Correlative Light and Backscattered Electron Microscopy of Bone —
Part II: Automated Image Analysis

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Summary: Detailed studies of biological phenomena often involve multiple microscopy and imaging modes and media. For bone biology, various forms of light and electron microscopy are used to study the microscopic structure of bone. Integrating information from the different sources is necessary to understand how different aspects of the bone structure interact. To accomplish this, methods were developed to prepare and image thin sections for correlative light microscopy (LM) and backscattered electron imaging in the scanning electron microscope (BSE-SEM). Images of the same fields of view may then be analyzed for degrees of relationships between specimen features not observed by LM or SEM alone. These methods are applied here to study possible associations between the degree of bone mineralization and pattern of collagen fiber orientation in the mid-shaft of the human femur. The "relational images" obtained allow us to examine the relationship between these two variables, both objectively and quantitatively.

Key words: image analysis, image comparison, circularly polarized light, scanning electron microscopy, human mid-shaft femur

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Introduction

The complex nature of biological structures necessitates the use of multiple analytical and imaging approaches. Our present knowledge of the biology of bone tissues has been greatly enhanced by the use of varied forms of light and electron microscopy that provide information about specific aspects of bone structure. A correlative approach in which information from multiple imaging sources is combined into a single dataset, could shed light on inter-relationships between different aspects of bone structure that might not be appreciated by any one imaging mode. With this in mind, we set out to produce a method to compare quantitatively same-field-of-view images of bone obtained from the light microscope (LM) and scanning electron microscope (SEM). We wanted to find out whether patterned differences in the orientation of collagen fibers, as determined by circularly polarized light microscopy (CPLM), could be detected in bone volumes of varying degrees of mineralization, as determined by backscattered electron microscopy in the SEM (BSE-SEM).

Both preferred collagen fiber orientation and mineralization density are thought to influence the mechanical strength of the femur (Boyle and Riggs 1990, Martin and Boardman 1993, Martin and (schlafl 1989); both affect bone microhardness as reflected in polishing resistance (Boyle 1984). However, their connection has only rarely been investigated (Riggs et al. 1993b, Viscosalli 1978), largely because acquiring these data requires two very different imaging techniques and two different types of sample preparation.

Collagen fiber orientation is efficiently examined through the use of circularly polarized light (CPL) (Boyle et al. 1984, Boyle and Riggs 1990, McMahon et al. 1995, Riggs et al. 1993b). When collagen fibers are more parallel with the plane of section, they appear brighter, and when they are more perpendicular they appear darker in CPL, although the sign of the birchimence of the bone salts crystals opposes that of the collagen and weakens the observed effect. Unlike the conventional linearly polarized light image of circumferential lamellar bone, CPL-imaged lamellae containing collagen with a component oriented
strongly parallel to the plane of section appear bright in their entirety, irrespective of their rotational position. Thus, areas in a cross section of the shaft of a long bone that appear bright in CPL represent areas in which collagen has been laid down in a more transverse orientation and may be better able to resist compressive forces. Longitudinally oriented collagen fiber bundles and crystallites are better able to withstand tensile forces and transversely oriented collagen fiber bundles and crystallites are better able to withstand compressive forces (Ascenzi and Bonucci 1967, 1968; Riggs et al. 1993).

Mineralization data have been obtained either through microradiography (e.g., Amprino 1958, Martin and Amprino 1985, Portigliatti-Barbossi et al. 1993, Vincentelli and Evans 1971) or BSE-SEM (e.g., Blochmann et al. 1997, Boyde and Jones 1983, Boyd and Jones 1996a, b, Grynpas 1993, Reid and Boyde 1987, Roodgers et al. 1998). In such images, variations in grey level relate to the degree of mineralization of the bone. Less or poorly mineralized bone appears relatively dark compared to more highly mineralized bone, which appears brighter.

Correlation between these two sets of information has rarely been attempted. Vincentelli (1978) found that in the human tibia, recent and poorly mineralized remodeling events (osteons), as determined by microradiography, tended to be more frequently transverse in orientation in older individuals, and more often longitudinal in younger individuals, indicating an age-related change in the predominant direction that collagen fibers are deposited. Portigliatti-Barbossi et al. (1983) studying the human femur, considered that most poorly mineralized canals could be found in cortices expected to be subject to tension during life, suggesting that bone remodeling may be more active in these areas where predominantly longitudinal lamellae can be found. Riggs et al. (1993b) related mineralization density, as determined by BSE-SEM, to collagen fiber orientation of the horse midshaft radius, but methodological requirements were such that analyzed sections were focused more than 100 microns apart and quantification of the relationship between variables was not attempted. However, the authors do report a higher mineralization density in the tensile cortex of the horse radius, suggesting a lower remodeling rate in this region of predominantly longitudinal lamellae. (Riggs et al. 1993b; see Boyd and Jones 1992) for quantitative BSE data). This result is contrary to that of Portigliatti-Barbossi et al. (1993b) but supported by comparisons of ash fraction and collagen fiber orientation by Mason et al. (1993).

The methods we present here render images comprised of a direct and qualitative relationship between the two variables in same-field-of-view images of bone, providing a clear visualization of such relationships.

Materials and Methods

Our image analytical method was developed and applied to sections of human mid-shaft femur. The specimens used in the development and initial applications of this imaging method derive from autopsy samples obtained through the Victorian Institute of Forensic Medicine and University of Melbourne, Australia (see Borelson et al. 1995 for description of the collection).

Mid-shaft femur blocks were obtained, marked for orientation, and cleared of soft tissue using a 1% Tergitol solution (Boyde 1984). Bone blocks were embedded, mounted on glass slides, and sectioned to 100 ± 5 μm using the methodology of Goldin et al. (1999). This method is pivotal to the same-field-of-view imaging of bone thin sections by CPLM and BSE-SEM required for the construction of our relational images. Uniform ± 2 μm section thickness was determined using an Edge R400 real-time three-dimensional (3-D) microscope (Edge Scientific Instrument Company, San Mateo, Calif., USA) fitted with precision stages in the "Z" direction. Slides were marked with a transmission electron microscope (TEM) locator grid outside the upper left and lower right corners of the section, which was used as registration for the CPLM and BSE-SEM imaging. Using these preparation methods, resulting sections could be first imaged in CPL and then be carbon coated prior to BSE imaging with the SEM.

Grey level images for the analysis of collagen fiber orientation were obtained using a Leica DMRX2 Universal Microscope (Leica Microsystems, Bannockburn, Ill., USA) configured with circularly polarized light filters and an automated Marshman stage. Lighting was adjusted and centered to a standard value prior to imaging each specimen. Digital images were transferred to a Leica Quantimet high-resolution image analysis system (Q600) via a Kodak Megaplus CCD camera (Kodak, Rochester, N.Y., USA). Tiled images (each 1024 × 768 pixels) were obtained at a low magnification (nominal 150X, field width 2.2 mm). Using this field size, entire cross sections could be acquired within approximately 250 images. A macro written within the Q600 program automated the field acquisition of images.

Digital grey level images for the analysis of bone mineralization were obtained using the LEO S440 SEM (Leo Electron Microscopy, Ltd., Thornwood, N.Y., USA) in BSE imaging mode. The magnification was set to accommodate the same field of view derived from the LM (size at a 1024 × 768 image size, 2.2 mm field width). The section was oriented to the exact position and rotation as established for the LM by superimposing a live BSE-SEM image of the TEM locator grid over the digital image obtained previously with the LM. Images of the region of interest were collected at a 15 mm working distance, 20 kV accelerating voltage, and approximately 750 pA beam current using a 10 mm filament that was allowed to warm and stabilize for 1 h before imaging. Leo Stage Scan software was used to provide automated tiling of BSE-SEM images across the entire region of interest in the same pattern as our LM imaging.

Dimethylglycol standards were used to monitor the stability of the electron beam during the imaging run and to
correct for any errors due to drift in the system that would increase grey level variability aberrantly within and between sets of tiled images. The standards are made from thermoset dimethacrylate esters derived from the reaction of halogenated phthalic acids and glycidyl methacrylate. C2H2O2Br (monobrom) has an electron backscattering coefficient (0.1159) less than that of the least densely mineralized bone tissue. C2H2O2Br (monochlor) has an electron backscattering coefficient (0.1519) higher than bone, and just higher than practically all calcified cartilage (Boyd and Jones 1996a, Boyd et al. 1998, Howell et al. 1997). An image of the standards was collected every 15 imaging frames. To standardize resulting images, the mode of the histogram peak for each standard was determined and bone images were stretched to a 0–255 grey scale based on those levels (Boyd and Jones 1996a, Boyd et al. 1998). The standardized images were saved and used for further image processing.

Tiled images obtained from our CPLM and BSE-SEM imaging sources were each automatically montaged using a dedicated software program developed using Visual Basic 6.0 (Microsoft) and Leadtools Imaging (v. 10, LEAD Technologies, Inc., Charlotte, N.C., USA).

Image Analytical Methods

Our image analytical methods were devised to allow direct comparisons between grey level images derived from different microscopy sources. We first describe a statistical method that outputs a continuous Red-Green-Blue (RGB) color scheme reflecting similarities and differences between a same-field-of-view image pair. This method allows for robust quantitative comparisons at the pixel level. We also describe a routine that utilizes color lookup tables (FLUT) to provide visual and quantitative comparisons between images at a lower resolution of detail (i.e., by grouping grey-level information into bins). Both of these methods allow for enhanced visualization of correspondence between the two image sources due to their use of colors, which are more readily discernable to the human eye than levels of grey. These image analytical methods can be used alone or in combination, depending on the level of analytical detail required for a particular application.

According to its physical definition, an image is a two-dimensional distribution of brightness, and can be described by the equation:

\[ I = F(x, y) \]

where \( I \) is the image, \( F \) is a function of \( x \) and \( y \), and \( x \) and \( y \) are coordinates specifying a point within an image frame.

Digital imaging uses information that represents sets of ready values of brightness at \( x \) for Eq. (1). In general, there are two ways to get such values: either by transducing physical properties of the specimen into numbers (i.e., assigning a grey level value to brightness at specified \( x \) coordinates), or by generating numbers by means of mathematical procedures. A combination of both of these procedures is applied in our methods.

Procedure

This method utilizes paired grey scale images, one from the LM and one from the SEM. These images are independent from each other, but use of the same field of view. We compare the grey levels in each image, pixel by pixel, and illustrate the degree of relationship between the two as a color image. To achieve a color image, three independent sources (the third being a constant of the processing environment) with nonidentical transfer functions are required, as follows:

\[ C = \Phi(l_1, l_2, l_3) = \Phi(F_1(x, y), F_2(x, y), F_3(x, y)) \]

where \( C \) is the color image, and \( \Phi \) is a function of three independent grey scale images of one object and the same field of view, which are represented as functions \( F_1, F_2 \), and \( F_3 \) (representing red, green, and blue, respectively). In our case two independent images are acquired digitally (from the CPLM and BSE-SEM, respectively), so that a third image is derived as a function of the first two:

\[ F_2 = E(F_1, F_3) \]

This third image is produced following a series of steps described below:

1. Grey level values of each pixel in the two original images are read (on a 0–255 grey scale) and matrices are produced for each image, based on the \( x \) and \( y \) coordinates of each pixel and their grey level value.
2. The mean grey level value and its standard deviation at each pixel is calculated for each of the two matrices.
3. The data are normalized using cumulative normalized distribution values (CND), with the properties of mean = 0 and standard deviation = 1. The normalization process is used to eliminate the impact of differences in the grey level histograms of the two images.
4. The differences between the CND values at each pixel are calculated for the matrices produced from the images. This results in a third matrix, which contains information on the similarities and differences in grey level values between the two original matrices. The mean grey level value and its standard deviation is also calculated for this resulting matrix of similarities and differences.
5. Finally, a composite color image according to Eq. (3) is produced as a representation of image resemblance.

This image is based on a composite matrix that contains RGB color values for each pixel, based on the three calculated matrices. This method provides a visual indication of the location of, and degree of association between, the two original images. In addition, the numerical matrix of RGB color values allows for later quantitative analysis.
All image processing and statistical procedures were initially undertaken using Mathead Plus 7.0 software (MathSoft, Inc., Cambridge, Mass., USA). Like other programs of its type, the Mathead program can open images, read image data into a matrix, and apply built-in customizable functions to matrices, element by element. It can also visualize such a matrix as a grey scale or color image. Although Mathead Plus 7.0 software provided a workable image processing solution for experimental purposes, the program did not provide the level of freedom and flexibility required for routine research use.

The numerical arguments used in our procedures are matrices or sets of matrices with about 10^6 or more elements. Image processing using the Mathead Plus 7.0 software routine was slow (about 15 min per image pair using a 200 MHz Pentium II (Intel Corporation, Santa Clara, Calif., USA) computer with 64 megabytes of RAM), and batch processing of images was not permitted. We therefore determined that a free-standing program, customized for the statistical routine we required, would be far more practical for the processing of numerous images quickly and efficiently. Fortunately, the latest programming concepts of component object model (COM) and rapid application development (RAD) relying on visual (“drag-and-drop”) programming made this solution feasible.

Such a program was developed around LEADTOOLS Imaging (16/32 ActiveX v. 10, LEAD Technologies, Inc., Charlotte, N.C., USA). The entire program was created in Visual Basic 6.0 (Microsoft, Inc.). It provides an automatic single- or multispot image comparison procedure, allowing for batch processing of images by reading file names from a list that it creates.

Procedure 2

For many applications, the enhanced visualization of relationships afforded by a pseudo-color LUT image, in which colors are specifically chosen to emphasize grey levels of interest, can be of additional benefit for understanding relationships between images. The collapse of grey level data into larger bins for analysis provides a robust, yet simple method of differentiating large scale patterned differences that may have biological significance (e.g., recognition of groups of low, medium, and highly mineralized osteons). The procedures described here were carried out in Adobe Photoshop 5.0 (Adobe Systems, Inc., San Jose, Calif., USA).

Individual image pairs, or entire montages, representing same fields of view, were opened in Adobe Photoshop and converted to indexed color images. A color LUT divided our CPL image into two grey level bins, devised to emphasize major differences in grey levels—usually bright pixels versus dark pixels. The color LUT for the BSE-SEM was devised to allow the recognition of four groups of relative degree of mineralization of bone tissue—from the least to the most highly mineralized. The limits of each bin were determined subjectively on the basis of observed mineralization density in combination with morphological features of interest.

These LUTs were applied to the original images, and the images were converted back to grayscale again. The images were then compared mathematically using the “difference” calculation function within Adobe Photoshop to produce a new grayscale image. The LUT’s colors were specifically chosen to emphasize regions of newly formed bone with preferred longitudinal or transverse collagen and then applied to this new image. Data could be quantified by calculating the percentage area of each resulting color in a given area of interest.

Results

Same-field-of-view BSE and LM images were digitally compared using the methods described above.

Procedure 1

Figure 1 shows an example of a same-field-of-view image pair obtained from our LM and BSE-SEM image sources and the resulting “relational” image produced by our imaging procedure. By preserving similarities and differences in grey levels between the two original images, for this analysis, grey level information from the CPLM image was assigned a blue color and grey level information from BSE-SEM image was assigned green. In this scheme, hot and/or bright colors indicate image grey level disparities. Color values in the neutral/gray range, therefore, indicate regions where the CPLM image is bright and the BSE-SEM image is dark (i.e., longitudinal collagen fibers in relatively highly mineralized bone), and values in the yellow range indicate domains where the CPLM image is bright and the BSE-SEM image is dark (i.e., transverse collagen fibers in relatively less mineralized bone—not seen in Figure 1). Cool and/or dark colors indicate similarities in grey level in the two original images. Color values in the aqua range therefore indicate regions where both images are bright (i.e., transverse collagen fibers in relatively highly mineralized bone), while color values in the yellow/orange range show areas in which both images are dark (i.e., longitudinal collagen fibers in relatively less mineralized bone).

Relational images retain numerical data indicating the degree of similarity or difference in grey levels between the two image matrices. This method is therefore particularly useful in high resolution situations where subtle changes in grey level relationships between nearby pixels are important.

Procedure 2

Our second imaging procedure was applied to entire cross-sectional montages for clear visualization of the overall distribution of bone tissue of differing mineralization and collagen orientation. Figure 2 represents the final LUT output of this routine applied to a montage of an entire femoral
mid-shaft cross section. In this 33-year-old female individual, much of the bone toward the endosteal surface is highly mineralized (represented by the predominance of the light grey color). Those more recent remodeling events close to the endosteal surface are predominantly transverse in orientation (represented by yellow in this image). An abundance of osteons with low to moderate mineralization is represented in the periosteal bone envelope of this individual. On the lateral aspect of the specimen, most osteons display a moderate degree of mineralization and contain longitudinal collagen fibers (showing as purple in this image). Bone that has been most recently deposited contains mostly longitudinal fibers as well (showing as blue in this image). On the medial aspect of the specimen, on the other hand, most of the moderate and low mineralization bone is transverse in collagen fiber orientation (yellow and green, respectively). This method clearly highlights the regional differences (in this case, medial versus lateral) in predominant orientations of recently deposited bone.

Using these imaging methods, it is possible to determine quantitatively the fraction of relatively less mineralized osteons (i.e., recent remodelling events), which have primarily longitudinal as against transverse collagen. Within individuals, it is possible to examine whether patterned differences in these relationships exist between cortices or between bone sites. Studies of variation both within and between populations (i.e., with age, gender, or population affinity) are also possible.

**Discussion**

Correlative image analytical methods were successfully applied to the study of bone architecture, allowing similarities and differences between matched grey level images derived from CPLM and BSE-SEM sources to be clearly visualized either in RGB relational images or in color LUT output. The RGB method (Procedure 1) is quantitative at the pixel level by employing matrix data obtained in the comparison process. The LUT method (Procedure 2) allows the user readily to visualize and quantify patterned relationships at the whole bone level. These techniques have the potential to elucidate relationships between different aspects of bone structure, such as preferred collagen orientation and mineralization density, that have not previously been quantified in tandem.

The need for quantitative comparisons of digital same-field-of-view images obtained from different microscopy sources has become more apparent in the field of bone biology as researchers call for studies of multiple aspects of bone structure in an effort to understand how bone responds to its mechanical environment (Martin and Boardman 1993). There are numerous microstructural features within bone that contribute to its overall strength and ability to withstand mechanical forces. Understanding how they interact with one another will enhance our knowledge of bone biology (Seeman 1997).
Although our method has already produced useful results, we shall continue to address some important analytical issues. The RGB comparison program, as currently configured, does not provide the means to eliminate background information from the analysis (i.e., non-bone areas such as pores, medullary cavity, and regions external to the bone cortex, which appear black in both imaging modes).

Images with a high background percentage therefore tend to be strongly influenced in the normalization process due to the higher incidence concentration of grey levels nearing 0. We are currently experimenting with masking techniques to eliminate from analysis all areas of non-bone. We are also considering the effect of the normalization procedures that are undertaken prior to the calculation of image matrices.

Fig. 2 Montage of entire young adult female mid-shaft femur cross section produced with look-up table image comparison method (Procedure 2). Specimen is oriented such that anterior is at top and lateral is to right. The colors were specifically chosen to provide clear differentiation between bone regions with differing mineralization/collagen fiber orientation relationships.
Analyses of whole bone cross sections require us to be especially concerned about the uniformity of LM illumination and the stability of SEM running conditions as they may be expressed visually (and quantitatively) within and between image montages. While tilting high-resolution images improves our ability to examine detailed bone microstructural relations, it also introduces errors between images. We correct for uneven LM illumination with a shade correction utility available within the Q600 Qwin Image Analysis software (Leica Imaging Systems, Ltd., Cambridge, U.K.). Stability of illumination is checked through the use of neutral density filters that are always set to a predefined grey level value prior to the imaging of a specimen.

We corrected for drifts in SEM instrument parameters with standards of known density according to the method of Boyd and Jones (1996a) and Howell et al. (1997). Such physical corrections permit the comparisons of image data matrices independent of microscope location and investigator. We confirmed this by imaging the same mid-shaft femur block specimen in two laboratories (HTRU, Hunter College, New York, N.Y., USA, and HTRU, University College London, England), the results of which yielded similar grey level distributions in the specimen.

The automated stitching of image tiles into a montage is only as good as the precision of movement of the microscope stage. Automated LM stages may have ± 0.1 μm movement precision and negligible accuracy variation over the stage platform, and stitching errors are unlikely to be observed on low magnification montages. The SEM motorized stages typically have ± 5 μm or smaller movement precision, but, in practice, variable accuracy over the stage platform may be on the order of ± 10–20 microns. If the error is linear, a software correction can be introduced. However, random cumulative error over the course of an imaging run may lead to differences between first and last images on the order of ± 50 μm. Our SEM stage produces a combination of these errors. Thus, we find it possible to obtain reasonable data at low magnifications using our analytical method, though high-resolution, pixel-to-pixel comparisons of tiled images require manual alignment of the BSE-SEM images against those from the LM in a program such as Adobe Photoshop 5.0 (Adobe Systems, Inc.).

We are currently experimenting with the use of automated montaging programs that use cross-correlational techniques to montage BSE-SEM images having a 20–30% overlap to achieve a better complement to the LM image montage (Dani and Chaudhuri 1995, Laroche 1998, Ott 1997). Another potential solution to this problem would be to correct for the predictable over- or understepping of the SEM stage using a software algorithm.

Other considerations that could potentially affect the ability to tile images include image distortion errors in both SEM and LM imaging, for example, parallel-sided field squashing, barrel and pincushion, nonuniform magnification across the field, etc. Therefore, no matter how good the stage controls in either instrument, there may always be mismatches at joints (tile edges). In the LM case, the real resolution element in our case is very roughly a few hundred cubic microns, since effects are summed through the section thickness and the lateral resolving power is of the order of one micron at the best focus plane, and worse everywhere else. Thus, potential bad joints in the LM case are blurred out. This error was minimized by our use of objective lenses (Leica 5×0.12 PL Fluotar objective) with flat field and insignificant, or at least undetectable, barrel, pincushion, fish-eye-lens effects. In the LM case, there would be no significant objection to warping any image field distortions back to shape: interpolating between pixel values would not be important when the recorded values already average data from a large volume within the sample. However, the same is not true for the SEM. Here, our aim was to make digital measurements of a sample property at single points. Deriving intervening values may be incorrect, since such values may not actually exist. Another serious problem exists for the SEM case, namely, the rotation of the image with focus. It is necessary to adopt a compromise where the scan rotation is adjusted to provide images that are “square” with the axes of stage motion. This is unlikely to be quite exact. In spite of all these theoretical problems, the present results show that, in practice, we have a very good match between CPL and BSE-SEM images.

Our imaging methods have the potential to be useful to research in realms beyond the applications illustrated in this paper; as more researchers move into digital imaging and analysis. Even within the bone field, such methods are not limited to images of circularly polarized light and BSE microscopy. The CPL images could just as easily be correlated with microradiographs as with BSE-SEM images. There is also potential use for this program, or similar programs, in the correlation of images obtained in conventional or confocal (auto-)fluorescence microscopy and the cathodoluminescence mode in the SEM.

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