

# QUANTIFICATION OF THE MICROSTRUCTURAL ANISOTROPY OF DISTRACTION OSTEOGENESIS IN THE RABBIT TIBIA

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## ABSTRACT

**A longitudinal orientation of fibers and trabeculae has been observed histologically within distracted callus. This study quantified the intensity and angle of orientation of trabeculae within a distracted callus. Sixteen New Zealand white rabbits underwent unilateral tibial callus distraction with an external fixator across a mid-diaphyseal osteotomy. Included were: a seven-day post-operative latency period, ten days of distraction at 0.5 mm every 12 hours, and 20 days of post-distraction consolidation before euthanasia. Tibiae were removed, stripped of soft tissue, sectioned, and processed for decalcified histology. Micrographs of mid-coronal sections of the callus were evenly divided into 12 regions and underwent Fast Fourier Transform (FFT) analysis of the digitized image to determine the angle and intensity of the orientation of the bony trabeculae within the callus. The microstructure of the regenerate callus demonstrated an angle of orientation that uniformly matched that of cortical bone in all of regions of the callus and an intensity of orientation which approached that of cortical bone.**

## INTRODUCTION

The unique mechanical environment of external fixation and periodic tension-stress exerts profound effects on the bony regeneration in distraction osteogenesis. Controversy has surrounded attempts to fit histological descriptions of the distracted callus into various defined modes of ossification.

The majority of studies in various animal models has found intramembranous ossification to be the predominant, if not exclusive, mode of ossification,<sup>1,4</sup> but some investigators also interpret the presence of cartilaginous islands or nodules as evidence of some concurrent endochondral ossification.<sup>5-9</sup> Most studies have agreed on the description of an early callus organization consisting of a fibrous radiolucent zone in the gap center, and two sclerotic zones of trabecular bone extending toward it from each of the distracted osteotomy surfaces. Post-distraction, as the callus consolidates, the sclerotic zones fuse to form a callus with trabecular bone distributed throughout.

In contrast, the callus of fracture healing has a very different organization. Fracture callus ossifies primarily on randomly dispersed fibers and throughout an expanded periosteal region before it then organizes the trabecular callus with remodeling. Markel et al. showed in 1991 that orientation of bony trabeculae changes through the maturation of fracture callus, having profound impact on the mechanical stability of the callus at various stages of healing.<sup>10</sup>

Especially given the trabecular character of the distracted callus early in consolidation, orientation has implications on callus strength. Recent studies assessing the strength of physiologically trabecular bone have indicated that trabecular orientation contributes significantly to its strength.<sup>11-13</sup>

With these potential implications on the strength of the distracted callus in mind, the longitudinal orientation of bony trabeculae after distraction needs to be quantified. The purpose of this study is to quantify not only the angle and intensity of the orientation of trabeculae within the consolidating distracted callus, but to quantify the uniformity and distribution of this anisotropy.

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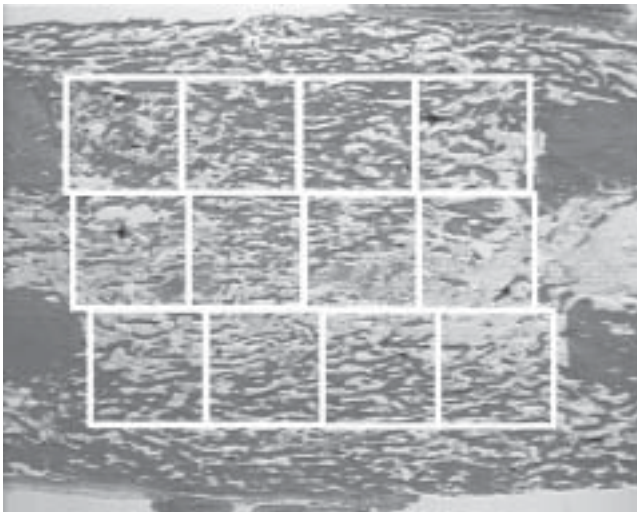


Figure 1. Photomicrograph of a mid-coronal section through the distracted callus of a rabbit tibia, and twelve areas for trabecular orientation analysis.

## METHODS

Sixteen skeletally mature (3.5 to 5.0 kg) New Zealand white rabbits underwent unilateral tibial callus distraction with an external fixator across a mid-diaphyseal osteotomy. A mid-diaphyseal osteotomy for lengthening was performed after an external fixation device was applied. Full weight bearing and unrestricted motion were allowed immediately after surgery. All procedures were approved by and performed in accordance with the Institutional Animal Care and Use Committee.

### Surgical Technique

Sterile surgical technique under controlled conditions was used to apply a unilateral external fixator (M-103, Orthofix, Verona, Italy) to alternating tibiae on 16 rabbits. Two 1.5-2.0 mm self-tapping, tapered half-pins were placed in pre-drilled 2 mm holes proximally and distally to attach the fixator parallel to the longitudinal axis of the tibial shaft and on the anteromedial aspect (with screws directed posterolaterally). Under saline irrigation for cooling, a transverse osteotomy was performed 1 to 2 mm distal to the tibio-fibular junction (between the two pairs of half-pins) using a Stryker reciprocating saw (Model 1370, Stryker Corporation, Kalamazoo, MI, USA).

### Callus Distraction and Consolidation

After a seven-day post-operative latency period, distraction across the osteotomy site was begun at a rate of 0.5 mm every 12 hours. Distraction at this rate was

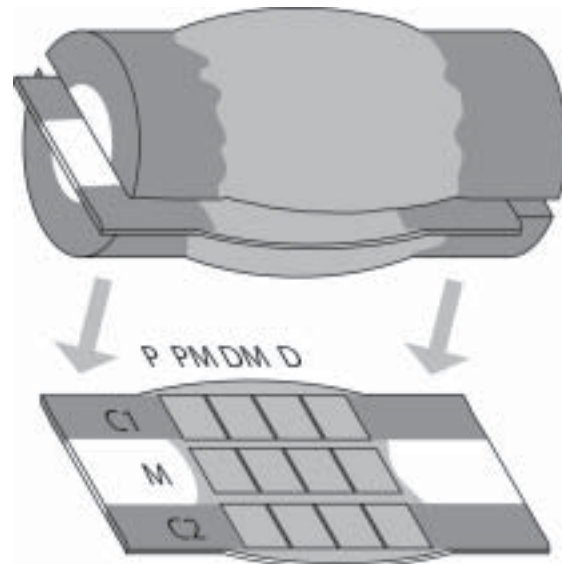


Figure 2. A 6µm mid-coronal plane section is taken from the distracted callus to be histologically prepared. The digitized photomicrograph of the section is then divided into twelve areas described by their coordinates in either cortical (C1 and C2) or medullary (M) longitudinal zones, and in the proximal (P), proximal middle (PM), distal middle (DM) or distal (D) transverse zones.

continued for ten days to achieve a 10 mm lengthening of each tibia. After a consolidation period of 20 days post-distraction, rabbits were euthanized, tibiae were harvested and the fixators were removed. After stripping the bulk of soft tissues, specimens were wrapped in saline-soaked gauze sponges and stored at -20 degrees Celsius.

### Histological Preparation

After decalcification and fixation, specimens were embedded in paraffin and a longitudinal section of 6µm was taken from the bone center in the coronal plane (Figure 2). These sections were fixed to slides and stained with toluidine blue, aldehyde fuchsin, and hematoxylin and eosin.

### Micrograph Digitization

Images of the callus, including the periosteal callus, oriented such that the longitudinal axis of the bone aligned with the horizontal edge of the image field, were digitized using a color Charged Coupled Device (CCD) camera (Model DXC-151, Sony, Japan) interfaced to a microcomputer. Digitized image data were transferred to a Silicon Graphics workstation (Indigo, Elan, Cupertino, CA).

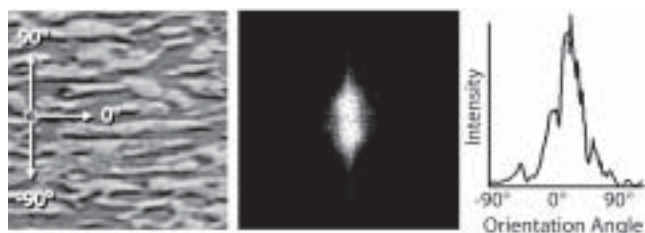


Figure 3. Once a field is selected from the digitized photomicrograph, the program gray-rescales the area (left), generates a Fast Fourier Transform power spectrum (middle), and finally produces an intensity histogram to graphically represent the distribution of oriented angles within the area (right).

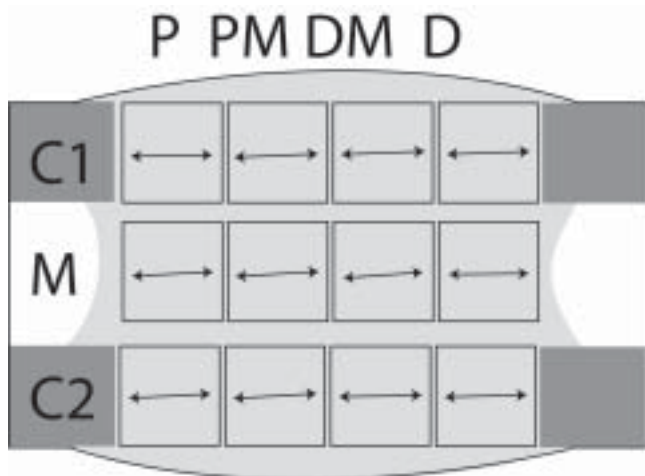


Figure 4. The averaged angle of orientation of the trabeculae within the 12 areas in the distracted callus, described by their coordinates in either cortical (C1 and C2) or medullary (M) longitudinal zones, and in the proximal (P), proximal middle (PM), distal middle (DM) or distal (D) transverse zones.

### Quantitative Analysis of Trabecular Orientation

For each digitized specimen image, the callus was divided into twelve approximately 2.5 mm by 2.5 mm areas determined by placement in one of the two neo-cortical zones or in the neomedullary canal zone transversely, and in the proximal, proximal-middle, distal-middle or distal zone longitudinally (Figure 2). A two-dimensional Fast Fourier Transform of a 256 x 256 pixel field which corresponded to each of the twelve areas on the histological specimen was calculated using custom software on the Silicon Graphics workstation according to the method previously described<sup>14</sup>. Briefly, the software first rescaled the gray levels of the selected field, then detected the directionality by transforming the power spectrum to a polar system using linear interpolation. The intensity of the orientation was then quantified by calculating the power spectrum within fan-shaped segments corresponding to one degree of orientation increment (Figure 3). The intensity of each orientation was represented as an index: 0 indi-

**TABLE 2**  
The mean and standard deviation of the intensity of orientation in the twelve areas within the callus

	Proximal Proximal	Proximal Middle	Distal Middle	Distal Distal
Cortex 1	0.80 ± 0.05	0.82 ± 0.04	0.82 ± 0.05	0.81 ± 0.04
Medullary	0.79 ± 0.08	0.81 ± 0.06	0.77 ± 0.04	0.78 ± 0.07
Cortex 2	0.80 ± 0.06	0.81 ± 0.03	0.81 ± 0.03	0.80 ± 0.04

cating a random orientation, and 1.0 indicating a completely parallel orientation. Periosteal callus areas were also measured when present.

### Data Analysis

The angle and intensity of orientation in the twelve averaged FFT areas from each specimen were compared by one-way analysis of variance. They were also compared with those in normal rabbit tibial cortical bone similarly prepared and measured by Student's t-test.

### RESULTS

Wherever bony trabeculae were present within the specimen sections, they were highly oriented along the longitudinal axis. All twelve averaged areas had a similar angle and intensity of orientation (Figure 1). The overall average for the angle of orientation was  $0.3 \pm 4.0$  degrees with a mean intensity of  $0.80 \pm 0.05$  (Figure 4, and Tables 1 and 2). While the angle of orientation in the extracortical callus was not significantly different from the angle of orientation in the twelve averaged areas ( $2.3 \pm 4.3$  degrees), the intensity of the orientation in extracortical callus regions, while still very high, was significantly lower than the mean intensity of orientation for the twelve areas ( $0.75 \pm 0.07$ ,  $p < 0.0001$ ). The averaged areas that had the lowest p-value in t-test comparison with normal rabbit tibial cortical bone (i.e., were oriented at angles most different from the longitudinal orientation of normal rabbit tibial cortical bone) were the two fields in the callus middle and in the medullary canal. One of these two areas also demonstrated the lowest intensity of orientation, significantly different from the average within the callus, excluding this region ( $0.77 \pm 0.04$ ,  $p = 0.037$ ).

### DISCUSSION

In this study, the angle and intensity of trabecular orientation within the distracted rabbit tibial callus was evaluated in a quantitative manner. The longitudinally well-oriented trabecular structure of distraction osteo-

TABLE 1

The mean and standard deviation of the angle of orientation in the twelve areas within the callus along with the p-value from a Student's t-test comparison to the mean of a sampling of normal tibial cortical bone, similarly prepared and evaluated

	Proximal		Proximal Middle		Distal Middle		Distal	
	Angle (deg)	p-value	Angle (deg)	p-value	Angle (deg)	p-value	Angle (deg)	p-value
Cortex 1	-0.9±4.3	0.480	0.6±2.8	0.684	0.3±3.1	0.927	0.1±4.6	0.952
Medullary	1.2±4.1	0.529	1.5±2.5	0.155	2.4±4.8	0.279	-0.1±4.3	0.433
Cortex 2	1.1±4.8	0.599	1.5±3.6	0.878	-0.7±3.8	0.364	-0.3±3.9	0.733

genesis in this rabbit model matched, after 20 days of post-distraction consolidation, the direction of cortical bone microstructural orientation. The trabecular structure also approached the orientational intensity of cortical bone. This anisotropy was uniform throughout the callus.

This quantification corroborates the histological observation that the fibers of the fibrous central zone, and the bony trabeculae of the sclerotic zones of the distracting callus are longitudinally aligned along the direction of distraction.<sup>2,3,5-8</sup> The tension across the callus during distraction aligns cells, as well as the fibrous extra-cellular matrix. Yasui et al. described the longitudinal arrangement of osteogenic cells, longitudinal columns of chondrocytes and mixed columns of osteogenic and chondrogenic cells also aligned along the tension vector.<sup>15</sup>

The tension-alignment of microstructures in distraction osteogenesis may have significance beyond the achieved mechanical alignment of the extracellular matrix. Mizumoto et al. reported that the expression of bone morphogenic protein-7 and many cytoskeletal proteins was upregulated in the callus during the period of active distraction.<sup>16</sup> They hypothesize that gene expression is regulated by the cytoskeletal response to alignment tension. If true, this hypothesis could explain in part the distraction pattern of ossification that has been distinguished from the mainly endochondral ossification of the fracture callus.<sup>1-9,15</sup>

The data reported in this study quantifies a callus that sharply contrasts the microstructural anisotropy of a fracture callus. Markel et al. quantified the anisotropy in 1991 in a fracture callus model, finding that while the intensity of orientation increased with remodeling over time, even after 12 weeks of healing, the intensity of orientation in the trabeculae of the callus was less than one-third that of cortical bone.<sup>17</sup>

In 1990, Markel et al. elucidated an implication of this alignment with the observation that once the fracture gap callus has remodeled to the point of longitudi-

nally oriented trabeculae, the mechanical properties of the callus have tighter correlations to non-invasive quantifications of gap tissue density, such as quantitated computed tomography, single photon absorptiometry and dual-energy x-ray absorptiometry (DEXA).<sup>10</sup> The specific correlation they found between DEXA and torsional strength after this remodeling to longitudinal microstructure had an  $r^2$  value of 0.51. For comparison, in 1998 Reichel et al. reported for distraction osteogenesis in a sheep model, a correlation between DEXA and torsional strength with an  $r^2$  value of 0.60.<sup>18</sup> The strength-predicting value of non-invasive densitometry such as DEXA depends on a tissue's approximation of the modality's assumption of material uniformity. While a distracted callus does not perfectly fulfill the assumption of material uniformity, the strong intensity of its orientation more closely approximates such, explaining the strong correlation between DEXA and torsional strength that Reichel et al. observed.<sup>18</sup>

It has been reported that highly oriented trabecular structure is beneficial to resist axial loading (on-axis loading), but vulnerable to the off-axis loading or shear loading.<sup>19</sup> Similarly, an increase in anisotropy associated with osteoporosis has been considered a risk factor for pathologic fracture.<sup>20</sup> With these findings, the distracted callus with highly oriented trabecular structure may be strong only for axial loading. Standard material testing in orthogonal directions will be required to elucidate the mechanical anisotropy of the distracted callus.

There are attempts to enhance callus maturation after distraction in applying controlled compression.<sup>21</sup> It might be important to measure the changes in trabecular anisotropy in addition to the changes in bone density to evaluate the effects of such modality. Further investigation is necessary to extend the microstructural resolution of the distracted callus into three dimensions. The orientation of fibrous and neovascular structures early in distraction also deserves further pursuit.

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