Master Thesis

Development of a framework to perform longitudinal micro-computed tomography monitoring on *ex vivo* embryonic chick femur cultures

Supervision

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Abstract

In the field of bone tissue engineering and regenerative medicine, *ex vivo* embryonic chick femur culture models have demonstrated their experimental advantages. The work reported in this thesis was dedicated to the development of a framework through a bioreactor approach in combination with the technology of micro-computed tomography (μCT) to conduct longitudinal studies on *ex vivo* embryonic chick femur cultures. The bioreactor chamber was designed to allow isolated embryonic chick femurs to be positioned at an air/liquid interface under a basic organotypic culture condition for μCT monitoring. By time-lapsed monitoring of the bone tissue, μCT evaluation provided the information on temporal and spatial changes of the chick femur model in a three-dimensional scheme. Different experimental groups were examined with respect to the frequency of μCT scans and the lengths of the period under organotypic culture. It was found that, after 10 days of culture, the femurs scanned every other day exhibited similar bone growth and bone dynamic morphometry as the femurs scanned only at the beginning and the end of culture. This demonstrated the multiple experimental manipulations and irradiation did not have a significant influence on the quantified bone parameters. However, considering transient bone biology, the registration method of superimposing two measurements from the interval of two days showed much more dynamic bone activity than the ones of the same femur registered from the corresponding two time points at the beginning and the end of the culture period of 10 days. Furthermore, for the femurs cultured for a longer period of 4 weeks, more bone formation and less bone resorption was shown during the first two weeks than during the second two weeks. The results indicated the importance of choosing the suitable length of culture periods and scan time points in order to study dynamic bone activity. It is therefore recommended that, in the future, a μCT monitoring scheme should be taken into consideration in a longitudinal study. In conclusion, by taking the bioreactor approach, a feasible model for longitudinal studies on *ex vivo* embryonic chick femur cultures could be successfully established.

Chapter 1 INTRODUCTION

The aim of this study was to develop a framework for *ex vivo* embryonic chick femur cultures. By taking advantage of using chick embryos to perform research, its ease of experimental manipulation, economical accessibility and rapid bone tissue development could be achieved. With a bioreactor approach, a systematic arrangement for an *ex vivo* culture framework combined with micro-computed tomography (μCT) imaging was expected to be a viable option for studying the bone development of chick femurs in a longitudinal manner. With this combination, further versatile experimental setups could be expected in the field of bone tissue engineering and regenerative medicine, to reduce animal models for *in vivo* studies, thereby lessening ethical concerns.

1.1 Chick embryo as a model system

A good model plays an important role in all areas of life. We set different types of models for investigating and discovering unknown spheres, passing our treasures of wisdom and discovery on to later generations. Approximately 2500 years ago, a written empirical setup for acquiring medical knowledge on embryology was found in the "Hippocratic Corpus" [1, 2]. By using chick embryos as a model comparable to human beings, this approach was at hand for studying biology and medicine. Until today, the work on chick embryos has resulted in many achievements in developmental biology [3, 4]. In bone research, it is helpful that the skeletal arrangement and bone cellular activities of a chick embryo conform to vertebrate, but with faster developing processes [5].

Although *in vivo* model systems have benefitted the progress of scientific research and modern medicine, especially for regenerative technologies, they are expensive, require complex systems and often give rise to ethical concerns. These drawbacks have led to the development of *ex vivo* model systems. A number of *ex vivo* model systems have been used in skeletal research; mouse, sheep, cow and rat have commonly been introduced [6-9]. In comparison to these animal models, the chick embryo is a useful model and has advantages in economical accessibility, ease of experimental manipulation and rapid development [10].

1.2 Ex vivo embryonic chick limb culture

Going back to the 1920s, the earliest experimental studies performed with chick embryonic limb-buds cultured *in vivo* and *in vitro* were described by Strangeways and Fell in 1926 [11]. These authors

were the first to describe the differentiation of embryonic tissue to skeletal tissue in culture. Not long after, by the observation of limb cartilage from 8-day old chick embryos cultivated in vitro under the microscope, Fell discovered the hard deposit of ingrowing connective tissue that was proven to be bone [12]. These simple experiments confirmed skeletal tissue development and growth outside the body in an artificial environment. A more sophisticated culture vessel was introduced in 1929, which allowed the culture of a chick femur in a watch glass enclosed in a Petri dish and carpeted with wet cotton wool to provide a moist environment. The anatomical development and metabolism of chick femur in culture were observed and recorded [13]. This led to the characterization of a chick bone culture system and promoted the studies on bone and cartilage formation [10]. In 1990, Roach developed a culture model by introducing millipore filters on stainless steel meshes at the interface between medium and air [14].

"Organotypic culture" is a term first introduced by Crain in 1966 [15] to describe neural tissues cultured outside the organism that differentiate in a surprisingly normal way. In organotypic cultures, the basic structure and function of the organ develop in an organized way. Thus, the specialized and characteristic properties of functioning tissues in the organ can be maintained [15].

An organotypic culture setup was introduced by Kanczler *et al.* [16]. A novel model for understanding the structural development of embryonic bone was established using Roach's approach, as described earlier [14] but without using the classical metal mesh. Embryonic chick femurs were dissected from 9, 10, 11, 12, 13, 15, or 17-day-old chick embryos (E9, E10, E11, E12, E13, E15, E17) and divided into two experimental groups: (i) noncultured (micro-computed tomography (μCT) scanned and histologically analyzed directly after dissecting) and (ii) organotypically cultured (μCT scanned and histologically analyzed after culturing for 10 days) with three different culture media: basal, chondrogenic and osteogenic tissue culture medium. The chick femurs were positioned on 0.4 μm filter well inserts, which were placed in well plates. These plastic well inserts provided a semi-porous membrane to create an air/liquid interface for culturing, giving the possible modulation on culture medium for tunable environmental stimuli. It has been discovered that the air/liquid interface is related to the increase of oxygen tension within the tissue, stimulating the viability for bone dynamic activities [6].

1.3 Bioreactor system

Although there is no exact definition for bioreactors, they were described by Martin *et al.* in 2004 [17] as follow: *"Bioreactors are generally defined as devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions (e.g. pH, temperature, pressure, nutrient supply and waste removal)".* In industries, bioreactor systems are commonly used in fermentation and food processing, wastewater treatment and production of pharmaceuticals, allowing high throughput and automatic control for reproducibility.

Till today, a number of bioreactor systems for *ex vivo* bone culture have been developed. For example, Davidson *et al.* in 2012 [18] created a novel perfusion bioreactor system for *ex vivo* bone culture studies, where a sufficient chemotransportation mimicking the original *in vivo* environment for bone tissue could be enabled by perfusion flow. The model used rat femurs being placed in the bioreactor and perfused with osteogenic medium, which was administered by a peristaltic pump. The perfusion flow was found to enhance the bone cellular viability after 14 days of culture compared to the fresh samples. In the study of Henstock *et al.* [19], a custom-designed bioreactor with the possibility to apply a cyclic hydrostatic pressure regime on cultured chick embryonic femurs was used. By using μCT to evaluate bone formation between the femurs cultured under the cyclic hydrostatic pressure regime and unstimulated control femurs, the results indicated that cyclic hydrostatic pressure promoted bone growth and mineralization.

1.4 Time-lapsed μCT monitoring for bone research

Conventionally, destructive methods for determining the quality and quantity of an engineered tissue in vitro demand pooled samples for adequate statistical analysis. Individual samples used for analysis are normally destroyed. To yield sufficient analysing data for representing the whole experimental group, many more samples are required. Moreover, the understanding of the dynamic bone tissue forming processes of individual samples is hindered. In comparison with the conventional methods, using μCT as a non-invasive way for the assessment of engineered bone tissues better satisfies the needs in scientific research [20].

The integral use of μCT for time-lapsed monitoring and quantification contributes to the effectiveness of high-quality evaluation for bone tissue engineering applications. A single sample can be scanned multiple times without disturbing culture conditions. In the study of Hagenmueller *et al.* in 2007 [20], the same scaffold constructs were scanned by μCT repeatedly after different days of culturing. The images from individual samples showed the gradual progression of bone tissue formation. Quantitative morphometric analysis, as performed for human bone biopsies, can be applied to tissue engineered bone as well; bone volume (BV), bone volume density (BV/TV), bone surface-to-volume ratio (BS/BV), trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp), trabecular number (Tb.No), structural model index (SMI) and tissue mineral density (TMD) of the constructs can be determined.

As bone is a living tissue it constantly undergoes modeling and remodeling. The dynamic activity relies on two distinct mechanisms to formulate bone tissue: bone formation and bone resorption. The advantages of μCT as a non-invasive technique as well as a time-lapsed imaging process for three-dimensional reconstruction make the dynamic quantification of bone biological activities possible and effective. Schulte *et al.* [21] introduced this technique to determine dynamic bone morphometric parameters, namely bone formation rate (BFR), mineral apposition rate (MAR), and mineralizing surface (MS). Not only could bone formation parameters be quantified, but resorption processes were also assessed quantitatively by calculating the eroded surface (ES) through processing μCT images. Mineral resorption rate (MRR) and bone resorption rate (BRR) were first

demonstrated in scientific research to be indicative parameters for quantifying bone dynamic activities. Compared to the traditional method, which uses the two-dimensional histomorphometry, the integral use of µCT as non-invasive, direct, three-dimensional, automated procedure to quantify dynamic bone morphology has distinct advantages [21].

The technique of µCT can be applied to investigate the temporal changes of individual samples in a longitudinal way. Using time-lapsed µCT, the mouse tail bone was observed *in vivo* as a model system to study postmenopausal osteoporosis. The results showed that ovariectomized mice exhibited an immediate increase in BRR and delayed increase in BFR. These temporal changes in bone remodeling rates were specified for monitoring transient bone biology [22].

1.5 Combination of bioreactor system and µCT monitoring

A well-designed bioreactor system combined with µCT monitoring provides an improved experimental option for bone culture systems. Previously, Hagenmueller *et al.* [23] demonstrated a bioreactor approach that was combined with online µCT monitoring and mechanical loading for bone tissue engineering applications. The custom-made bioreactor offered a regulated environment for culturing cells. During culture, the chambers enabled the experimental demand of cyclic mechanical loading and time-lapsed µCT monitoring. The culture chambers were kept in an incubator with controlled temperature, gas and humidity. For µCT imaging or mechanical loading, the chambers were taken out temporarily. This setup proposed an approach to investigate the relationship between different external stimuli in a longitudinal manner [23].

1.6 Objectives and rationale of the thesis

This thesis aimed at developing a feasible framework for longitudinal studies on *ex vivo* embryonic chick femurs. The work was based on the insights given by the forerunners in the field [13, 16, 19-21, 24]. Their knowledge and well-developed techniques laid the foundation for the present research, allowing the progression to continue in this interdisciplinary field of bone research.

The development of an *ex vivo* animal model is expected to compensate for the drawbacks of *in vivo* animal models, which are expensive, complex and often give rise to ethical concerns. With the advantages in economical accessibility, ease of experimental manipulation and rapid growing processes, the chick embryo is a favourable and approachable animal model in scientific research with a long history of investigation in the field of biology and medicine. The current *ex vivo* embryonic chick femur culture models give rise to promising perspectives on bone tissue engineering and regenerative medicine. The organotypic chick femur culture models demonstrate a novel system for investigating the influence of environmental stimuli on dynamic bone activity [10, 16, 24, 25].

Well-designed bioreactor systems provide environments for different experimental setups to study bone in various aspects for the purpose of planned research [26, 27]. Bioreactor systems can be combined with the integral use of μCT, thus providing a non-invasive way for assessing the spatial and temporal changes of bone formation and resorption. With the help of μCT, longitudinal studies, which aim at investigating the temporal changes of the individual samples, can be conducted by time-lapsed monitoring. The technique of μCT provides static and dynamic morphologies of bone tissue, thus allowing analyzing methods to be used [20-22].

The present developments of a bioreactor system and μCT technology were necessary for developing a feasible framework to study *ex vivo* chick femur cultures in a longitudinal and non-invasive way. The work was to provide such a methodology used for *ex vivo* embryonic chick femur cultures. The steps taken to accomplish such a framework are as follows:

- A bioreactor chamber was designed to enable the basic condition of *ex vivo* embryonic chick femur culture under time-lapsed μCT monitoring
- To validate the developed bioreactor, three experimental groups were examined with respect to the frequency of μCT scans and the lengths of the period under organotypic culture.
- The integral use of μCT was made to provide three-dimensional images and quantifications for analyzing the bone tissue developments among the experimental groups.

Overall, the established framework was expected to provide an experimental option by using an *ex vivo* embryonic chick femur model, and to demonstrate its attainable and cost-effective advantages for current bone research.

Chapter 2 MATERIALS AND METHODS

2.1 Egg incubation

Chick eggs were supplied by Wüthrich Brüterei AG (Belp, Switzerland), and incubated for 18 days within an IncuView™ egg incubator supplied Incubator Warehouse (Fruitland, ID, U.S.) (Figure 2.1). During the incubation, the air temperature was maintained at 37.5°C, the air was circulated with an inner fan, and substantial humidity was controlled by keeping water in the reservoirs of the base in order to prevent the eggs from dehydration. The rack for placing the eggs moved automatically every hour to turn the eggs. The turning prevented young embryos from becoming stuck to the membranes and helped to provide sufficient nutrient supply from albumen [28]. The eggs needed to be aligned with the rack for a feasible turning process, which was checked every weekday and tilted eggs were realigned by hand.

Figure 2.1 **IncuView™ egg incubator with eggs on the turning rack (retrieved from http://incubatorwarehouse.com/)**

2.2 Bioreactor chamber design

The bioreactor chambers used in the experiments were designed by using computer-aided design software Solidworks (Waltham MA, US) for time-lapsed micro-computed tomography (µCT) monitoring. Individual embryonic chick femurs were scanned for multiple times over the whole experimental period. Polysulfone was chosen as a suitable material for the bioreactor chambers due to its low radio opacity allowing an essential passage of radiation [23]. The bioreactor chambers were custom-designed in order to maintain the same setup of the organotypic culture described later (Chapter 2.3). In each chamber one individual well plate and insert could be fit (Figure 2.2 a). During µCT-scanning, the chambers were closed (Figure 2.2 b and c). During transition, e.g. the preparation for µCT scanning, the lid of the chamber could be semi-opened (Figure 2.2 d), thus providing sufficient air circulation with controlled environmental parameters, temperature, CO_2 concentration and humidified air, inside the incubator (Figure 2.2 e).

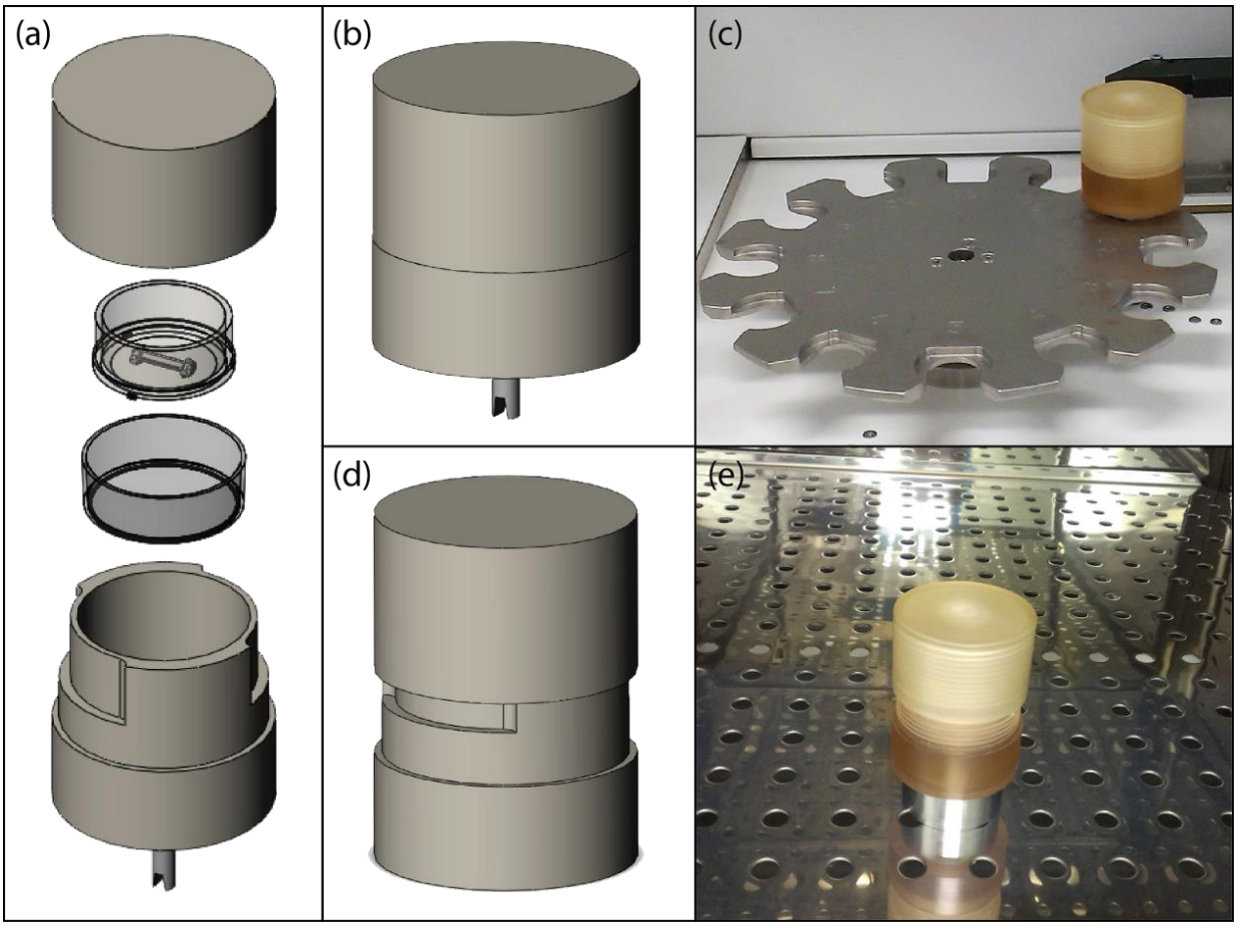

Figure 2.2 **Overview of the bioreactor design: (a) a schematic view of a bioreactor chamber designed to hold one well plate and well insert; (b) the closed bioreactor chamber; (c) the closed chamber was put on the carousel plate of µCT machine (d) the semi-open bioreactor chamber; (e) the semi-open bioreactor chamber inside the incubator.**

2.3 Preparation of embryonic chick femurs for organotypic cultures

18-day-old chick embryos were dissected using surgical scalpels and tweezers, and the femurs were culled carefully to remove the adherent muscle and soft tissue. The isolated femurs were then organotypically cultured in basal tissue culture medium, following the method described by Kanczler *et al.* in 2012 [16]. The basal culture medium contained: α-minimum essential medium (α-MEM; #BE02-002F, Lonza, Basel, Switzerland), penicillin, streptomycin (100µg/Ml; #17-602E, Lonza, Basel, Switzerland) and ascorbic acid 2-phosphate (100µM; A5960, Sigma-Aldrich, Saint Louis, MO, U.S.). Accordingly, the isolated femurs were placed on well inserts with a 0.40 µm pore size membrane (#PICM03050, Merck Millipore Corporation, Darmstadt, Germany). Then, the inserts with the femurs were positioned into six-well tissue culture plates (#92006, TPP, Trasadingen, Switzerland) with 1mL basal culture medium per each well. The whole setup provided an interface between air and liquid for the femur cultures (Figure 2.3). During the culturing period, the whole setting was maintained in an incubator at a controlled temperature of 37 °C and a CO_2 concentration of 5% in humidified air. The culture medium of 1mL was changed daily.

Figure 2.3 **Organotypic culture setup: isolated embryonic chick femurs were put on the micro-pore size membrane of the well inserts inside the well-plates with 1 mL culture medium in each well.**

2.4 Experimental groups

There were three experimental groups in this study (Table 2.1). Depending on the frequency of μCT scanning, codes were assigned to the M (monitoring) group for μCT monitoring every other day and to the NM (non-monitoring) group for μCT monitoring at the beginning and at the end of culture period (day 0 and 10). Group NM was set as the control group for group M in order to examine the influence of irradiation and multiple experimental manipulations within the 10-day culture period (n=8 femurs per group). The group L (long) served as a long-term culture group (n=7 femurs per group), and μCT monitoring took place every two weeks on day 2, 16 and 30.

Table 2.1 Overview of three experimental groups

Group	Culture period	μCT scanning	The days of scan	Number of femurs
M	10 days	Every other day	Day 0, 2, 4, 6, 8, 10	8
NM	10 days	The beginning and the end of culture	Day 0, 10	8
L	30 days	Every two weeks	Day 2, 16, 30	7

2.5 Micro-computed tomography

A μCT imaging system (Scanco Medical μCT50, Brüttisellen, Switzerland) was employed to monitor the bone tissue of embryonic chick femurs. A voxelsize of 35 μm was chosen for isotropic resolution. The energy was set at 55 kVp with an integration time of 200 ms, and two-fold frame averaging was performed.

2.6 Image processing

Mineralized tissue was segmented from nonmineralized tissue using a global thresholding procedure [29]. The threshold was chosen by eye for singling out mineralized tissue at 15.5% of the maximal image grey value from the initial 2-dimensional images of the scans (Figure 2.4). A constrained Gaussian filter was applied to all specimens for reducing image noise with a filter width of 1.2 and filter support of 1. Binary data from serial scans was registered after thresholding and Gaussian filtering for the assessment of dynamic bone morphometry.

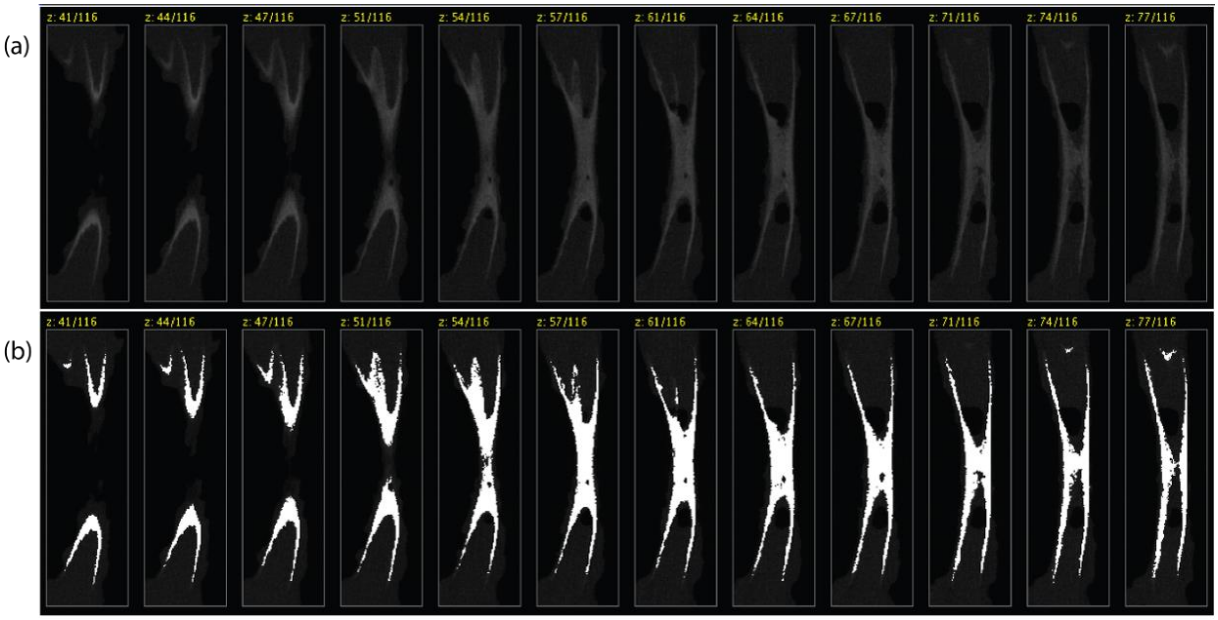

Figure 2.4 **Thresholding procedure for segmenting the bone tissue from the original µCT images of the embryonic chick femur (specimen #M01, day 0): (a) 2-dimensional images before threshold segmentation with consecutive z-position layers; (b) mineralized tissue was distinguished from background by eye at a threshold of 15.5% of the maximal image grey value from the initial 2-dimensional images.**

Afterwards, by following the method to acquire the sites of formed bone and resorbed bone introduced by Waarsing *et al.* [30], the measurements from µCT scanning at two time points were superimposed. Thus, the formed bone was considered as the gained bone areas from the later measurement only and the resorbed bone was the bone areas present in the earlier measurement only. Then the superimposed measurements of the same specimen from two different time points were registered at the most comparable position [21], thereby allowing the visualization and dynamic assessment of bone microstructure within multiple time points. For the registration, the algorithm proposed by Thevenaz *et al.* [31] was applied to minimize the data differences between two registered measurements. The bone direction of later measurements were rotated to match the bone sites of earlier measurements. From these registered images, different volumes were displayed with three colors for formed bone (orange), resorbed bone (blue) and constant bone (grey) (Figure 2.5).

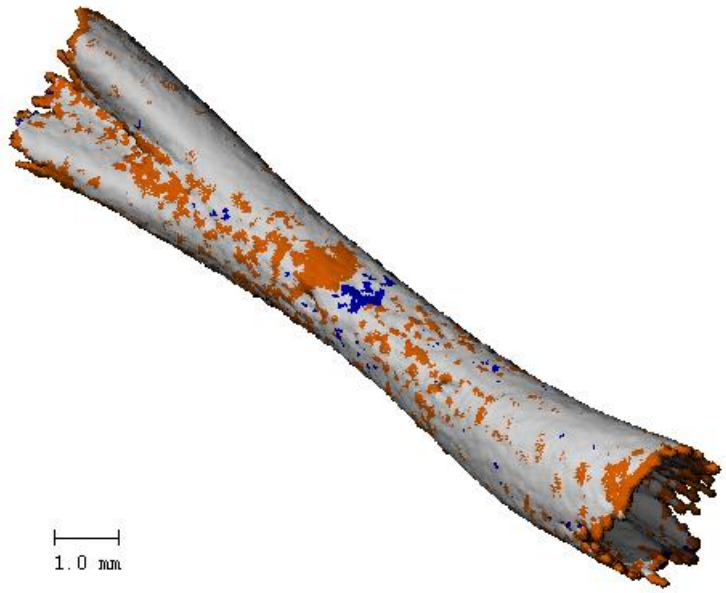

Figure 2.5 **Registered images of two time points: formed bone in orange, resorbed bone in blue and constant bone in grey on a scale bar of 1 mm.**

2.7 Bone morphometry

Using registered time-lapsed μCT images, the procedure to calculate dynamic bone morphometric parameters was described and validated previously [21]. For bone formation, bone formation rate (BFR) is defined as the formed bone volume per original bone volume per day with unit %/day; mineralizing surface (MS) is the percentage of formed bone surface per total bone surface with unit %; mineral apposition rate (MAR) is defined as the mean thickness of the formed bone volume divided by the number of days between the scans with unit μm/d. On the other hand, for bone resorption, analogously, bone resorption rate (BRR) is the resorbed bone volume per original bone volume per day with unit %/day; eroded surface (ES) is the amount of resorbed bone surface divided by original bone surface with unit %; mineral resorption rate (MRR) is the mean thickness of the resorbed bone volume per day with unit μm/d. Bone volume (BV) and bone surface (BS) of every measurement were calculated from binary data for comparison and understanding among consecutive scans.

Using Microsoft Excel (Redmond WA, US), all the calculated data of each experimental group is presented as mean ± standard deviation. Linear regression calculation was applied on the consecutive mean data of BV and BS, with r-squared values for understanding the trends of linear progression. Student's t-test was used to evaluate the data between two experimental groups. When considering more than two groups, analysis of variance (ANOVA) was done by SPSS (Chicago IL, US). ANOVA was followed by post-hoc assessment using the least significant different method. Values of $p \leq 0.05$ were considered significant, and those of $p \leq 0.01$ were considered highly significant.

Chapter 3 RESULTS

3.1 Egg incubation and chick femur dissection

After egg incubation for 18 days, the hatching rate for growing to the appearance of 18-year old chicks was 82%; 14 eggs were successfully incubated from a total of 17 eggs. The dissecting process for isolating chick femurs was manipulated by hand, and the success rate was 82%; 23 femurs were successfully dissected from a total of 28 femurs.

3.2 The function of designed bioreactor chambers

The individual femurs were manipulated to be put inside and outside the bioreactor chambers at different time points, maintaining their basic culture condition during the periods of μCT scans. In this study, the bioreacter chambers designed for *ex vivo* embryonic chick femur culture successfully functioned under time-lapsed micro-computed tomography (μCT) monitoring. This was confirmed by the clear μCT images of individual samples in different experimental groups. However, during the periods of μCT scans (35 mins per scan), the environmental setup could not be controled in the incubator. To enhance the convenience between the waiting periods of μCT scans in queue for several samples, the semi-open bioreactor chambers were functioned for air circulation inside the incubator.

3.3 Effects of irradiation and experimental manipulations

The effects of several experimental manipulations and irradiation over the whole culture period (10 days) were investigated in experimental groups—M and NM (Table 2.1).

The μCT measurements of day 10 at the end of the culture period were superimposed after thresholding on the measurements of day 0 at the beginning of culture. The superimposing technique provided the information on bone dynamic activity during the registered period. The voxels were not present on the binary images of day 0, which represented the formed bone volume on day 10 comparable to day 0. After image processing, the bone formed between day 0 to day 10 was shown in orange (Firgure 3.1). Alternatively, the bone resorbed from day 0 to day 10 was shown in blue (Figure 3.1). The formed and resorbed bone volume provided substantial information for further quantitative analysis of bone morphometry. Two representative images of groups M and NM

both had more formation sites than resorption sites, indicating that the samples experienced bone formation between day 0 and day 10. The three-dimensional images after processing provided structural information; formation sites were concentrated at the two ends and on the central surface of the whole bone volume.

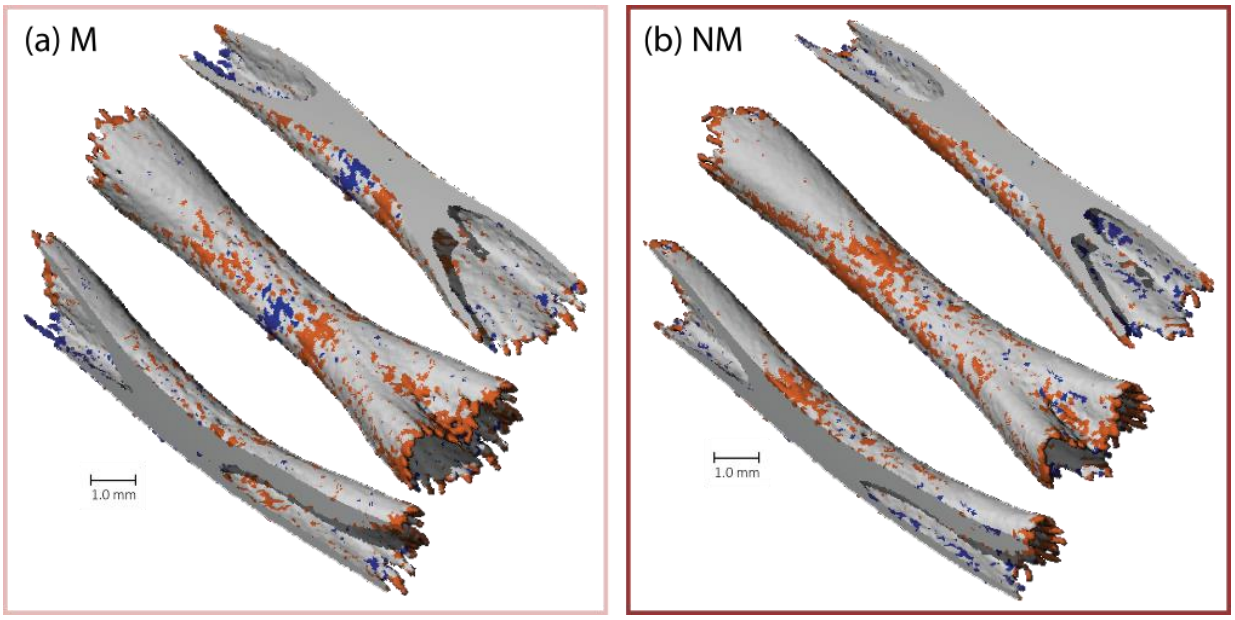

Figure 3.1 **Registered three-dimensional images: the µCT measurement after thresholding of day 10 was superimposed on the one of day 0, where the orange volume represents formed bone, the blue volume represents resorbed bone and the grey volume represents constant bone. (a) Representative sample #M03 of group M and (b) representative sample #NM03 of group NM.**

From the registered measurements, in which formed bone tissue, resorbed bone tissue and constant bone tissue were determined, bone volume (BV), bone surface (BS) and bone thickness were analysed to determine bone dynamic activities between two time points of scans in accordance with the existing formulas [21]. Bone dynamic morphometry was compared between the groups M and NM.

On average, group M had 26 mm^3 of BV at the beginning of culture and grew to 27.4 mm^3 of BV at the end of culture. By comparison, BV of group NM grew from 25.2 mm^3 to almost 26.6 mm^3. Apparently, both groups experienced almost the same amount of bone volume growth, although group NM had a lower average BV than group M. Furthermore, both groups had similar BS values at the beginning and the end of culture (Figure 3.2).

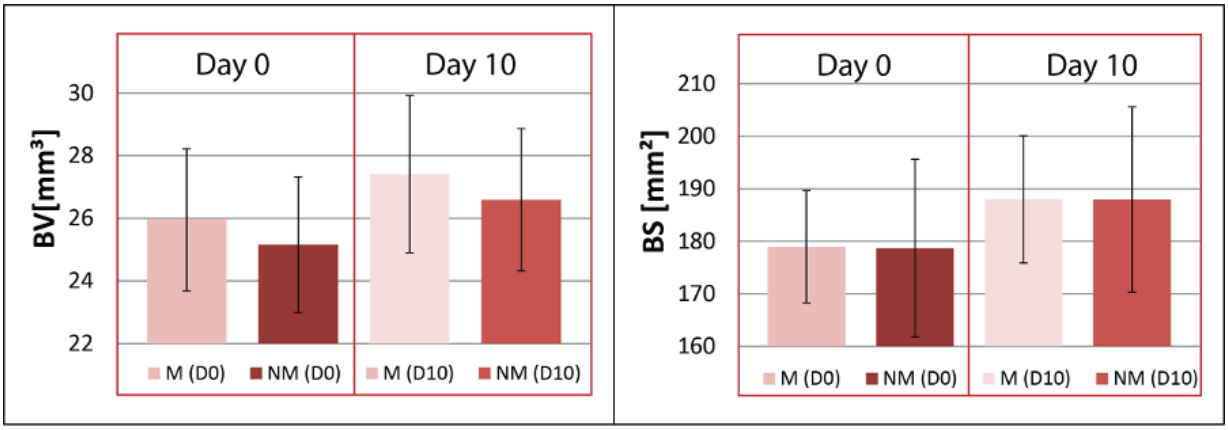

Figure 3.2 **Static bone morphometry of group M and group NM (n=8 femurs per group): bone volume and bone surface evaluated from µCT measurements of day 0 and day 10 of organotypic culture. (All values are means ± standard deviation)**

Bone dynamic morphometry between the groups M and NM also showed comparable results. For bone formation, the BFR of both groups exhibited almost the same rate per day. The MS was slight different between the groups M and NM at around 46 %. The MAR had differences of 0.8 µm of the mean thickness of formed bone per day between the two groups. In brief, two groups showed no significant differences in the bone formation parameters (Figure 3.3). On the other hand, for bone resorption, although there was a significant difference between the BRR of the groups M and NM (p-value < 0.05), ES and MRR presented the analogous data with no significant differences between the two groups. When comparing the columns of bone formation and bone resorption, it was obvious that the chick femurs experienced much more bone formation activities than bone resorption activities (Figure 3.3).

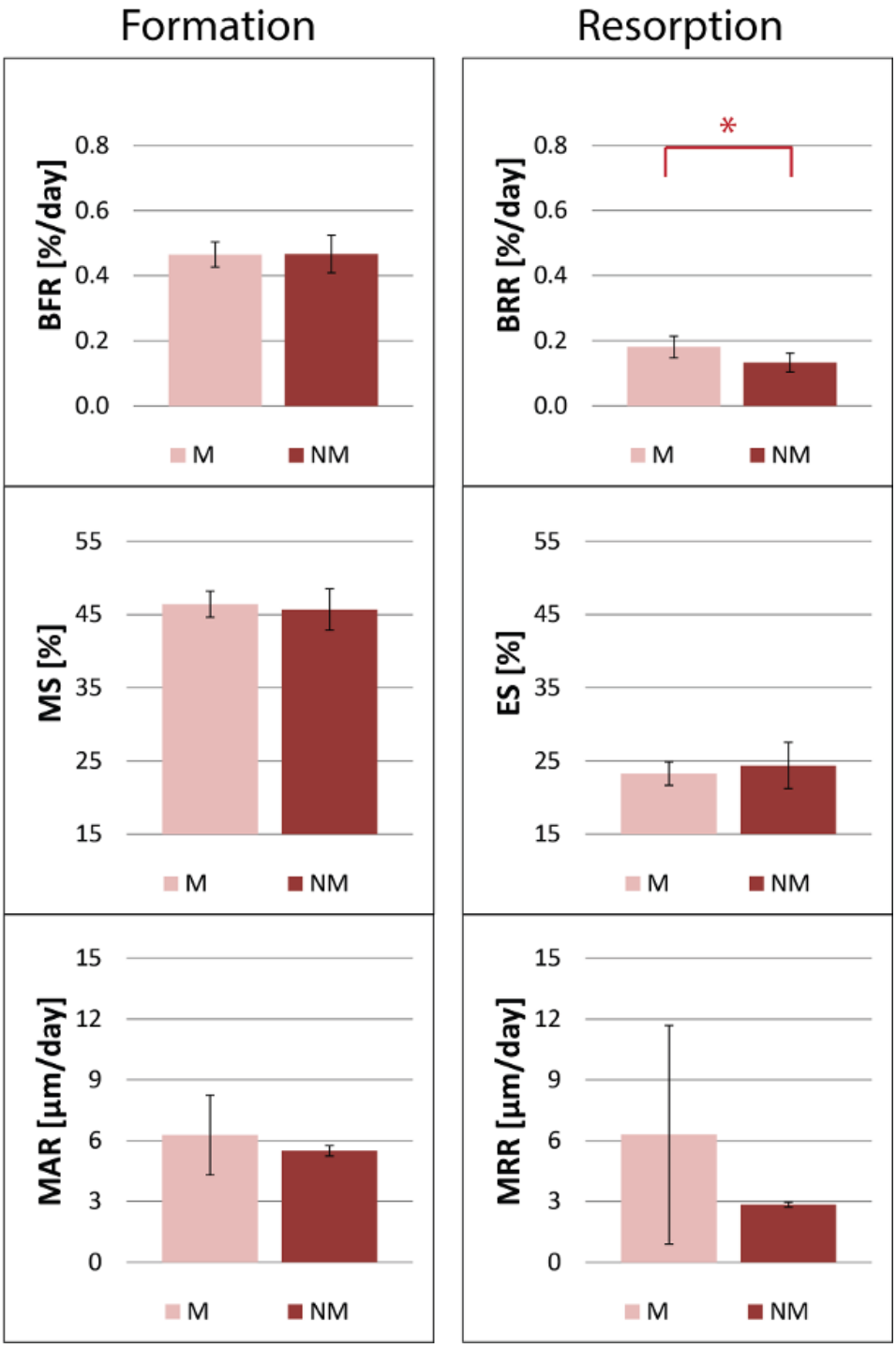

Figure 3.3 **Dynamic bone morphometry of group M and group NM (n=8 femurs per group): BFR, BRR, MS, ES, MAR and MRR evaluated from the registered measurements of day 0 and day 10 of organotypic culture. (All values are means ± standard deviation; * p-value < 0.05)**

3.4 Effects of registered measurements from different time points and duration

For the experimental group M, every femur was µCT monitored every two days over the culturing period of 10 days. The µCT measurements provided additional information for bone activities on the interval of 2 days, as compared to the total period of culture for 10 days. In Figure 3.4 obviously, BV and BS gradually increased from day 0 to day 10 of culture. BV and BS showed progressive increases almost linearly with high r-squared values, 98.61% for BV and 95.46 for BS.

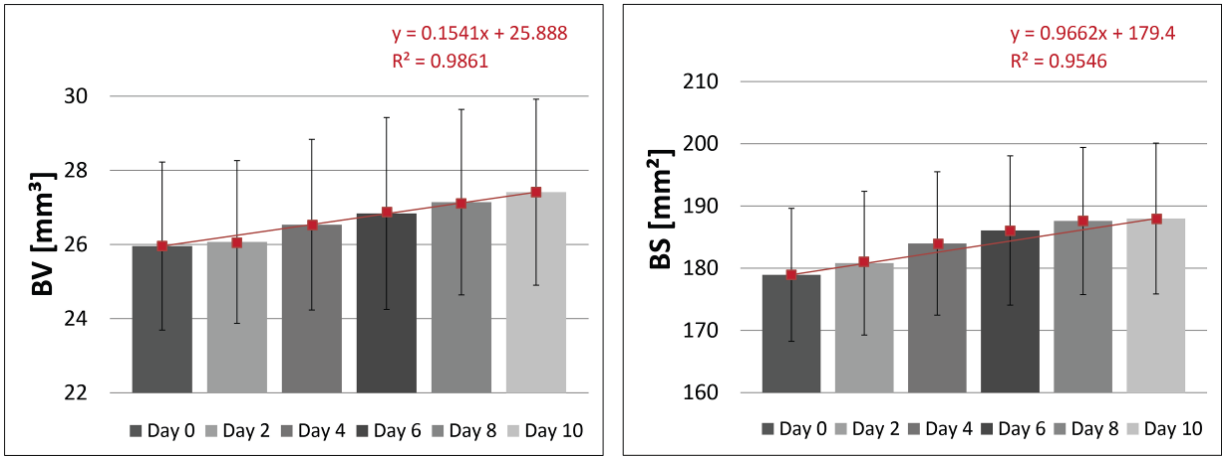

Figure 3.4 **Static bone morphometry from the measurements of consecutive µCT data monitored every other day (group M): Bone volume (BV) and bone surface (BS) at day 0, 2, 4, 6, 8 and 10 of organotypic culture with the regression line calculated by average values (All values are means ± standard deviation; n=8)**

Considering dynamic bone morphometry in Figure 3.5, the registered measurements of consecutive scans from the interval of two days are displayed by the grey columns , while the registration over the whole culture period is shown by the pink columns. The differences between the grey and pink columns were found in BFR, BRR, MS and ES. The registration of two time points gave different results on dynamic bone activities. When images registered from the interval of every two days of culture, BFR, BRR, MAR and MRR calculated per day, were higher than those registered from the beginning to the end of the culture period. When the values within the registration periods were calculated in percentage, MS showed a higher percentage of 46.4% and ES a lower percentage of 23.3%, as indicated by the pink columns (registered from day 0 to day 10) as compared to the grey columns (registered scans of every 2 days). The results provided important information that bone modeling and remodelling were dynamic and active within short periods of time, while the registration over a longer period of time could not provide such information.

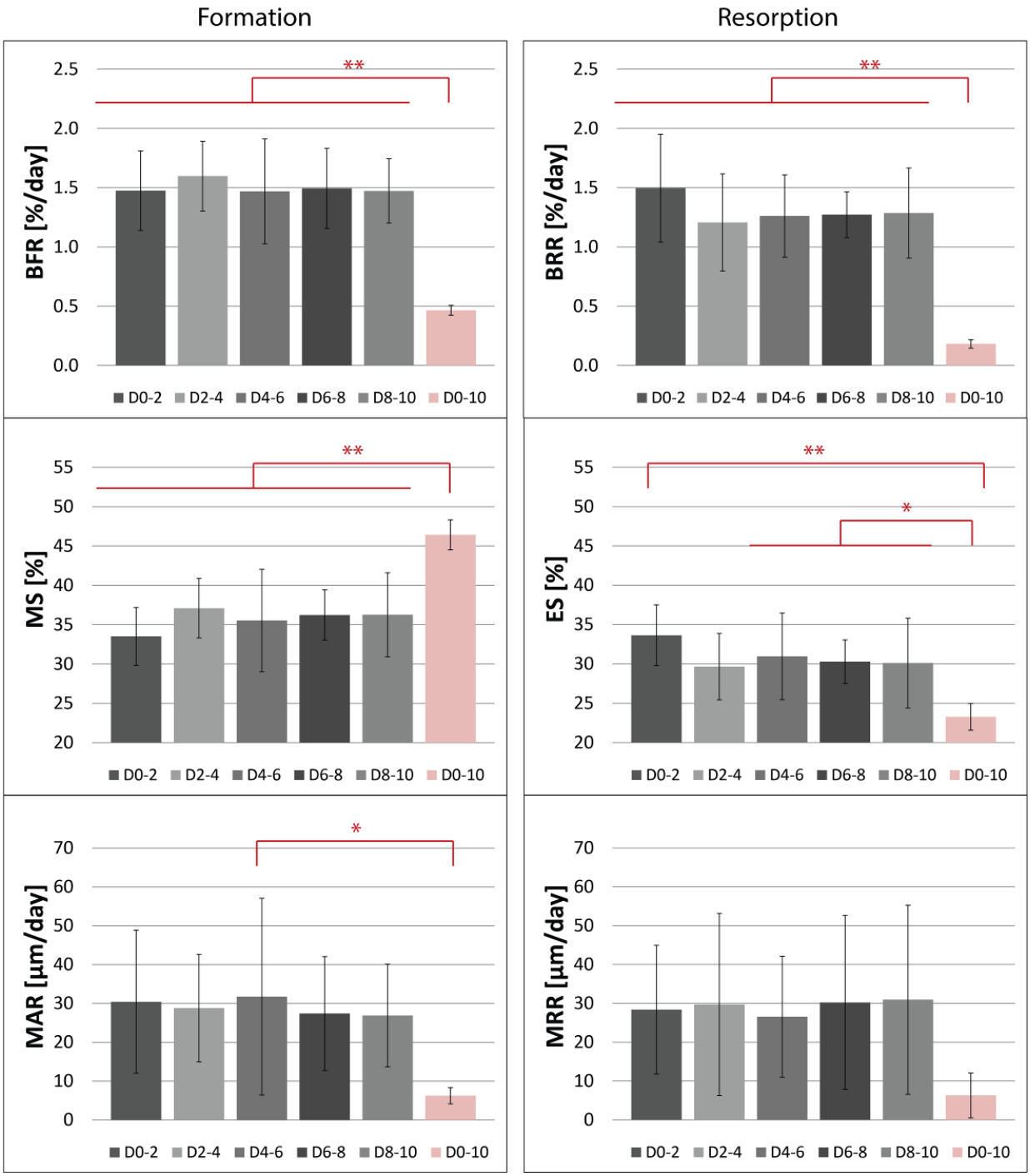

Figure 3.5 **Dynamic bone morphometry from the measurements of consecutive data µCT monitored every other day (group M): BFR, BRR, MS, ES, MAR and MRR of different consecutive time points evaluated from registered measurements. (All values are means ± standard deviation; ** p-value < 0.01; * p-value < 0.05; n=8)**

3.5 Results of the organotypic long-term culture

Over long-term culture, 7 femurs in the experimental group L were organotypically cultured for 30 days and monitored every two weeks. The registered images of bone tissue from week 0 to week 2 showed more bone formation than those from week 2 to week 4 (Figure 3.6 a and b). Also, the registration was done over the whole culture period between week 0 to week 4 (Figure 3.6 (c)).

Figure 3.6 **Registered three-dimensional images, where the orange color represents formed bone, the blue represents resorbed bone and the grey represents constant bone (representative sample #L03). (a) The µCT measurement of week 2 superimposed on the measurement of week 0. (b) The µCT measurement of week 4 superimposed on the measurement of week 2. (c) The µCT measurement of week 4 superimposed on the measurement of week 0.**

Although only three measurements were examined, it was evident that BV and BS values did not change as linearly as the culture over a short period mentioned earlier (Figure 3.7). In the first two weeks of culture, the femurs experienced more bone formation activity than in the last two weeks of culture; bone resorption activity was higher in the last two weeks of culture.

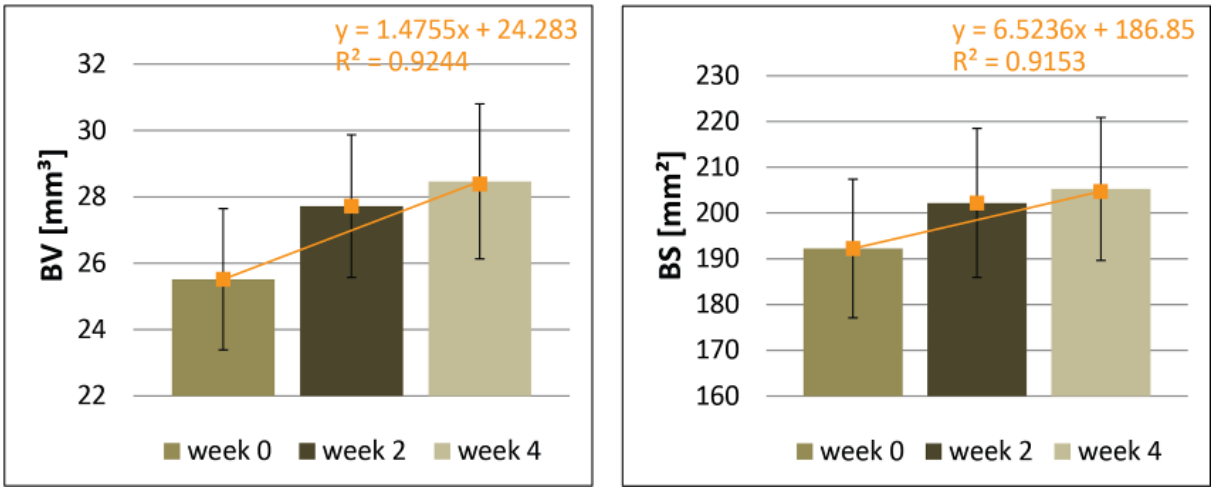

Figure 3.7 **Static bone morphomotry from the measurements of consecutive data µCT monitored every two weeks (group L): bone volume (BV) and bone surface (BS) at week 0, 2 and 4 of organotypic culture with the regression line calculated by average values. (All values are means ± standard deviation; n=7)**

The quantitative data also indicated that bone formation activity was higher than bone resorption activity (Figure 3.8). Dynamic bone morphometry revealed that the bone tissue of femurs was growing during the whole period of *ex vivo* culture, which was in agreement with static bone morphometry (BV and BS). The differences between the registrations over the whole period of culture and the ones registered in the interval of 2 weeks were similar as mentioned afore in Chapter 3.2, where the longer period of registration could not represent the dynamic bone activities over a shorter period (Figure 3.5).

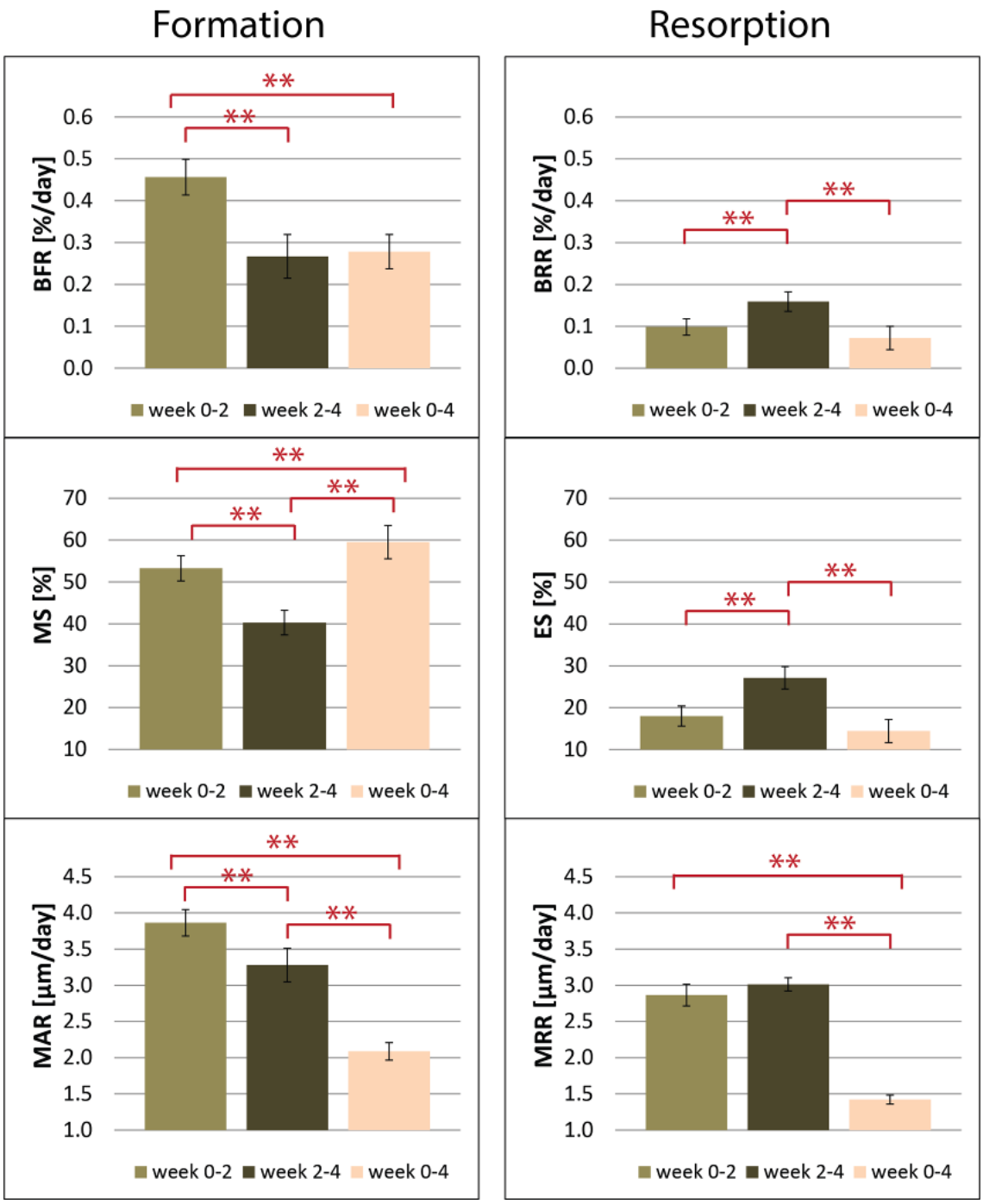

Figure 3.8 **Dynamic bone morphomotry from the measurements of consecutive data µCT monitored every two weeks (group L): BFR, BRR, MS, ES, MAR and MRR of different consecutive time points evaluated from registered measurements. (All values are means ± standard deviation; ** p-value < 0.01; * p-value < 0.05; n=7)**

Chapter 4 DISCUSSION

4.1 Egg as a cost-effective model for performing bone research

Eggs are economical sources as popular human food for nutrient supply. In addition, eggs have served as numerous scientific models since the first written work traced back to 2500 years ago [2]. The attributes of accessibility and sizes made eggs helpful for ancient people to understand biology and medicine. This study made use of these advantages of eggs. The embryonic chick femurs extracted from eggs were introduced for developing a methodology in contemporary bone research.

For incubating the eggs and dissecting the femurs, the conventional ways were followed [16]. In this study, the rates for successful hatching and dissecting the femurs were both 82%. These rates were not predictable, as it was the first time for the author to conduct the research on the chick femur model. For the quantity of usable femurs, these rates were not good for planned experiments, as they resulted in 67% usable femurs from the total number of femurs. However, like the training for surgeons, with more practical exercise on the manipulation between surgical scalpels and chick models, the rates of successful dissection could be higher. The rates for obtaining usable chick femurs need to be taken into consideration when the experimental schemes are planned.

Compared to other *ex vivo* models, chick femurs demonstrated quantitative and qualitative advantages. For example, bone samples from rat, mouse and cow were commonly found in bone research [6, 9, 10, 32]. In order to healthy breeding and raising of these animals, the models are more expensive and complex than the way of hatching chick eggs to provide a qualified number of bone samples. Also, the simple experimental manipulation on embryonic chicks was manifested by their attainable sizes and clear skeletal structure. With the matured skills obtained from more experiments, the advantages of using chick egg as a model will be demonstrated more in its cost-effectiveness for bone research.

4.2 Bioreactor system facilitating the experiments for bone organ culture

Bone organ culture systems can provide original complex structural environments and maintain the original interactions between diverse cells and extracellular matrix. Within these three-dimensional biomimetic environments, *ex vivo* bone culture systems have their own advantages with respect to the ease of environmental modulation and experimental control, in addition to capturing the organic responses similar to *in vivo* study. Recently, *ex vivo* organotypic embryonic chick femur culture has demonstrated to be a powerful and high throughput method to study bone development [16]. By combining the embryonic chick femur model with the technique of μCT, its effectiveness on three-

dimensional spatial patterning of fundamental bone development has been demonstrated. The present study followed this existing method of organotypic culture of embryonic chick femurs and provided a way to perform longitudinal μCT monitoring on individual femurs over the whole culture periods.

A well-developed bioreactor system has proved its advantages for the diversity of bone research [23, 33]. In this study, the bioreactor chamber was designed for one single femur cultured under the same culture condition as described in a previous study at an air/liquid interface [16]. On purpose, the adjustable lid of the bioreactor chamber functioned as a convenient device for the interval periods before and during μCT scanning (Figure 2.1). The concern was that the environmental condition was not controlled as in the incubator during the period of μCT scanning. To facilitate the preparation for μCT scanning, the semi-open lid of the designed bioreactor chamber enabled cultured femurs in the incubator under environmental control. To meet the aim of this study, this bioreactor approach feasibly granted μCT monitoring on individual samples in a longitudinal way.

As to the use of a basal tissue culture medium, its components were suggested in a previous study. It was found that the influence of the medium on bone growth was the least among other culture media [16]. Thus, the basic maintenance of the bone developing process could be expected to validate this framework. Unlike the previous study using the culture medium of 2 mL per femur in each well [16], the present study used the culture medium of 1 mL to provide minimal liquid contact with femur samples at the air/liquid interface. In order to meet the need of longitudinal μCT monitoring, the femur samples and their culture medium were moved between six-well plates and the bioreactor chamber. This caused unseen loss of corresponding culture medium among the movement. However, from the values of BV and BS obtained in this study (Figure 3.4), the progressive bone growth confirmed that the bioreactor approach for μCT longitudinal monitoring on *ex vivo* femur cultures could maintain the basic bone developing process.

4.3 The integral use of μCT on ex vivo bone model

Normally, to evaluate an *ex vivo* bone model, the histological assays and monitoring were done at the end of culture [6, 19]. In order to effectively capture the bone developing process at the different stages, conventionally, a considerable number of bone samples was needed to meet the experimental requirements [16]. This limitation could be overcome by performing a longitudinal study on a single sample throughout the whole culture period. Using μCT time-lapsed monitoring, the longitudinal studies were carried out by scanning the individual samples at several time points [20]. The *ex vivo* chick femur models presented here provides detailed three-dimensional information on the long bone growing procedures.

The superimposed images presented clear structural changes on the bone tissue where formed bone and resorbed bone were shown from the differences between the scans of two time points. From the results of this study, the bone was above all formed at the two ends and on the central surface of the whole bone volume. This dynamic structural information on the bone tissue development was first

time shown in an chick femur model. Previously, the μCT longitudinal assessment on *in vivo* mouse tail model was performed [22]. The *in vivo* mouse bone model successfully showed that the postmenopausal osteoporosis could be investigated by the analysed information from μCT time-lapsed monitoring. While longitudinal studies on an *ex vivo* bone model awaited to be developed in bone research, the present established framework was expected to be one of the forerunners to expand this methodology on the *ex vivo* bone model.

To effectively understand the bone adaptation processes with corresponding cellular activities, the combination of μCT time-lapsed monitoring and image processing have proven as an advantageous tool [20]. The earlier works validated an effective way to have a quantitative assessment of bone formation and bone resorption [21]. In this study, either from the superimposed images or quantified parameters, it was obvious that femurs dissected from 18-day-old embryonic chick experienced much more formation activity than resorption activity, which means that, at this stage, chick femurs experienced bone growth. Although the basal culture medium was shown to be less influence for bone growth [16], the progressive bone formation still existed to a certain extent in the present study. It suggested that the use of embryonic chick femur for bone organ culture was productive for investigating the progression of bone mineralization. At this stage, the embryonic chick femurs experienced rapid bone growth in order to be mature for hatching [28]. The *ex vivo* culture showed its advantages in modulating external stimuli to study the bone developing process [16, 18, 19, 34]. With the integral use of μCT, an evaluation of these external stimuli could be made.

On the other hand, the informative parameters provided the understanding of the bone adaptation process. These dynamic bone parameters with different units should be considered to have further interpretations. For example, for bone formation, BFR and MAR were calculated on the time scale of days compared to MS calculated in % over the whole culture period scanned at two time points, resulting in different results. Also, it gave critical information on the μCT monitoring schemes that will be discussed later in Chapter 4.4.

4.4 The importance of μCT monitoring schemes and duration of culture

During the experimental manipulation, it was possible that the femurs moved or culture medium was lost when a femur under organotypic culture was transferred from six well plates to the bioreactor chamber for μCT monitoring. Thus, the experimental groups M and NM were set for investigating multiple μCT scans over the whole culture period compared to the one monitored only at the beginning and the end of the culture period. This also gave the information on the influence of irradiation from consecutive μCT scans on individual femurs. Although the risk of ionizing irradiation is present by multiple μCT scans, a previous study demonstrated that the integral use of μCT could be recognized as a safe tool to investigate the temporal and mineralization events [20]. However, the aim of setting up these μCT monitoring schemes was not to mainly investigate the influence of irradiation, but to develop an effective methodology to study *ex vivo* bone model in a longitudinal way.

After μCT evaluation, the results showed similar bone growth and dynamic bone morphometry between the groups M and NM. With respect to the multiple experimental manipulations and the influence of irradiation, these similar results demonstrated less influence on the bone development between the two groups, thus presenting an opportunity for this framework to be used in longitudinal studies. Although the bone tissue of individual femurs extracted from μCT images appeared to be different in their three-dimensional patterns, the influences of bone tissue dynamic activities quantified by the evaluated bone morphometry were found to be insignificant between the two groups. This validated that the use of quantification for bone morphometry remained to be an effective and efficient approach to understand bone organic activities. It provided a preliminary confirmation for the framework developed by the combined techniques for this study.

Bone is a living tissue going through formation and resorption continuously to meet the environmental and homeostatic needs. The evaluations of bone tissue from different time points were performed to get the informative bone parameters, related to dynamic bone activity [21]. Because of the multiple consecutive μCT measurements every other day in group M, the two time points for superimposing registrations was taken from the interval of two days' culture period and also from the time points of the whole culture period. The evaluations demonstrated the limitations of the technique for transient biology. From the quantified bone morphometry, the registered images from a short interval apparently demonstrated more dynamic activities than those registered over a longer period. The differences indicated that the measurements done at longer intervals were not able to capture bone activity compared to shorter intervals. For example, it could be speculated that the constant bone shown on the screen may have already experienced numerous cycles of bone formation and resorption between two time points. Hence, the understanding of bone activities can be obtained from sufficient μCT monitoring schemes to identify an optimum period for investigating bone development. The period between two μCT measurements should be considered to serve the aims of experiments and to obtain the informative data about bone activities. With sufficient research plan, the present framework could provide a solid base on which longitudinal studies for multiple experimental setups could be conducted.

Samples in another experimental group L were scanned every two weeks. From the time points of μCT scans, the registered measurements were divided into the first half and the second half of the culture period. During the first half of the culture period, more bone formation and less bone resorption were observed compared to the second half of the culture period. This confirmed that a deterioration period of bone formation began after a culture period of 8 to 10 days, as suggested in the contemporary study on *ex vivo* chick femur cultures [10]. Thus, it indicated that the limitation of *ex vivo* bone for long-term culture indeed existed. With this framework, in the future, a longitudinal study with a short interval between μCT scans for a long-term culture is suggested to obtain the period of stable bone dynamic activity. Thus, an optimum period of *ex vivo* embryonic chick femur culture can be known more precisely by the quantified bone parameters. Also, with current bioreactor knowledge, flow perfusion was found to maintain *ex vivo* bone viability for bone research [35]. This is the niche; the current *ex vivo* chick femur models can be advanced and stabilized to a longer longitudinal scale.

4.5 Applications of the established framework

Using μCT monitoring of bone tissue, the established framework for *ex vivo* embryonic chick femur cultures is promising for future bone research. Within this framework, creating a defect model on *ex vivo* embryonic chick femur culture could be practical for evaluating biomaterials applied in the field of bone tissue engineering. With the rapid development of chicks and the ease of control, the *ex vivo* culture system is an accessible way for testing biomaterials at the initial stage of screening as possible biomaterial candidates for further clinical studies. Introduced by Smith *et al.* in 2014 [24, 25], using a sterile scalpel blade, 2mm segmental defects were created in embryonic 11-day-old chick femurs. They tested hydrogels with different growth factors in the segmental chick femoral defect under organotypic cultures. The results showed bone integrated to the sites where a novel growth factor was applied. However, in 11-day-old chick femur, the bone development was still at a preliminary stage, showing little bone volume. In the present study, 18-day old embryonic chick femurs were incubated for 3 days before the eggs would hatch (day 21). At day 18, the bone tissue already grew to a considerable volume and the immune system had been developed [10].

Thus, the efficacy of biomaterials for bone tissue engineering can be evaluated with critical defect sizes, beyond which bone cannot heal by itself. With the help of the established technique of μCT, three-dimensional structure and bone morphometry can be obtained to understand overall bone cellular activities, especially the defect is created on the sites of mineralized bone tissue. To investigate bone repair, the ways to create different types of defects are possible, resulting in the investigating work to build up an effective defect model for the research on bone repair and regeneration [36]. Another issue is that the bone growth within different kinds of culture medium needs to be taken into consideration [16], and it can be one of the factors influencing the evaluation of biomaterials. When planning the experiments, one should set a comparable control group, which is important for investigating the effectiveness of the testing biomaterials.

Another prospective application of the developed framework of the present study is the bioreactor system. For bone tissue engineering, different kinds of bioreactor systems have been developed to mimic the environments that the bone activities may take place in real situation [17, 26, 37]. A cyclic hydrostatic pressure regime proved to enhance bone growth for embryonic chick femur cultured in a custom-made bioreactor system [19]. In the present study, the technique to provide an air/liquid interface for whole bone static culture was used and the bioreactor chamber was designed to fit for simultaneous μCT monitoring and organotypic culture. This framework can be modulated through bioreactor system design for versatile experimental needs. For example, different types of mechanical loading or dynamic culture condition may be applied to study environmental influences on bone tissue [18, 34]. Moreover, how to maintain the bone viability to a certain extent is important to have an optimum period for the research to be conducted on *ex vivo* bone models [18]. In the future, by current bioreactor knowledge, the sophisticated arrangements are expected to provide culture environments for sustaining and stabilizing the *ex vivo* bone studies to another niche.

The skeletal arrangements of a chick embryo conforms to other vertebrates, where the long bones represent the same structural support among these kinds [5]. Although there are differences between chick femurs and human femurs, the basic bone cellular activities of developed chick femurs, osteoblasts for bone formation and osteoclasts for bone resorption, can supply substantial

information for studying bone. The *ex vivo* embryonic chick femur model has proven to be an easy experimental manipulation and economical source with less ethical concerns than *in vivo* models. In the future, it is expected to be a high throughput method to evaluate biomaterials in a simple and short period system. Combined with μCT monitoring, the framework developed has shown to be a feasible model in the field of bone tissue engineering and regenerative medicine.

Chapter 5 CONCLUSIONS

Fundamental bone development is important in bone research to understand the orchestrated processes that determine the three-dimensional appearance and corresponding dynamic activities. The integral use of micro-computed tomography (μCT) facilitates the investigation of bone development, where μCT analysis of mineralized bone tissue at different time points offers reliable three-dimensional structure and quantification for bone morphometric parameters. Longitudinal studies on bone models are important to acquire corresponding bone activities over the whole culture periods. Among animal models, the embryonic chick femur has served as a cost-effective and accessible model for performing skeletal research. In order to validate the framework developed in present study, the experiments on *ex vivo* embryonic culture were performed with different μCT monitoring schemes.

This is the first developed framework to perform μCT longitudinal monitoring on *ex vivo* embryonic chick femur cultures. From this study, the critical information was revealed and confirmed:

Ex vivo embryonic chick femur cultures provided a way to capture the original complex environment for the development of bone tissue. Moreover, they introduced easier experimental manipulation and less ethical concerns than *in vivo* studies. With practical training on incubating and dissecting embryonic chicks, chick eggs were economical resources to perform skeletal research with their attainable sizes, clear skeletal structures and rapid developing processes. A well-developed bioreactor system facilitated longitudinal studies on *ex vivo* chick femur cultures. However, an optimum period of *ex vivo* chick femur cultures was limited by its corresponding viability in an artificial environment.

Using μCT time-lapsed monitoring, the reconstruction of μCT measurements to determine bone dynamic activity confirmed current bone biology that bone experiences modeling and remodeling although the structural appearances change slightly. Based on the purpose of research, μCT monitoring schemes are important for the balance between the investigation of transient bone biology and experimental laboring. As an experimental option, the established framework is a feasible methodology for the applications of bone research.

Chapter 6 RECOMMENDATIONS

In the present study a framework to perform longitudinal micro-computed tomography (µCT) on *ex vivo* embryonic chick femur cultures was established. Some constraints were found in the preliminary stage for acquiring usable chick femurs due to the lack of experiential practices on incubating eggs and dissecting chicks. Although using chicks as models is a high throughput method compared to other large animal studies, the beginners need to notice the quantity and quality of specimen control when planning the experimental schemes.

The developed bioreactor design enabled the µCT scans of individual chick femurs at several time points over the whole culture period. Due to the limitations of current registering techniques to investigate dynamic bone morphometric parameters, the absolute bone cellular activities between two µCT scans could not be exactly captured. In this case, between the scope of investigating transient bone biology and labouring on multiple experimental manipulations, the µCT longitudinal monitoring schemes have to be taken into consideration.

Current strategies in bone tissue engineering introduced a number of environmental setups for the investigation of different biomaterials. Before the clinical use of bone grafting products, the efficacies of the strategies on developed biomaterials and environmental arrangement in bone regeneration and repair are still under investigation and development. By the combination of multidisciplinary techniques of the integral use of µCT and the bioreactor approach, the present framework has helped to provide potential opportunities for versatile experimental setups. For example, chick femur defect models could be effective for evaluating the efficacy of new biomaterials. Bioreactor system designs make the multiple culture arrangements possible to understand the external influence on the process of bone development. By arranging more sophisticated culture environments, maintaining bone viability with its corresponding dynamic activity is expected during a longer period of culture in order to study bone in a more longitudinal way. By the applications of the established framework, the investigation in the field of bone tissue engineering and regenerative medicine is awaited to yield fruitful outcome.

Based on the established framework directly, the preliminary experiments of future research lines are suggested:

- *Ex vivo embryonic chick femur defect models:*
  To investigate the bone repair and regeneration within the defect sites created by different means (e.g. drill, segmental, wedge…etc.) and their corresponding critical sizes to build up optimal defect models comparable to tested biomaterials

- *Bioreactor systems:*
  To investigate the environmental influence using the bioreactor technology in order to find means to maintain stabilized bone activity during long-term *ex vivo* chick femur cultures

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APPENDIXES

APPENDIX I Processed 3-dimensional images

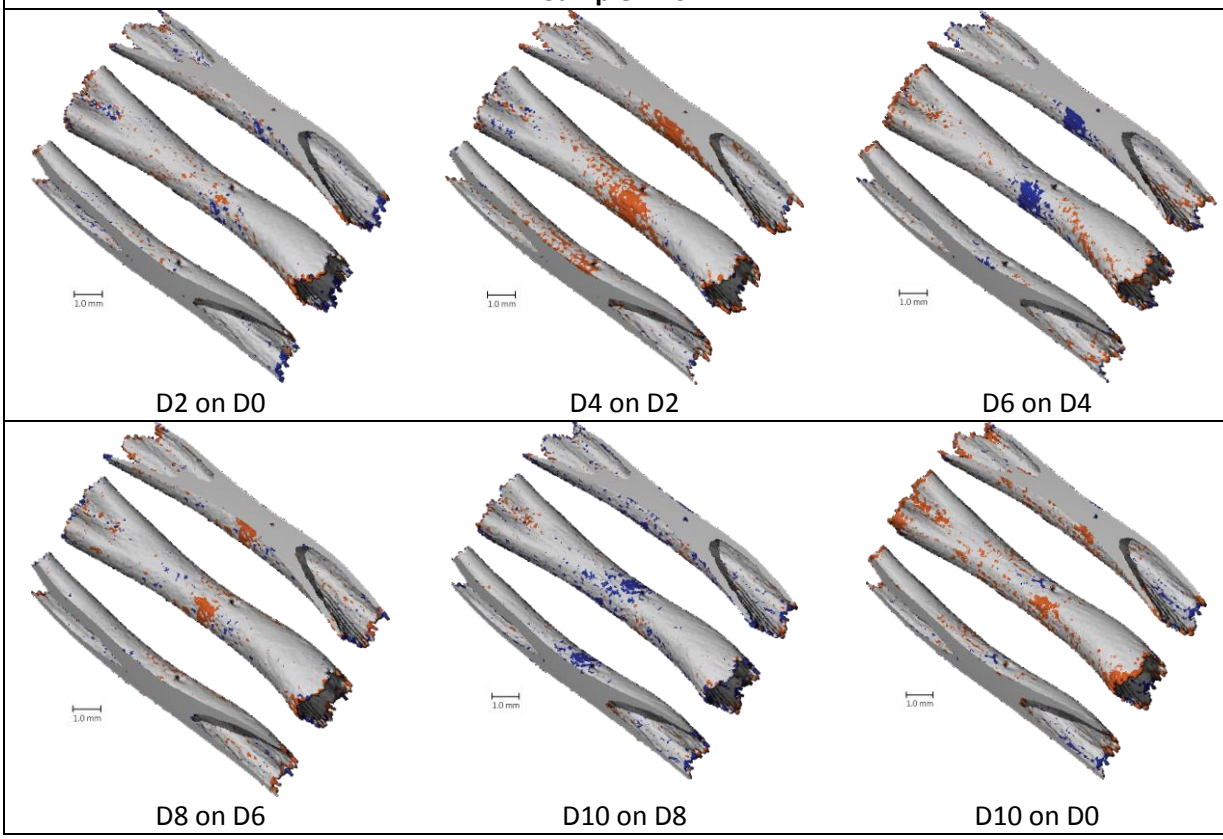
I.1 Group M

µCT scanned on day 0, day 2, day 4, day 6, day 8 and day 10 of culture (D0, D2, D4, D6, D8 and D10).

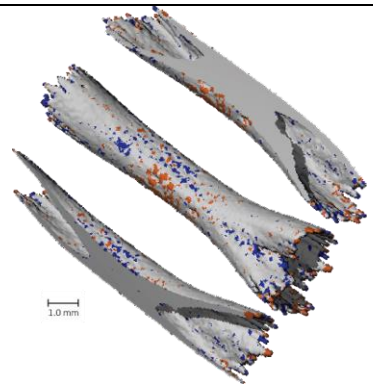
The differences between two scanning measurements were obtained by registering images after thresholding and Gaussian filtering of the original reconstructed images from µCT scans. For example, "D2 on D0" means the binary data from day 2 of the culture period registered on the image of the scan on day 0. The voxels present only in the later measurements are shown in orange and represent the formed bone, while the voxels present only in the first measurement are shown in blue and represent the resorbed bone. The constant bone is shown in grey.

- **Formed bone**
- **Resorbed bone**
- **Constant bone**

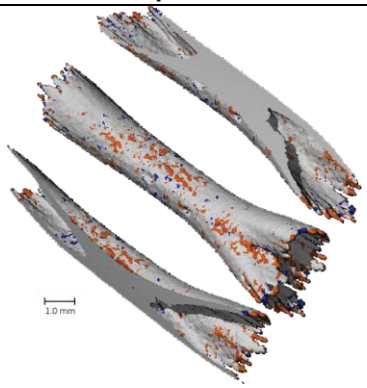
Sample: M01



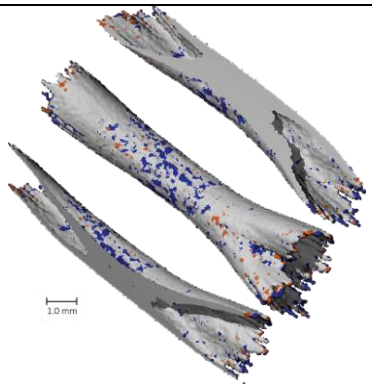
Sample: M02



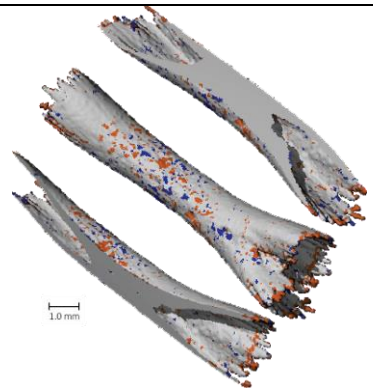
D2 on D0



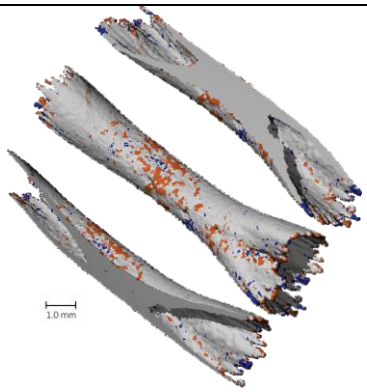
D4 on D2



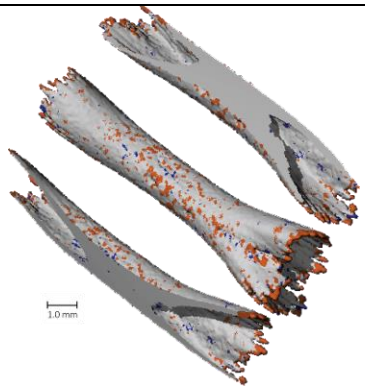
D6 on D4



D8 on D6

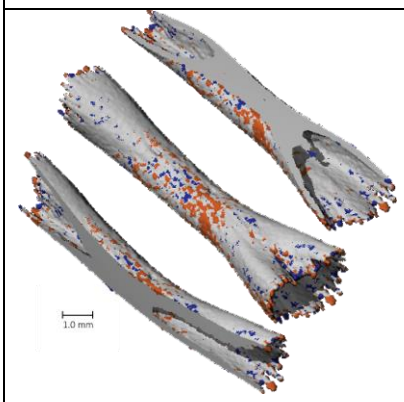


D10 on D8

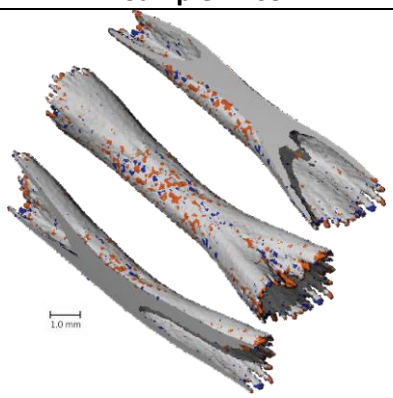


D10 on D0

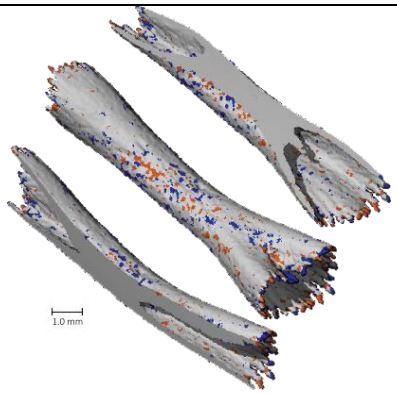
Sample: M03



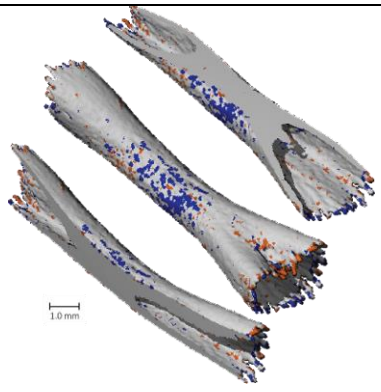
D2 on D0



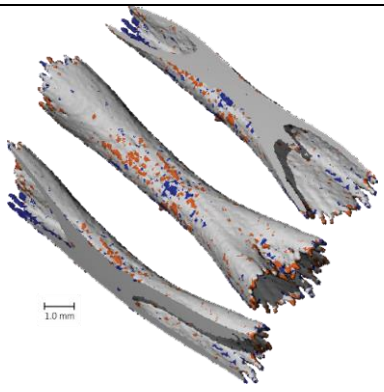
D4 on D2



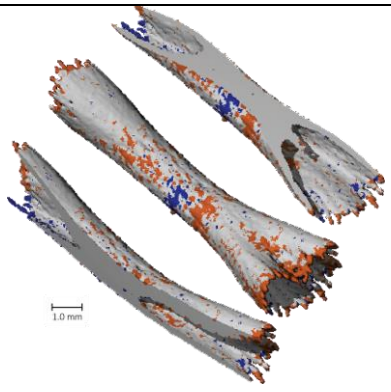
D6 on D4



D8 on D6

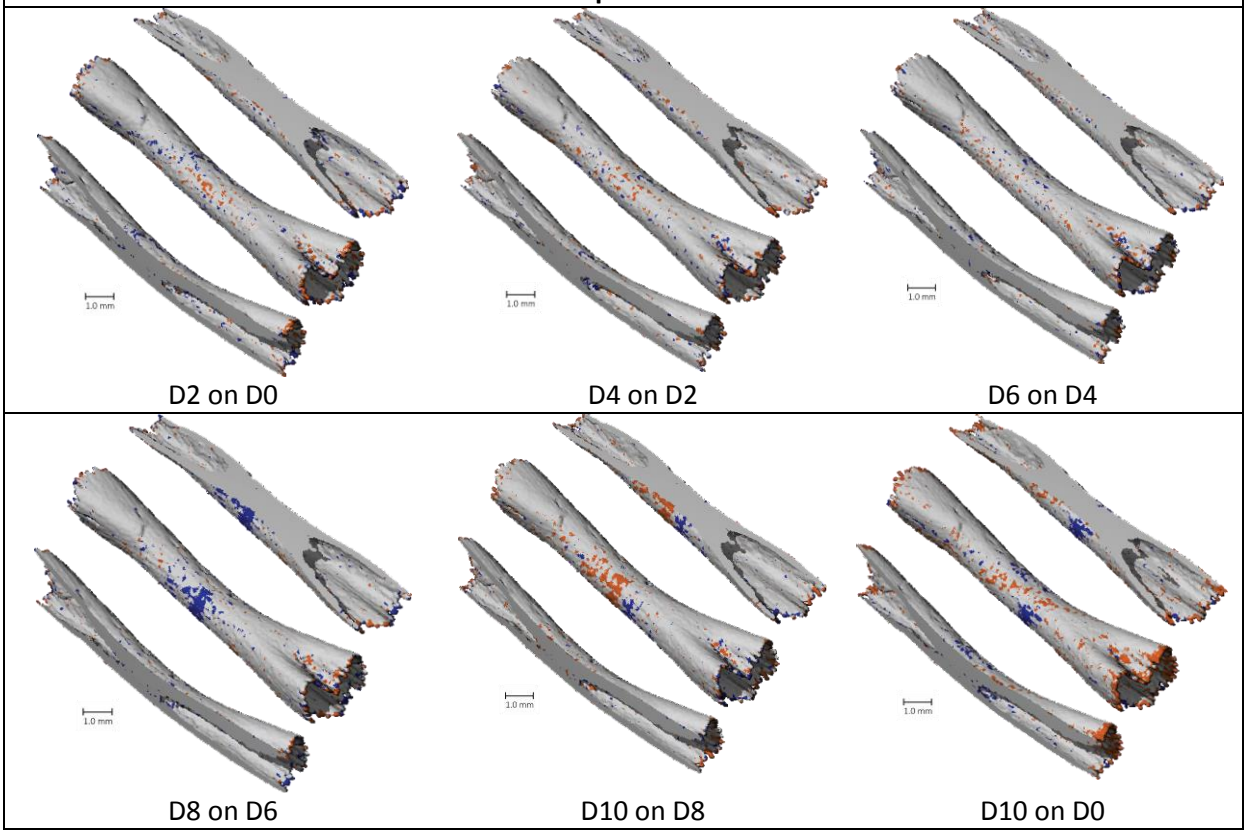


D10 on D8

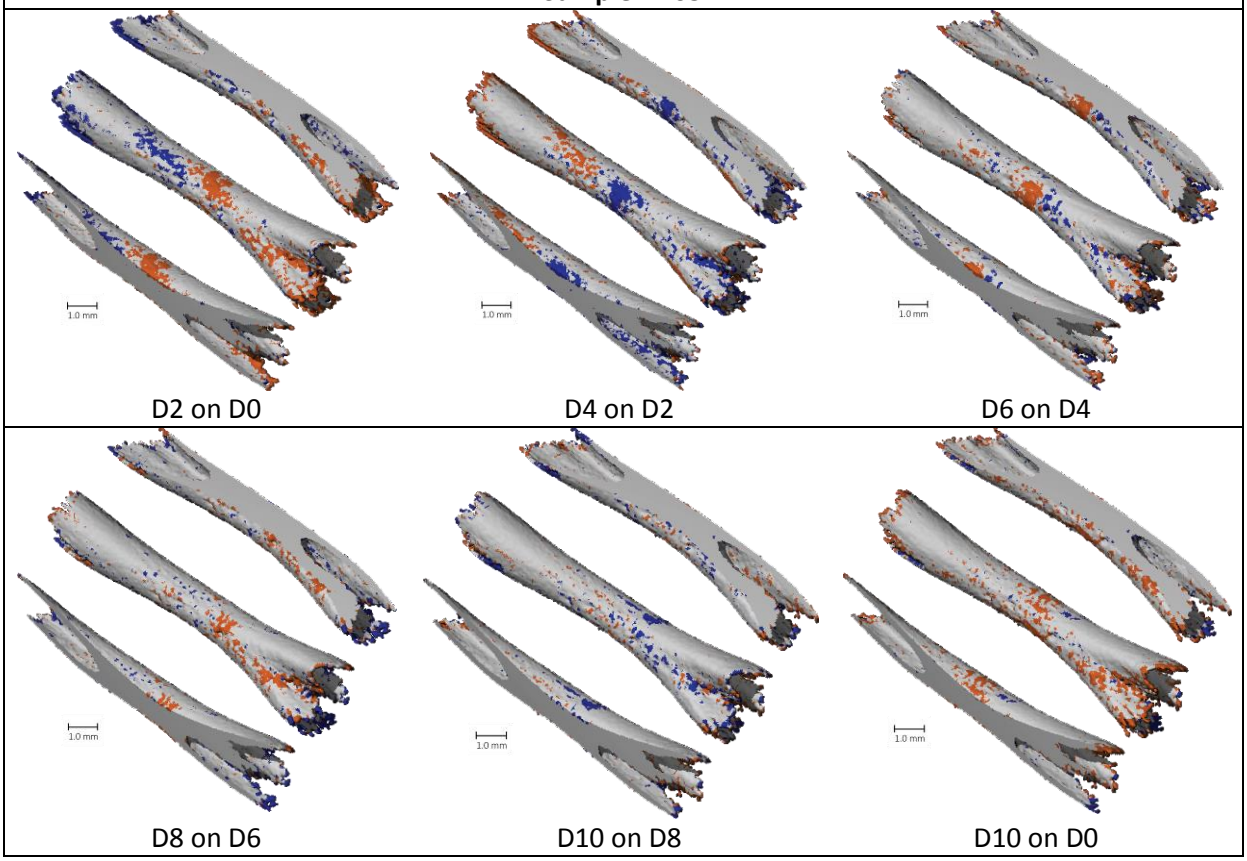


D10 on D0

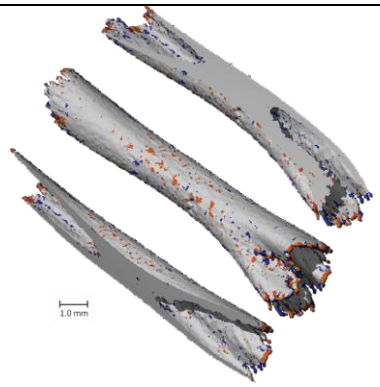
Sample: M04



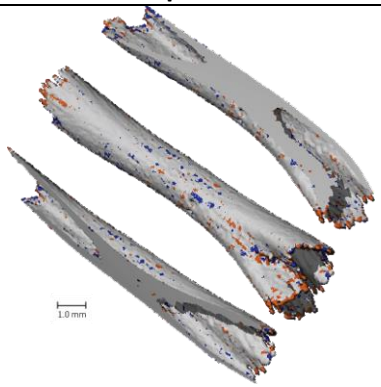
Sample: M05



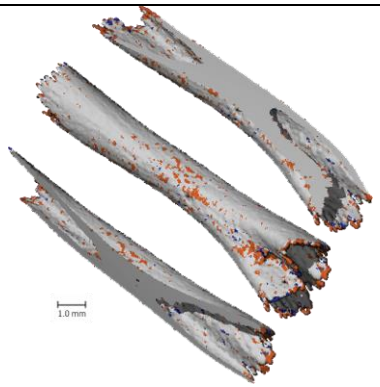
Sample: M06



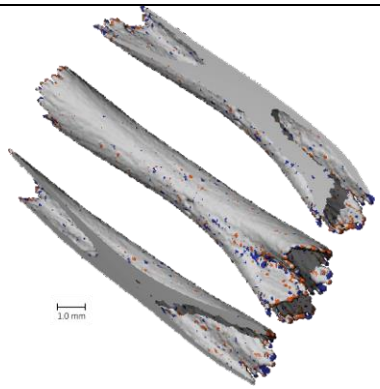
D2 on D0



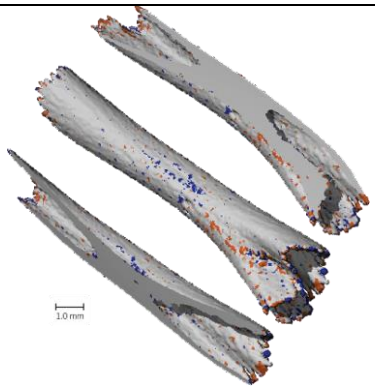
D4 on D2



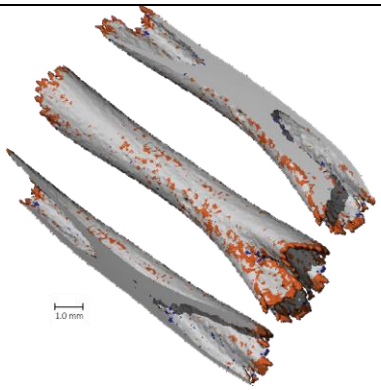
D6 on D4



D8 on D6

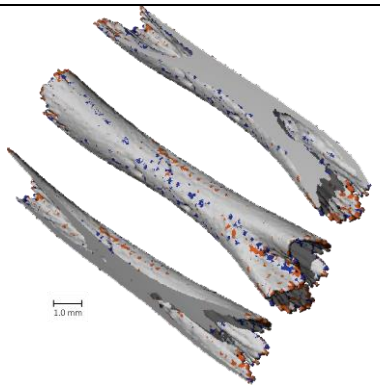


D10 on D8

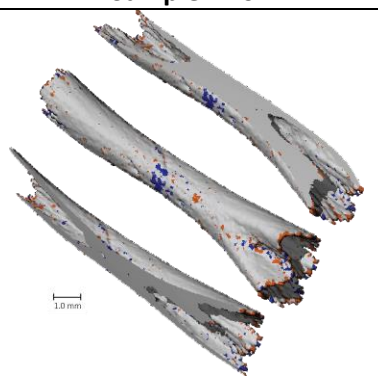


D10 on D0

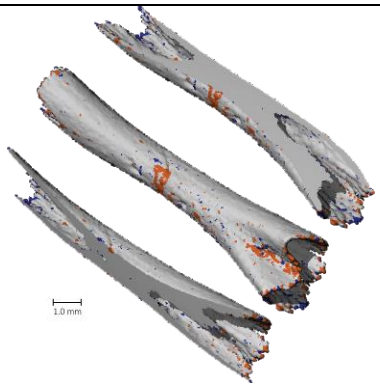
Sample: M07



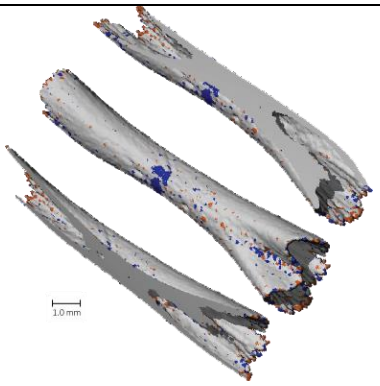
D2 on D0



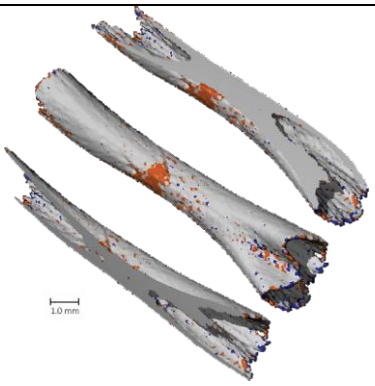
D4 on D2



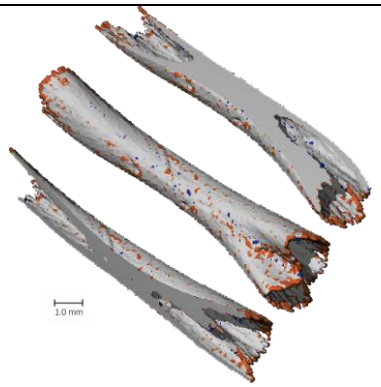
D6 on D4



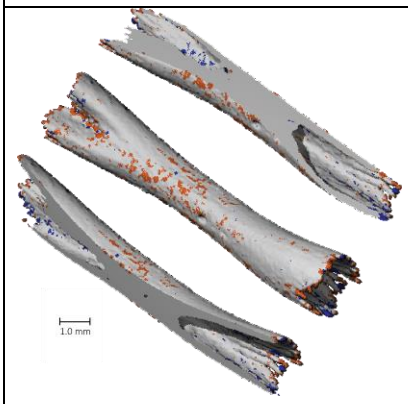
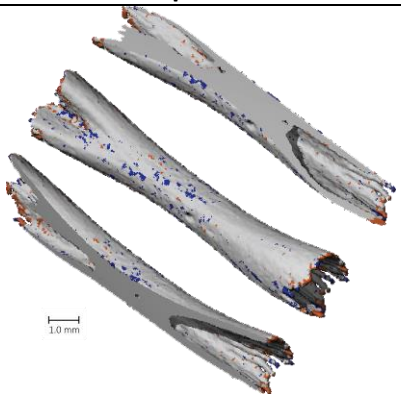
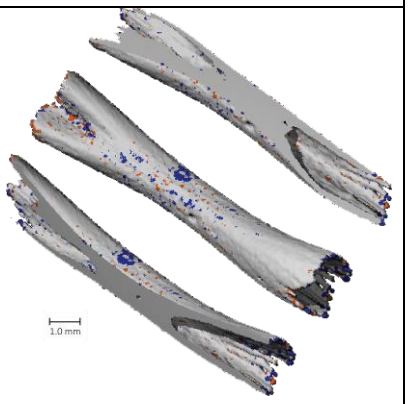
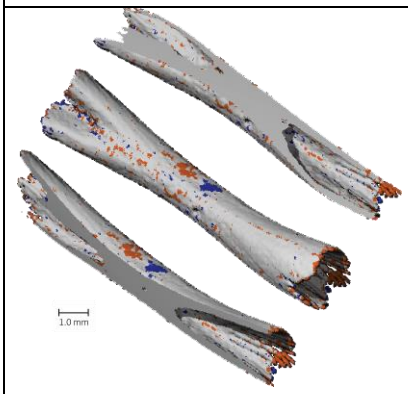
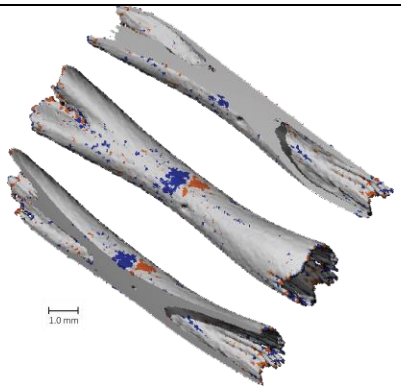
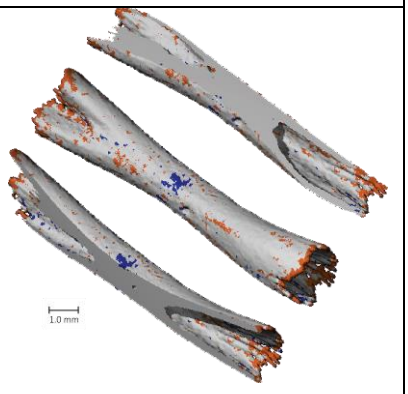
D8 on D6



D10 on D8



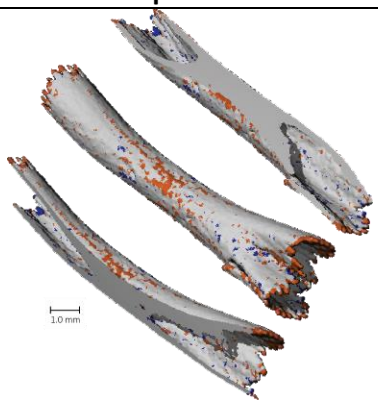
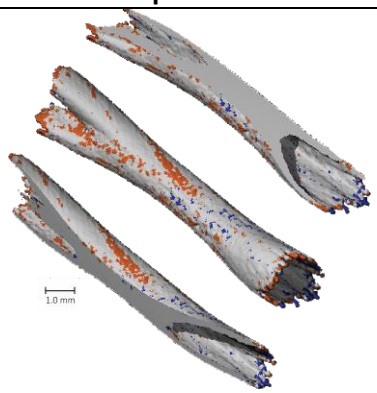
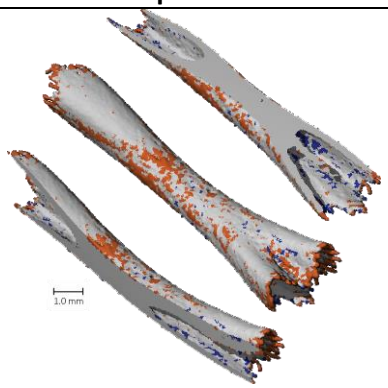
D10 on D0

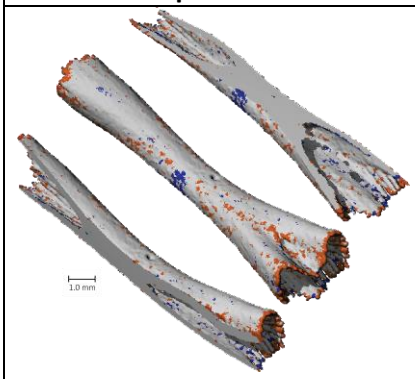
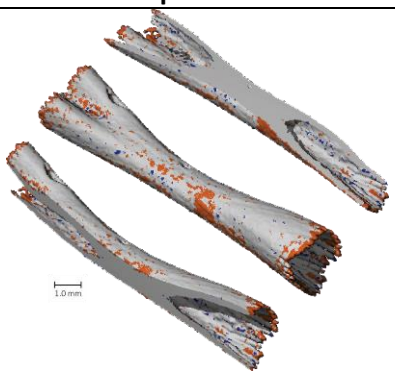
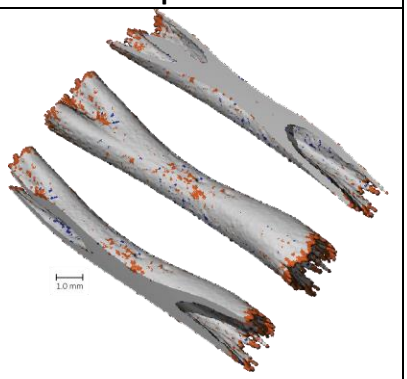
Sample: M08		
		
D2 on D0	D4 on D2	D6 on D4
		
D8 on D6	D10 on D8	D10 on D0

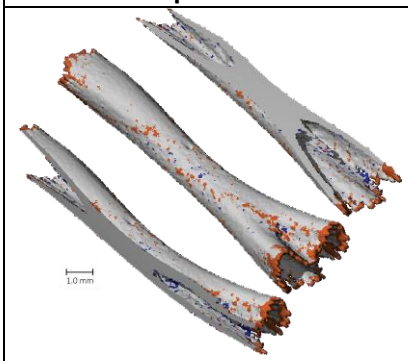
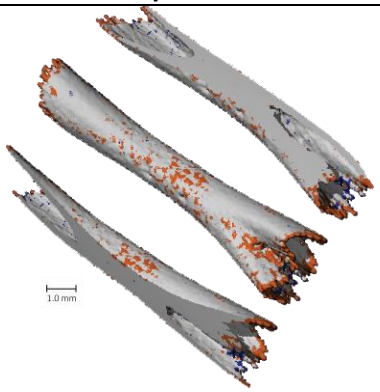
I.2 Group NM

µCT scanned on day 0 and day 10 of culture. (D0 and D10)

- **Formed bone**
- **Resorbed bone**
- **Constant bone**

Sample: NM01	Sample: NM02	Sample: NM03
		
D10 on D0	D10 on D0	D10 on D0

Sample: NM04	Sample: NM05	Sample: NM06
		
D10 on D0	D10 on D0	D10 on D0

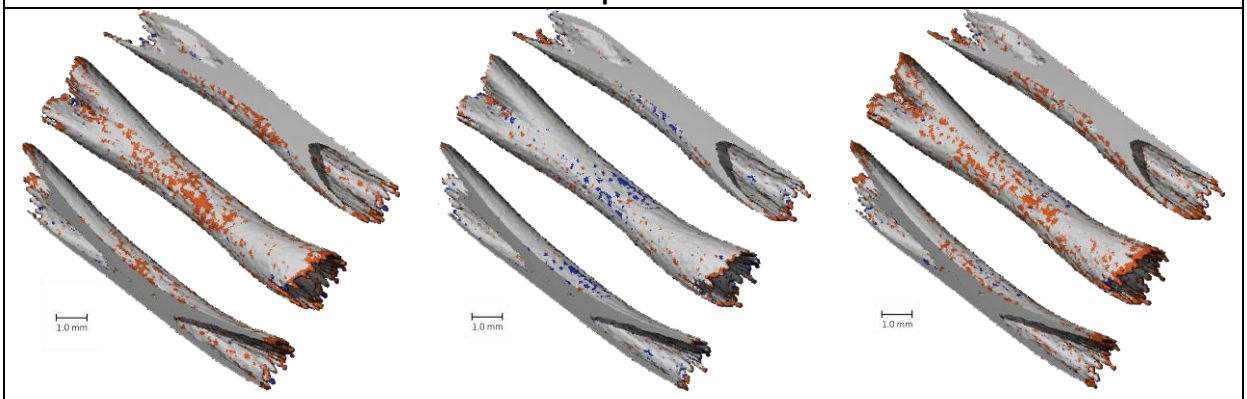
Sample: NM07	Sample: NM08
	
D10 on D0	D10 on D0

I.3 Group L

µCT scnned on day 2, day 16 and day 30 of culture. (D2, D16 and D30)

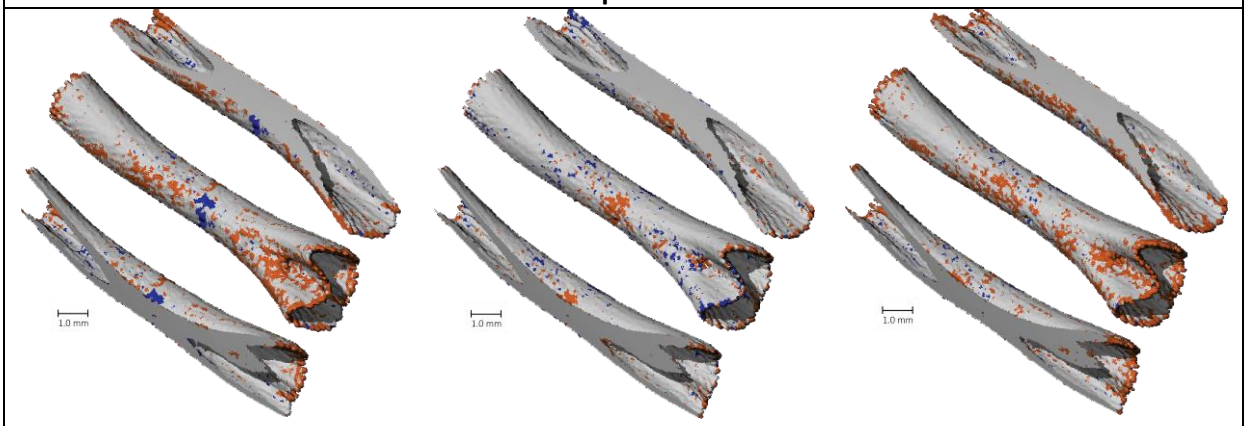
- **Formed bone**
- **Resorbed bone**
- **Constant bone**

Sample: L01



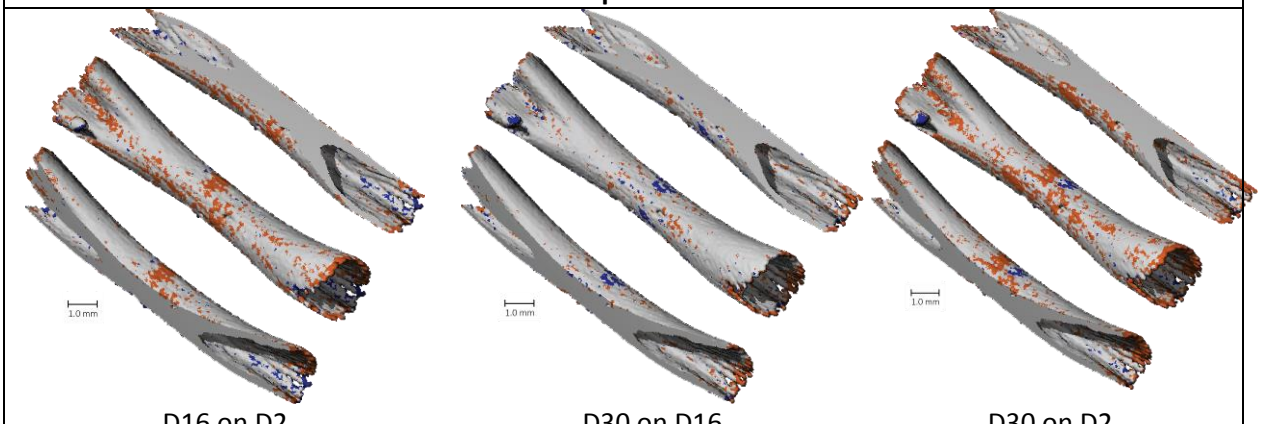
D16 on D2 | D30 on D16 | D30 on D2

Sample: L02



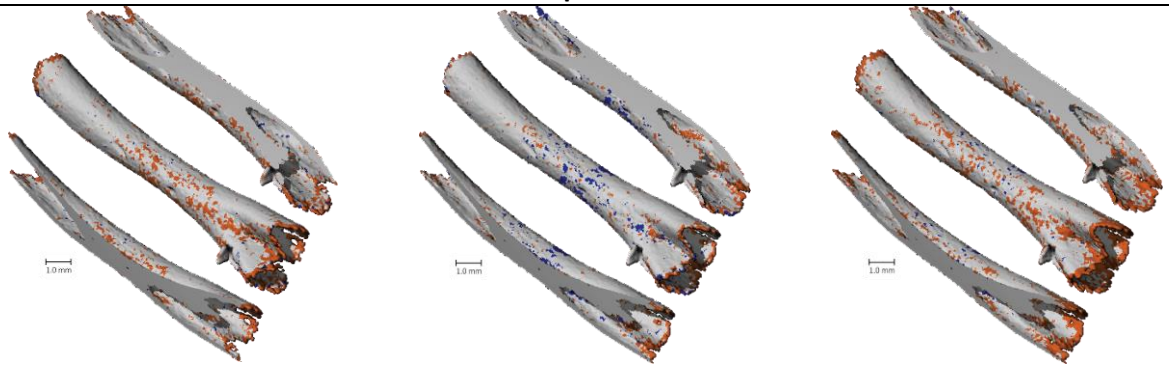
D16 on D2 | D30 on D16 | D30 on D2

Sample: L03



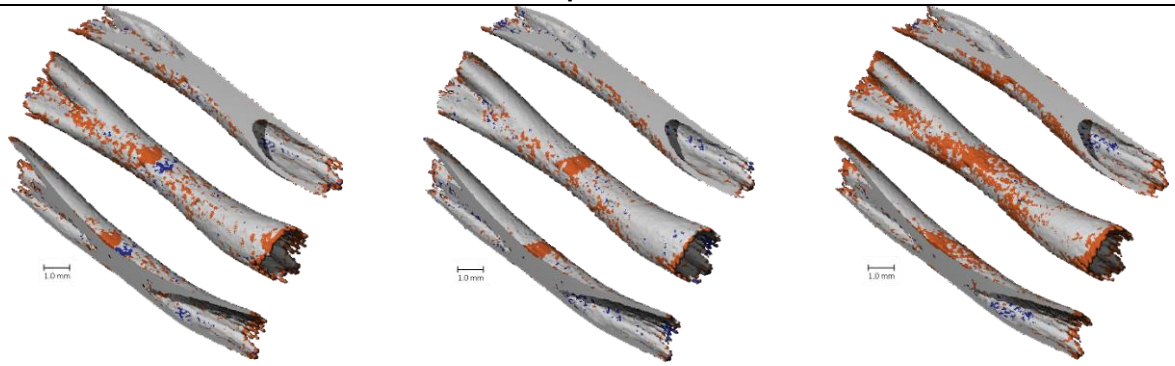
D16 on D2 | D30 on D16 | D30 on D2

Sample: L04



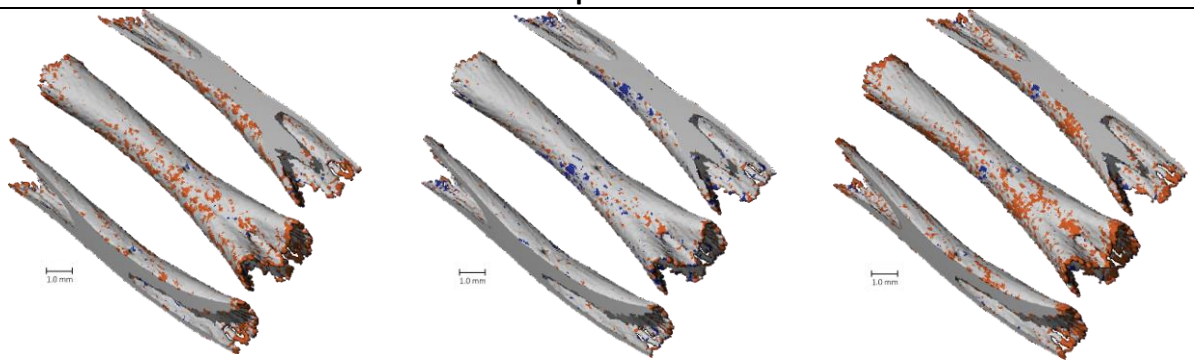
D16 on D2 | D30 on D16 | D30 on D2

Sample: L05



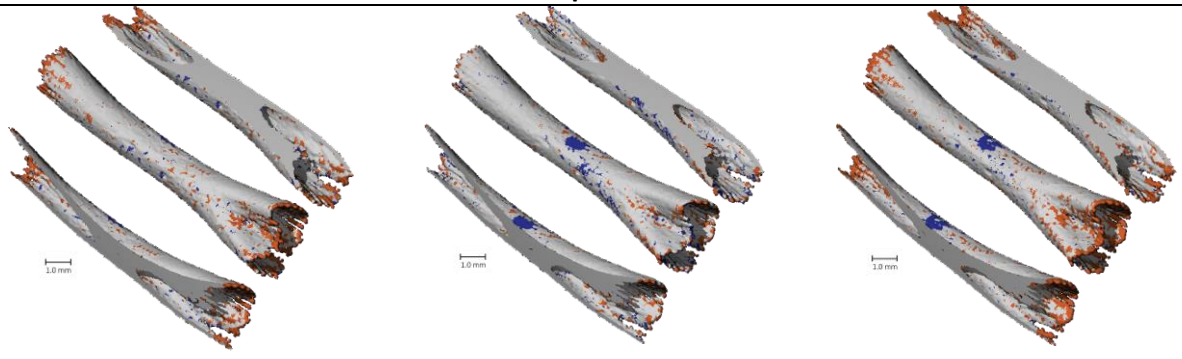
D16 on D2 | D30 on D16 | D30 on D2

Sample: L06



D16 on D2 | D30 on D16 | D30 on D2

Sample: L07



D16 on D2 | D30 on D16 | D30 on D2

APPENDIX II Bone morphometry

Bone morphometry: bone volume (BV), bone surface (BS), bone formation rate (BFR), mineralizing surface (MS), mineral apposition rate (MAR), bone resorption rate (BRR), eroded surface (ES), and mineral resorption rate (MRR).

II.1 Group M

II.1.1 Static bone morphometry

BV [mm³]						
Sample	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
M01	26.981	27.032	28.113	28.648	29.083	28.288
M02	27.126	26.924	27.703	26.977	27.671	28.565
M03	26.136	26.626	27.223	27.271	27.293	27.598
M04	26.653	26.598	26.885	27.153	27.105	27.832
M05	22.377	22.280	22.659	23.083	23.451	23.548
M06	29.784	29.705	29.986	31.552	31.648	31.989
M07	24.539	24.443	24.954	25.360	25.440	26.091
M08	24.066	24.939	24.754	24.659	25.434	25.375
Average	*25.958*	*26.068*	*26.535*	*26.838*	*27.141*	*27.411*
STD	*2.267*	*2.196*	*2.301*	*2.587*	*2.502*	*2.510*

BS [mm²]						
Sample	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
M01	179.396	181.825	184.173	187.577	189.354	187.904
M02	175.816	176.766	180.089	179.649	184.372	184.192
M03	177.407	181.619	184.099	185.390	186.231	186.905
M04	179.498	181.666	183.618	184.165	185.583	186.491
M05	159.273	158.676	164.017	166.724	166.767	168.150
M06	198.773	201.284	206.610	210.148	210.455	212.788
M07	181.166	181.798	185.502	187.860	189.826	189.873
M08	180.188	182.929	183.724	187.029	188.128	187.593
Average	*178.940*	*180.820*	*183.979*	*186.068*	*187.589*	*187.987*
STD	*10.686*	*11.547*	*11.516*	*11.986*	*11.839*	*12.131*

II.1.2 Dynamic bone morphometry

Measurement registration: Day 2 on Day 0						
Sample	BFR [%/d]	MS [%]	MAR [um/d]	BRR [%/d]	ES [%]	MRR [um/d]
M01	1.287%	31.347%	23.671	1.369%	32.742%	22.469
M02	1.117%	30.068%	75.754	1.460%	36.321%	68.472
M03	1.722%	36.496%	25.196	1.237%	31.105%	21.764
M04	1.161%	31.767%	20.703	1.298%	34.969%	20.623
M05	1.956%	34.623%	27.155	2.537%	35.779%	31.028
M06	1.273%	31.492%	23.904	1.530%	37.071%	21.384
M07	1.375%	31.244%	23.030	1.525%	35.707%	20.557
M08	1.903%	40.992%	23.951	1.008%	25.407%	20.627
Average	*1.474%*	*33.503%*	*30.421*	*1.496%*	*33.638%*	*28.366*
STD	*0.336%*	*3.682%*	*18.408*	*0.455%*	*3.866%*	*16.577*

Measurement registration: Day 4 on Day 2						
Sample	BFR [%/d]	MS [%]	MAR [um/d]	BRR [%/d]	ES [%]	MRR [um]
M01	1.915%	42.329%	24.985	0.804%	23.786%	19.692
M02	1.755%	40.426%	62.543	0.888%	26.404%	87.615
M03	1.591%	38.511%	24.100	1.020%	27.362%	21.247
M04	1.315%	35.226%	21.305	1.049%	30.600%	19.610
M05	2.046%	37.879%	28.768	2.046%	35.140%	25.153
M06	1.462%	35.717%	23.627	1.341%	31.992%	22.311
M07	1.517%	36.800%	21.832	1.005%	26.821%	20.424
M08	1.179%	29.797%	23.315	1.499%	35.096%	21.565
Average	*1.598%*	*37.086%*	*28.809*	*1.206%*	*29.650%*	*29.702*
STD	*0.295%*	*3.777%*	*13.819*	*0.409%*	*4.215%*	*23.468*

Measurement registration: Day 6 on Day 4						
Sample	BFR [%/d]	MS [%]	MAR [um/d]	BRR [%/d]	ES [%]	MRR [um]
M01	1.474%	37.782%	22.678	1.066%	27.184%	21.799
M02	0.794%	24.383%	94.328	1.736%	39.936%	64.924
M03	1.383%	34.099%	22.309	1.463%	34.379%	21.666
M04	1.238%	35.509%	20.364	0.989%	30.274%	19.781
M05	1.777%	37.769%	24.163	1.424%	29.996%	22.000
M06	2.312%	47.473%	25.406	0.697%	21.620%	19.671
M07	1.517%	35.811%	22.081	1.126%	29.401%	20.483
M08	1.259%	31.372%	22.630	1.584%	34.843%	22.014
Average	*1.469%*	*35.525%*	*31.745*	*1.261%*	*30.954%*	*26.542*
STD	*0.442%*	*6.507%*	*25.331*	*0.348%*	*5.512%*	*15.539*

Measurement registration: Day 8 on Day 6						
Sample	BFR	MS	MAR (um)	BRR	ES	MRR (um)
M01	1.361%	35.569%	22.923	1.015%	27.934%	20.792
M02	1.617%	39.535%	63.628	1.018%	27.328%	85.546
M03	1.371%	34.237%	22.345	1.506%	33.037%	23.282
M04	1.101%	33.319%	20.450	1.287%	32.984%	21.611
M05	1.837%	37.688%	23.788	1.513%	30.143%	23.873
M06	1.245%	33.744%	21.482	1.181%	32.306%	21.144
M07	1.305%	33.736%	21.252	1.355%	32.310%	21.197
M08	2.113%	41.942%	23.243	1.299%	26.222%	24.179
Average	*1.494%*	*36.221%*	*27.389*	*1.272%*	*30.283%*	*30.203*
STD	*0.338%*	*3.184%*	*14.685*	*0.193%*	*2.773%*	*22.401*

Measurement registration: Day 10 on Day 8						
Sample	BFR	MS	MAR (um)	BRR	ES	MRR (um)
M01	0.931%	25.884%	21.697	1.917%	41.635%	22.890
M02	1.770%	42.335%	59.531	0.886%	23.699%	90.942
M03	1.573%	37.275%	23.116	1.478%	29.861%	25.767
M04	1.540%	40.917%	21.493	0.783%	24.672%	20.121
M05	1.511%	34.630%	23.013	1.548%	31.757%	24.316
M06	1.425%	37.485%	21.998	1.199%	30.003%	21.831
M07	1.749%	39.455%	22.747	1.032%	26.397%	20.507
M08	1.278%	32.049%	21.641	1.440%	32.823%	21.030
Average	*1.472%*	*36.254%*	*26.904*	*1.285%*	*30.106%*	*30.926*
STD	*0.271%*	*5.337%*	*13.199*	*0.380%*	*5.700%*	*24.327*

Measurement registration: Day 10 on Day 0						
Sample	BFR	MS	MAR (um)	BRR	ES	MRR (um)
M01	0.457%	44.801%	5.830	0.199%	24.976%	4.197
M02	0.413%	46.158%	11.429	0.130%	21.705%	20.535
M03	0.506%	47.565%	5.701	0.237%	24.579%	5.117
M04	0.420%	45.631%	4.965	0.196%	25.325%	4.219
M05	0.447%	45.087%	5.283	0.196%	23.552%	4.392
M06	0.529%	50.330%	5.985	0.136%	20.564%	3.987
M07	0.496%	47.085%	5.506	0.159%	22.095%	3.809
M08	0.450%	44.608%	5.508	0.194%	23.399%	4.130
Average	*0.465%*	*46.408%*	*6.276*	*0.181%*	*23.274%*	*6.298*
STD	*0.041%*	*1.906%*	*2.106*	*0.036%*	*1.694%*	*5.766*

II.2 Group NM

II.2.1 Static bone morphometry

	BV [mm³]		BS [mm²]	
Sample	Day 0	Day 10	Day 0	Day 10
NM01	23.782	24.801	175.36	183.48
NM02	23.944	24.788	173.61	178.64
NM03	22.889	24.354	163.10	170.55
NM04	27.912	29.163	202.79	210.62
NM05	26.416	28.300	201.52	213.44
NM06	27.126	28.868	179.72	191.42
NM07	26.910	28.449	179.81	191.48
NM08	22.296	24.015	153.68	163.93
Average	*25.159*	*26.592*	*178.70*	*187.94*
STD	*2.165*	*2.276*	*16.94*	*17.62*

II.2.2 Dynamic bone morphometry

Measurement registration: Day 10 on Day 0						
Sample	BFR	MS	MAR (um)	BRR	ES	MRR (um)
NM01	0.394%	42.259%	5.233	0.140%	26.738%	2.876
NM02	0.439%	42.089%	5.125	0.177%	28.780%	3.089
NM03	0.530%	47.353%	5.633	0.149%	24.463%	2.903
NM04	0.454%	43.898%	5.289	0.160%	27.333%	2.827
NM05	0.549%	47.597%	5.492	0.120%	23.053%	2.755
NM06	0.452%	46.820%	5.787	0.110%	22.929%	2.689
NM07	0.404%	45.243%	5.840	0.117%	22.723%	2.897
NM08	0.513%	50.174%	5.571	0.089%	18.977%	2.760
AVE	*0.467%*	*45.679%*	*5.496*	*0.133%*	*24.375%*	*2.849*
STDV	*0.058%*	*2.827%*	*0.261*	*0.029%*	*3.150%*	*0.123*

II.3 Group L

II.3.1 Static bone morphometry

BV [mm³]			
Sample	Week 0	Week 2	Week 4
L01	22.494	24.604	24.972
L02	27.347	29.655	30.097
L03	28.669	30.751	31.637
L04	26.055	28.297	29.321
L05	25.897	28.311	29.691
L06	23.747	26.113	26.815
L07	24.399	26.322	26.732
Average	*25.515*	*27.722*	*28.466*
STD	*2.131*	*2.155*	*2.341*

BS [mm²]			
Sample	Week 0	Week 2	Week 4
L01	160.613	167.863	172.755
L02	202.816	212.284	213.129
L03	199.590	207.378	209.435
L04	201.654	213.797	217.250
L05	202.485	214.038	218.398
L06	189.443	199.602	202.928
L07	189.008	200.376	203.045
Average	*192.230*	*202.191*	*205.277*
STD	*15.155*	*16.290*	*15.613*

II.3.2 Dynamic bone morphometry

Measurement registration: week 2 on week 0						
Sample	BFR [%/d]	MS [%]	MAR [um/d]	BRR [%/d]	ES [%]	MRR [um/d]
L01	0.466%	58.001%	3.670	0.072%	15.084%	2.851
L02	0.453%	52.691%	3.951	0.119%	19.981%	2.916
L03	0.393%	52.379%	3.603	0.095%	19.661%	2.791
L04	0.432%	51.943%	3.901	0.086%	17.174%	2.787
L05	0.509%	54.634%	3.934	0.111%	17.296%	3.180
L06	0.510%	54.937%	3.845	0.083%	15.300%	2.728
L07	0.432%	48.345%	4.146	0.123%	21.410%	2.807
AVE	*0.456%*	*53.276%*	*3.864*	*0.098%*	*17.987%*	*2.866*
STDV	*0.043%*	*3.005%*	*0.183*	*0.019%*	*2.425%*	*0.150*

Measurement registration: week 4 on week 2						
Sample	BFR [%/d]	MS [%]	MAR [um/d]	BRR [%/d]	ES [%]	MRR [um/d]
L01	0.198%	36.409%	3.130	0.152%	29.762%	2.882
L02	0.236%	38.423%	3.124	0.172%	29.609%	3.052
L03	0.240%	43.074%	3.103	0.127%	24.561%	2.974
L04	0.317%	41.992%	3.547	0.158%	25.875%	3.172
L05	0.350%	43.938%	3.675	0.145%	24.187%	2.967
L06	0.277%	40.817%	3.226	0.159%	25.437%	2.987
L07	0.250%	37.283%	3.146	0.202%	30.338%	3.061
AVE	***0.267%***	***40.276%***	***3.279***	***0.159%***	***27.110%***	***3.014***
STDV	***0.052%***	***2.939%***	***0.233***	***0.023%***	***2.679%***	***0.092***

Measurement registration: week 4 on week 0						
Sample	BFR [%/d]	MS [%]	MAR [um/d]	BRR [%/d]	ES [%]	MRR [um/d]
L01	0.247%	60.860%	1.983	0.030%	13.292%	1.351
L02	0.247%	57.030%	2.005	0.079%	15.762%	1.356
L03	0.247%	61.078%	1.948	0.066%	13.730%	1.477
L04	0.299%	60.165%	2.218	0.073%	13.572%	1.508
L05	0.345%	63.523%	2.199	0.068%	12.524%	1.387
L06	0.315%	62.252%	2.034	0.066%	11.927%	1.410
L07	0.248%	51.740%	2.227	0.124%	20.065%	1.467
AVE	***0.278%***	***59.521%***	***2.088***	***0.072%***	***14.410%***	***1.422***
STDV	***0.041%***	***3.976%***	***0.122***	***0.028%***	***2.767%***	***0.062***

APPENDIX III Procedures for data analysis (VMS system)

1. Rotation

Rotate the chick femur to the vertical position on the 2-dimensional μCT images

a) Start “uct_evaluation” with consecutive 2-dimensional images

b) Angle tool: decide a rotating angle to the vertical position on the screen

c) Start “ibt_project”: register a new angle, reconstruct the new angle

2. Create AIM file to project disc (ur)

a) Start “uct_evaluation”

b) Contouring tool: covering all bone volume

c) “Start evaluation”: AIM and GOBJ files in measurement disc (ud)

d) Segmentation settings (follow the manual for serial measurements)

e) Start “ibt_project”: transfer aim file to project disc (ur)

f) Check the files in project disc

3. Thresholding for bone tissue

a) Start “viz2d”: choose a grey value for bone tissue by eye

b) Change all the command data to the value of the threshold (e.g. MEAS1, MEAS2, …)

4. Registration the measurements of two time points

a) Start “ibt_project

b) Command “MEAS1” → “MEAS2” and so on (follow the manual for serial measurements)

c) Change all the name of “thrcl.aim” files created from the previous step to “ufilt.aim” in project disc (ur), and delete other files or put them to other documents.

d) Run again the step b)

5. The images of constructed bone tissue with formed and resorbed bone shown

a) Start “uct_3d”: choose the files with ending “_m1m2_cl_tri.aim”

b) Choose color for: formed bone in orange, resorbed bone in blue and background in white

6. Get the data for calculating bone morphometry

a) Start “ibt_project

b) Command “OMASK”: choose the files with ending “_thres

c) Command “QUANT”: when the registered measurement of m2 on m1, for example, the ending change to “m1m2”, “12” and m2 on m1, and the mask change to “_m1_f_temp3”

7. Calculation for bone morphometry

Download the csv files and follow the manual of serial measurements to get BV, BS, BFR, BRR, MS, ES, MAR and MRR.