Three-dimensional, Sub-micron Imaging Of Bone And Fluorescent Markers Of Bone Formation At The Bone-implant Interface

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Introduction: Rapid osseointegration of an orthopaedic implant is key to short- and long-term implant success. A number of methods have been proposed for improving fixation of a metallic implants including coatings and nano-scale surface texture treatments [1]. Quantitative evaluation of metallic implant fixation is traditionally performed using plastic embedded specimens that are sectioned using ultramicrotomy (for SEM) or polished to 50-200 μm in thickness (for evaluation of bone formation markers). A limitation of both techniques is that they are two-dimensional in nature and therefore provide only a limited sample of bony ingrowth relative to the location of a coating, surface treatment or surface microstructure (particularly when inserted into regions of cancellous bone). Micro-computed tomography could provide such three-dimensional images, but image artifacts around metal implants prevent assessment of bone within three voxels of the implant surface (the region of most interest) [2,3]. Here we demonstrate the use of an imaging method known as serial milling for obtaining three-dimensional images of the bone-implant interface and fluorescent markers of bone formation.

Methods: To demonstrate feasibility of the approach we examine a single specimen from an animal experiment. Stainless steel threaded Kirschner wires (1.6-mm-diameter, Zimmer Inc., Warsaw, IN, USA) were surgically inserted into the caudal vertebrae of a three-month-old female Sprague-Dawley rat. The animal was part of a larger experiment that was approved by the local IACUC. The K-wires were allowed to heal for three weeks after which the animal was anesthetized and a cyclic compressive load between 25N and 100N was applied transversely to the K-wire at 0.5 Hz (6 minutes of loading, Fig. 1A). Loading was repeated on two consecutive days (three loading bouts in total). Fluorescent bone formation markers were applied at 2 and 7 days after loading and the animal was euthanized 11 days after loading. The seventh caudal tail vertebra with K-wire intact was collected, cleaned of soft tissue, fixed in formalin and embedded undecalcified in methyl-methacrylate.

Images of the specimen were collected using serial milling. Serial milling is a destructive imaging approach in which the top 5 μm of the specimen is removed with an endmill by a computer controlled milling machine (a device designed to cut metal) and epifluorescent images of the newly revealed surface are collected [4] (i.e. the surface of the embedded block is imaged, no sectioning is performed). The process is repeated until a three-dimensional set of images is achieved. Images were collected using three different fluorescent excitation/emission filters to allow automated identification of bone, and fluorescent bone formation markers. This method achieved three-dimensional images with a voxel size of 0.7 x 0.7 x 5.0 μm. Images were thresholded manually and the two bone formation labels were connected and merged into individual objects of new bone during post-processing using image closing. Image analysis was performed in two different regions of interest (Fig. 1B): 1) the region of the cortex in which the K-wire was inserted (a cylinder extending 1 mm in radius from the center of the K-wire and
0.5 mm in height); and 2) the small callus formed at the periosteal insertion location of the K-wire during the four weeks between surgery and specimen collection. In the region of the cortex, bone-implant contact (BIC) and bone volume fraction (BV/TV) between the threads were determined. Automated measurement of bone-implant contact at such high resolutions requires a distance used to defined “contact” between the implant and bone [3]. Hence, BIC was expressed as the percent of implant surface area that has bone within a specified distance. Additionally, the size of the callus region and the bone formed in the callus region in the week following mechanical loading was determined.

![Fig. 1. (A) K-wires were placed in the caudal (tail) vertebrae of the rat and a mechanical load was applied (arrows). (B) A cartoon illustrating the region around the 7th caudal vertebra. Regions of interest examined included the region around the K-wire insertion in the cortex as well as the small callus formed outside of the cortex.](image)

**Results:** Under UV fluorescence the K-wire appeared black, bone appeared bright and the plastic embedding media (including the marrow space) appeared gray (Fig 2A,B). Multi-level thresholding was used to segment the three different objects within the image. At locations of the bone-implant interface, bone tissue was observed within one pixel (0.7 μm) of the implant surface (Fig. 2B). Three-dimensional renderings showed contact between the K-wire threads and bony ingrowth (Fig. 2C). The K-wire showed very strong integration within the cortex. Bone implant contact across in the cortex region of interest was 3% within 1 pixel (0.7 μm) of the implant surface and increased to 10% within 4 pixels of the implant (2.8 μm). The bone volume fraction within the threads of the implant was 32.4%. The K-wire was integrated with the bony callus formed outside the periosteum (Fig. 3A). Bone formation in the week following mechanical loading was observed in the callus region. Bone formation predominately occurred on the side of the callus in which loading was applied (Fig. 1, 3B). The callus region at the bone-implant interface was 0.046 μm³ in volume and 41% of the bone volume in the callus had formed in the week following applied mechanical loading.
Fig 3. (A) An image of the bony callus extending out from the cortex with the K-wire is shown. (B) The same image presented without the K-wire. Images were collected 5 weeks after insertion of the K-wire. Green regions indicate bone formation over 3-9 days prior to specimen collection.
**Discussion:** Here we demonstrate the first three-dimensional images of the interface between a metallic implant and bone within one micrometer (0.7 μm) of the implant surface. Visualization and quantification of the interface between a metallic implant and bone has, to date, been limited to two-dimensional assessment which cannot provide information about the spatial relationship between regions of new bone formation and microstructural characteristics of the implant (location of coatings, surface texture treatments) or local mechanical environment. Serial milling avoids image artifacts generated by metal implants (which limit visualization of bone close to the implant [3]) and additionally provides images of fluorescent markers such as bone formation markers. A disadvantage of serial milling is that it is destructive in nature. Theoretically the serial milling approach could be applied to any size implant and any combination of bioinert (metallic, polymer) or bioactive scaffolds, although the greatest utility is in pre-clinical evaluation of implant coatings, surface treatments and microstructural modifications.

**Significance:** Rapid implant fixation and osseointegration is key to preventing implant loosening and failure. Surface coatings/treatments have been proposed to improve fixation of metallic implants. Here we demonstrate a three-dimensional imaging method capable of visualizing the bone and fluorescent markers within 1 μm of the metallic implant.

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