

Chapter 12

Ethics and Resources



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12.1 Introduction

In the opening sections of this text, we noted that often students and technologists are sent to a confocal microscope to collect images without a full understanding of how the system works. Unfortunately, an additional factor affecting image collection is that often the students and technologists are given a preconceived idea of what the final image or data should look like. This may put pressure on a confocal microscope user to produce data to match expectations rather than that which is seen through the microscope. It is fairly easy with a confocal system to increase the apparent labeling by changing the sensitivity of a detector or increasing the laser intensity so more photons are generated. These factors can make an image appear brighter than that seen through the microscope and result in the wrong interpretation of the data. Several other factors such as summing images and slowing the scan speed have similar effects.

It is also possible to mishandle digital images resulting in the inadvertent creation of artifacts. It is essential that accepted practices and care be taken when processing confocal images and that guidelines presented below are followed.

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12.2 Imaging Ethics

The problem of biased collection of images due to a preconceived idea of how data should appear is not a new problem as illustrated by a quote from the 1742 book *The Microscope Made Easy* (shortened title) by Henry Baker: “When you employ the microscope, shake off all prejudice, nor harbour any favorite opinions; for, if you do, ‘tis not unlikely fancy will betray you into error, and make you see what you wish to see.” With the sophistication of today’s microscopes and the power of digital imaging techniques, accurate presentation of data and avoiding the temptations of seeing what we wish to see and of “cleaning up” images have never been more important. As noted by North (2006), “All data are subject to interpretation,” and many errors are introduced in complete innocence. A perfect example, as discussed throughout this text and by North, is whether the presence of yellow in a red/green merged image represents true colocalization. Hopefully at this point, it is recognized that many factors affect and alter the colors (signal) in a fluorescence image.

Unfortunately, it is not always in complete innocence that images are used inappropriately. The number of cases involving questionable images has been on a consistent upward trend since the first Department of Health and Human Resources Office of Research Integrity (ORI) Annual Report reporting period of 1989–1990 when only 2.5% of the cases involved questionable images (Krueger 2005). Perhaps the most famous case of unethical use of digital images is that of Woo Suk Hwang’s manipulation of digital images of stem cells in his 2005 science paper (Hwang et al. 2005). As noted in the first edition of this book, other examples of questionable image manipulation, including confocal images, have been reported as detailed in the 2008 ORI Annual Report (Federal Register Volume 73 Number 196 page 58968). The 2009 ORI Annual Report further indicates that 68% of the cases opened by the ORI involve cases of questioned images (Krueger 2009). Unfortunately the trend for research misconduct resulting from image manipulation continues. From 2011 to 2015, there were 45 cases investigated by the ORI where findings of research misconduct associated with image manipulation were confirmed. While these were not categorized by type of image manipulation, they did involve cases of deleting or inserting parts of micrographs or reusing and relabeling unrelated images (<http://ori.hhs.gov> March 2017 newsletter 24:1).

With the ease of image collection in confocal microscopy, continual diligence in handling confocal digital images through the various steps of collection and processing in Photoshop, AMIRA, and other programs is essential. Our images are our data, and just as it would not be acceptable to vary or alter pipetting volumes when loading a Western blot, it is not acceptable to vary or alter pixel data. Equally important is that early in training all are taught the importance of maintaining detailed laboratory notebooks for experiments, but few are taught the importance of the proper handling and archiving of digital images. An article by Goldenring (2010) emphasizes the importance of keeping original image files collected from the microscope and for maintaining non-flattened archival Photoshop files of all image manipulations. It was only through proper archiving of all images and image

processing that Dr. Goldenring and members of his laboratory were able to disprove reviewer and editorial charges of misconduct regarding confocal images.

Maintaining records of all original files and instrument parameters emphasizes a very important point concerning archiving of confocal data. While the editor-in-chief of *Microscopy and Microanalysis*, the journal of the Microscopy Society of America, Dr. Price had a case where a very qualified reviewer refused to review a manuscript until all metadata collected with the images in the manuscript were provided. Once the metadata were provided, the paper was accepted for publication emphasizing the importance of having this information available. Although often cumbersome, it is essential when collecting a confocal image that an original copy of the data in the proprietary format of the manufacturer that includes all collection parameters such as laser intensity, detector settings, scan parameters, etc. is stored and available for review. Should questions concerning the data integrity arise, this is the only mechanism to demonstrate all data has been processed properly. Many institutions are now requiring the archiving of all data in centralized data banks, and this has become an important component of image publication (Price 2014).

12.3 Journal and Office of Research Integrity Guidelines

Most journals have published guidelines for acceptable processing of digital images. The ORI has also published a series of guidelines for handling digital images that are essential for acquisition and publication of confocal images (<http://ori.dhhs.gov/products/RIandImages/guidelines>). While many of these topics have already been discussed, because of the importance of the topic, some redundancy is justified, and the full list of 12 guidelines is given below. Expanded discussions of each topic are available on the ORI website as well as in a paper by Cromer (2010) entitled *Avoiding Twisted Pixels: Ethical Guidelines for the Appropriate Use and Manipulation of Scientific Digital Images*. Cromey's paper gives many specific examples of how digital images should be processed and manipulations reported in a manuscript.

1. Treat images as data: Scientific digital images are data that can be compromised by inappropriate manipulations.
2. Save the original: Manipulations of digital images should always be done on a copy of the raw image. The original must be maintained.
3. Make simple adjustments: Simple adjustments to the entire image are usually acceptable. Reasonable adjustments using software tools like brightness and contrast, levels, and gamma are usually appropriate.
4. Cropping is usually acceptable. Legitimate reasons for cropping include centering an area of interest, trimming empty space around the edges of an image, and removing debris from the edge of an image. Questionable forms of cropping include editing which can create bias such as removal of dead or dying cells leaving only healthy cells. Motivation for cropping should always be a primary consideration. Is the image being cropped to improve its composition or to hide something?

5. Comparison of images should only involve images that have been collected under identical conditions of preparation and acquisition, and any post imaging processing should be identical for all images involved.
6. Manipulation should be done on the entire image. It is not acceptable to alter one area of an image to enhance a specific feature.
7. Filters such as smoothing and sharpening functions degrade data and are not recommended. If filters are used, this needs to be reported in the Methods and Materials for the paper.
8. Cloning or copying pixels from other images or a different area of the same image should not be done. Copying pixels to create structures in an image which did not exist is research misconduct.
9. Intensity measurements are difficult to perform and must be done on image pixels collected and processed in an identical manner. Measurements should always be performed on the raw data.
10. Avoid the use of lossy image compression formats. TIFF (Tif) is the most widely accepted format for images, but always check the journal format prior to submitting images. In general, the JPEG format should never be used for collection of scientific images.
11. Confocal images include X, Y, and Z dimensions, and digitally altering the size (magnification) in any of these directions will alter the data. Care must be used to sample or collect images according to the Nyquist Theorem. If doubt exists concerning Nyquist sampling, then oversampling should be performed.
12. Altering the number of pixels in an image to make images fit a page can result in software interpolation of data which will create a new resolution and possibly intensity value for pixels. This can result in aliasing artifacts.

As noted by Rossner and Yamada (2004), each image should be an accurate representation of what was observed through the microscope. Manipulating images to make them more convincing can change data that others might be interested in or interpret differently.

12.4 Microscopy Society of America Statement on Ethical Digital Imaging

All of the above considerations have led the Microscopy Society of America to issue a Statement on Ethics in Digital Imaging. Although some of the terminology concerning storage media is outdated due to the rapid development of technology and very large “Big Data” files, the basic premise of the statement provides guidance on how to properly handle confocal images:

Ethical digital imaging requires that the original uncompressed image file be stored on archival media (e.g. CD-R) without any image manipulation or processing operations. All parameters of the production and acquisition of the files, as well as any subsequent processing steps, must be reported to ensure reproducibility.

Generally acceptable (non-reportable) imaging operations include gamma correction, histogram stretching, and brightness and contrast adjustments. All other operations (such as unsharp masking, Gaussian blur, etc) must be directly identified by the author as part of the experimental methodology. However, for any image data that is used for subsequent quantification, all imaging operations must be reported.

Even when using the simplest and generally acceptable imaging operations, one should always be aware of the changes in pixel and voxel values. Figure 12.1 illustrates the changes in pixel value when performing a simple adjustment of contrast and brightness, an image manipulation function most, if not all of us, routinely use. These functions involve grouping a range of values at the low or high end of the histogram and reassigning all of the values within the range a “0” or “255,” respectively. This creates more black or white in the image. Since values are changed, it is essential that any quantitative analysis is completed prior to performing image enhancement functions. Cromer (2010) provides a number of other examples illustrating the effects of acceptable imaging operations on pixel and voxel values and how these may change the data.

If care is taken in the preparation and collection of confocal images and all of the above guidelines are followed for processing the images, few problems concerning the ethics of how your data was collected and processed should arise. Always remember the Confocal Commandments and keep an original unaltered archived file of your data and perform all image manipulations using a copy of the original file.

12.5 Available Resources

There are a number of websites maintained by manufacturers that are excellent resources for fluorescence and confocal microscopy. Among these are:

Nikon Microscopy U: <http://www.microscopyu.com/articles/confocal/>

Olympus Microscopy Resource Center:

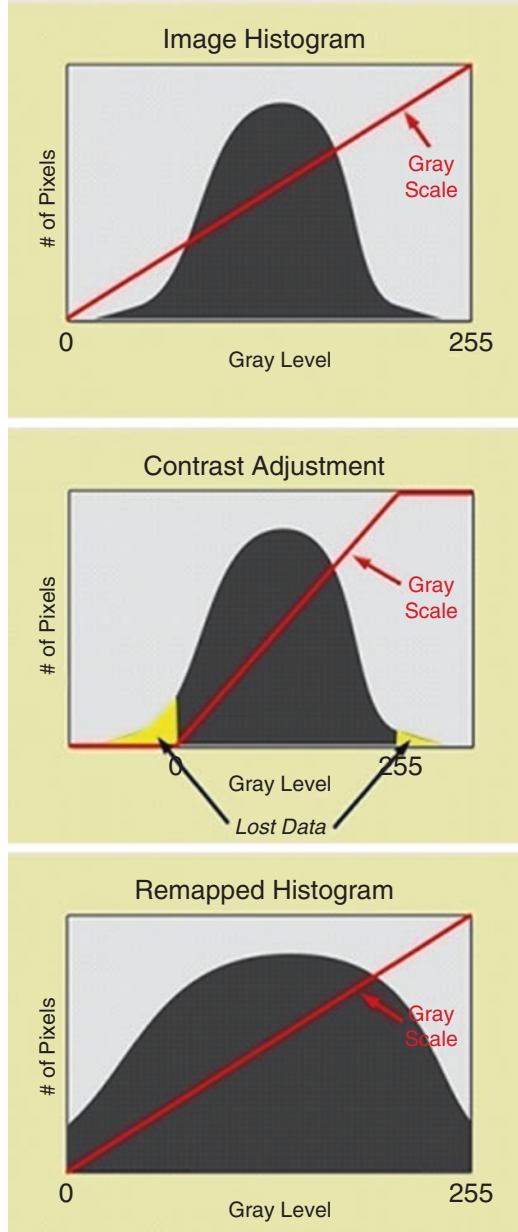
<http://www.olympusmicro.com/primer/techniques/fluorescence/fluorhome.html>

Zeiss Online Campus: <http://zeiss-campus.magnet.fsu.edu/tutorials/index.html>

The Confocal Microscopy List Serve (confocalmicroscopy@lists.umn.edu) is dedicated to topics in confocal imaging, while the Microscopy Society of America also maintains a List Serve (<http://www.microscopy.com/>) addresses topics in confocal as well as all other forms of microscopy.

There are also a number of books available which cover many aspects of confocal imaging. However, many of these are relatively old and/or cover various techniques and applications rather than basic information on how to operate a confocal system. The rate at which confocal technology is being developed also makes it difficult to keep up with advances in confocal microscope hardware. A chronological listing of some of the books we have found very useful is as follows:

Fig. 12.1 (Top) Histogram representing an image that does not use the full dynamic range with few pixel values near “0” or “255” so few black or white values would be present. (Middle) By using the contrast and brightness controls the gray scale can be changed to group a large number of values near the low and high ends of the image. (Bottom) When these grouped values are remapped to “0” or “255” values, data is lost on both ends of the histogram



- Stevens JK, Mills LR, Trogadis JE (eds) (1994) *Three dimensional confocal microscopy: volume investigation of biological systems*. Academic Press, 507 pp. (Good practical information; nice section on spinning disk microscopes)
- Gu M (1996) *Principles of three-dimensional imaging in confocal microscopes*. World Scientific Publishing Company, Inc., 352 pp. (Very technical but good for advanced students)
- Paddock SW (ed) (1999) *Confocal microscopy (Methods in molecular biology volume 1212)*. Humana Press, 464 pp. (Very good practical protocols as well as basics)
- Alberto D (ed) (2001) *Confocal and two-photon microscopy: foundations, applications and advances*. John Wiley and Sons, Inc., 576 pp. (Excellent treatise of some advanced confocal imaging techniques)
- Matsumoto B (ed) (2002) *Methods in cell biology volume 70: cell biological applications of confocal microscopy*, 2nd edn. Academic Press, 507 pp. (Information on system hardware and applications to some specific biological organisms and systems.)
- Hibbs A (2004) *Confocal microscopy for biologists*. Springer, 474 pp. (Good for beginners and advanced; great appendix of information; live cell imaging)
- Pawley JB (ed) (2006) *Handbook of biological confocal microscopy*, 3rd edn. Springer, 988 pp. (A very good comprehensive review of advanced confocal microscopy).
- Michael Conn P (2010) *Techniques in confocal microscopy*, 1st edn. Academic Press, 544 pp. (Presents a range of uses for confocal imaging).
- Price RL, Gray Jerome W (2011) *Basic confocal microscopy*. Springer, 302 pp. (The first edition of the current book)
- Paddock S (ed) (2014) *Confocal microscopy. Methods and protocols*. Humana Press, 375 pp. (Presents a range of techniques using various biological samples)
- Liu J, Tan J (2016) *Confocal microscopy*. Morgan and Claypool, 90 pp; eBookISBN 9781681743387 (Primarily covers techniques used in industrial metrology and scale resolution in bio-imaging)
- Gonzalez S (2017) *Reflectance confocal microscopy of cutaneous tumors*, 2nd edn. CRC Press, 535 pp. (Describes the use of reflectance confocal imaging in the examination of skin tumors)

Hopefully the book you are currently reading will be added to your list of useful resources for confocal imaging.

Additional Literature Cited

- Baker H (1742) *The Microscope Made Easy; or I. The nature, uses, and magnifying powers of the best kinds of microscopes described... for the instruction of such, particularly, as desire to search into the wonders of the minute creation... II. An account of what surprising discoveries have been already made by the microscope... And also a great variety of new experiments and observations...* London, R. Dodsley. 311pp

- Cromer DW (2010) Avoiding twisted pixels: ethical guidelines for the appropriate use and manipulation of scientific digital images. *Sci Eng Ethics* 16:639–667
- Goldenring JR (2010) Innocence and due diligence: managing unfounded allegations of scientific misconduct. *Acad Med* 85(3):527–530
- Hwang et al. (2005) Patient specific embryonic stem cells derived from human SCNT blastocysts. *Science* 308:1777–1783
- Krueger J (2005) Confronting image manipulation of digital images in science. *ORI Newsletter* 13(3):8–9
- Krueger J (2009) Incidences of ORI cases involving falsified images. *ORI Newsletter* 17(4):2–3
- North AJ (2006) Seeing is believing? A beginners' guide to practical pitfalls in image acquisition. *J Cell Biol* 172(1):9–18
- Price RL (2014 Aug) 2014. Editorial: data repositories, meeting proceedings, open access/online publications, and first reporting issues. *Microsc Microanal* 20(4):996–998. <https://doi.org/10.1017/S1431927614012926>
- Rossner M, Yamada KM (2004) What's in a picture? The temptation of image manipulation. *J Cell Biol* 166(1):11–15