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Original Article

# Biological application of focus ion beam-scanning electron microscopy (FIB-SEM) to the imaging of cartilaginous fibrils and osteoblastic cytoplasmic processes

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ABSTRACT

*Objectives:* The aim of this study was the biological application of focused ion beam-scanning electron microscopy (FIB-SEM) to obtain serial sectional images of skeletal tissues that showed the ultrastructure of 1) cartilaginous extracellular fibrils and 2) osteoblastic cytoplasmic processes.
*Methods:* Seven-week-old female wild-type mice were fixed with half-Karnovsky solution and then OsO4, and tibiae were extracted for block staining prior to observation under transmission electron microscope (TEM) and FIB-SEM.
*Results:* TEM showed the fine fibrillar but somewhat amorphous ultrastructure of the intercolumnar septa in the growth plate cartilage. In contrast, FIB-SEM revealed bundles of stout fibrils at regular intervals paralleling the septa's longitudinal axis, as well as vesicular structures embedded in the cartilaginous matrix of the proliferative zone. In the primary trabeculae, both TEM and FIB-SEM showed several osteoblastic cytoplasmic processes on the osteoid, in greater numbers than those seen in the bone matrix. FIB-SEM revealed the agglomeration of cytoplasmic processes beneath osteoblasts that formed a tubular continuum extending from those cells. Based on these findings, we postulated that osteoblasts not only extend their cytoplasmic processes to the bone matrix, but also stack these cell processes on the osteoid of the primary trabeculae.
*Conclusion:* Taken together, these data suggest that FIB-SEM imaging of serial bone sections may facilitate new insights on the ultrastructure of cartilage and bone tissues.




## 1. Introduction

Focused ion beam-scanning electron microscopy (FIB-SEM), also referred to as ion abrasion scanning electron microscopy [1–3] may be one of the most valuable techniques to assess tissue morphology at the ultrastructural level, especially in cartilage and bone research. Usually, FIB-SEM employs epoxy resin-embedded specimens that are ground by a gallium-focused ion beam [4]. The resultant surface images are visualized by scanning electron microscopy (SEM) backscattering, a process known as “slice-and-view” [5,6]. Although targeting specific regions of a given tissue sample can be challenging by transmission electron microscopy (TEM), FIB-SEM may overcome such limitations by employing faster visualization of ultra-thin serial sections. However, few studies have explored the use of FIB-SEM for the ultrastructural assessment of bone and cartilage [7].

Histologically, developing growth plate cartilage comprises three major zones: the reserve or resting zone, the proliferative zone, and the hypertrophic zone [8]. Chondrocytes in the

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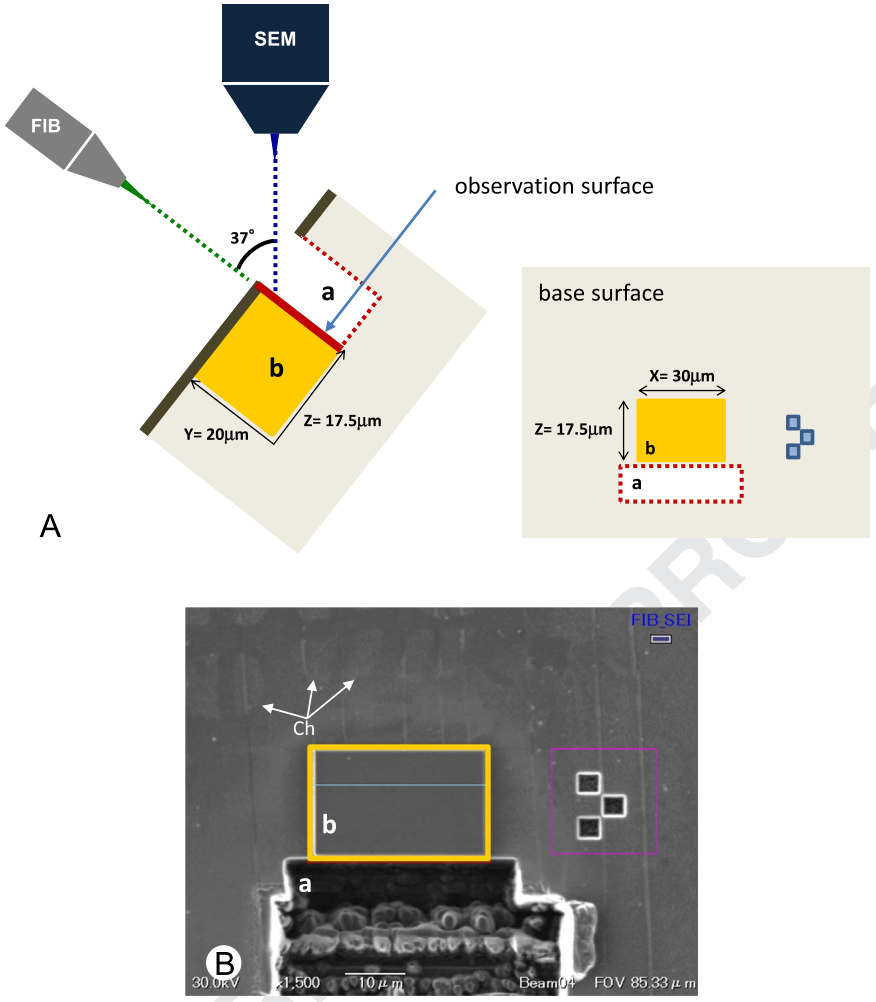


**Fig. 1.** *Region of interest (ROI) for focused ion beam-scanning electron microscopy (FIB-SEM) observation.* Schematic design of the FIB-SEM principle (A). The ROI was 30 μm × 20 μm × 17.5 μm, as indicated by the boxed area *b*. Boxed area *a* is a pre-milled region used to observe the block surface and release the resin piece. The specimen was derived from the tibial metaphysis and included the growth plate (B). Panel B shows the naked-eye view of the superficial layer sputter-coated with platinum. Ch: chondrocyte.

proliferative zone proliferate and synthesize the cartilaginous matrix simultaneously [9]. Cells in that zone become flattened proliferative chondrocytes, align themselves in columns, and then develop a hypertrophic phenotype characterized by a translucent and enlarged cell body [8,10]. The cartilaginous extracellular matrix in these zones can be divided into two regions: the intercolumnar septa, which parallel the longitudinal cell columns, and the transverse partitions within these columns [11]. However, if external mechanical loading is applied to the growth plate cartilage, the ultrastructural relationship between the chondrocytes and extracellular fibrils can be dramatically affected, disrupting the chondrocytic columns [12]. Therefore, it is important to determine the normal ultrastructure of extracellular cartilaginous fibrils through FIB-SEM observations so that any existing disorders can be readily identified.

As a consequence of normal endochondral ossification [8,13], osteoblasts extend their cytoplasmic processes into the bone matrix of the primary trabeculae [14,15]. Since bone formation rate is extremely high in the primary trabeculae [16–18], one may wonder whether an osteoblast can produce new cytoplasmic processes at the same rate that it forms new bone. Alternatively, is it possible that cytoplasmic processes are synthesized in advance and "stocked" beneath the osteoblasts until needed?

In this study, we present the ultrastructure of 1) cartilaginous extracellular fibrils and 2) osteoblastic cytoplasmic processes by means of FIB-SEM. In addition, we present a modification of the block staining procedures reported by Thomas et al. [19] for adequate contrast in backscattered SEM images.

## 2. Materials and methods

### 2.1. Tissue preparation for TEM and FIB-SEM

All animal experiments were conducted under the Hokkaido University Guidelines for Animal Experimentation (approval No.15-0041). Seven-week-old female wild-type mice (Jcl: ICR, CLEA Japan) were used in this study (n=6).

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital and perfused with half-Karnovsky solution (2% paraformaldehyde and 2.5% glutaraldehyde diluted in a 0.067 M cacodylate buffer, pH 7.4) through their left heart ventricle. After perfusion, all tibiae were stripped of soft tissues and immediately immersed in the same fixative for 48 h at 4 °C. After decalcification with a solution of 4.13% EDTA for 2 months, the specimens were cut into 2 mm × 1 mm × 1 mm pieces that included the metaphyseal growth plate. Specimens were subjected to block staining as described below and were dehydrated with ascending concentrations of acetone before being embedded in epoxy resin (Taab, Berkshire, UK) [20]. Semi-thin sections were

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