

## Stained Slides vs Noninvasive Imaging: Strange Bedfellows in Bone Research?

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### Introduction

Most veteran histologists have heard the following at one time or another: “You will be replaced by a machine; Histology will be supplanted by imaging modalities.” In the clinical arena circa 1980, it was the advent of the cell sorter. In the 1990s in the orthopaedic research field, it was various evolutions of x-ray and computed tomography (CT). In each venue, however, quality histologic preparations are paramount for accurate disease diagnosis and the identification of cellular mechanisms.

Bone research combines aspects from a number of disciplines. Histology lends variants of paraffin microtomy and staining for decalcified bone and connective tissue, and polymer or resin microtomy for undecalcified bone. Systems for cutting and grinding of resin-embedded bone containing metallic implants have been developed for the study of tissue growth into and around orthopaedic prosthetic devices. The discipline of radiology contributes film, and more recently, digital single-plane x-rays. Body composition and bone mass are now measured with dual-energy x-ray absorptiometry (DEXA). Standard CT analysis has morphed into peripheral quantitative computed tomography (pQCT) and micro computed tomography (micro CT), allowing for the three-dimensional evaluation of bone in vivo or ex vivo. Positron emission tomography (PET) scan instruments are now being utilized in bone infection and metastasis studies. Micro MRI units are available in orthopaedic research core laboratories for imaging all aspects of the musculoskeletal system. More recently, in vivo imaging systems (IVIS) that image bioluminescence and fluorescence have become standard members of the orthopaedic research armada. In addition, biomechanics, the study of forces and strains on the development and structural integrity of bone, is still a prominent focus in orthopaedic research.

Bone is a complex tissue that undergoes remodeling throughout life in order to maintain skeletal integrity. As a result, bone can both adapt to altered loading and

repair fractures. Defects in integrity may be expressed in terms of mineral density, cellular deficiency (either in numbers or function), or in combinations of these. It is this multiplicity of factors that makes bone research challenging. The biology of the osteoclast, or teams of osteoblasts, needs to be considered not only independently but also in relationship to one another, and relative to the physical load in an environment of specific biochemical and endocrine entities being produced by the body. The best studies will include many of the investigative modalities previously mentioned, employing the imaging devices as monitoring tools, and the histology for obtaining high resolution and mechanistic information. And in the end, the bottom line for all this remains: How can this information be used in the prevention of disease and improvement of quality of life for those already afflicted?

A number of important bone pathologies are characterized by loss of bone, either systemically, as in osteoporosis, or locally, as in periodontal bone loss, rheumatoid erosions of the joints, cancer-mediated bone destruction, and periprosthetic loosening. Conditions such as rheumatoid arthritis and osteoarthritis (OA) involve both bone and cartilage.<sup>1</sup> Intense research efforts have provided considerable insight into the mechanisms of bone turnover in health and disease, and have led to the identification of many of the molecules involved in these processes. However, it is clear that a more complete understanding of bone diseases, and the development of improved therapies, will require the coordinated use of all current experimental techniques.

### Current Studies: Histology Is the Foundation

Basic science studies facilitate investigation into cell biology and sophisticated skeletal phenotyping, as well as conventional orthopaedic pursuits, including biomechanical evaluation of implant function.<sup>2</sup> Many of these in vivo studies utilize animal models, including osteomyelitis in rabbits<sup>3</sup> and distraction osteogenesis in rats and mice.<sup>4,5</sup> Combination studies with these platforms include antibiotic efficacy, drug therapies, therapeutic agent delivery, and implantation.<sup>6</sup>

Rats and mice provide an excellent platform for the study of basic bone growth, healing, and response to injury. The immunologic compatibility between human and murine specimens, combined with precision processing of bone samples, has fostered studies on the effects of growth factors, diet, and specific diseases on bone growth and healing. The noninvasive imaging-to-histology pathways in these studies have crossovers and evolutions that closely parallel human clinical diagnostic conduits.

After all, many of the basic studies are developed to answer questions spawned by events seen in the emergency room, operating room, and in other patient populations. If correlations cannot be made, then the value of the information gleaned at the laboratory bench may not have significance for the patient. The primary divergence in this correlative approach can be in the

confirmation of effect. In basic research, once a modality's result is *surmised* by electronic imaging, it is optimal to *confirm* the imaging data via postmortem histology. Clinicians must have far more faith in electronic imaging and serum testing because in the clinical arena, postmortem histologic analysis would not be an acceptable option. Once the clinician is satisfied that a bone infection is eradicated based on results from serology, x-ray, CT, or MRI, therapeutic intervention can potentially be ceased. But in a laboratory evaluation of the efficacy of a new infection eradication regimen, investigation by x-ray, CT, and MRI should be backed up by histological assessment of the tissues to ensure that there are no harbors of bacterial sequestrum present, or that there is no incidental cellular modification as a result of the regimen. In the orthopaedic clinic, a DEXA scan can interpret relative bone density in the osteoporotic patient. In the basic study of osteoporosis prevention, the DEXA and CT scans can reveal which treatments promote superior bone density and quality.<sup>1</sup> But no imaging modality can provide the cellular ratios of osteoclasts vs osteoblasts, the rates of lamellar bone formation, or information on osteoblast and osteocyte apoptosis. For this, techniques such as a histologic workup comprising undecalcified slides stained with methyl methacrylate (MMA) trichrome and von Kossa, as well as unstained slides for dynamic tetracycline labeling analysis, are required.

### Electronic Imaging Tools

A cabinet-style, closed system, variable kilovolt x-ray unit is a two-dimensional x-ray tool that provides, by today's standards, low resolution images of the relative density of the material within the specimen by using long exposure times to yield images of differing contrast.

DEXA is a two-dimensional x-ray-based method for measuring bone density. What sets DEXA apart from plain film x-rays is that DEXA technology can be used to quantitatively estimate the actual mineral content of tissues, thus providing a more accurate determination of bone density in vivo. For comparative purposes, longitudinal repeat measures of bone density are possible.<sup>7</sup>

Peripheral quantitative computerized tomography generates three-dimensional measurements of volumetric bone density in the same manner as a clinical CT scanner. The three-dimensional data acquired facilitate the calculation of bone volume by combining data from serial images down to 0.11 mm in thickness. Whereas the two-dimensional instruments can provide information about the presence or absence and density of bone healing post-trauma, the pQCT provides differential volume of cortical vs trabecular bone either in vivo or ex vivo. Due to its enhanced sensitivity, drug efficacy can be evaluated at earlier timepoints. This concept is becoming increasingly important as in vivo studies of experimental treatments are demanding shorter experimental turnaround and improved resolution.

Micro CT is a closed system unit similar in appearance to a closed system x-ray unit except that this instrument produces volumetric CT data from serial images down to 10 microns in thickness.<sup>8</sup> At this level of resolution, quantitative static histomorphometric indices yielding precise estimates of strength are possible. This is valuable to the orthopaedics researcher because these quantitative indices are achieved without processing or staining of MMA sections. The nondestructive micro CT methodology for the determination of strength translates to no more “bone breaking” on the MTS 858 Bionix materials test system (MTS Systems Corp., Eden Prairie, MN). With this technology, a rat tibia in an osteogenesis study can be evaluated for strength and then submitted for histological evaluation with all the cellular architecture preserved, which means that significantly fewer animals will be needed throughout the course of the study.

**Figure 1** shows a radiograph (x-ray) demonstrating bone loss due to breast cancer metastasis (MDA-MET) in a murine model. Although the presence of bone resorption is evident by x-ray, visualization of the mechanism by which this occurs calls for a tartrate resistant acid phosphatase (TRAP) stain performed on decalcified paraffin sections. As shown at 40X, osteoclasts are recruited and activated by the tumor and are responsible for the bone destruction observed on x-ray.<sup>9</sup>

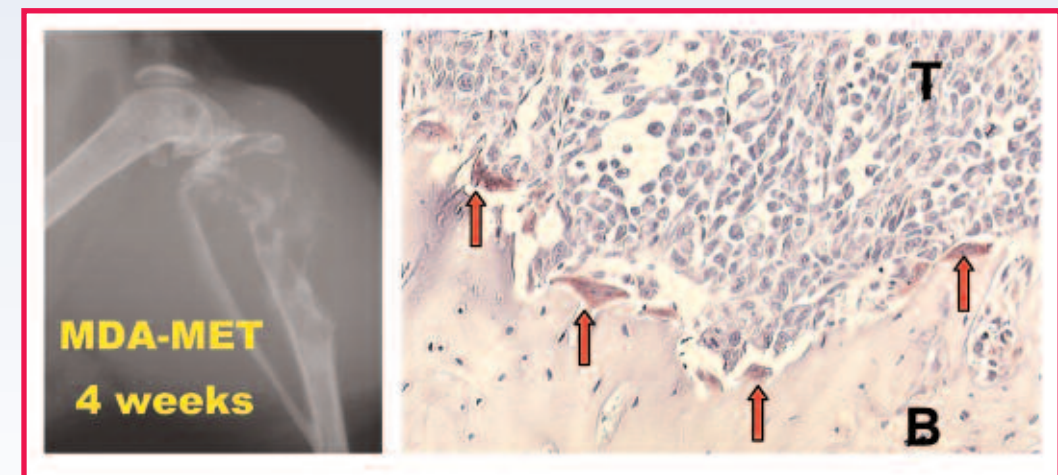


Fig. 1. T=tumor; B=bone.

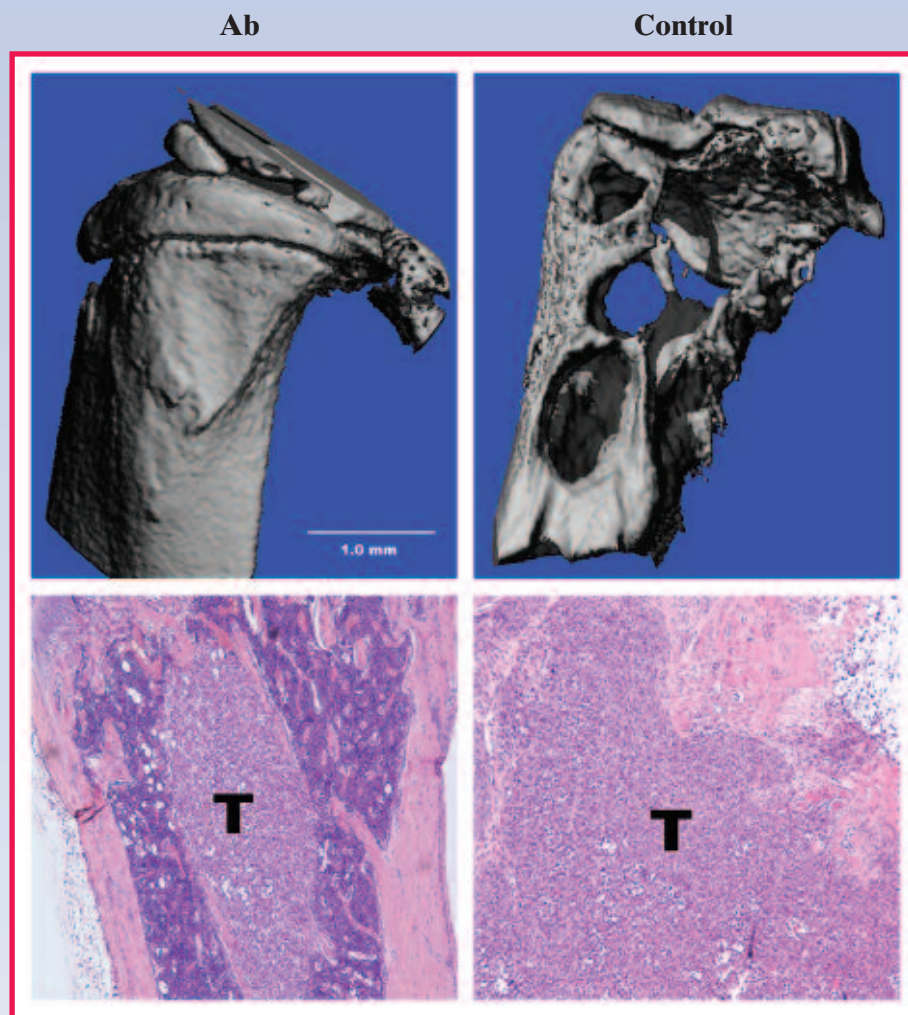


Fig. 2.

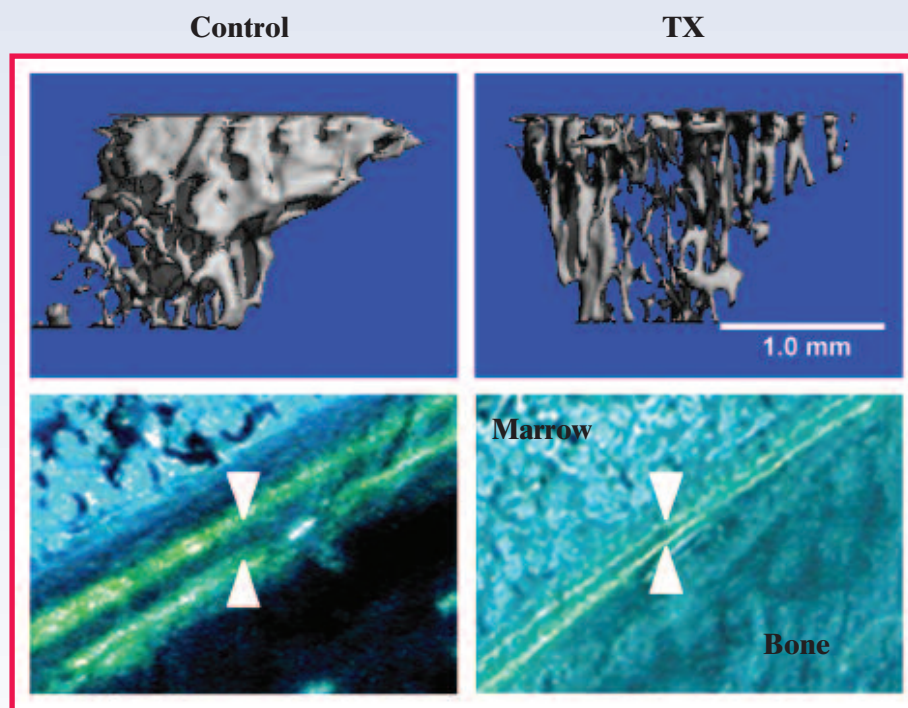


Fig. 3.

**Figure 2** shows micro CT renderings of the tibiae of mice injected with tumor and treated with an experimental antibody treatment (Ab) or a control antibody (Control). While the micro CT image associated with Ab treatment shows maintenance of bone, the image of Control shows extensive bone destruction. However, the postmortem H&E staining of decalcified paraffin sections (Ab, 400X; Control, 100X) shows the presence of tumor (T) in both specimens that was not visualized by micro CT.

**Figure 3** depicts micro CT renderings comparing bone micro architecture of a control mouse (Control) vs a mouse subjected to an experimental drug therapy (TX). While the images clearly depict a loss of bone as a result of the therapy, postmortem histologic analysis of a double-labeled single fluorochrome from undecalcified, MMA-embedded unstained sections indicates the mechanism of the bone loss; the primary reason for the lack of bone depicted by micro CT was not bone resorption as suspected, but rather suppression of normal bone formation<sup>10</sup> (arrows) (Control, 100X; TX, 40X).

**Figure 4** shows TRAP-stained osteoclasts seen in an undecalcified MMA section of bone (400X). Specific labeling locates osteoclasts (arrow) in situ, and their location relative to the cortical bone interface helps assess activity associated with bone resorption analysis.

**Figure 5** shows specific immunohistochemical staining for a human chondrocyte-specific protein in a decalcified, paraffin-embedded section of human bone (arrows) (100X). Proper fixation and precise endpoint decalcification allow for a wide range of IHC procedures.

**Figure 6** shows tetracycline double labeling as viewed in an unstained section of undecalcified MMA-embedded murine bone (200X). Tetracycline is absorbed by newly forming bone at the time of administration. Two pulses of tetracycline at the appropriate times can assist in the evaluation of rates of mineral apposition and bone formation, calculated from the distance between the two labels (arrows).

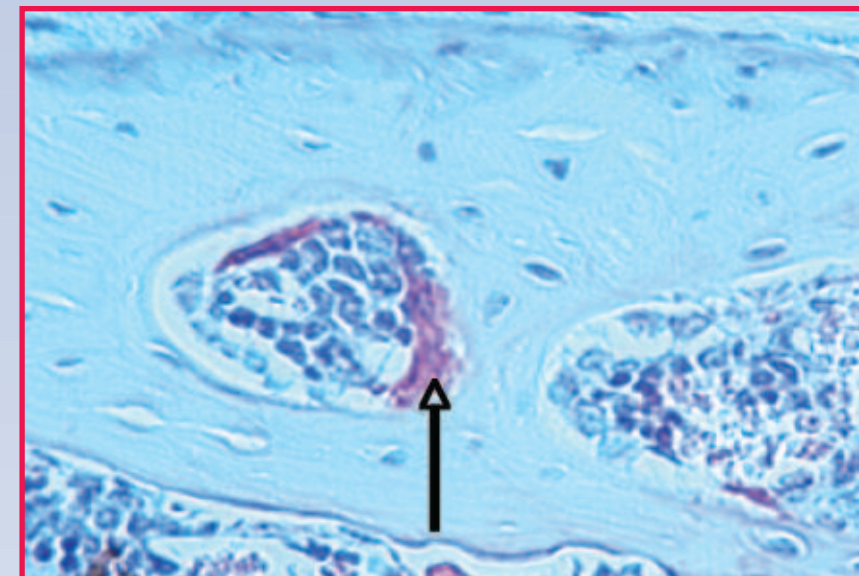


Fig. 4.

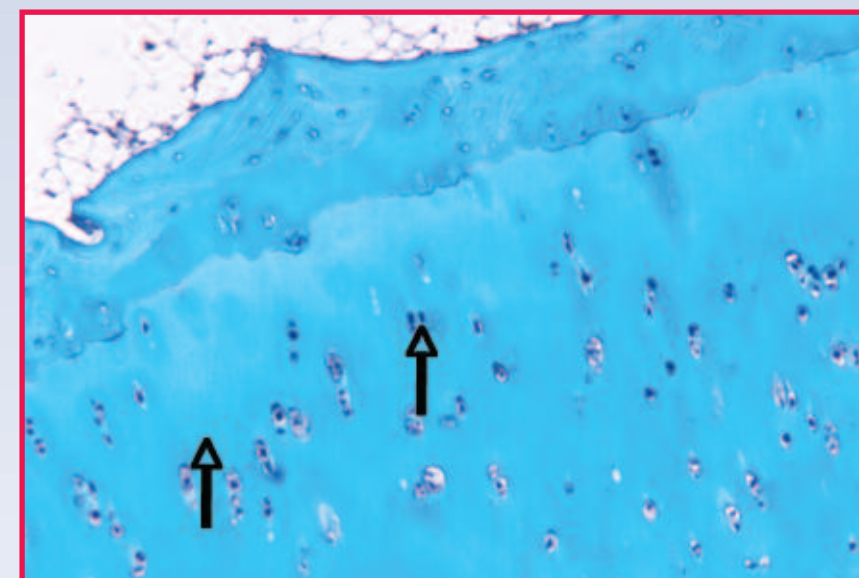


Fig. 5.

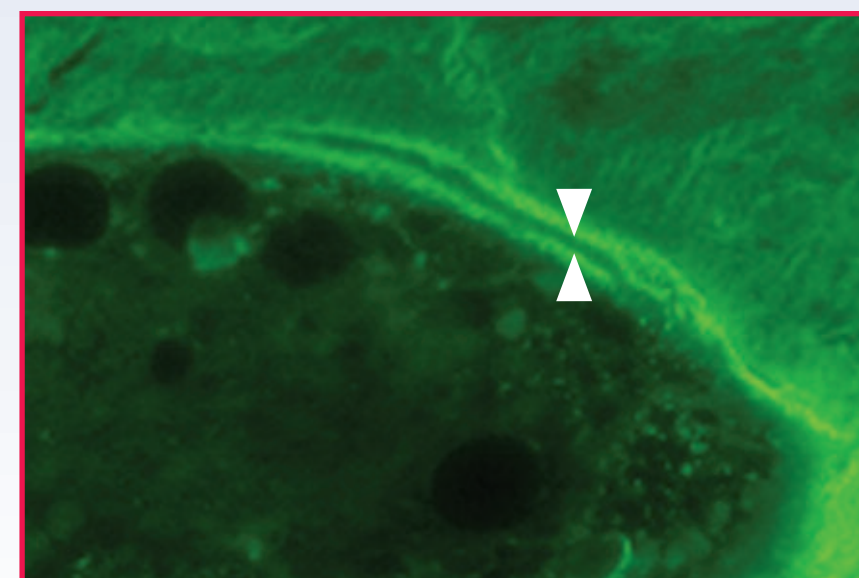


Fig. 6.

## Discussion

So does this constantly evolving electronic imaging technology spell the end of histology in orthopaedic research? Not if there is a need for static and dynamic histomorphometric indices, double fluorochrome labeling, or bone cell histology. These techniques require a great deal of expertise and familiarity with a wide range of methodologies. For example, small samples derived from cartilage explants are processed through glycol methacrylate and sectioned and stained appropriately. Undecalcified samples containing metallic implants are embedded in specialized resins and submitted for “cut & grind” histologic preparation.<sup>11</sup> Throughout the myriad of studies in the orthopaedic research lab, precise decalcification, along with specific processing protocols and refinements in reagents and antibodies allow for application of antibodies such as VEGF (vascular endothelial growth factor), VEGF R1 and R2 (receptor 1 and 2), collagen types I, II, and III, BMPs (bone morphogenic proteins), and others. Apoptosis studies using TUNEL (terminal deoxynucleotidyl transferase biotin-d UTP nick end labeling) or caspase 3 staining are routinely performed in plastic, as well as studies involving RT-PCR and in situ techniques.<sup>12</sup>

There are numerous examples of the power of combinatorial experimental approaches that help to elucidate complex biological questions. We have used computed tomography to measure osteolysis around total hip replacement implants and its progression. Strong associations exist between the volume and progression of osteolysis and the polyethylene (PE) wear that occurs after implantation, suggesting that PE particles may be involved in bone loss. Tissue sampled from zones of osteolysis during implant revision surgery showed abundant PE particles, often within giant multinucleated cells. Immunohistochemistry and in situ hybridization demonstrated that cytokines that activate osteoclasts, such as receptor activator of NF-kappa B (RANK), RANKL (RANKL), and tumor necrosis factor alpha (TNF $\alpha$ ), were strongly expressed by large multinucleated cells containing PE debris.<sup>13</sup> A strong correlation was found between the following four parameters: volume of bone loss, PE particles, RANK

expression, and TNF $\alpha$  expression. Importantly, correlative in vitro studies revealed that RANKL and TNF $\alpha$  synergize to increase the volume of bone resorbed. This suggests that the interaction of TNF $\alpha$  and RANKL promotes osteoclast activity associated with polyethylene wear.<sup>13</sup> These (and other) data suggest that therapies targeting TNF activity may be useful in treating peri-implant osteolysis.<sup>14</sup>

Another example of a combinatorial approach is in experiments designed to elucidate the etiology of osteoarthritis. While this disease results in the destruction of articular cartilage, the subchondral cancellous bone also shows characteristic histological and histomorphometric changes. Using state-of-the-art molecular technologies, we have investigated the expression of skeletally active genes in OA.<sup>15-17</sup> Significantly, some of the changes in gene expression can be directly related to structural changes, suggesting important genetic regulation of the bone changes seen in OA. We are currently using a combination of genomic and proteomic approaches to further investigate the basis of this disease. These modern approaches directly relate to the tissue level changes, and have led to the development of a field that we have termed molecular histomorphometry. All histomorphometric indices are measured including osteoid, trabecular bone area, volume, forming and resorbing surface, and cell numbers. Several of these histomorphometric indices can also be determined using micro CT and appear to correlate well with direct histological measures. However, dynamic histomorphometric measurements are only possible from unstained fluorescent labeled plastic sections. Conventional histomorphometric data remain the gold standard for the assessment of the cellular mechanisms of bone turnover. It is the marriage of the two techniques that provides the complete picture of bone turnover.

### The Future of Orthopaedic Research

This blend of new technology with established histologic procedures has produced a highly desirable situation for the basic research community. And there is a great deal more to be learned from the careful application of these approaches. For example, very few transgenic animals produced around the world have been examined with respect to their skeleton. If they have, it usually involves only a cursory glance at the gross anatomy. Another example is the study of metastatic cancer in bone and why it is so difficult to treat. It requires a much better understanding at the tissue and cell level regarding the establishment, proliferation, and osteolytic behavior of cancer cells in bone. The vision of developing these systems to the point where they are more, rather than less, involved in skeletal research, as well as an integral part of the clinical diagnostic repertoire, must be pursued. In some institutions where basic science-derived histology is a staple, the addition of new technology has actually increased histologic workload. The investigation of bone and musculoskeletal

pathology, whether using animal models or in vitro approaches, combined with powerful imaging and molecular tools, must eventually be related to diseases of the human musculoskeletal system. Results from the potent experimental armamentarium we have at our disposal are beginning to answer questions about what *does* happen rather than what *may* happen. Many recent grant submissions involving the new technologies described here have ultimately hinged on the correlation of histologic data with the imaging data acquired. If this trend continues, with the introduction of new equipment and the development of new technologies, the role of the histotechnologist will be necessary and remain prominent.

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