In this application note, we detail how the planar X-ray image and the Bruker Molecular Imaging Bone Density Software module can be used to quantify changes in bone parameters in genetically modified animal models. The software module enables the measurement of linear bone parameters, e.g. length and width, as well as bone mineral and bone surface density. Bone density is an important biomarker in health sciences to discriminate healthy bones from diseased ones and often used in \textit{in vivo} microCT analysis. Thus, it is widely applied in monitoring diseases such as osteoporosis, arthritis and other musculoskeletal disorders. With the availability of genetically modified animals, the impact of genes of interest on skeletal development can be monitored relatively easily and economically with systems such as the Bruker In-Vivo FX PRO.

**Introduction**

In the past few decades, advances in technology and research have enabled scientists to gain increasing insight into fields such as genetics, pharmacology, and pathophysiology, with a main focus toward disease prevention, treatment and management. Many studies address issues of human disease and disorders, by employing the use of animal models. However, as the demand for genetically modified animals in the field of research increases, so does the need to study pathological effects over time. Technology-adapted and non-invasive testing equipment, such as the Bruker In-Vivo FX PRO or its successor the In-Vivo Xtreme II imaging system has been developed for this purpose.

The Bruker In-Vivo FX PRO imaging system is a multimodal optical/X-ray imaging device often used for imaging bioluminescent, fluorescent and Cherenkov as well as non-Cherenkov signals. The FX PRO is also equipped with an X-ray unit that encompasses most functions of an X-ray machine used for human imaging. In order to produce images of a similar quality as that produced by a standard (i.e. for human) X-ray machine, the resolution of the FX PRO has been amplified and the X-ray energy lowered significantly, to accommodate the size and weight of small animals (Vizard et al. 2010a).
Moreover, the X-ray unit uses a phosphor screen to convert the ionizing X-ray energy, ranging from 10 to 35 (45*) kVp (energy levels recommended for small animals), into emitted light, which is then captured by a camera, generating a digital image. The resulting digital image contains an array of pixels with different brightness, depending on the attenuation coefficient of the tissue captured in the image. For more technical specifications regarding the FX PRO please refer to the United States Patent documentation No.: US 7,706,501 B2 (Vizard et al. 2010a).

In contrast to micro CT equipment like the Bruker SkyScan 1276 μCT, which enable the very precise volumetric measurement of bone density parameters, the Bruker In-Vivo FX PRO does the same but in 2D, and with a focus on longitudinal bones. Here, the Bruker Molecular Imaging Bone Density Software module enables estimation of bone density parameters by using an algorithm, which creates a non-linear least squared fit of transverse density profiles from a “region of interest” (ROI) selected on the X-ray image (Fig. 1a) on an area of a long bone which is geometrically, approximately cylindrical in shape.

The transverse density profiles are calculated based on a somewhat similar principle to the DEXA scanner (Kelly et al. 1998; Lochmuller et al. 2001), where pixel brightness is dependent on the X-ray attenuation coefficient of the material (in this case, bone, marrow and surrounding soft tissue); the denser the tissue/material, the brighter the pixels in the area of interest (Vizard et al. 2010b) (Fig. 1b).

The Bruker Bone Density Software was designed specifically for in vivo X-ray analysis, and therefore contains an array of calibrated tissue attenuation coefficients between which it can discriminate. For example, the calibration for the bone tissue X-ray attenuation coefficient, has been computed based on the X-ray attenuation coefficient of aluminium, which has the same X-ray attenuation coefficient as the calcium phosphate and hydroxyapatite present in bone (Fig. 2) (Vizard et al. 2010b). The surrounding tissue is estimated according to the water attenuation coefficient, based on the fact that rodent bodies consist of 75 % water (Vizard et al. 2010b).

After the pixel profile has been established for every tissue, a mathematical model is used to calculate the density parameters of the bone within the ROI. Although, the general methodology of calculus is significantly different between the Bruker Molecular Imaging Bone Density Software module, QCT, and DEXA, the resulting density estimates lie within reasonable agreement (Vizard et al. 2010b).

For more detailed information about the quantitative bone analysis that can be performed with the Bruker Molecular Imaging Bone Density Software module, the reader is referred to the white paper titled “Analytical radiography for planar radiographic images implemented with a multi-modal system” (Vizard et al. 2010b).

In this application note, we present the methodology and results of a study that used the Bruker In-Vivo FX PRO and

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*newer systems like the Bruker In-Vivo Xtreme II are equipped with ionising X-ray energy up to 45 kVp
Bone Density Software module to characterise the skeletal phenotype of the genetic metabotropic glutamate receptor 1 (Grm1) knockout mouse model (Musante et al. 2016).

The studied subjects were BALB/c/Pas strain mice, with the cerevelet 4 – (crv4) mutation, which disrupts the genetic splicing process of the metabotropic glutamate receptor 1 coding gene Grm1, resulting in a mouse model (Grm1^{crv4/crv4}) with a constitutive deficiency of this gene (Conti et al. 2006). Gross analysis of the Grm1^{crv4/crv4} mouse model indicates profound phenotypic changes compared to the healthy wild-type mice, including ataxia, intention tremor, reduced weight and size, skeletal anomalies, motor control deficits (Conti et al. 2006; Rossi et al. 2013), reduced bone size and bone mass (Musante et al. 2016), and renal failure (Puliti et al. 2011). These characteristics are also specific for the SpinoCerebellar Ataxia, autosomal Recessive 13 (SCAR13) disorder in humans, thus the Grm1^{crv4/crv4} mouse model is considered an appropriate model to study the equivalent human disease.

Glutamate receptors play a major role in the process of bone formation (Brakspear and Mason 2012; Chenu et al. 1998; Cowan et al. 2012; Hinoi et al. 2007; Itzstein et al. 2001; Mason et al. 1997; Takarada and Yoneda 2008) and SCAR13 patients have been reported to suffer from impaired skeletal growth (Davarniya et al. 2015; Guergueltcheva et al. 2012). Analyses of the bones from Grm1^{crv4/crv4} knockout mice using the Bruker In-Vivo FX PRO and Molecular Imaging Bone Density module revealed significant growth retardation and bone mineralisation defects in the homozygous Grm1^{crv4/crv4} knockout animals which were not present in heterozygous mice (Musante et al. 2016).

Methods:

The right femurs of a total of 10 adult female mice (5 Grm1^{crv4/crv4} and 5 wild-type), aged between 12-24 weeks were used for analysis. Bone length and bone mineral density measurements were performed on femur bones, as their size, and somewhat uniform shape of the diaphysis facilitates an accurate selection for region of interest (ROI) analyses.

For the purpose of bone mineral and bone surface density measurements, only the bones of the older, 24 weeks, females were analysed, as at 12 weeks of age, demineralisation effects from gene ablation were not apparent.

The bones were measured ex vivo, as the tissue was later used for histological analysis. The hind limbs of the mice were removed, still attached to the pelvis, to prevent damage to the femoral heads, and fixed in formaldehyde for >1 week. After being detached from the pelvis, and stripped of skin and muscle, the bones were stored immersed in PBS. Each bone was X-rayed once. In each case, the bone was placed on a petri dish on its lateral side, with the femoral head facing upwards, positioned on the magnification stage of the Bruker In-Vivo FX PRO.

Images were acquired using the X-ray source energy at 35 kVp - with an X-ray calibrated energy of ~13 keV. Binning was set at 2 X & 2 Y, with a 0.4 mm aluminium filter, f-stop set to 2.8 and exposure times of at least 10 seconds, which provided sufficient grey scale values for acquiring an image quality suitable for bone mineral density measurements (Vizard et al. 2010b).

Images were processed using the Bruker Molecular Imaging software version MI 5.3.2 with the Bruker Bone Density Software Module. In order to ensure consistency between measurements, images were analysed, using the Image window in the navigation panel, so that all the femurs were perpendicular to the top of the window, with the femoral head pointing to the left (see Fig. 4).

For femoral length analysis, a line tool in the manual ROI window of the navigation panel was used and placed at each end of the femur (Fig. 3). The line ROI was set in a straight, fully horizontal manner, and ranged from the most external pixel of the condyles to the most external pixel of the femoral head. The images were analysed using the “Analysis” tool and perimeter length was set to mm.
Further, for bone density analysis, the rectangular ROI tool in the “Manual ROI” window was used and placed in the middle of the diaphysis (Fig. 4). Care was taken to place the ROI in approximately the same position on each analysed bone. The ROI was aligned with the bone and a sufficient amount of background area was included in the ROI. While the bone density model used assumes that bone regions are surrounded by soft tissue, we obtained meaningful results for ex vivo analysis using a ROI that included a relatively thin (2-3 pixels) region of flanking background (Fig. 4). The model fit was carefully maintained by using an ROI with a Chi-squared between 8 and 20. (Note: Reliable measurements must have a Chi-squared of under 50, as this parameter represents the value of the quality measure for the cylindrical fit). For further guidance regarding software features please refer to the Bruker Bone Density Software Module Quick Start Guide (Bruker BioSpin, MA, USA).

The resulting measurements were stored in an Excel file and processed for statistical analysis in Matlab. Two-sample T-tests and boxplots were generated to assess statistically significant differences between the measured bone parameters of the wild-type and the \textit{Grm1}^{crv4/crv4} mice.

Results & Discussion:

The X-ray module of the Bruker In-Vivo Imaging System in combination with the Molecular Imaging Bone Density software, allowed us to observe highly significant differences in bone density and femoral length between the two mouse genotypes (Fig. 5). Femoral length in wild-type animals ranged from 15.01 to 15.56 mm with an average of 15.32 mm, whereas femoral measurements in sex- and age-matched \textit{Grm1}^{crv4/crv4} mice ranged from 13.54 to 14.85 mm, with an average of 14.08 mm. The two sample t-test showed that these differences were statistically significant with a p-value < 0.01 (see Fig. 5a).

These findings coincide with other evidence from the original study, where histological analysis confirmed a premature fusion of the epiphyseal growth plate in the Grm1crv4/crv4 mice (Musante et al. 2016).

The bone mineral density estimates identified ranges between 2.17 and 2.54 g/cm³ (average: 2.39 g/cm³) for wild-type mice, and between 1.84 and 2.04 g/cm³ (average: 1.97 g/cm³) for \textit{Grm1}^{crv4/crv4} mice (see Fig. 5b). Similarly to femur...
length, a two sample t-test showed statistically significant differences between bone mineral density estimates of the 24 weeks old mice from the two genotypes, with a p-value < 0.01.

Furthermore, the bone surface density estimates, ranged between 0.15 and 0.17 g/cm² (average: 1.6 g/cm²) for wild-type mice, and between 0.11 and 0.14 g/cm² (average: 0.13 g/cm²) for Grm1<sup>crv4/crv4</sup> mice (see Fig. 5c). In a similar manner to the previous two bone parameters, the two sample t-test showed statistically significant differences between bone surface density estimates of the 24 weeks old mice from the two genotypes, with a p-value < 0.01.

Further, prior cell culture experiments performed on the two mouse genotypes revealed an impaired osteoblast (bone forming cells) function in cells cultured from the Grm1<sup>crv4/crv4</sup> mutants, and altogether a different maturation process and distribution of the bone forming cells in mutant mice compared to wild type (Musante et al. 2016).

These latter findings concur with the reduced bone density and femoral length observed in the Grm1<sup>crv4/crv4</sup> mice, demonstrating that the Bruker In-Vivo FX PRO and Molecular Imaging Bone Density module, is an effective technology for identifying differences in bone parameters, between mouse populations.

The bone density model employed here assumes in vivo bone measurements with adjoining soft tissue (i.e. water) densities. We obtained reproducible bone density results imaging ex vivo samples in air. While we do not have a complete explanation for the successful application of this method at the moment, it may be based on the relatively narrow region of flanking background included in our ROI analysis which supplies slightly higher backgrounds counts than more distal flanking background. Moreover, the accuracy of the ex vivo results appear to be maintained, as long as care is being taken that the Chi-squared weighed averages include all measured profiles (if not, the experimenter should re-select ROI and re-compute) and that the Chi squared value remains below 50. Others have replicated background tissue counts for imaging/analysis by submerging ex vivo bone samples in PBS (unpublished). Further studies will be needed to define the reproducibility of using the existing algorithm for ex vivo bone studies.

Beyond its previously established use for in vivo bone density analyses, the Bruker In-Vivo FX PRO X-ray scanner and its bone density software package may also appropriate for ex vivo sample sets. Such analyses are often of value as part of an end-of-study analysis, and are of value in providing definitive proof of prior time point observations, based on in vivo data sets. Furthermore, the use of the X-ray machine can extend to analysis of other tissue and organs, for example for the analysis of ectopic and/or impaired calcifications.

Figure 5: Boxplots representing statistically significant differences between bone measurements acquired using the Bruker Molecular Imaging Bone Density module for wild-type and Grm1<sup>crv4/crv4</sup> female mice. A. Differences in femoral length between the two genotypes, where N = 5 per genotype; B. Differences in bone mineral density (BMD) between the two genotypes, where N = 3 per genotype; C. Differences in bone surface density (BSD) between the two genotypes, where N = 3 per genotype. Where genotype 1 represents wild-type and genotype 2 represents Grm1<sup>crv4/crv4</sup>; ** P<0.01 by Student t test.
References