, prior to surgery, and at week 4 and week 14 post-operation. All resulting data sets were analyzed using software from the 3D-Calc project. Further, from each group a typical animal was selected of which the data sets for the different time points were overlaid using image registration software.

All of the OVX animals showed a big loss of trabecular bone (53% after 14 weeks) and a big reduction in connectivity of the trabecular network. Interesting is that beside loss of bone, also bone apposition can be seen: in each animal, the remaining trabeculae slowly increased in thickness. Image registration even showed the creation of new trabecular structures.

The sham group also exhibited a (much smaller) loss of trabecular bone, up to 16% loss after 14 weeks. Again, in each animal the loss of trabecular bone is accompanied by an increase in trabecular thickness.

In the tibolone group, the ovx related bone loss was reduced to 38% at week 14. And again, bone loss was accompanied by an increase in trabecular thickness.

All groups exhibited similar structural changes, although they differed strongly in size. Even in the sham group bone loss and decrease in connectivity followed the same pattern as in the OVX group, and followed the known relation between bone volume fraction and connectivity, even though the reason for the bone loss is quite different.

Although a similar, but weaker, relation is known between volume fraction and trabecular thickness (thicker trabeculae correlate to high volume fractions), our data show contradictory relations within individual animals. After bone loss, compensatory mechanisms might try to maintain bone strength by increasing trabecular thickness.

Using in-vivo longitudinal methods to analyze structural bone changes within individual rats strongly increase the statistical power of the analysis. The inter-individual variation between groups at a certain time-point could largely be explained by variation in base-line measurements. By combining in-vivo micro-CT with image registration techniques, structural changes within a bone can be followed in time, even at the level of single trabeculae.

**Use of MicroCT and Finite Element Models to Determine the Matrix Modulus of Various Species**

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The mechanical properties of trabecular bone are affected by the bone mass, trabecular architecture and matrix properties (ie. bone quality). Conventional mechanical compressive testing of core samples can only measure the conglomerate effect of these three factors. By combining microCT scanning with finite element models resolved at the scale of individual trabeculae the effects of bone mass and trabecular architecture can be separated from those of matrix properties. In this ongoing study we are using this combined CT/FE method to measure the mechanical properties of bone from 3 species: cows, pigs and ostriches. Architectural stiffness and matrix modulus are being investigated independantly. These results will be compared to matrix mineralization measures made using backscatter scanning electron microscopy. Challenges faced during scanning will be addressed including scanner stability and choice of proper thresholds.

## **Analysis of cross-sections in biomedical applications**

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### **1. Separation software for binary images.**

Software that works with binary images was developed to assist in separation of necessary parts of the cross-sections that have no absorption difference with the unwanted details. This software was tested on mixture of bones when one bone was separated and the rest were deleted. It was also applied to extract air channels in the inner ear of a mouse (void space). In both cases the process of separation was automated and the speed of data processing was increased dramatically.

### **2. MicroCT and histology.**

To validate the use of micro-CT for biomedical applications a comparison with histology was made for bone material. Good correlation was found although some of artifacts were detected. In both histological slices and micro-CT slices some trabeculae did not coincide. Also a skew caused by the knife during histology was detected.

### **3. Contrast agents for soft tissues**

In order to expand micro-CT capabilities into soft tissues investigation contrast media were applied to joints, pancreas, noses of embryos, mature animals and human material. Hg chloride, paraffin, vegetable oil, resin, immersion oil, oil for ultracentrifuge, U acetate and Os tetroxide were tested. Considerable improvement in contrast was detected.

## **Calibration of thickness and BMD measurement in micro-CT imaging of bone**

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### **A. Thickness**

In micro-CT measurements, surface finding or segmentation is frequently performed using a global grey level threshold. However, as Ito et al. (1998) pointed out, any structure thickness can be obtained by changing the threshold value. It is therefore necessary to calibrate thickness measurement.

An approach to solving the problem of the dependence of measured thickness on selected global threshold is presented. This consists of:

1. Not using global grey level thresholding. Instead adaptive local thresholding (Canny 1986) is employed which also corrects the thickness biasing and under-reporting of thin structures associated with global grey-level thresholding.
2. Aluminium foils are measured to calibrate the adaptive local thresholding.

Aluminium provides a suitable material for calibration of micro-CT measurement of bone structure thickness. Al has a similar x-ray opacity to cortical bone, and is also materially uniform on a micron scale, unlike hydroxyapatite preparations at densities similar to cortical bone, which is important for precise calibration of thicknesses of microns to tens of microns.

Calibration with 20 and 250 micron thick Al foils showed that both thicknesses could be measured accurately simultaneously. Thickness of aluminium and bone was demonstrated to be measurable by micro-CT where structures are at or above about 3 pixels in width. The thickness calibration with 20  $\mu\text{m}$  thick Al foil was found to be stable over the range of magnifications of  $\times 40$  and higher, or pixel sizes 6.8 microns and lower.

### **B. BMD**

The method for bone BMD calibration reported here is a first approach to the problem which after testing and input from users, can be refined and improved.

The calibration of BMD measurement in bone micro-CT scanning will refer here to two measurement scenarios: (a) measuring BMD in a medullary region containing bone trabeculae and marrow (wet or dry) and (b) measuring BMD in cortical bone.

The problem: Object size and geometry and the distribution of x-ray densities in an object will affect the reported CT density within an object. The two most important reasons for this are:

1. Beam hardening (where the x-ray beam is polychromatic), that is, the more rapid removal of soft x-rays than hard x-rays, changing the energy spectrum of the beam passing through the object,
2. Sigmoid surface density gradients causing under-reporting of density of thin objects.

The solution: To create measurement phantoms that closely reproduce the geometry and density distribution of your study objects, so that both the above factors 1 and 2 influencing reported CT density are controlled in the measurement.

Note that an Al filter (1mm) should be used for bone measurements to narrow the x-ray energy spectrum, reducing the low energy component, thus reducing beam hardening.

Consider the example of mouse bone measurement. Scientists employing the mouse (or rat) model most frequently examine certain key bone sites, namely the hindlimb long bone ends at the knee (distal femur and proximal tibia) and the lumbar vertebra. These sites can be approximated as having a trabecular-plus-marrow medullary region about 2 mm in diameter and a surrounding cortical wall of 100-150 micron thick cortical bone. The mouse bone phantom (set of phantoms) consists of a cylinder of 2mm diameter, 2 cm length, composed of a mix of epoxy resin and hydroxyapatite (HA) with a small grain size optimised for micro-CT, produced by CIRS, Almeda, USA, with HA concentration from 0 (soft tissue equivalent) to 250 mg.cm<sup>-3</sup>. Surrounding the epoxy-HA rod is a layer of aluminium foil 100 micron thick, covering one half only (1cm length) of the 2cm long epoxy-HA cylinder. The surrounding aluminium layer simulates the beam hardening effect of the cortical wall surrounding the mouse bone sites.

When measuring the epoxy-HA cylinder one should exclude the 20-30 µm layer adjacent to the Al shell. All scan and reconstruction parameters should be the same as used for calibrated experimental mouse bone samples. To measure medullary BMD, the Al-surrounded part of the phantoms is measured using four HA concentrations: 0, 50, 150, 250 mg.cm<sup>-3</sup> of Ca-HA. A calibration curve is created by obtaining the grey level distribution and mean grey value from each phantom, and this can be applied to trabecular-marrow medullary volumes imaged in the mouse bones (excluding 20-30 µm adjacent to cortical bone).

Inspection of scans of the epoxy-HA phantoms shows clumping on HA in dark spots. However the clumped HA accounts for only a small part of the density distribution of the phantom which is predominantly normal.

For cortical bone, one should scan and measure the part of the four phantoms uncovered by aluminium, excluding the 10-20 µm surface layer. The calibration curve is applied to cortical bone by extrapolation upward to the measured cortical bone density. Again when selecting cortical bone in the experimental mouse bone samples, the surface layer of 10-15 µm of bone should be excluded.

## **MicroCT evaluation of bone biopsies: repeatability test and comparison between four different acquisition conditions**

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Treatments for microCT examination of bone specimens usually found in literature are the inclusion in PMMA (Engelke et al. 1993, Kuhn et al. 1990) or the bone put in alcohol (Rüegsegger et. al. 1996). The inclusion of the bone biopsies in PMMA is a necessary step in histology. Because the MicroCT does not change the structure of the examined bone specimen, the inclusion in PMMA is not strictly necessary. The embedding process is time consuming and it worsens the signal to noise ratio. It is not clear which effect the inclusion has on the morphological parameters compared to the results obtained with the bone put in air. A comparison is required, to see if there are differences in the morphological calculations obtained in the two cases. A second important point are the necessary X-ray acquisition images, expressed in terms of rotation step. Rüegsegger reports for the MicroCT acquisition a rotation step of 0.36 degrees over 216 degrees, while the constructor (Skyscan) of the MicroCT used in LTM gives as default rotation step 0.90 degrees over 185 degrees full rotation. Looking at the reconstructed 2D images, specially for bone embedded in PMMA, noise in the images is noticed. Taking half the rotation step (0.45 degrees) this effect tends to disappear. We tried to investigate the question, if the 0.45 degrees rotation step produces significantly different morphological measurement results than the 0.90 step, especially for the embedded bone. A smaller step has the drawback of more files to handle, bigger disk space usage and slower image reconstruction.

In this work we did comparisons of MicroCT acquisitions-reconstructions between four different acquisition conditions: First, bone in air scanned at 0.45 degrees rotation step; second, bone in air scanned with 0.90 degrees rotation step; third, bone embedded in PMMA scanned at 0.45 degrees; fourth, bone embedded in PMMA scanned at 0.90 degrees rotation step. Before doing this, a repeatability test for MicroCT analysis has been performed on three representative specimens of different bone fraction (8%, 16%, 25%).

The total of 17 human bone samples of different bone fraction (5%...33%) have been examined. The samples are biopsies taken from the absorted femoral neck of different patients submitted to total hip arthroplasty. After three day formalin-fixation they were cut to a suitable size to fit a 18mm diameter glass cylinder. The specimens had variable size and shape, in the order of 15mm in diameter and 6mm height. After acquisition and reconstruction (cone-beam algorithm), the images have been thresholded using a "local threshold algorithm". The morphological parameters extracted for the comparisons were: Bone Volume fraction (BV/TV), Trabecular Number (Tb.N.), Trabecular Thickness (Tb.Th.), Trabecular Spacing (Tb.Sp.) using the plate model (Parfitt 1987). Also the model independent mean thickness (TbTh\_vol, Hildebrand 1995) and the Structure Model Index (SMI, Rüegsegger et. al. 1997) have been calculated.

The repeatability test over the three specimens showed that the whole microCT examination procedure has a variation coefficient smaller than 3% for all the parameters taken into account. Most of the morphological parameters for the bone in air had a smaller variation coefficient (e.g. for BV/TV it is about 1%) than those for the embedded ones (e.g. for BV/TV it is 1.67%). If the difference in some parameter has to be found in an experiment, the respective variation coefficient has to be taken in account.

Comparison between the four acquisition conditions: The visual comparison showed that bone samples put in air had the trabeculae better distinguishable from the rest, than those put in PMMA. In the case of the embedded bone specimen it is nearly impossible to separate the marrow from the PMMA. The 0.45 degrees acquisitions in air gave the sharpest images as expected, whereas the 0.90 acquisitions with the bone included in PMMA in some slices present thin stripes across the whole image, specially for the samples with big bone fraction.

Linear regression and the paired t-test have been used for statistics. The paired t-test indicated the differences found in most of the comparisons as statistically significant ( $p < 0.05$ ). It has to be outlined, that the single differences found in the comparisons are very close to the variation coefficients of the measures obtained in the repeatability test. Therefore care has to be taken in saying that there is an evidence of overestimation or underestimation of the observed parameters, based on the statistical tests that have been made. If comparisons between included bone or bone in air have to be done, the linear regression should be used to recalculate the value for the examined case. The same way can be argued for comparisons between the two rotation steps.

Bone specimen dataset on the internet: A full dataset of a human bone specimen acquisition and reconstruction obtained at the LTM is available at the BEL repository, integrated in the digital library supported by the Living Human Project. It is freely downloadable and could be helpful for microCT users to make comparisons with the acquisitions-reconstructions achieved from other laboratories and with different microCT scanners.

The internet address of the dataset is:

[http://www.tecno.ior.it/VRLAB/researchers/repository/BEL\\_repository.html#MicroCTscans](http://www.tecno.ior.it/VRLAB/researchers/repository/BEL_repository.html#MicroCTscans)

## **Influence of experimental conditions and image processes on microarchitecture study by micro QCT.**

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Trabecular bone microarchitecture study by X-ray micro-computed tomography ( $\mu$ CT) of small animals and especially rats and mice is of the utmost importance to understand and modelize the disease or drug effects on trabecular bone. The aim of this study is to quantify the influence of experimental conditions and image processing in order to optimise the rat trabecular bone microarchitecture analysis. The influences of the rotation step and the frame averaging on the image acquisition were analyzed. Then the image process effects including the image segmentation from 12 to 8 bits, the threshold and the Region Of Interest (ROI) location were quantified.

An ex-vivo rat femur containing cartilage, cortical bone and trabecular bone was imaged by the  $\mu$ CT Skyscan 1072. We compared two experimental conditions of radiographic image acquisition. The X-ray tube parameters were the same (tension of 75kV, intensity of 100 $\mu$ A) and an aluminum filter was used. The first acquisition was performed using the standard parameter set for trabecular bone study : 200 radiographic images were acquired over an angular range of 180° (angular step: 0.9°) with a frame averaging of 2. For the second acquisition 400 radiographic images were acquired over an angular range of 180° (angular step: 0.45°) with a frame averaging of 4.

The rotation step and the frame averaging influenced the grey level histogram used for the segmentation from 12 to 8 bits. We obtained a better segmentation histogram with the angular step of 0.45° and a frame of 4, so we work with those acquisition parameters. A cursor shifting of one increment for the segmentation values induced the following changes for the microarchitectural parameters : 3.66% for BV/TV, 3.11% for TbN, 1.79% for TbTh, 9.1% for TbSp.

Shifting of the VOI of 4 slices for the location of the cartilage-trabecular limit induced changes of : 1.38% for BV/TV, 1.88% for TbSp, 0.35% for TbTh, 1% for TbN.

Changes of two grey levels values for the threshold induced : 2.17% for BV/TV, 1.63% for TbN, 3.9% for TbSp, 0.35% for TbTh.

In case of rat femur analysis, the optimum segmentation and threshold values varied between two rat femurs and moreover from one to another section of a same rat femur. The variations within the same femur are due to the variation of bone diameter, cortical thickness and trabecular bone quantity from the diaphysis to the epiphysis.

In order to define the best compromise for each femur, histograms of the metaphysis and one from the diaphysis were used for the choice of segmentation and threshold.

In conclusion, the improvement of the acquisition process (versus threshold conditions) can lead to obtain a better characterization of trabecular bone, but represents a time consuming process.