Bone Histology of Fossil Tetrapods

Advancing Methods, Analysis, and Interpretation

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Image Standardization in Paleohistology

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WHAT IS DIGITAL MICROGRAPHY?

Workstations for digital photomicrography of the 1970s included a microscope, a video camera, and a computer-controlled image analyzer the size of an American refrigerator! Since that time, the microscopes have changed little, but tremendous advances in computer and camera digitalization technologies allow microscopists to record their images with increased ease and at higher resolution for all of their scientific and aesthetic purposes.

More than 40 years ago, the CCD, or charge-coupled device, was invented. A CCD is simply an integrated circuit, a chip of perhaps 2.5 cm² and containing a set of thousands of extremely small image elements known as pixels. The light from each pixel is converted into an electric charge, which is proportional to the light intensity and from which is assigned a value (i.e., a number) corresponding to the level of gray or color in the video signal generated by the chip. The light signal detected by the device converts the entire set of numbers into a digital representation—that is, an image.

Microscopists do not have to make a choice between scientific accuracy and aesthetic quality: What you retrieve from a digital imaging system is a set of numbers, and this set must be perceived visually and understood for the image to be useful. Microscopists convert these numbers, in the range between 0 and 255 of gray or the red-green-blue (RGB) scale, into images that are observed and often measured for explanations and perspectives about the tiny world they see and deliberate upon. However, although many microscopists do not feel it is particularly relevant or important for their images to have aesthetic value, this is exactly what
digital artists attempt to express with these same numbers, and an aesthetic perspective can also benefit science. Artists integrate imagery into the human experience to evoke emotions and a sense of aesthetic value, and scientists can use the same imagery to communicate information.

Many of us are glad for time spent in the darkroom in the days when film cameras decorated our microscopes, learning in analog and alchemy what is now accomplished by digital capture devices and computer processing. It is important to note, however, that the amount of time and effort it takes to acquire an original image of high scientific content and artistic properties has not changed. Every image we produce and give to the public contains the most powerful scientific content and aesthetic appreciation that our experience allows. It is in everyone’s best interest to do so, because the care taken to capture good photomicrographs demonstrates to observers that you have taken care with the science too; moreover, your attention to the aesthetics of good photomicrographs is what will make the scientific points all the more clear and powerful.

**IMAGE STANDARDIZATION**

In the performance of digital photomicrography, it will be useful for paleohistologists to follow a standard, enabling the community to interact more freely and to compare results. The following guidelines are meant to establish such a standard.

**Orientation**

It is important to know the position where a skeletal tissue sample is obtained. For instance, is the bone cross section derived from the endosteal or periosteal cortex, a necessary datum for interpreting growth direction and other histological attributes? Does the image derive from a proximal, middle, or distal location (some investigators use a specific percentile of position along the length of a bone shaft when possible), and is it from the medial, lateral, anterior, or posterior cortex, and from the dorsal or ventral aspect?

**Absolute Scale**

The most common foible of digital photomicrography is the reporting of scale. Though it is necessary to provide details of the lens used to acquire an image (see the following), it is not appropriate to provide an “x” value as an indication of magnification (e.g., 10X). An image may be projected or printed at any size, so this value has no meaning in respect to the actual magnification of an image.

However, there are two useful ways to present information about magnification. The first is to embed a scale bar in the image itself, reporting its length along with the other details of an image (e.g., “scale bar = 100 μm”). The second method is to report the field width (FW), or the left-to-right length across the published
image in a convenient unit (e.g., FW = 156 μm). If the image is a macro view of a specimen, then specimen width, or the maximum left-to-right width of the specimen, can be used instead (e.g., specimen width = 74 mm). Of course, you can provide both a scale bar and the FW (or specimen width), because viewers differ in their preference for one or the other. One drawback of providing FW is that an editor or someone who subsequently uses the figure may crop it or otherwise may not reproduce its entire width.

A third method of reporting scale applies specifically to images available online for downloading, and is related to FW. This value is a unit measure per pixel (e.g., 3.7 μm per pixel). Digital images have a specific pixel resolution, the values of which are sometimes given as image size (e.g., 1024 x 768), and are stored in the file properties and image metadata. If given the unit measure per pixel (and assuming the image has not been down-sampled or binned in reproduction), anyone working with your downloaded image can calibrate their imaging software (e.g., Adobe Photoshop) and make measurements. Of course, if FW is reported, someone wishing to download your image and make measurements can always calculate the unit measure per pixel themselves, and vice versa.

Resolution

There is no firm standard that can be applied to image resolution; this remains a murky subject. It serves no purpose to specify a minimum pixel resolution for acquiring images, because this value contains no information about image detail, which is the true physical attribute of your image that counts for anything. However, if memory storage and computer speed are not constraints, then a general rule of thumb is to acquire an image at the highest pixel resolution possible. This will capture the most image detail afforded by your image acquisition system. Should it be more resolution than you need, it will nevertheless provide you some ancillary advantages over a lower resolution image.

The highest resolution image should be your original (unedited) image, even if this is not the one you present and publish. The original should also be the one you provide to an image database. Acquiring images at the highest resolution with which you can be comfortable (in terms of the constraints that time, digital space, and software will permit) allows your images to be used as a resource beyond your present needs. Keep in mind that it is far better to eliminate image detail that you don’t require now with image processing algorithms than to find yourself needing detail you don’t have at a later date.

Print publication standards vary, but most specify a pixels per inch (ppi) resolution to which your images must adhere, usually in the range of 200 to 600 ppi, and a minimum image width (e.g., 6 in). There are important reasons for this, so it is best to understand what you should and should not do to meet this specification. For example, Figure 5.1 is an image of the femoral cortical bone of "Lucy"; a
three-million-year-old early hominin representative of the taxon *Australopithecus afarensis*. Information in the Adobe Photoshop "Image Size" window for this image indicates that it has pixel dimensions of 2160 x 1584 and a file size of 9.79 megabytes (MB). At a resolution of 72 ppi, the document is 30 in. wide and 22 in. high. The publisher requests that this image must be at least 200 dots per inch (dpi) and 10 in. wide. Note: Publishers sometimes report their specification in dpi, but they mean ppi.

In Adobe Photoshop, the first step must be to uncheck the "Resample Image" box. Then we enter 10 in. for document width, and see what happens. By unchecking the Resample Image box, we are careful not to change the file size, thus keeping all of the original resolution to the image at this file size. Luckily, in this case, to maintain the original resolution, Adobe Photoshop increases the ppi to 216, which fulfills the minimum requirement of the publisher (see Figure 5.2). However, what if the publisher requested an image of 600 dpi (or ppi) at 10 in. wide? Only after having reduced the document size to 10 in. and discovering that the ppi was too low (i.e., 216) would we then check the Resample Image box and enter 600 ppi (see Figure 5.3). Because document size is constrained to 10 in., the consequence of increasing ppi while reducing the width and height of the image must be to increase dramatically the number of pixels (expressed as "Pixel Dimensions" in the window) and the image file size. Where did all of this resolution come from? Nowhere; the program creates it. It is self-evident that no new image detail can
be created, and that the image will look no different than before we had made the transformation at the same print size. Be aware, however, that such a request by a publisher's is not without merit; image specifications are designed to relate directly to the dpi of a given printing technology, so conforming to their requirements will help to preserve your image properties. Just remember, to prevent any undue artifacts arising from image transformations such as this, resize your image as a first step before you start adding empty resolution.

Resolution for images meant to be viewed online and on screen only should be treated differently from images reproduced in print. First, the desired document size must be decided, keeping in mind that most desktop computer screens are able to view a reasonably large area. Then, reduce the ppi to 72, which is the pixel resolution of computer screens today. This will generate a rather small file size suitable for online viewing only.

**IMAGE ACQUISITION SYSTEM FOR LIGHT MICROSCOPY**

Microscopists should record and provide for observers several details about the image acquisition system: information about the light, microscope, and camera system used. Information about the nature of the light used—transmitted or reflected, polarized or not, and so on—is necessary for understanding image
properties. The manufacturer and model of the microscope are important, as are the specific details about the lenses used. Concerning the latter, both the magnification rating and the numerical aperture (NA), or the value of a lens that determines its light-gathering capacity, are critical determinants of image quality. The convention is to provide the lens magnification followed by the NA (e.g., 40/0.70 means 40x and an NA of 0.70). Finally, the camera manufacturer and model plus the image acquisition software are important determinants of the quality of your image. The following is an example of how such a description of the image acquisition system might appear in the methods section of a scientific paper:

Histological “ground” thin sections were coverslipped with ethylene glycol and imaged in conventional transmitted light (LM) and circularly polarized transmitted light (CPL) using a Leica-Leitz DMRX/E Universal Microscope configured with a Marzhauser motorized stage and CPL filters and using Leica PL Fluotar 40/0.70 (enamel) and PL Fluotar 20/0.50 (bone) objective lenses. LM and CPL images were acquired with a JVC KYF55B color video camera using Syncroscope Montage Explorer software. (Synoptics Inc., Frederick, Maryland)

A figure caption appropriate for a light microscopy image may be found following Figure 5.1.

Note that although all compound microscope lenses include magnification and NA on the body of the lens casing, this is not always the case for stereo zoom light microscope lenses, in which case one must be as complete as one can be with the description of the microscope.

IMAGE ACQUISITION SYSTEM FOR SCANNING ELECTRON MICROSCOPY

The image reporting requirements of scanning electron microscope (SEM) digital photomicrography vary from those of light microscopy. An electron beam is manipulated differently than a beam of light, and some novel image-acquisition variables need to be recorded. Fortunately, all digital SEMs are able to embed a data zone on the image that can be set up to provide all of the necessary information, including a scale bar and FW (see Figure 5.4).

In the example of an image of Lemur sp. molar enamel (see Figure 5.4), this novel information includes the accelerating voltage (extra-high tension, or EHT, measured in kilovolts), probe current (e.g., measured in picoamperes, or pA), working distance (WD, measured in millimeters), and the operating or signal mode (e.g., secondary [SE] or backscattered electron [BSE] mode; the latter is represented as CZ BSD in Figure 5.4). The microscope manufacturer and model must also be reported, which is a Zeiss EVO 50 SEM in the case of Figure 5.4. Because this is an environmental microscope, and the specimen shown in Figure 5.4 was imaged at high pressure and without a conductive coating, the vacuum pressure
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adjust white balance or focus? Did the software focus automatically, and how often? You should also report the settings used during image acquisition, especially if the format, size, or resolution changed later in the process. Were the images first saved in RAW, JPEG, or TIFF format? Were the JPEGs 8 bit or 16 bit? What settings for image size, compression, and quality were used? An example of this type of reporting might resemble this portion of a published methods section:

These digital photomicrographs were taken as 8-bit jpgs (quality = fine, compression = optimal quality, image size = large/4288x2848 pxl). The interface program for these images was Camera Control Pro 2 (Nikon Inc.) running on a Windows 7 (64-bit; Microsoft, Redmond, Washington) computer (IPP, Palo Alto, California). (Werning 2012)

**Photomontaging Software**

The recent publication of powerful and relatively cheap panoramic/photomontaging software has provided histologists with the opportunity to create images that show large sections of tissue in great detail and at high magnification. These programs take multiple overlapping images and digitally stitch them together to create a larger composite image, called a photomontage, panorama, or pano. Depending on the number of component images and their resolution, the resulting photomontages may enter the gigapixel range and are often computationally intensive to create. Furthermore, the algorithms used to rotate and align component images, correct lens errors, and project the final composite image vary by program and user setting. Changes in these settings affect the size and shape of the photomontage produced, so it is important to report them. Additionally, settings related to the original images can determine the quality of the panorama (for example, amount of overlap in adjacent images), and these also should be reported.

An example of this type of reporting might resemble this portion of a published methods section:

For the cross-sectional images of the specimens/elements listed above, overlapping digital images (overlap 50% by eye in X and Y directions) were taken under regular transmitted light at 5X total magnification using a D300 DSLR camera (Nikon Inc.) through an Optiphot2-Pol light transmission microscope (Nikon Inc.) . . . I then assembled photomontages of the full cross-sections using Autopano Giga 2.0 64Bit (Kolor, Challes-les-Eaux, France), with the following settings: Detection settings: detection quality = high, layout = free. Optimization settings: strong (for partial cross-sections) and gigapixel (for full cross-sections), optimizer stages: local approach, strong algorithm, first optimization, clean up control points or links, keep only control points below the error RMS = 2.0, final optimization, advanced distortion. Panorama settings: preferred projection = automatic, preferred extend = clamp to panorama content, initial type of anchor = mono transfer function. Render settings: size = 100%, algorithms: interpolating = bicubic, blending = smartblend, format = jpg, depth = 8 bits, layers = none, DPI = 72. (Werning 2012)
Image Editing Software

The reporting of image editing software generally includes a brief mention of the programs used to manipulate the images, basic information about the type of manipulation done (e.g., cropping images or adjusting brightness, contrast, or color), and the extent of the manipulation. However, this approach occasionally ignores the true extent of manipulation, which may begin even before a researcher views the image.

For example, panoramic software easily creates images that are too large to be opened by most available image editing programs, whether because of pixel dimension limits (many programs cannot process gigapixel images, and thus limit image size to a 30,000 pixel maximum length or width) or file type constraints (e.g., the TIFF file format was only restandardized to allow 64-bit offsets). Before 2011, they were limited in size to 4GB, the maximum file size when using 32-bit offsets, or RAM limits (e.g., programs designed for 32-bit systems cannot access more than 4GB of RAM). These constraints may necessitate additional software to reduce pixel dimensions and file size before the image can be viewed and edited, or software plug-ins for other programs to open the image and/or save it in a different format. If used, this software should be reported.

The full name and edition of the software used for image editing always should be reported, for the simple reason that program capabilities vary with edition. For example, Adobe Photoshop CS5 can open file formats and sizes that Adobe Photoshop CS2 cannot, and Adobe Photoshop CS5 Extended has additional analytical tools that Adobe Photoshop CS5 does not. Additionally, one may opt to use various third-party filters and plug-ins that are not part of the typical install. These also should be reported. The extent of the image manipulation is also important to note, as is any ultimate changes in file format. An example of this type of reporting might resemble this description (not previously published):

The original uncompressed tiffs were edited in Photoshop CS5 Extended (Adobe Systems, Inc.) to remove the background and to add a scale bar. Brightness and contrast were adjusted uniformly across the image using the Autoleveling filter in the FoveaPro 4.0 Image Adjustment package (Reindeer Graphics) in Photoshop. The image was then resized to 50% of the original pixel dimensions and resaved as a jpg to comply with journal submission requirements.

Note that noncommercial programs and filter packages used for image editing and analysis often require citation. This may be as simple as including a link to the software/filter download, but may include published references (e.g., when a package is developed for an academic open-source program such as NIH Image).

Reporting these settings and programs may seem cumbersome, but it is the only way to ensure reproducibility of results. If publication text limits are a concern, these methods can be reported as part of online supplements.
CONTEXT DEPENDENT ATTRIBUTES OF IMAGES

All imaging conditions are either controlled or constrained by a context determined by the characteristics of your sample and the purpose of its investigation. The following guidelines, although not meant to establish specific standards, concern additional reporting requirements for your records and for images provided to the viewing public.

Light

The manipulation of a light beam in light microscopy is ordinarily designed to provide image contrast. Straightforward transmitted or reflected white light presents an image whose contrast is determined strictly by adjusting the obliquity of the incoming beam(s) (e.g., by manipulating the back aperture of the microscope or the orientation of fiber optic lights) and by the size and absorbing attributes of objects in your sample. This image is usually the starting point of any microscopical investigation, and from there more contrast-enhancing techniques are used, such as polarizing microscopy, phase contrast, and differential interference contrast, which must be described in the records and reports of images. Transmitted or reflected polarizing microscopy is the most common method for enhancing contrast in specimens paleohistologists observe.

Because manipulations of light beams generate images that vary depending upon the attributes of a specimen, it is critical to understand how light signals return to the eye and the camera in order to interpret images correctly. Inferences about the microanatomy of a specimen must always bear in mind this principle, so we will briefly mention methods for enhancing contrast that paleohistologists popularly use.

There are two fundamental ways of enhancing contrast: One is by means of optical components that alter the path of a beam of light, and the other is by treating the sample with preferentially light-absorbing materials.

The back aperture of a light microscope is the most common method for obtaining contrast. Adjusting the diameter will determine the extent to which the light beam is focused to infinity (parallel) or not (oblique). Oblique light is either absorbed by specimen details or it interacts with objects at angles that internally reflect light away from the optical axis.

Light beams are also commonly manipulated by filters. Plane-polarized light is the most common method for obtaining contrast from histological thin sections. Photons in a beam of light propagate in all conceivable sinusoidal vibration planes (i.e., when the light is unpolarized). When the light is linear or plane polarized with an arrangement of two polarizing filters, one vibration direction is isolated. The first filter (the polarizer) is positioned between the light source and the specimen, permitting light to pass in only one vibration direction. The second filter (the analyzer) is positioned between the specimen and the eyepiece objectives or
recording system, with its vibration direction at 90 degrees to that of the polarizer. This arrangement of filters is often referred to as “crossed polars” or “crossed plane polarizers.” Some polarization microscopes use a pair of special calcite crystals to generate plane-polarized light, and their arrangement as “crossed nics” is identical to that previously described for polarization filters.

In the absence of a birefringent material in the optical path, the analyzer blocks all light emanating from the polarizer: the image therefore appears black, or extinct. When a birefringent specimen is placed between the two filters, the optical properties of that material interact with light exiting the polarizer to change its vibration direction to that of the analyzer such that the light may pass. Bone histological thin sections are most commonly observed with polarized light because the collagen they contain is birefringent. Indeed, the presence of image contrast in polarized light microscopy of fossil bone may sometimes be a clue to the presence of relatively intact collagen remnant in the sample. Bronage et al. (2003) described in detail the justification for converting a linear polarizing microscope into one providing circularly polarized light, because this simple innovation manipulates the light in such a way as to avoid artifacts commonly present in images obtained by linear plane-polarized light microscopy.

Another device paleohistologists commonly use is the neutral density filter, which limits the intensity of light over the whole visible light spectrum. These filters come in a variety of light-absorbing powers, and are typically used when overall light intensity is high. Light intensity may be high for several reasons: Section thickness may be very thin, and the lamp at its lowest setting continues to offer more than the desired amount of light; the specimen may be especially clear; and/or the desire for a shallow depth of field, and thus a fully open back aperture, renders too much light. Good-quality neutral density filters suppress all wavelengths of light equally, so colors should remain faithful. However, when some parts of a histological section are suitably illuminated, other parts may be too bright and/or too dark. When this occurs, a series of images taken at different light intensities using lamp controls and/or neutral density filters may be subject to high dynamic range, or HDR, imaging software. This software will preferentially use the brightest images of the darkest objects and the darkest images of the brightest objects to present a final image with the most even illumination intensities possible across the field of view.

A second form of contrast enhancement is achieved by applying a light-absorbing material, which we observe to preferentially bind to certain microanatomical details in the histological section. Nature sometimes unwittingly generates such histological contrast as a result of mineral precipitates formed during diagenesis (e.g., manganese “staining”). It must be possible to simulate such contrast, but it is very uncommon for paleohistologists to apply surface stains or other light-absorbing substances to their histological thin-sections. We have no reason to expect that such experimentation will be fruitless.
Finally, in the case that a contrast-enhancing method is used and a significant portion of the light is lost to the technique, it is recommended that images also be published in transmitted light (or reflected light as the case may be) so that observers can have an unfettered appreciation for the details of the natural specimen.

*Color versus Grayscale Images*

There are sometimes very explicit reasons for obtaining images in either color or gray scale, particularly when color or gray image-processing algorithms are used in image-analysis applications. Except in these situations, it is generally recommended that you acquire and provide images in color, adjusting when possible the camera color settings to match the subjective image observed directly from the eyepieces. Color images have the advantage of providing information about important attributes such as diagenesis. Moreover, because the human eye–brain complex is designed to use color for discriminating image detail, observers will always be more fully aware of such details that you describe in narratives of your images that contain color.

*Section Thickness*

Reporting section thickness in paleohistology is uncommon, but it is important information and should be reported whenever possible. A 100 ± 5 μm ground section thickness is standard in many mineralized tissue investigations because a known and uniform section thickness is critical for quantitative analyses of the three-dimensional distribution of image detail, collagen fiber orientation, and so on. However, we know too well that requiring this standard in paleohistology is unsustainable because of the vagaries of specimen constraints. We must nevertheless be aware that differential brightness within and among specimens may be purely a result of the optical path difference by which light passes through the section, and thus to properly compare one section with another, we must know and be able to compare section thicknesses among samples. It is obvious that as section thickness diminishes, spaces in the section void of tissue increase and average brightness in, say, a polarized light image, will cause an increase in background extinction (i.e., darkness), which must be considered in comparisons of brightness among specimens. It is also obvious that section thickness is critical when performing classical stereology, in which we numerically estimate image content from discrete 3D volumes within histological thin sections.

As knowledge of section thickness is important, so too is the care that must be exercised in measuring thickness. Enough measurements should be made at intervals around the section to ensure section uniformity or to map the variation in thickness. If you are lucky to be using a microscope fitted with factory-calibrated precision staging in the Z-direction, then a determination of section thickness is trivial. With such microscopes, the focal plane of a reasonably high-magnification
lens (e.g., 20x or 40x) is positioned at the top of the histological sample, and then a number of microns is read in the travel to the last in-focus plane at the bottom of the section. That is, focus on the top surface and then, while moving the stage/specimen upward, count the microns traveled in the Z-direction until the bottom surface of the section is in focus (image details or surface imperfections—e.g., scratches—can be used to confirm when the focal plane is at the top and bottom surfaces). Because this measurement reading is foreshortened by the refractive index of the tissue, mounting/embedding materials, and often a glass coverslip, this value must be multiplied by the composite refractive index. We routinely use the value 1.52 for fresh mineralized tissue preparations, which, although not perfect, gives a value more true than we would obtain with a hand micrometer (i.e., the number of microns traveled in the Z-direction multiplied by 1.52 equals section thickness).

Experience with such optical, noncontact measurement techniques has demonstrated that measurements made with a grade of hand micrometer conventionally used for determining section thickness (e.g., 10 µm interval micrometer) frequently differ on the order of ± 10 µm away from our optical measurements (even when achieving excellent precision). This, we believe, is due primarily to microscopic debris distributed on the section, the micrometer tips, or both, and/or very small variations in the timing, speed, and pressure applied to the micrometer spindle. Some of us can attest to greater than 10-µm diameter particles in local tap water, so rinsing in distilled water or some other particulate-free solution is also a variable to consider prior to measurement with a hand micrometer. Thus, attention to section cleanliness, dynamic variables, repeatability, and perhaps measurement averaging are extremely important when using these instruments. Digital micrometers will improve the reliability of measurements, but considerable care in their operation is still warranted.

**Future Research Needs from Images**

Integrative scientific research is lauded as a major source of new knowledge, an approach that blurs borders among fields and miscible subject areas. Moreover, cooperation is a requirement of integrative research, even before any collaboration. To promote cooperation and data sharing, even if we do not collaborate, it will be useful, to the extent that we can be giving of our time, effort, and funds, to provide images that may contribute to future research agendas. To that end, we consider the following.

**Field of View**

Particularly since the advent of motorized microscope staging and photomontaging in both the X- and Y-axes, there is the possibility to obtain high-resolution images of extremely large fields of view (e.g., 20,000 to 40,000+ pixels in both
X- and Y-directions). From these images, we may crop the desired field of view in postacquisition processing for our specific research needs. If such large fields of view are available, future researchers may find new potential in your images. Note: When acquiring large montages, it is particularly useful to record the unit measurement per pixel (e.g., μm per pixel), because this value will be needed for giving an absolute scale to cropped portions of the montage.

Field of view can also be extended in the Z-axis. Sometimes we may acquire an image stack—also called a through series—for the purpose of reconstructing an image of the 3-D distribution of structures within a histological section or of a microsurface topography. Montaging in the Z-axis may also be performed so that all in-focus content can be collapsed into one sharp 2-D image. In either case, it will be extremely useful for your research and the future research of others if the source files of image stacks are placed in an accessible database.

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LITERATURE CITED
