



Research integrity: VIB guidelines

Acceptable scientific image manipulations

Introduction

Images represent a very important source of information in scientific articles. Much of what we conclude comes from what we see in a picture. In our area of life sciences we use images of gels, blots, organisms, cells, organelles and much more. ***Images can be manipulated to help us better see what is in the image.*** Contrast, grey scale and brightness can be adjusted, filters can be used, images can be cropped¹ or compressed, the number of pixels altered, lanes or zones can be cut away, etc. Certain alterations are linear, others are non-linear. Not all manipulations are scientifically correct. There are manipulations that fall within the scope of scientific misconduct², because they result in misrepresentation of the data. Such misrepresentation makes it impossible for others to interpret the data correctly, or worse, leads to conclusions that are not correct. Scientific misconduct is generally seen as a very serious offense that is likely to harm a scientific career, and may result in severe sanctions (see VIB policy and procedures on scientific misconduct³).

Below a guideline and recommendations are given on what constitutes acceptable and unacceptable image manipulations. Much of what is given below has been copied or adjusted from:

- The guidelines for image manipulations of Rockefeller University Press;
- The Council of Science Editors White Paper on Promoting Integrity in Scientific Journal Publications, 2012 Update.
- The online education tools of the Office of Research Integrity of the Department of Health and Humans Services, U.S.A.

VIB standard for acceptable image manipulations

VIB supports and maintains the following standard from the Rockefeller University Press and Journal of Cell Biology on image manipulations:

- ***No specific feature within an image may be enhanced, obscured, moved, removed, or introduced.***

¹ Cropping refers to the removal of unwanted outer parts of an image or the changing of the aspect ratio of an image (which is the ratio between the height and the length of an image).

² Scientific misconduct is defined within VIB as fabrication of data, falsification (e.g. through the undisclosed selection and suppression of unwanted results or through the manipulation of a representation or illustration), plagiarism, or other practices that seriously deviate from those that are commonly accepted within the scientific community for proposing, conducting, or reporting research. It does not include honest error or honest differences in interpretations or judgments of data.

³ VIB document: Research misconduct; principles and procedures, VIB intranet.

- ***Adjustments of brightness, contrast, or color balance are acceptable if they are applied to the whole image and as long as they do not obscure, eliminate, or misrepresent any information present in the original.***
- ***The grouping of images from different parts of the same gel, or from different gels, fields, or exposures must be made explicit by the arrangement of the figure (e.g., dividing lines) and in the text of the figure legend.***
- ***Nonlinear adjustments (e.g. changes to gamma⁴ settings) must be disclosed in the figure legend.***

Inappropriate image manipulation

Inappropriate manipulation refers to adjustment of image data that violates the above guidelines but does not affect the interpretation of the data. Examples include adjustments of brightness/contrast to a gel image that completely eliminate the background (so the reader cannot tell how much of a gel is shown) or that obscure background smears or faint background bands. Another example is the splicing of images from different microscope fields into a single image that appears to be a single field.

Fraudulent image manipulations

Fraudulent manipulation refers to adjustment of image data that does affect the interpretation of the data. Examples include deleting a band from a gel to “fix” a negative control that did not work or adding a band to a gel to indicate the presence of product that was actually not there. Other examples include adjustments of the contrast level of an image such as:

- Changing the contrast of an experimental compared with a control photo.
- Changing the contrast of an individual panel in time series.
- The use of different contrast levels when making merged images compared with the original images.

It is also not allowed to change the number of pixels to be greater than that in the original image. This is because the computer then has to create data that were not present in the original. By doing so, the resulting image becomes a misrepresentation of the original data.

Note: The above is only a number of concrete examples of unacceptable image manipulations and does not represent a limitative list.

Acceptable image manipulations

Certain adjustments such as background subtraction or using a filter or digital mask may be needed to extract information accurately from complex images. Reporting the details and logic of such manipulations that are applied to images as a whole should resolve concerns about their use. Leaving out certain parts of an image (e.g. leaving out one or a few non relevant lanes or zones of a gel), or combining certain parts or whole images into one image, may be acceptable, but should be clearly mentioned and demarcated, preferable by using boxes that delineate the different parts of the image.

⁴ Gamma, or gamma correction is the name for non-linear coding and decoding of the luminescence of an image. This coding and decoding is done when linear data recorded by electronic sensors are transferred into a file format. Non-linear encoding (gamma compression) of the intensities of the primary colors of a photographic are then made. In most computer systems images are encoded with a gamma of about 0.45 and decoded with a factor of 2.2. A gamma factor greater than one makes the dark parts of an image darker, and a gamma factor of smaller than one makes the dark parts of an image lighter.

Further recommendations

It is recommended that all authors of a manuscript review images intended to support their manuscript against the original image data prior to submission of their manuscript for peer-review. It is also recommended that authors report how image data were manipulated, even if the image manipulations are considered acceptable practice, or state that image data were not manipulated. In order to not misrepresent image data, always use TIFF as the format to store your image data in.

ANNEX: Guiding principles on image manipulations from the Office of Research Integrity of the Department of Health and Human Services of the USA.

<http://ori.dhhs.gov/education/products/RlandImages/guidelines/list.html>

1. Treating Images as Data

Scientific digital images are data that can be compromised by inappropriate manipulations.

- Digital images are composed of a grid of individual elements called pixels.
- Each pixel has a specific location relative to its neighbors, it has a scale (size in microns) relative to the instrument used to capture the image and it has an intensity value based on the amount of light (energy) that was collected when the entire image was captured.
- Intensity values can be expressed as numerical values, often these are 8 bit numbers (integers). In the case of a three color image, this means numbers between 0-255 for each color; red, green and blue.
- Because images are a grid and each position in the grid has numerical values, digital images share many characteristics with a spreadsheet.
- All of the different types of image manipulations are simply mathematical functions that change the underlying numbers in the image.
- It is easy to conceptualize that if you change the numbers, you would change the image. It is important to remember that when you manipulate the image in software, you are changing the underlying numerical values.
- A careful scientist working with a spreadsheet of numerical data would document any mathematical functions that were applied to the data in a uniform manner. The scientist would be unlikely to modify the data in ways that were non-uniform.
- Careful scientists document their image manipulations for the same reasons and avoid performing manipulations that are non-uniform.
- Careful scientists are aware of the physical, electronic and software limitations of their acquisition instrument. Every technique has limitations and caveats that must be accounted for when interpreting image data.
- Acquisition settings on a particular instrument can compromise image data from the very beginning.
- Users often want to see bright images. By aggressively adjusting the gain on instruments like confocal microscopes, portions of the image can become over-saturated. Over-saturation truncates the values of the brightest areas in the image such that every pixel has a value of 255 and any subtle differences between pixels are missing. Over-saturation of the image during acquisition means that the subtleties of the data in those areas are lost and cannot be recovered at a later time.
- Users are also tempted to reduce the background level settings to ensure that they acquire a clean-looking image. An image with a background level that has a uniform black color (e.g., in a confocal image) is highly unlikely. The presence of background signal is the hallmark of a real biological image.
- Post-acquisition manipulations of image data using software can also create over-saturation and/or abnormally clean backgrounds in an image. Users should learn how to use and interpret the intensity histogram tool to ensure that they are not over-manipulating their image data. Our eyes can perceive perhaps 30 greyscales and at most a few thousand colors. The intensity histogram tool allows us to look at the data in a different way to ensure that we are manipulating it correctly.

2. Saving the Original

Manipulations of digital images should always be done on a copy of the raw image data. The original must be retained.

- The raw image data file is an investigator's safety net.
 - Processed images can be compared to unprocessed images to ensure that important data was not lost from, or inadvertent artifacts added to, the processed images.
 - A copy of the unprocessed image is an extremely important protection for an investigator in the event that an accusation of scientific misconduct is made.
 - Given the trend towards making online publications the journal of record, as well as the use of supplemental data appendices, it is possible that an investigator's image data will be available for further analysis by others for many years after publication. Proper archiving of raw data will continue to protect the investigator in the event that his/her analysis or manipulation of the data is questioned at some future date.
 - A growing number of journals have indicated in their instructions to authors that the raw data may be required from the authors in the event that there are questions about the image processing or data interpretation.
 - Keeping a copy of the raw image data file is also a user's greatest protection against an inadvertent mistake in image processing.
 - The Microscopy Society of America has recommended that all image data be written to read-only media, such as CD-R or DVD-R, as soon after the experiment as is possible.
 - Because of the possibility of bad media, creating a second copy of a disc is often advisable. Discs should be properly labeled and stored; kept out of extremes of heat or cold, and should not be exposed to direct sunlight.
- The raw image data may initially be created in a vendor's proprietary file format.
 - In some cases the proprietary file format may include metadata that describes the image acquisition conditions.
 - Do not discard the files containing the metadata, it is vital if the experiment is to be repeated under similar conditions.
 - Some journals require that data about the acquisition conditions be provided for online supplementary materials.
 - When converting image data from a proprietary format, users should save their image data as TIFF (tagged image file format) files.
 - The TIFF file format is recommended by the Microscopy Society of America. TIFF is a loss-less file format that supports a wide range of bit-depths.
 - Do not use the JPEG file format. JPEG uses a lossy form of compression and is inappropriate for most scientific images (see guideline 10).
- In certain industries the retention of the raw image data file is mandatory.

3. Making Simple Adjustments

Simple adjustments to the entire image are usually acceptable.

- Digital images, like photographic negatives, sometimes need some help so that the viewer can appreciate all the information that is present in the image.
- “Reasonable” adjustments using software tools like brightness and contrast, levels, and gamma are usually appropriate manipulations for digital images.
- There is some difference of opinion about the use of gamma, which adjusts the mid-tones of an image more than the brightest and darkest pixels, but if the changes that are applied with any of these tools are explained and documented there should not be a problem.
- Users should avoid using software tools that do not allow them to document the changes that were made to the image. Beware, many of the automatic image adjustment tools that are available in commercial software are far too aggressive for scientific images.
- As was mentioned in guideline #1, avoid manipulating the image in a way that will cause the data to truncate (be cut off) at either the lightest or darkest end of the intensity range (i.e., over-saturate or create a no-noise background).
- Brightness and contrast should be used in moderation.
 - Increasing the brightness shifts the entire intensity histogram to the right.
 - Increasing the contrast causes the intensity histogram to expand.
- Using a contrast or histogram stretch is a legitimate way to make low contrast images appear to be higher contrast.
 - This technique works best with low contrast images that do not have “outlier” values in the intensity histogram.
 - The darkest pixel is remapped to equal black (intensity = 0)
 - The brightest pixel is remapped to equal white (intensity = 255)
 - The overall shape of the intensity histogram is retained; however, it is elongated to fill the entire intensity range. There are gaps that occur in the histogram after this technique is performed.
 - The increased contrast in the image comes because the eye now sees larger differences between the intensity values.
 - Do not be confused by the similar sounding “histogram equalization”. This is a very different form of image processing and is non-linear.
- Adjusting the gamma on an image applies a curve-based function to the image.
 - Positive gamma (>1) increases the intensity of the mid-tones in the image more than the darker or lighter parts of the image.
 - Negative gamma reduces the intensity of the mid-tones more than that of the darker or lighter parts of the image.
 - Because gamma adjustments are non-linear, they should be declared in the figure legend or the methods section of a paper.

4. Cropping is usually OK

Cropping an image is usually acceptable.

- The word Crop can be defined as “to trim” or “cut back”.
- The Crop tool in most image processing programs is used to trim off the outside edges of a digital image. Cropping can be used to make an image smaller (in pixels) and/or to change the aspect ratio (length to width) of the image.
- Photographers have historically cropped images to direct the viewer’s eye to a particular subset of a larger image. This can be very useful in art photography, but the same technique used in news or scientific photography has the potential to be misleading.
- Example: A tightly cropped image of a political protest can give the impression that there was a large crowd at the event. The reality may be that it was a small group that was crowded together to make it appear in the photo as if there were more people present.
- Acquiring scientific images requires us to consider what is important in the sample. The act of selecting a specific area on a sample and the magnification at which to capture the image is a form of cropping. Scientists must be careful to avoid bias, such as selecting images that represent what they think the results of the experiment “should” look like.
- Acquiring adequate numbers of representative images of all treatment groups and controls allows the user and their colleagues to carefully review the image data away from the microscope and avoid bias.
- Cropping an image for a publication figure is usually considered acceptable.
- Scientists should consider their motivation for cropping the image. Is the image being cropped to improve its “composition” or to hide something that disagrees with the lab’s favored hypothesis?
- Legitimate reasons for cropping include:
 - Centering an area of interest
 - Trimming “empty” space around the edges of an image
 - Removing a piece of debris from the edge of the image
- Questionable forms of cropping would include removing information in a way that changes the context of what remains in the image. Examples:
 - Cropping out dead or dying cells, leaving only a healthy looking cell
 - Cropping out gel bands that might disagree with the hypothesis being proposed in the paper
- Beware of cropping images too much. Most journals require a minimum of 300 dots per inch (DPI) for images. This means that if an image is to be 3.5 inches wide in the journal, it needs to have 1050 pixels.
- Enlarging an image with a small number of pixels can lead to artifacts (see guideline 12).
- Do not let image manipulation software replace good science.

5. Comparing Images

Digital images that will be compared to one another should be acquired under identical conditions, and any post-acquisition image processing should also be identical.

- Accurate comparison of treated and control digital images is difficult or impossible if the images are not acquired under similar conditions.
- Several types of instruments, like confocal fluorescence microscopes, allow users to adjust the signal amplification settings on every image.
 - With signal amplification, small features may appear larger or smaller than they do in other images solely due to the degree of amplification and the artifacts of aliasing (see also Nyquist sampling as discussed in guideline 11).
 - Investigators would do well to quiz their students, staff and post-docs about image acquisition settings. Instrument core facilities are more often frequented by students and staff. The investigator is typically not involved in the decision making about the instrument settings, however, they are often intimately involved in the interpretation of the image data.
 - Many newer instruments store metadata regarding the instrument settings used for each image. This should allow investigators to review the instrument settings.
- When a group of images is to be compared to one another, the processing of the individual images should be identical.
 - This would include acquisition techniques such as background subtraction and white-level balancing. These techniques should be documented in the methods section of published research.
 - This would include groupings of images that will be published together as publication figures.
 - If all the images in a figure have been processed in the same manner, then the viewer can better understand how each image relates to the others in the group.
 - If there is a bona fide reason that the images in a figure were processed differently, the author must explain the rationale in either the methods section or the figure legend. Failure to disclose this information means that the authors are providing misleading information to the viewer.

6. Manipulating the Entire Image

Manipulations that are specific to one area of an image and are not performed on other areas are questionable.

- This guideline refers to adjustments that ordinarily might be applied to an entire image. An example would be the photographic technique of dodging and burning. (Dodging and burning - a darkroom technique where a small portion of a photographic print is exposed to less or more light (respectively), than the rest of the print.)
- This guideline is not referring to techniques such as cloning and copying. These are discussed in guideline 8.
- Purists believe that selective enhancement of specific areas of an image should never be allowed. For the most part they are correct; however, there are a few rare and legitimate exceptions to the rule.
- Currently available print and display technologies cannot deal with a range of colors beyond that of 8bits per color. 8bits is 256 shades (or greyscales). 8bits each of red, green, and blue is called 24bit, or 16.7 million possible colors. In reality, it is difficult to correctly display and impossible to print, all of the potential colors in an image using current technologies.
- Some scientific instruments and cameras are able to acquire images at higher bit depths (e.g., 10, 12, 16 bits). As an example, 16bit is 65,536 shades (or greyscales). 16bits each of red, green, and blue would be 48bit, or 281 trillion possible colors. This is a tremendous amount of information to attempt to communicate in an image.
- Possible exception – suppose that a scientist obtains 16bit greyscale images that contain two groups of important features. One set of features is very bright and the other is dim. Simply converting these images to 8bit greyscale might not allow the viewer to appreciate both sets of features due to the wide dynamic range of the images. A scientist could carefully and selectively enhance the dimmer set of features before the conversion to 8bit to ensure that the viewer can see the features. All the images submitted for a journal article would need to be enhanced in a uniform manner (guideline 5) and the figure legend or methods section would need to be explicit about what was done.
- Almost all other forms of selective enhancement would most likely be considered inappropriate. This includes selective enhancement of specific bands or lanes within an image of a gel.
- If selective enhancement was performed and properly declared in submitted manuscript, then it would be up to the editors and reviewers to decide if it is appropriate on a case-by-case basis.
- Users need to be aware that software filters can selectively enhance specific areas of an image. Guideline 7 discusses some of the issues related to software filters.

7. Filters Degrade Data

Use of software filters to improve image quality is usually not recommended for biological images.

- Commercial digital image manipulation software was primarily written for graphics specialists in the design and print industries, not for scientists. Software filters found in commercial software cannot be trusted to appropriately manipulate scientific image data.
- Software-based digital image filters are mathematical functions called convolution kernels. To see how a convolution kernel works, see this web page at the Florida State University Molecular Expressions website (Suggestion - press the AUTO button).
- Software filters typically change the numerical intensity value of every pixel in the image.
 - Convolution kernels perform their mathematical functions using the numerical intensity values in small regions of the image (typically 3x3, 5x5, 7x7 pixel areas).
 - This means that the amount of change to the intensity of an individual pixel that is caused by the convolution kernel can be different in different parts of the image. While the mathematical function is uniform, its effect is different in different parts of an image.
 - Under certain circumstances, convolution kernels can create an artifact or multiple artifacts in an image. An artifact is defined as “a structure or substance not normally present but produced by an external agent or action...”⁵
 - If users do not carefully compare a filtered image with the original image, it is possible that artifacts could be incorrectly interpreted as meaningful data.
- If software filters must be used on scientific image data, the filters should be noted in an article’s figure legends or methods section. The notation should include the software version, filter name(s) and any special settings that were used.
- On a related note: software filters (guideline 7) and to some extent the techniques in guidelines 6 (selective enhancement) and 8 (cloning and copying) have sometimes been used to “clean up” the background in images. Depending on the sample, backgrounds may be present in a digital image due to; non-specific staining, a less-than pristine preparatory technique (dirt) or electronic noise from the detector.
- Scientists should keep in mind the possibility that someone will look at their data in a way they had not considered. Perhaps the collagen matrix, support media, interface between two structures, or other “unimportant” features in the image contain information that will spark an idea for a reader’s research. If the author changes the “unimportant” parts of an image to enhance the aspects in the image that they think are important, the author has lied to the reader and possibly removed the opportunity for a serendipitous finding.
- “Data beautification”⁶ is a form of misrepresentation even when it doesn’t completely cross the line into outright falsification.

⁵ Excerpted from The American Heritage Dictionary of the English Language, Third Edition Copyright © 1992 by Houghton Mifflin Company.

⁶ Data beautification is a term that comes from an editorial in the journal Nature. The journal defines beautification as “the digital manipulation of properly acquired data for the purpose of making a figure clearer, more perfect and more consistent with the best images yielded in such experiments. Removing dust from a digitized photo with the erasure tool, cropping bands from gels, and playing with fluorescence micrographs to enhance a particular effect are all attempts to show better results than were actually achieved in that run. In all these cases the data are legitimately acquired but then processed to yield an idealized image.” Nature 439, 891-892 (23 February 2006)

8. Cloning Degrades Data

Cloning or copying objects into a digital image, from other parts of the same image or from a different image, is very questionable.

- Users are often tempted to use tools like Photoshop's clone stamp tool to "clean up" a dirty preparation.
 - The clone stamp tool takes a small sample of a region in an image, which is then applied over other regions of the same image or a different image. When the clone stamp tool is used by a skilled operator the changes to the image are often not visible by eye, although they can be seen with image processing.
 - The rationale for using the clone stamp tool is usually to cover up imperfections in the image. If an image requires this much specialized processing, the best solution is to capture another image from the specimen, or from a new specimen prepared under the same conditions.
 - Use of the clone stamp tool, or other retouching tools (e.g., the Adobe Photoshop Spot Healing Brush, Healing Brush, Patch, Dodge, Burn, Smudge and Sponge tools), is a form of selective image processing, and is inappropriate for scientific images.
- The use of cloning or copying techniques to specifically create objects in an image that did not exist there originally is research misconduct (falsification, fabrication).
 - According to ORI investigator John Krueger, cloning and copying of data has frequently been used to falsify images. Because of the historical misuse of these tools, the undeclared use of cloning in a published image could lead to charges of research misconduct.
 - Examples of misconduct would include copying gel bands into an existing gel image to create a new result, and/or any other image "seamlessly" created from the combination of portions of two or more images.
- In some instances the combination of two images into a single publication figure is appropriate.
 - The figure needs to make it obvious to the editor, reviewers, and journal readers that the two parts came from separate images.
 - A common example would be aligning lanes from two or more gels into one figure. Most journals require a line or small gap between the combined images to clearly show that they are from separate gels.

9. Making Intensity Measurements

Intensity measurements should be performed on uniformly processed image data, and the data should be calibrated to a known standard.

- Intensity measurements are difficult to perform in an appropriately rigorous and scientific manner.
 - Many variables must be considered and controlled for, before the data can be regarded as meaningful. This would include things like the uniformity of sample preparation techniques, as well as optical aberrations and electronic noise to name a few.
 - Intensity measurements would include the analysis of colocalization data, which is essentially the comparison of intensity data from two different wavelengths.
 - Unfortunately, many publications do not provide sufficient detail to reassure the reader that the intensity data were correctly gathered and interpreted.
 - A particular difficulty with intensity measurements is the need for a robust and repeatable standard to calibrate or normalize the data. Unfortunately appropriate standards are not always available for purchase. Developing home-made standards is not a trivial task.
 - Intensity measurements of light-absorbing materials (also known as densitometry) are typically more straight-forward to perform than measurements of light-emitting materials (i.e., fluorescence). This is because light-absorbing materials (i.e., DAB staining used in immunohistochemistry) do not change intensity appreciably after exposure to light.
 - Light-emitting materials, such as fluorescent dyes, are prone to fading after exposure to light, making them difficult to use when measuring intensity values.
 - The many types of microscopes that are used to capture fluorescence images are subject to a number of known fluctuations over time, as well as other physics/electronics limitations. Scientists who are unaware of, or cannot account or control for, the limitations of the acquisition instrument, should not perform intensity measurements.
- In general, intensity measurements should be performed on raw data to avoid potential artifacts.
 - If normalization, calibration, or any other image processing is performed on the data, it should be performed uniformly across all the data, and the procedures should be carefully described in the methods section of the publication.
 - Scientists should be extremely cautious about using software filters on digital images that will be used for intensity measurements
 - Software filters may introduce unexpected artifacts in the images (see guideline 7), possibly leading to the measurement of artifacts rather than real data.

10. Lossy Compression Degrades Data

Avoid the use of lossy compression.

- Image file compression comes in two basic types.
 - Loss-less file compression reduces the size of the image file while maintaining the integrity of the image data. An example of loss-less file compression is the compressed Tiff file format, which uses the LZW algorithm. This type of file compression is appropriate for scientific data, although compressed Tiff files are not universally supported by imaging software.
 - The most common form of lossy file compression is that provided by the Jpeg file format. Jpeg is an ISO/ITU standard file format for storing images that uses the discrete cosine transform (a complex mathematical algorithm) to compress the file. The Jpeg file format is not suitable for scientific images because of the changes that the transform makes to the image data. The discrete cosine transform changes the spatial (XY) resolution of the image and the intensity value of any given pixel.
 - The Joint Photographic Expert Group (JPEG) says that “many aspects of scientific and industrial usage involve subsequent processing of a digital image, for example to enhance features or count items. Using any form of lossy compression for images in this context may create problems - after all the information thrown away during lossy compression is generally that information that is imperceptible to a human eye - not necessarily showing the same characteristics as computer image processing software”.
 - Dr. John Russ adds; “The reason for recording images in scientific studies is not to keep remembrances of familiar objects and scenes, but to record the unfamiliar. If it is not possible to know beforehand what details may turn out to be important, it is not wise to discard them. And if measurement of features is contemplated (to measure size, shape, position or color information), then lossy compression, which alters all of those values, must be avoided”.
 - The Journal of Cell Biology simply states; “It is tempting to acquire your image files in JPEG format to save disk space, but doing so compromises your data. Always use TIF format”.
 - TIFF is the only file format that is recommended for scientific images by the Microscopy Society of America. TIFF is a loss-less file format that supports a wide range of bit-depths.
 - An excellent tutorial demonstrating the issues with using Jpeg for scientific images is available at Florida State University’s Molecular Expressions website. See: <http://micro.magnet.fsu.edu/primer/java/digitalimaging/processing/jpegcompression/>
 - Other well known image file formats (additional information and links available at: http://en.wikipedia.org/wiki/Image_file_formats)
 - GIF image files are limited to 256 colors or shades of grey. Saving images with higher bit-depths to this file format will result in a loss of information.
 - PNG image files use loss-less compression and can store bit depths of up to 48bit color or 16bit greyscale.
 - PSD image files are native Adobe Photoshop files. These support high resolutions and bit depths, but are uncompressed files.
 - RAW image files can support high resolutions and bit depths, but are uncompressed files. This is not a fully standardized file format yet.
 - If you must use the JPEG file format — for example, to post an image on a web site — save the image into the JPEG file format as the final step and not before.

- With most image manipulation programs, opening and saving a JPEG image multiple times applies the compression algorithm to the image repeatedly. This degrades the image each time it is re-saved.
- Users should be aware that other computer programs use lossy compression.
 - The Adobe Acrobat program is often configured by default to apply Jpeg compression to images embedded in documents that are being transformed into PDF files.
 - Microsoft Powerpoint is used by some labs to lay out figures for publication. Powerpoint is undoubtedly simpler to use than most bitmap manipulation programs (e.g., Adobe Photoshop, Corel Photo-Paint, ImageJ); however, resizing images in this program can cause the loss of data (see guideline 12) and compressing the PPT file is believed to apply Jpeg compression to the embedded images.
 - Powerpoint is designed to prepare presentations at screen resolutions (72 dpi), rather than print (typically 300 dpi for images, 1200 dpi for text and line art).
 - Powerpoint is not the best tool for assembling figures for print, and many journals will not accept files in the PPT format.

11. Issues with Magnification

Scientific digital images are data that can be compromised by inappropriate manipulations.

- Digital images of real world objects sample the object such that each pixel in the image has a scale. This scale may be in meters per pixel for satellite images, or in tenths of microns per pixel for microscope images.
- Ideally, the scale is the same in both the X and Y dimensions; however, this is not always the case.
 - In confocal microscopy and other sectioning techniques, the XY pixel also represents a volume, because the image includes a Z dimension. The Z dimension in confocal microscopy is typically twice that of the XY resolution, an issue that can lead to misinterpretation if not accounted for.
 - It is imperative that the scale of the pixels in XY and Z be maintained so that features in the image can be correctly interpreted.
- The magnification of an image is determined by the difference between the original scale of the pixel and the scale of the pixel in its final form (e.g., paper printout, projected on the wall of a large lecture hall). Since it is often impossible to know in advance what the final magnification will be, a scale bar of known size is the best way to express the magnification.
 - Journals may resize a figure to fit the page. Resizing the image makes any magnification number provided in the figure legend incorrect, whereas a scalebar resizes along with the image.
 - The all-too-common practice of stating the magnification of a microscope objective in the figure legend—without taking into account other instrument optics and image processing—is sloppy science, and omits important information.
- Microscopes can, in several imaging modes, visualize objects smaller than the diffraction-limited resolution of the instrument.
- This does not mean that the microscope can resolve the structures, since visualizing sub-resolution structures only works if the objects are well separated from other objects in the image field. Resolution is defined as the ability to separate two closely adjacent objects, and is limited by the diffraction of the objects.
- Sub-resolution objects typically appear to be the same size as objects that are at the actual diffraction-limited resolution; however, this is an artifact. Because of this problem, measurements of the size of objects that are at, or near, the diffraction limit are very suspect.
- Another important issue with sampling small objects using digital image capture is the need to correctly oversample the object.
 - The Nyquist sampling theorem suggests that a point object should be oversampled at least two times in X and Y. Because adequate contrast is essential to correctly resolve structures in microscopy, a 2.5-3 times oversampling is more appropriate.
 - Higher levels of sampling (hyper-sampling) can yield increased accuracy of feature measurements (particularly for larger objects); however, there is often a resulting loss of contrast. Loss of contrast can make it difficult to determine where the edges of an object are, and it can make resolving two closely adjacent objects nearly impossible.
 - Scientists studying live cells in time should also consider oversampling on the temporal scale to avoid artifacts.
 - The reason for 2.5-3x oversampling is to avoid image artifacts.
 - Undersampling an image—using too few pixels to accurately describe a small feature—can yield artifacts that masquerade as real structures, which can lead to misinterpretation of the image data.

- Oversampling does not lead to artifacts; however, it does not increase the diffraction-limited spatial resolution data to the image.
- When in doubt, oversample.

12. Issues with Pixels

Be careful when changing the size (in pixels) of a digital image.

- All the care described in guideline 11 to correctly acquire digital images can be for naught if the image is processed injudiciously.
- Because images may not fit the journal page while in their original size, users typically reduce or enlarge the image. Changing the size of an image (the number of pixels in X and Y) can introduce aliasing artifacts.
 - Decreasing the image size in pixels reduces the spatial (XY) resolution in an image.
 - If the size reduction is not by a power of two, the software program performs an interpolation to create both a new XY resolution as well as the intensity values of each pixel.
 - If the image has regular, repeating structures, size reductions can create moiré artifacts.
 - Similarly, increasing the image size causes the software program to interpolate the new XY resolution and pixel-intensity values. Enlarging an image does not increase the spatial resolution; to the contrary, it may make specific features more difficult to resolve because aliasing artifacts tend to make the edges of features less distinct.
 - In either case, users should insert a magnification scale bar prior to changing the total number of pixels in an image (magnification may be nearly impossible to calculate afterwards).
 - Users should change the image size only once. This prevents the resizing artifacts from compounding one another.