

Blank-field correction for achieving a uniform white background in brightfield digital photomicrographs

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Color digital images are rapidly becoming the medium of choice for the publication of photomicrographs in scientific literature. For example, the proportion of pages with brightfield photomicrographs published in color in *Veterinary Pathology* (issue 3 of each year) increased from 25% in the year 2000, to 50% in 2003, and 60% in 2006. The use of color is especially valuable for immunohistochemistry, where subtle color differences are important for image interpretation. The full application of color digital imaging is limited by several variables—two of the most challenging are color balance and even illumination.

An image with balanced color has equal illumination by all colors of the spectrum; in red, green, and blue (RGB) mode, the histogram peaks of red, green, and blue overlap, resulting in an image with a white or grey background. Modern digital cameras designed for photomicroscopy have a white balance feature that eliminates most problems with color balance, but these cameras can cost more than U.S. \$10,000. Digital consumer cameras adapted for photomicroscopy cost about one-tenth as much, but they might not have a white balance feature that adequately compensates for the needs of brightfield microscopy.

Achieving even illumination throughout the field is problematic with color or black and white images, even with the best brightfield microscopes. The most obvious problem—especially at low magnification—is vignetting, in which the outer edges of the image are significantly darker than the center. Some modern digital cameras designed for photomicroscopy have a shading correction feature that decreases

problems with vignetting; also, image processing software can be used to partly correct vignetting of a single image (1). However, vignetting is most effectively eliminated through use of a corrective blank field (2,3). The process involves capturing a tissue image,

locking the aperture and exposure time, and then capturing a second image after moving the stage to an area of the slide that contains no tissue, but is otherwise identical to the image (i.e., same lighting, coverslip, and mounting medium). The blank image captures all aberrations in color balance and illumination that are not inherent in the stained tissue. Intuitively, color and illumination aberrations in the tissue image will be eliminated by subtraction of identical aberrations in the blank image; the resultant image will include only the tissue with a uniform white background. However, the actual image processing to achieve this goal is not as intuitive, requiring division of information in the tissue image by the blank image, followed by multiplication by a constant (2,3).

Methods available for blank-field correction all have limitations that prevent their widespread use. The free program Image Arithmetic 2.5 (2) works well, but it cannot process tagged image file format (TIFF) files. Regitnig et al. (2) provided instructions for image division using the free program Scion Image (the Microsoft® Windows® version of NIH image), but their instructions did not work on Scion Image version Alpha 4.0.3.2. Also, Scion Image cannot process Joint Photographic Experts Group (JPEG) files. Another limitation of free

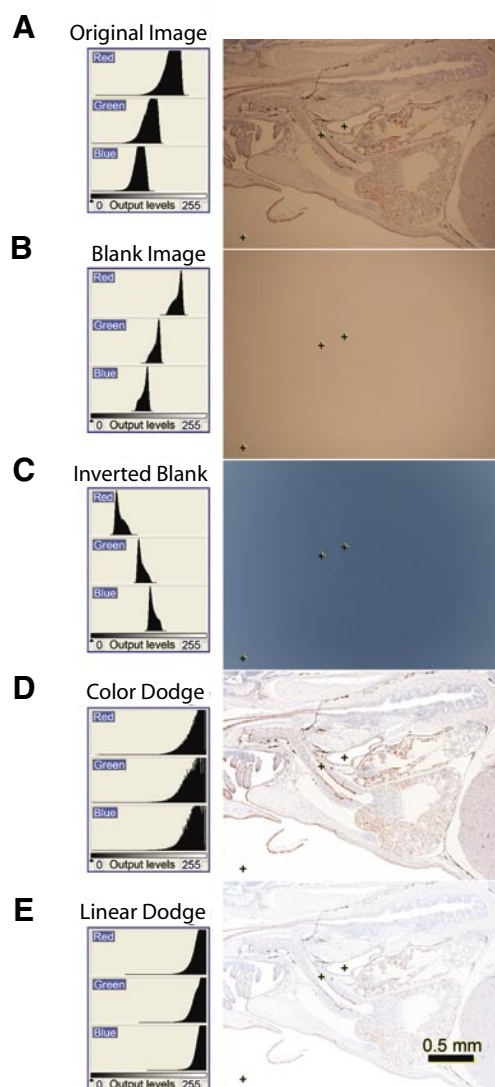


Figure 1. Images for achieving blank-field correction described in Protocol 1 (A–D). When the (A) original tissue and (B) blank fields were photographed, the color temperature of the light source was too low, and the white balance feature of the camera was turned off. (C) Panel B, inverted. (D) The best final image for most purposes used the color dodge (image division) feature of Adobe Photoshop to yield this image. (E) Alternatively, some types of quantitative analysis might require a final image achieved through image addition, using the linear dodge feature of Adobe Photoshop. The plus symbols mark the pixels compared in Table 1. The image is a mid-sagittal paraffin section of the heart and surrounding structures of a pink salmon alevin exposed to weathered crude oil during early development; the section was stained with a monoclonal antibody specific for cytochrome P4501A with a hematoxylin counterstain (5). Magnification is the same for all images (12×).

Benchmarks

programs is that it can be expensive to get them tested and approved for loading onto networked computers. Also, blank-field correction has not been widely adopted, because the process has not been described for the image-processing program that most people actually use: Adobe® Photoshop®.

Color balance and even illumination can both be achieved with blank-field correction using image division that is part of the color dodge feature of either the full version (tested versions 5.5, CS, and CS2) or the less expensive consumer version (tested Photoshop Elements 3.0) of Adobe Photoshop (Figure 1, Table 1, and Protocol 1). All tested versions of Photoshop can process a wide variety of file formats, including JPEG, bitmap, and TIFF files. The protocol works equally well with black and white images. Images in Figure 1 and Figure 2 were captured using a Nikon DS-5M camera with a DS-L1 control unit and a Nikon Plan Apo 4× objective lens, numerical aperture (NA) 0.2. Images were captured at 2560 × 1920 pixel resolution and saved as bitmap files (file size 14.4 MB). The original tiled image used to create Figure 2B has a pixel resolution that can be printed at a 300-dots per inch (dpi) resolution at a full poster size of 127 × 31 cm. In Figure 1, the color dodge feature of Adobe Photoshop changes the bright areas of the image progressively more than the dark areas, resulting in a wider range of color intensity in the final image (Table 1). By comparison, the linear dodge feature uses image addition to produce a final image with a range of color intensity that is usually less than the original image, and the resulting final image appears overexposed. Differences in these two features are most obvious for dark staining tissues (Table 1). For presentation of brightfield photomicrographs, image division (color dodge feature) results in the best final image. Image addition (linear dodge feature) might be the best choice when quantitative image analysis is used to determine the absolute difference between bright and dark areas of an image.

Blank-field correction significantly decreases artifacts in digital images using an intuitive method that is readily repeatable and scientifically authentic

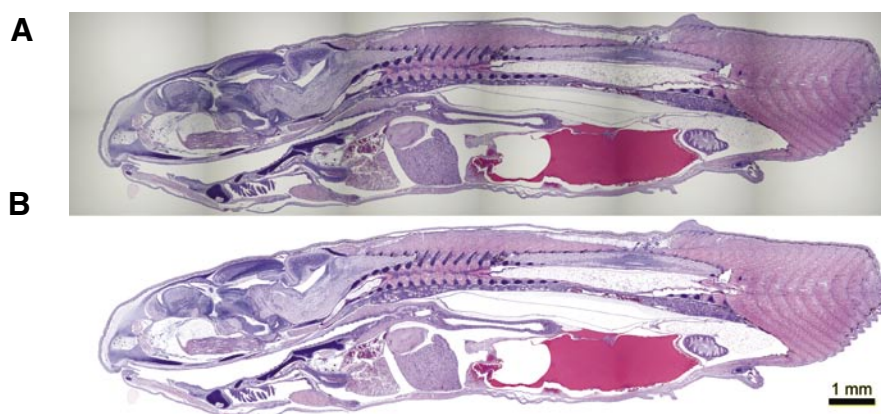


Figure 2. Blank-field correction eliminates vignetting within a 6 × 2 tiled image of a normal pink salmon alevin, mid-sagittal paraffin section with hematoxylin and eosin (HE) stain. The white balance feature of the camera was turned on for this demonstration. (A) Vignetting at the margins of each tiled image is most obvious around the head. (B) Margins of the 12 original images are undetectable after each image was corrected using Protocol 1.

(4). Another advantage of blank-field correction is that it allows for seamless stitching of multiple tiled images. This process can be used to produce high-resolution large images for posters, and the greater resolving power of higher magnification objective lenses used for each tile increases the resolution of the final overview image. Several vendors offer systems for automated image tiling or slide scanning, but these expensive systems do not produce better results than manual blank-field correction and stitching.

The use of blank-field correction for digital microscopy also has at least two challenges. First, it is difficult to find a completely clean blank image on a microscope slide, particularly when using low-power objective lenses. Because artifacts on the blank image would introduce artifacts into the tissue image (i.e., they would incorrectly make dark pixels brighter), it is best to remove discrete artifacts (such as dust, human epidermal cells, etc.) from the blank image (e.g., using the Photoshop rubber stamp tool) before inverting the blank image; to document these modifications, both the original and corrected blank images can be saved. Similar discrete artifacts should not be removed from the tissue image, because their removal might alter the data contained in the tissue image. An option for capturing a clean blank image is to remove the slide from the microscope

stage and photograph the blank image without a slide (2).

The second challenge of blank-field correction is that slight variations in image intensity between the blank and tissue images will mean that in the final image about half of the pixels with no tissue will be off-scale, limited to the maximum value of 255 on the RGB scale. Off-scale loss of information can be prevented by slightly brightening the blank image before it is inverted; however, this problem might not be worth correcting, because pixels that contain tissue rarely go off-scale after correction.

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COMPETING INTERESTS STATEMENT

The author declares no competing interests.

Table 1. Red, Green, and Blue (RGB) Values For Pixels From Identical Pixel Coordinates

Image (from Figure 1)	Red Channel			Green Channel			Blue Channel		
	Bright, No Tissue	Dark, No Tissue	Dark Red	Bright, No Tissue	Dark, No Tissue	Dark Red	Bright, No Tissue	Dark, No Tissue	Dark Red
A. Original	196	171	141	145	129	32	120	104	20
B. Blank	198	172	194	148	130	148	122	105	121
C. Inverted blank	57	83	61	107	125	107	133	150	134
D. Color dodge correction ^a	252	254	185	250	253	55	251	253	42
E. Linear dodge correction ^b	253	254	202	252	254	139	253	254	154

Pixels are from (i) the center of the field with no tissue (bright, no tissue); (ii) the corner of the field with no tissue (dark, no tissue); and (iii), the center of the field where the tissue stains dark red (dark red). Intensity of each color in the RGB scale can range from 0 (dark) to 255 (bright) (see Figure 1, +).
^aColor dodge divides the pixel value of the original image by the pixel value of the blank image, and multiplies the result by 255.
^bLinear dodge adds the pixel values of the original and inverted blank images.

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Protocol 1. Steps to Achieve a Uniform White Background in Brightfield Digital Photomicrographs

Before starting, adjust the microscope at the desired magnification for Koehler illumination and white balance the camera.

1. Capture tissue image and save image.
2. Lock aperture and exposure. Set auto exposure (AE) lock on the camera to ensure that exposure conditions are the same for steps 1 and 3. On some camera systems, AE lock can be set before step 1.
3. Capture blank image. Move to a blank area of the slide (i.e., coverslip, but no tissue) and expose blank image (save as in step 1).
4. Process using Adobe Photoshop (see Figure 1).
 - a. Open the tissue image and blank image.
 - b. Invert the blank image (use Control-I for Windows, Command-I for Macintosh).
 - c. Go to the inverted blank image; on the program menu, click Layer → Duplicate Layer → Destination Document. Choose original image filename; click OK.
 - d. Go to the window with the tissue image (it should now be covered with a layer containing the inverted blank image).
 - e. In the Layers Palette, click the pull-down menu, and then click on Color Dodge. The image now has a uniform white background.
 - f. Flatten image (click Layer → Flatten Image).
 - g. Save the final image.