

Fundamentals of Image Acquisition and Processing

Albert Cardona^{a*}, Pavel Tomancak^b and Andy Oates^b

^aMolecular Cell Developmental Biology, University of California Los Angeles, 621 Charles E. Young Dr. South, 90015 CA, USA.

^bMax Plank Institute for Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany.

ABSTRACT

To plan an experiment one needs to know what results are possible. This course focuses in optimal image acquisition, noise and background removal, color management, multiple plane focusing and image composition for publication.

1 IMAGE BASICS

- **Digital image:** a list of numbers, each number representing light intensity at a particular coordinate in the plane.
- **Histogram:** a statistical representation of an image, namely the distribution of pixel intensities (X axis) plotted against the number of pixels (Y axis).
- **Look-up table (LUT):** a guide for color representation for a list of numbers representing a digital image.
- **Color:** provided by the look-up table, color is always false. Color perception in humans changes throughout the visible spectrum, with green the best detected color. In the human retina, red-green-blue are but three overlapping ranges of the visible spectrum detected by three different receptors. Because of red-green-blue different wavelength ranges, each is focused at different depth in the retina. In humans, green is the best for feature recognition and contrast generation, followed by red and distantly by blue. For color blind people, copying the red channel into the blue channel when only two channels are present (typical FITC and Texas Red fluorescent labels in confocal imaging) enables their differentiation of both channels.
- **Pixel depth (8-bit, 16-bit, 32-bit, RGB):** the size and structure of the pixel intensity range.
 - 8-bit: from 0 to 255
 - 16-bit: from 0 to 65025
 - 32-bit: from 0 to 1 with 16 decimal precision
 - RGB: three 8-bit channels, for red, green and blue
 - CMYK: four 8-bit channels, for cyan, magenta, yellow and black.

The relevance of pixel depth lays in the granularity in which the light intensity is acquired. Thus, there is much higher measurement precision between 16-bit image regions than between 8-bit image regions.

In addition, CMYK is the format that printers use. When an RGB image is sent to a printer it will be automatically transformed to CMYK. It's better then to do so oneself before printing, adjusting as needed.

*Contact: cardona@ucla.edu

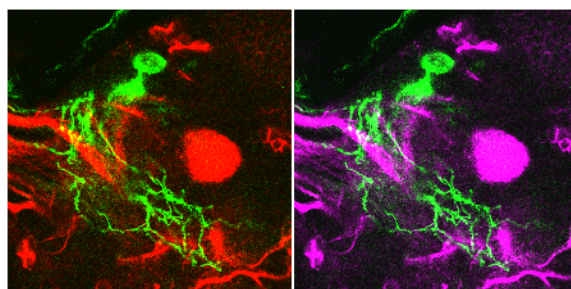


Fig. 1. Compare red and green channels only (*left*) versus red channel copied on the empty blue channel (*right*) for the benefit of the color blind.

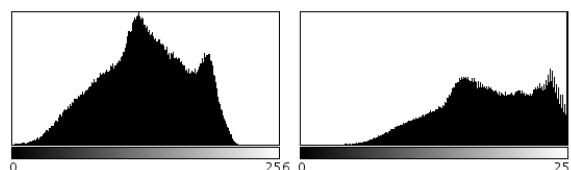


Fig. 2. Compare a proper histogram (*left*) versus the histogram of a saturated image (*right*). Note that information has been lost, compressed at the high end.

2 IMAGE ACQUISITION

2.1 Histogram usage

Human eyes perform automatic background subtraction and correct for uneven illumination, which leads to suboptimal image acquisition. The solution is to analyze the image using an histogram, which is a plot of the pixels' distribution. By adjusting the microscope and camera settings while looking at the histogram and not at the image to acquire, the experimenter can optimize the dynamic range (to avoid saturation) and avoid color imbalance.

2.2 Illumination

In conventional optical microscopes, the distribution of color intensity depends on the temperature of the lamp illuminating the sample and the filters applied to it before and after hitting the sample. Microscopes are equipped with a condenser, a shutter, several filters to reduce maximum illumination, bandpass color filters mainly for fluorescent samples, and condenser and objective prisms to generate interferential contrast in combination with a polarized light filter (analyzer).

2.3 Noise correction: brightfield and darkfield images

The set of filters and prisms in a microscope may be dirty, leading to constant noise overlaid on the sample. In addition the lamp may not generate homogeneously distributed light. The acquisition of a brightfield image, i.e. an image with equal illumination but no sample, enables the removal of constant noise. In addition, digital cameras have hot pixels, that is, even when no light is present some pixels are non-zero. Acquiring a darkfield image, i.e. an image with no light projected on the camera, enables the correction of hot pixels.

3 IMAGE PROCESSING

3.1 How to remove the background

- To remove microscope noise, camera hot pixels and uneven illumination (as in DIC images), apply:

$$result = \frac{image - darkfield}{brightfield - darkfield} * average$$

where *average* is the image average intensity. The above formula can be applied trivially with the ImageJ plugin Calculator Plus.

- To remove/correct undesired and/or uneven illumination, when a brightfield image is not available: rolling ball algorithm ("Subtract background" command in ImageJ).

3.2 How to enhance signal to noise ratio

By using a brightness and contrast dialog with an histogram, we can adjust the maximum and minimum of the image LUT range, zeroing pixels under the minimum and pushing all pixels above the maximum to the LUT maximum value. For all pixels with intensities within range, their intensity is scaled proportionally.

3.3 How to focus multiple planes from thick samples

Acquire several images at X micron intervals and then apply a stack focusing algorithm that flattens into a single image all sharp regions ("Stack Focuser" ImageJ plugin).

3.4 How to overlay DIC and fluorescent images

Convert the DIC image to RGB, separate its channels and then paste the fluorescent image into the desired color channel with the 'Max' option (which chooses the largest value for each pixel, between the desired color channel of the DIC image and the pasted fluorescent image).

3.5 Image math

Consists in applying a pixel-wise operation between two images to generate a combined image. Operations can be of any sort, typically

being purely arithmetical such as addition, subtraction, multiplication and division, and boolean such as AND, OR, XOR, Min and Max. Such operations are useful for image overlaying, channel bleeding correction and background subtraction, among others.

4 IMAGE FORMATS: LOSSLESS AND LOSSY COMPRESSION

There are several kinds of image formats. Some preserve all data without compression (TIFF), others extract and store rules and then reconstruct the pixels either directly without loss (PNG, TIFF-LZW) or by interpolation (JPEG). The relevant decision to make is whether a lossless or a lossy format should be used. As a general rule, images for analysis must be stored in lossless formats (PNG or TIFF-LZW), and images for internet broadcasting should be compressed (PNG or JPEG).

4.1 Tips and issues

- Don't save images in lossy formats (such as JPEG) if further editions are necessary. At every open/save iteration, some information is lost.
- When in doubt, store images in general purpose lossless formats such as uncompressed TIFF. To reduce the size, zip files together with an archiver. In this way, images will remain compatible in years to come with new software, while preserving all information in the minimum possible space.
- Don't store grayscale images as RGB. All three 8-bit channels contain the same data, unnecessarily.
- JPEG compression is lossy (destructive), but it's much higher than the maximum level of PNG compression (which is lossless), and thus advisable for web pages.

5 RESOURCES

- ImageJ: <http://rsb.info.nih.gov/ij/>
- ImageJ documentation and image processing tutorials: <http://imagejdocu.tudor.lu/>
- ImageJ WCIF manual for image processing: <http://www.uhnresearch.ca/facilities/wcif/imagej/>
- Image Calculator Plus plugin for background removal: <http://rsb.info.nih.gov/ij/plugins/calculator-plus.html>
- Stack focuser plugin for multiple plane focusing: <http://rsb.info.nih.gov/ij/plugins/stack-focuser.html>