

Quantification of Bone Histomorphometric Parameters Using the Weibel Technique in Animals

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ABSTRAK

Pengukuran histomorfometri tulang diperlukan untuk memahami kesan penyakit tulang dan juga keberkesanan rawatan pada metabolisme tulang dalam satu tempoh masa. Kajian sebelum ini menggunakan teknik Weibel sebagai kaedah stereologikal kuantitatif untuk menentukan perubahan sel dan dinamik tulang. Walau bagaimanapun, tidak ada penerangan tentang bagaimana teknik ini telah digunakan. Kajian ini bertujuan untuk memberikan gambaran lengkap tentang penggunaan teknik Weibel untuk mengukur indeks histomorfometri tulang statik dan dinamik. Kepakaran teknikal, pemprosesan optimum sampel tulang, perawakan hirisan tisu trabekular dan jumlah imej teranalisa yang mencukupi untuk setiap bahagian adalah diperlukan untuk mencapai keputusan yang boleh dipercayai dengan kemungkinan ralat yang rendah.

Kata kunci: histomorfometri tulang, pembentukan semula tulang, teknik Weibel

ABSTRACT

Bone histomorphometric measurements are required to understand the efficacy of treatment on bone remodelling. Previous studies used the Weibel technique as a quantitative stereological method to determine bone cellular and dynamic changes. However, there was no description on how this technique was applied. This study aimed to provide a full picture about the utilization of the Weibel technique to measure static and dynamic bone histomorphometric indices. Technical expertise, processing of bone samples, randomization of the trabecular sections and an adequate number of analysed images for each section are required

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to achieve reliable results with a low possibility of errors.

Keywords: bone histomorphometry, bone remodelling, weibel technique

Bone is a unique dynamic tissue whereby its components undergo continuous resorption and re-deposition by a process known as bone remodelling. Bone remodelling is a lifelong process which starts from the very earliest foetal bone formation and continues until death. In brief, this process involves the breakdown of mature bone (bone resorption) by osteoclasts and the activation of osteoblasts to build new bones (bone formation). Therefore, bone remodelling is a result of osteoclasts and osteoblasts activities (Abdul-Majeed et al. 2013). Bone resorption and formation are balanced in a homeostatic equilibrium to continuously replace old bones by new ones and to guarantee that there is no net loss or gain of bone; this coordinated balance is called coupling. The primary function of bone remodelling is to maintain bone health. However, uncoupling leads to unbalanced bone remodelling process and results in serious bone diseases like osteoporosis (Abdul-Majeed et al. 2015).

Bone histomorphometry is a quantitative method to obtain information on the process of bone remodelling at the level of the basic multicellular units. Static bone histomorphometry is used to examine bone histology and quantitatively evaluate the activity of bone cells at a specific time. Bone is a dynamic organ. Therefore, dynamic bone

histomorphometry is the standard method for evaluating changes in cellular activity and bone remodelling over time (Vedi & Compston 2003; Kulak & Dempster 2010).

The Weibel technique is a quantitative stereological method for morphometric cytology and utilizes the M42 grid which is also known as a multipurpose test-system. Moreover, it can generate quantitative data on the internal structure of cell, tissue and organ (Weibel et al. 1966; Freere & Weibel 1967).

Several preclinical studies had used the Weibel technique to determine static and dynamic bone histomorphometric parameters in intact rat and osteoporotic rats. Moreover, these studies used this technique to investigate the efficacy of antiosteoporotic therapies (Nazrun et al. 2005; Shuid et al. 2007; Hermizi et al. 2009; Mehat et al. 2010; Hermizi et al. 2011; Aktifanus et al. 2012; Fathilah et al. 2012; Hussan et al. 2012; Muhammad et al. 2012; Soelaiman et al. 2012; Abdul-Majeed et al. 2012; Chin et al. 2014; Parvaneh et al. 2015). These studies have referred to (Weibel et al. 1966; Freere & Weibel 1967) in performing the protocol without a detailed description on how did they evaluate the parameters. Therefore, the aim of this paper is to provide a solid reference for those who will be doing the same analysis.

In this present paper, we describe the utilization of the Weibel

technique to measure static and bone histomorphometric indices. Processing of decalcified and undecalcified bone sample is also discussed. In this brief technical report, the trabecular bone of rat femora was used to describe the technique. However, this technical report may be applied to other types of bone.

PROCESSING OF BONE SAMPLES

Three adult *Sprague Dawley* rats were purchased from the Laboratory Animal Research Unit, Universiti Kebangsaan Malaysia. The rats were kept two per cage under 12 hrs light-dark cycles. The rats were fed commercial rat chow and tap water ad libitum. After one week of acclimatization, the rats were sacrificed and the left femur of each rat was dissected out and cleaned of soft tissue. It was fixed in 70 % alcohol for at least 72 hrs and the distal third was divided sagittally using bone cutting tools with a low-speed saw. The first half was decalcified and stained with haematoxylin and eosin for static bone histomorphometric analysis while the second half was processed for dynamic histomorphometric analysis (Figure 1-4). The study protocol was obtained from the UKM Animal Ethics Committee, (PP/FAR/2011/IMA/27-JANUARY/352-JANUARY-2011–DECEMBER-2012).

PRINCIPLES AND GENERAL CONSIDERATION OF WEIBEL TECHNIQUE

The stereological test system is composed of line system (21 lines) and

point system (42 points) standards which are the basis for applying the principles of stereology. The line system represents the surface measurements while the point system reflects the volume measurement (Figure 5). The M42 grid was adjusted on the captured image and the parameters were calculated based on the Freere & Weibel (1967) formulas. The computed values were obtained in ratio or percentage.

i) The relative surface area (S_a) was calculated as follows:

$$S_a = 2N/Lt$$

N =number of interactions between the test line and the surface of interest

Lt =total number of the lines (21)

ii) The relative volume (V) was calculated as follows:

$$V = P/Pt$$

P =number of interactions between the points and the surface of interest

Pt =total number of points (42)

QUANTIFICATION OF BONE CELLULAR HISTOMORPHOMETRY USING WEIBEL TECHNIQUE

Bone static histomorphometric parameters that were measured using the Weibel technique are osteoblast surface/bone surface (ObS/BS %), osteoclast surface / bone surface (OcS/BS %), eroded surface/bone surface (ES/BS %), osteoid surface/bone surface (OS/BS %) and osteoid volume/bone volume (OV/BV %) (Figure 6-7; Table 1).

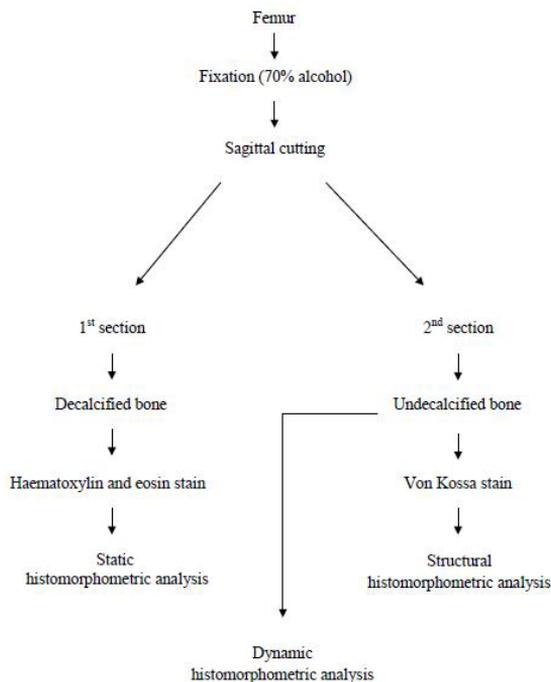


Figure 1: General protocol for bone histomorphometric analysis.

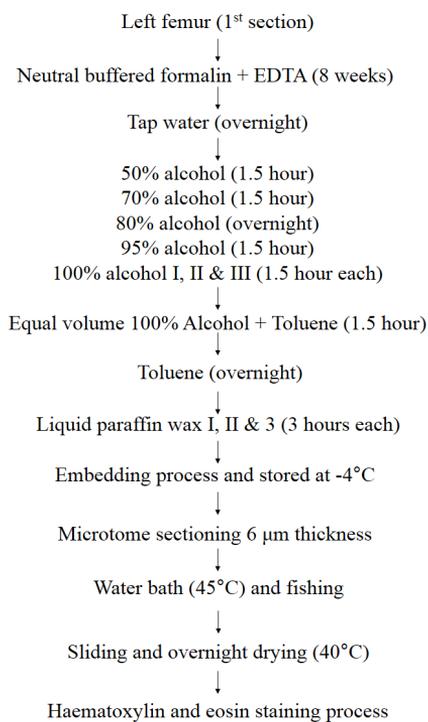


Figure 2: Processing of decalcified bone samples

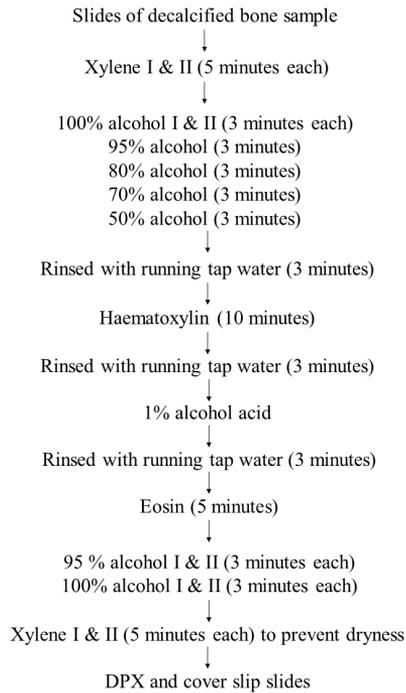


Figure 3: Haematoxylin and eosin staining protocol

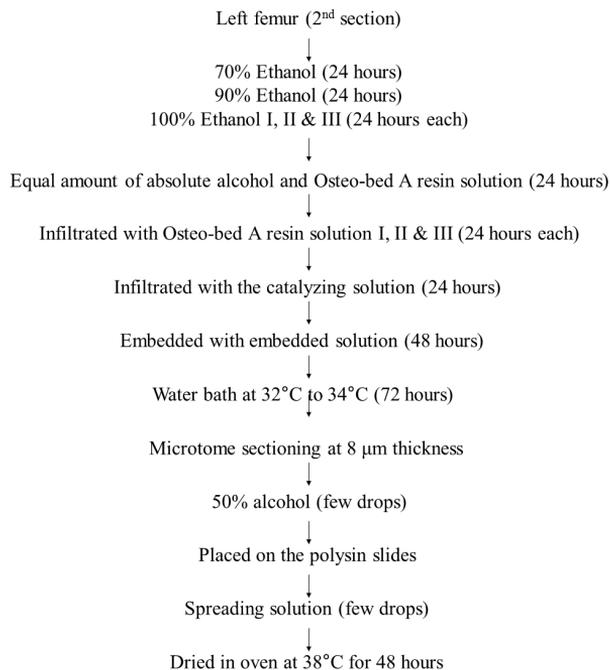


Figure 4: Processing of undecalcified bone samples

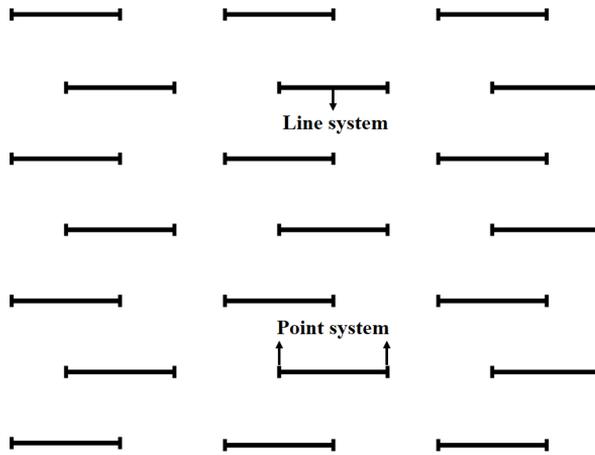


Figure 5: The Weibel grid composed of line system (21 lines) and point system (42 points)

i) Osteoblast Surface / Bone Surface (ObS/BS)

$$\begin{aligned}
 ObS/BS &= \frac{2NLOb/Lt \text{ (osteoblast)}}{2NLTb/Lt \text{ (trabecularbone)}} \\
 &= \frac{\sum NLOb/Lt \text{ (osteoblast)}}{\sum NLTb/Lt \text{ (trabecularbone)}} \\
 &= \frac{NLOb \text{ (osteoblast)}}{NLTb \text{ (trabecularbone)}}
 \end{aligned}$$

NLOb=number of interactions between test lines and osteoblast cells
 NLTb=number of interactions between test lines and trabecular bones

ii)Osteoclast Surface/Bone Surface (OcS/BS)

$$\begin{aligned}
 OcS/BS &= \frac{2NLOc/Lt \text{ (osteoclast)}}{2NLTb/Lt \text{ (trabecularbone)}} \\
 &= \frac{\sum NLOc/Lt \text{ (osteoclast)}}{\sum NLTb/Lt \text{ (trabecularbone)}} \\
 &= \frac{NLOc \text{ (osteoclast)}}{NLTb \text{ (trabecularbone)}}
 \end{aligned}$$

NLOc=number of interactions between test lines and osteoclast cells

NLTb=number of interactions between test lines and trabecular bones

iii) Eroded Surface / Bone Surface (ES/BS)

$$\begin{aligned}
 ES/BS &= \frac{2NLES/Lt \text{ (erodedsurface)}}{2NLTb/Lt \text{ (trabecularbone)}} \\
 &= \frac{\sum NLES/Lt \text{ (erodedsurface)}}{\sum NLTb/Lt \text{ (trabecularbone)}} \\
 &= \frac{NLES \text{ (erodedsurface)}}{NLTb \text{ (trabecularbone)}}
 \end{aligned}$$

NLES=number of interactions between test lines and eroded surfaces
 NLTb=number of interactions between test lines and trabecular bones

iv) Osteoid Surface/Bone Surface (OS/BS)

$$\begin{aligned}
 OS/BS &= \frac{2NLO/Lt \text{ (osteoid)}}{2NLTb/Lt \text{ (trabecularbone)}} \\
 &= \frac{\sum NLO/Lt \text{ (osteoid)}}{\sum NLTb/Lt \text{ (trabecularbone)}} \\
 &= \frac{NLO \text{ (osteoid)}}{NLTb \text{ (trabecularbone)}}
 \end{aligned}$$

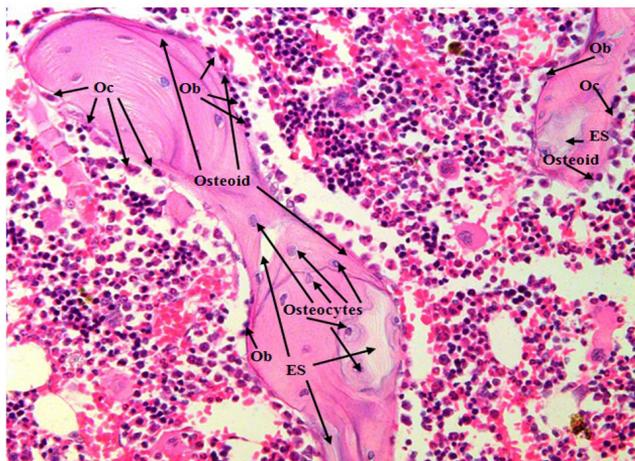


Figure 6: Micrographic photos of decalcified trabecular bone stained with haematoxylin and eosin (20x magnification) demonstrating cellular bone histomorphometric parameters. Oc, osteoclast; Ob, osteoblast; and ES, eroded surfaces.

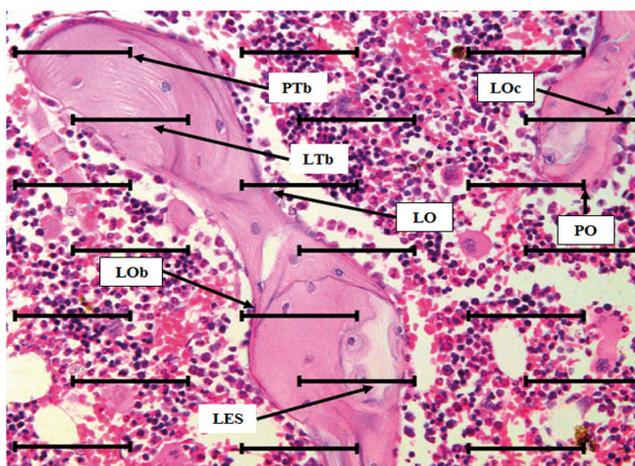


Figure 7: Sample of trabecular section stained with haematoxylin and eosin (20x magnification) superimposed with multipurpose test-system (M42 grid). LTb, area of interaction between trabeculae and the test line; PTb, area of interaction between trabeculae and the test point; LOb, area of interaction between osteoblast cell and test line; LOc, area of interaction between osteoclast cell and test line; LES, area of interaction between eroded surface and test line; LO, area of interaction between osteoid and test line; PO, area of interaction between osteoid and test point.

Table 1: Description of bone static histomorphometric parameters

Parameter	Symbol	Unit
Osteoblast surface / bone surface	Obs / BS	%
Osteoclast surface / bone surface	OcS / BS	%
Eroded surface / bone surface	ES / BS	%
Osteoid surface / bone surface	OS / BS	%
Osteoid volume / bone volume	OV / BV	%

NLO=number of interactions between test lines and osteoids

NLTb=number of interactions between test lines and trabecular bones

v) Osteoid Volume/Bone Volume (OV/BV)

$$\begin{aligned}
 OV/BV &= \frac{2NPO/Pt \text{ (osteoid)}}{2NPTb/Pt \text{ (trabecularbone)}} \\
 &= \frac{2NPO/Pt \text{ (osteoid)}}{2NPTb/Pt \text{ (trabecularbone)}} \\
 &= \frac{NPO \text{ (osteoid)}}{Pt \text{ (trabecularbone)}}
 \end{aligned}$$

NPO=number of interactions between points and osteoid

NPTb=number of interactions between points and trabecular bones

QUANTIFICATION OF BONE CELLULAR HISTOMORPHOMETRY USING WEIBEL TECHNIQUE

In order to quantify the dynamic histomorphometric parameters the femurs need to be fluorescent-labelled with intraperitoneal injections of 20 mg/kg calcein at day 9 and day 2 before the rats were sacrificed.

Bone dynamic histomorphometric parameters that were used as example are single-labelled surface/bone surface (sLS/BS %), double-labelled surface/bone surface (dLS/BS %), mineralizing surface/bone surface (MS/BS %), mineral apposition rate (MAR $\mu\text{m}/\text{day}$) and bone formation rate (BFR $\mu\text{m}^3/\mu\text{m}^2/\text{day}$) (Figure 8, Table 2). Only sLS/BS % and dLS/BS % will be calculated by using the M42 grid while the rest of the parameters will be

derived (Figure 9).

Single-labelled surface is formed after the first calcein injection (at day 9 before sacrifice) while the double-labelled surface is formed after the second calcein injection (at day 2 before sacrifice). Double-labelled surface is darker than single-labelled surface. In general, sLS/BS parameter corresponds to poor bone formation while dLS/BS, MS/BS and MAR correspond to bone formation and mineralization.

i) Single-labelled surface/bone surface (sLS/BS)

$$\begin{aligned}
 sLS/BS &= \frac{2NLsLS/Lt \text{ (single-labelled surface)}}{2NLTb/Lt \text{ (trabecular bone)}} \\
 &= \frac{2NLsLS/Lt \text{ (single-labelled surface)}}{2NLTb/Lt \text{ (trabecular bone)}} \\
 &= \frac{NLsLS \text{ (single-labelled surface)}}{NLTb \text{ (trabecular bone)}}
 \end{aligned}$$

NLsLS=number of interactions between test lines and single-labelled surfaces

NLTb=number of interactions between test lines and trabecular bones

ii) Double-labelled surface/Bone Surface (dLS/BS)

$$\begin{aligned}
 dLS/BS &= \frac{2NLdLS/Lt \text{ (double-labelled surface)}}{2NLTb/Lt \text{ (trabecularbone)}} \\
 &= \frac{2NLdLS/Lt \text{ (double-labelled surface)}}{2NLTb/Lt \text{ (trabecularbone)}} \\
 &= \frac{NLdLS \text{ (double-labelled surface)}}{NLTb \text{ (trabecularbone)}}
 \end{aligned}$$

NLdLS=number of interactions between test lines and double-labeled surfaces

NLTb=number of interactions between test lines and trabecular bones

iii) Mineralizing Surface / Bone Surface (MS / BS)

$$MS/BS = (sLS + \frac{1}{2}dLS)/BS$$

iv) Mineral Apposition Rate (MAR)

$$MAR=X/Y$$

X=the distance between the two labels

Y=days between the two calcein injections (7 days)

v) Bone Formation Rate/Bone Surface (BFR/BS)

$$BFR/BS=(MS/BS)*MAR$$

MS/BS=mineralizing surface

MAR=mineral apposition rate

A vast amount of literature showed that the use of the Weibel technique is a very effective stereological test system and give reliable results on the analysis of bone static and dynamic histomorphometric parameters (Nazrun et al. 2005; Shuid et al. 2007; Hermizi et al. 2009; Mehat et al. 2010; Hermizi et al. 2011; Aktifanus et al. 2012; Fathilah et al. 2012; Hussan et al. 2012; Muhammad et al. 2012; Soelaiman et al. 2012; Abdul-Majeed et al. 2012; Chin et al. 2014; Parvaneh et al. 2015). However, a detailed description of the protocol had not been mentioned. According to our knowledge this is the first technical paper describes the implementation of Weibel technique in the assessment of bone histomorphometric indices.

However, there are several types of errors that might lead to false results such as processing of bone samples, the distribution of parameter of interest over

the trabecular and the identification of each parameter of interest in the trabecular sections. Fixation of bone samples, thickness of trabecular sections and standardized staining protocol are the errors that affecting the quality of the of bone samples. Optimized technical skills could overcome the errors that might occur during the processing of bone samples (Weibel et al. 1966). Errors due to distribution of the parameter of interest over the trabecular bone can be overcome by randomizing the trabecular sections of each bone sample and quantifying at least three images for each trabecular section. Understanding the mechanism of bone metabolism can achieve good identification of parameter of interest. However, this method can easily be applied to bone histomorphometric measurements at the microscopic levels. This paper was not designed to compare this technique with other techniques.

A combination of bone histomorphometric measurements with the assessment of bone mineral density, micro computed tomography, bone radiography, serum levels of biochemical markers and histochemical measurements might give a better overall indication about bone health status and may be useful to predict the risk of fractures.

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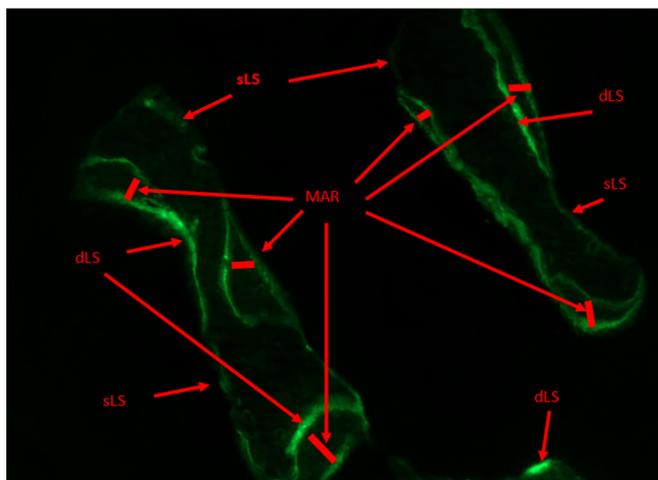


Figure 8: Micrographic photo of decalcified trabecular labelled with calcein (20x magnification) demonstrating dynamic bone histomorphometric parameters. dLS, double-labelled surface; sLS, single-labelled surface; MAR, mineral apposition rate.

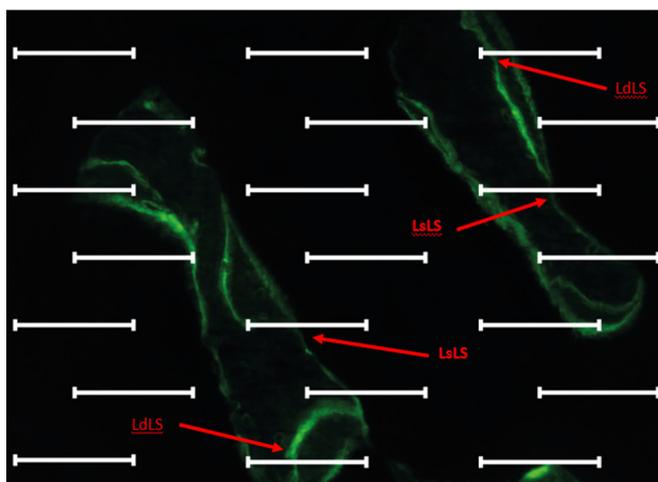


Figure 9: Sample of trabecular section labelled with calcein (20x magnification superimposed with multipurpose test-system (M42 grid). LsLS, area of interaction between single-labelled surface and test line; LdLS, area of interaction between double-labelled surface and test.

Table 2: Description of bone dynamic histomorphometric parameters

Parameter	Symbol	Unit
Single-labeled surface / bone surface	sLS / BS	%
Double-labeled surface / bone surface	dLS / BS	%
Mineralizing surface / bone surface	MS / BS	%
Mineral apposition rate	MAR	m / day
Bone formation rate/ bone surface	BFR / BS	m ³ / m ² / day

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