



Curso Binacional 2023
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Microscopía para el Estudio de Biofilms Bacterianos

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Curso Internacional Microscopía para el Estudio de Biofilms Bacterianos

Actividad Práctica 1 - **Procesamiento de Imágenes** - Deconvolución y Procesamiento

Guía de Protocolo [versión en inglés]

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Image Processing and Manipulation

The purpose of this module is to introduce basic image processing tools for fluorescence image deconvolution and analysis tasks. This module will be separated in two main topics: DECONVOLUTION and GENERAL IMAGE PROCESSING.

1. DECONVOLUTION

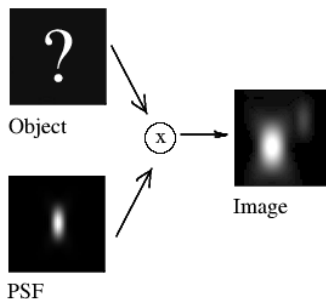
A. THEORETICAL BACKGROUND

Deconvolution Definition

Deconvolution is a mathematical operation used in Image Restoration to recover an image that is degraded by a process than can be described with a convolution. The Image Formation process is mathematically described by a Convolution equation of the form

$$g = h \otimes f, \quad (\text{Eq. 1})$$

where the image g arises from the convolution of the real light sources f (the object) and the Point Spread Function (PSF) h . In other words, the microscope yields an image g , which is a degraded version of the object "f". The degradation is caused by blurring (convolution) by the PSF. Wider PSFs lead to more severe blurring. The PSF can be measured for example by Recording Beads, or by a theoretical calculation based on knowledge of the Microscopic Parameters. This is outlined in the Figure 1.

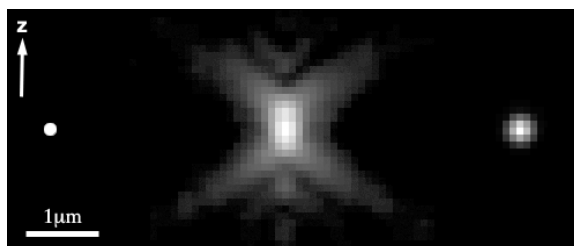


Our aim is to recover the original object "f", and we do it by doing deconvolution.

www.svi.nl/HuygensDeconvolution, www.svi.nl/DoingDeconvolution

Figure 1. Convolution process.

Here is an X-Z projection of a stack of a point source object, a (convolved) image, and deconvolved image.



Left: drawn representation of a Molecular Probes PS-Speck Green 175nm diameter bead to scale. The pixel size on the microscope is 95nm (X,Y) and the Z slice interval is 100nm (i.e. roughly cubic voxels). **Centre:** image of the bead (1.4NA/100x Zeiss PlanApo/Widefield) with gamma correction adjusted to emphasize the darker areas (actually a Huygens PSF generated from an 80 slice stack of one bead). **Right:** MLE Huygens deconvolution output of a single bead image using this PSF (also with the gamma similarly adjusted).

Signal to Noise ratio, Definition

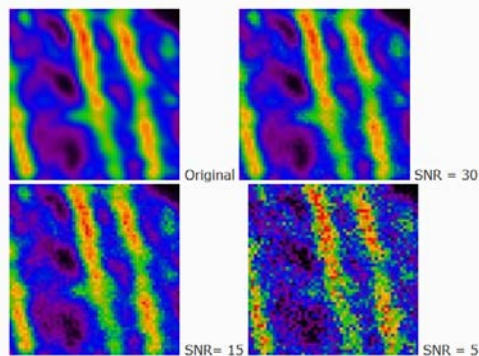
Throughout the Huygens Software the Signal-to-Noise ratio (SNR or S/N) of a digital microscopic image is defined as **the square root of the number of photons** in the brightest part of the image. The idea behind this is that a Poisson distribution is assumed for the Photon Noise. In this statistics, the "error" σ is the square root of the expectancy value P . Thus, the SNR would be

$$\text{SNR} = P/\sigma = \frac{P}{\sqrt{P}} = \sqrt{P}$$

If you know the conversion factor c (also called sometimes *system gain*) of your detector, calculating the SNR with the above definition is direct. When you don't have this information, estimating the SNR is based on the idea of establishing the pixel value corresponding to a single photon 'hit'. Afterwards, one can calculate how many photons are involved in the most intense voxels of the image. If you have clipped images you may not be able to properly determine the SNR, because the maximum or minimum intensities present in the image are not realistic.

Background Note

Do not confound "noise" as defined here with other spurious signals, like the background. To calculate the SNR you do not have to divide "signal" by "background", that would be something else (we can call that signal-to-background ratio SBR). Look at the sample images below: they clearly have different levels of noise, but the intensity averages in the high and low intensity regions are always the same, and therefore they have the same SBR. The SNR measures the roughness or granularity of your image, and this is independent of the relation between signal and background.



Estimation of the SNR - Experimental measurement

The correct method to estimate the SNR must find the Standard Deviation (STD) around the maximum level, the "noise" around what is defined to be our "signal". Ideally, one would introduce in the sample a large homogeneous high intensity plateau, where (because of the present noise) some standard deviation would be measured. Unfortunately this is experimentally difficult, and you never have a large volume with high constant intensities. The maximum in a real sample is usually very much localized (in some features of the objects) and intensity varies quickly around it, therefore you can't assume that its STD is due to noise only.

If you still imagine that you can do such experimental preparation, then the ratio between maximum (averaged) value and its STD would provide the SNR. (We focus on the number of photons, not on recorded intensity, but the two values are ideally related by a constant c , the camera conversion factor). The STD around the maximum intensity M in such 'calibration' plateau would be, in absence of other experimental artifacts, caused by the intrinsic Poisson statistics of the photon-electron events. And in such statistics, the STD is (proportional to) the square root of the number of photons. This means that

$$\text{SNR} = \frac{M}{\text{STD}} = \frac{c P}{c \sqrt{P}} = \sqrt{P}$$

Because this experimental approach is almost never possible, we need another more practical one. Estimating the SNR in noisy images is surprisingly easy: we will outline the procedure in step-by-step form below. In low noise images it is much more difficult. Fortunately, in such cases the establishment of the precise SNR is not very important for the ICTM or MLE restoration method.

The high noise case

Most dots in the background originate from single photon hits. Estimating the SNR is based on the idea to establish the pixel value corresponding to a single photon 'hit'. In many microscopes the hits will not be located in a single pixel but instead distributed over two or three pixels adjacent in the fastest scanning direction, usually the x-direction. Follow these steps to compute the SNR:

1. Zoom up the image in the Expand viewer (slicer page) until you can clearly see individual pixels.
2. Establish the value of the dark areas by pointing the cursor at these locations and reading off the voxel value in the Slicer page.
3. Look for dots in the background with low intensity. There should be many dots with similar intensity, each of them the result of a single photon hit. In most cases the intensity of the photons will be spread over two or three adjacent voxels. Sum these values to get the total intensity of a single photon hit. Depending on the design of the data acquisition system you will see the spreading of the intensity in an asymmetric way (with a tail in the forward scan direction), or in a well designed system in a symmetric way.
4. Establish the value of the voxel with maximum intensity, for example by asking Huygens for the image statistics.
5. Subtract the Black Level from both the single hit intensity and the maximum intensity, to obtain the good values i_{single} and i_{max} .
6. Compute the number of photons in the maximum value voxel by dividing its intensity i_{max} by the single hit intensity i_{single} . The S/N ratio is now

$$SNR = \sqrt{\frac{i_{max}}{i_{single}}}$$

The low noise case

In low noise images you cannot see the individual photon hits anymore. Methods exist where the energy in the image outside the band limit (which can only be due to noise) is compared to the in-band energy. Other methods rely on information gathered during the averaging of single scan lines, and must therefore be integrated in the microscope image acquisition software. In principle one could establish the SNR accurately from a series of images (sections) taken at the same z-position.

Fortunately both the ICTM and MLE methods need only a rough guess of the true SNR. As a rule of thumb good quality confocal fluorescence images have a SNR between 20 to 40. Images recorded with a cooled CCD camera on top of a conventional microscope can easily have SNR ratios above 50, even above 100. In the Deconvolution Recipes describing the use of the MLE and ICTM methods we outline a simple procedure to experiment with various settings of the SNR parameter.

The Signal-to-Noise ratio (SNR or S/N) is used in the Huygens Software as a regularization parameter, i.e. as a parameter that controls the sharpness of the restoration result. The higher this value, the sharper your restored image will be. Therefore you should not think about the SNR as a parameter describing your original image, but as a tunable parameter that controls the deconvolved result, like the "number of iterations" does.

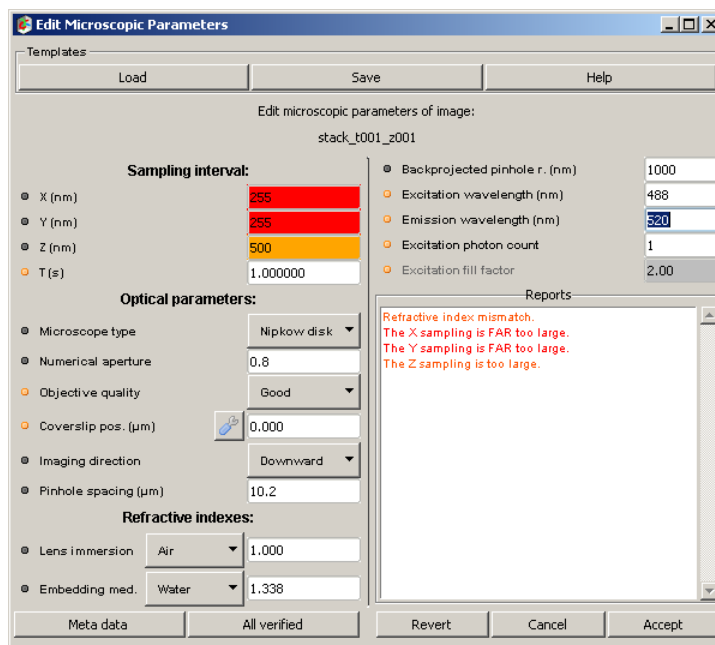
Using a too large SNR value might be risky when restoring noisy originals, because you could be just enhancing the noise. A noise-free widefield image usually has SNR values higher than 50. A noisy confocal image can have values lower than 20.

B. PRACTICAL STEPS

1. **Deconvolution using Huygens Professional software.** Launch the software, and then open one of the images stacks downloaded.
2. **Introduce image parameters.** Select the opened image (seen as a blue square around the image). Then go to the Deconvolution/Operations window. In the "Parameters" section write the pixel/voxel size dimensions, and the time sampling interval if necessary. Next, select the used microscope type, the numerical aperture (NA) of the objective, the imaging direction, and the pinhole spacing (only for spinning disc microscopes). Next, you have to enter the refractive indexes of the objective and the embedding medium. Finally, you have to set the back projected pinhole radius, and the excitation and emission wavelengths of each channel of the image. You can save all the values as a template for posterior runs.

Set the parameters according to the following table:

Microscopic parameters	Bacterial Biofilms	Zebrafish Pineal Complex
Stack image name	B1_pre-bExM_Pm2ddc_WGAAF488_xy64,4nm-z200nm_63x-W-opto_#1 5 B2_post-bExM_Pm2ddc_WGAA488-SYTO61_xy64,4nm-z200nm_63x-W-opto_#1 5+5	E1_zebrafish_pinealcomplex_32hpf-flhGFP-pMyo568-lesslices E2_zebrafish_pinealcomplex_26hpf-Tubac488-flhmcherry-lesslices
Voxel size $\Delta x/\Delta y/\Delta z$ (nm)	64.38 / 64.38 / 200	105 / 105 / 500
Microscope type	Spinning disk	Spinning disk
Objective (& optics) / N.A. / Lens Immersion medium	B1 63x & optovar1.6x / 1.2 / water B2 63x & optovar1.6x / 1.2 / water	63x / 1.2 / water
Mounting medium	Acrylamide-acrylate co-polymer* (select "water" for now)	Water
Excitation / emission wavelength (nm)	B1 ch0 = 488 / 520 B2 ch0 = 488 / 520 ch1 = 647 / 650	E1 ch0 = 568 / 603 ch1 = 488 / 510 E2 ch0 = 568 / 610 ch1 = 488 / 525
Back projected pinhole radius (nm) / pinhole distance (μm) (www.svi.nl/BackprojectedPinholeCalculator)	248 / 2.50	396.8 / 4.02



3. **Introduce deconvolution parameters.** In the “Operations” section, you must enter/ select the following: We will use the Classic Maximum Likelihood Estimation (CMLE) protocol. In “destination” you can choose the window **a**, **b** or **c** to display the result. The Signal/Noise can be estimated by following the instructions given in this document. For “maximum iterations” we can start with 60. We will set “Search for background” as Manual, and background per channel will be estimated as an average of a selected region where background is present. “Bleaching correction” will be off, and the “iteration mode” classical. All the remaining parameters will be let as default.
4. **Deconvolution run.** Just click on the “Run” button and wait a few minutes.
5. **Evaluate the results.** Choose the corresponding window by clicking on it and save the results as 8-bit TIFF format (it is useful to write the SNR and background values in the name for further comparisons). In the conversion mode question window, we will choose “contrast stretch” option. Then we will use FIJI/ImageJ software to analyze in detail the resulting images. We will do this iteratively until happiness.


2. IMAGE PROCESSING

The objective of this practical work is to Process 2D images in time using ImageJ/FIJI, beginning with the basic image manipulation (to see the image information, recognize the image histograms, adjust brightness/contrast, apply basic filters and automatically segmentation of structures inside the images). For this, the two previous deconvolved images will be use.

A. PRELIMINARY STEPS

1. Install the free & open source software ImageJ from <http://rsb.info.nih.gov/ij/download.html> or its extended version FIJI (<https://fiji.sc>). You may need to install or configure the Java runtime (java.sun.com).
2. Download the two images to be used in this session to your local storage drive.

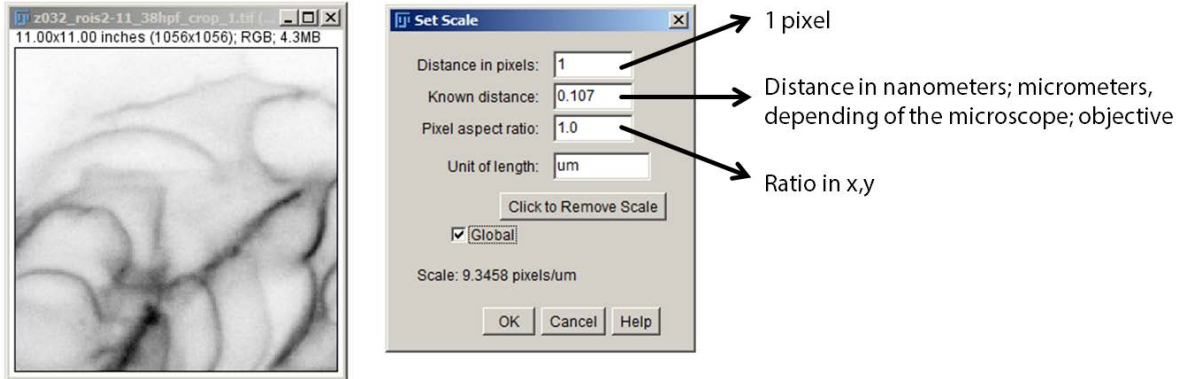
B. IMAGE BASIC MANIPULATION

- 1) To open de images, go to the **File/Open/** menu. Review each image data, for this, select **Image/Show Info y Properties**. (The supported formats in ImageJ include TIFF, PNG, GIF, JPEG, DICOM, BMP, PGM, FITS, LUT). Additionally, you can download plug-ins to open special formats. FIJI includes a large set of import plug-ins for common microscope vendors that allow reading files in their proprietary formats.
- 2) Modify the image type, go to **Image/Type/8 bits**. You can also change the image color selecting **Image/Lookup Table**.
- 3) Modify the size of the Image, go to **Image/Scale**. Select the half of the size (X scale: 0.5; Y scale: 0.5). You can also modify the size cutting the image, for this, make a rectangular selection using the rectangular tool () and go to **Image/Crop**.
- 4) Modify brightness and contrast of the images, go to **Image/Adjust/Brightness and Contrast**.
- 5) Visualize the image histograms, for this go to **Analyze/Histogram**.
- 6) Make the negative of the images, for this go to **Edit/Invert**. You can review the histogram of the inverted images, go to **Analyze/Histogram**.

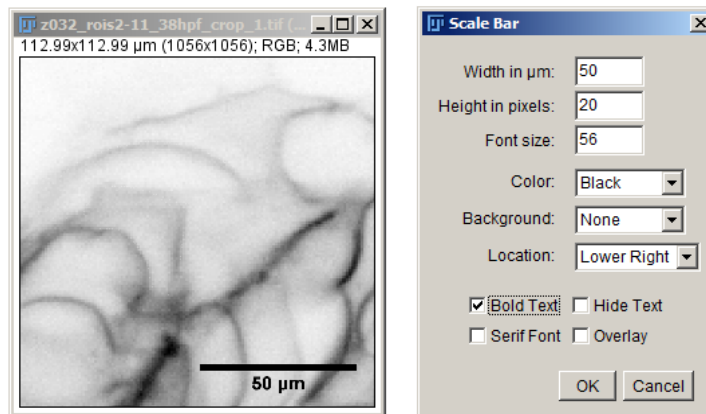
*** Keep in mind: there is not always an "undo" option in FIJI/ImageJ (and sometimes it may not work properly). It is highly recommended to save partial results in order to prevent mistakes and data loss. Use the menu option "save as".**

C. CALIBRATION OF THE IMAGEJ/FIJI SOFTWARE FOR IMAGE MESUREMENTS

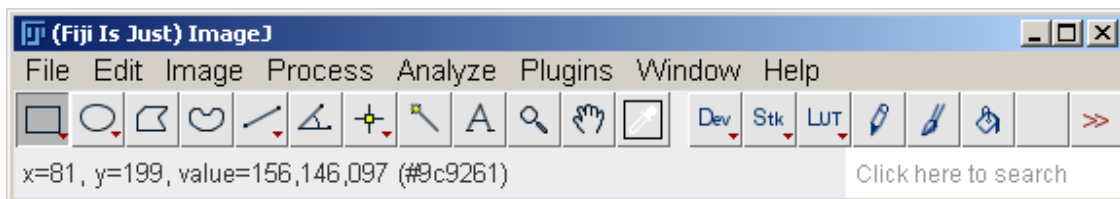
- 1) Open the image to calibrate, and transform it to 8-bit. Second, go to **Analyze/Set Scale**.
- 2) Fill the data requested. If you know the size of the pixel, you can fill as below:



- 3) Scale Bar: To insert a scale bar to your image, go to **Analyze/Tools/Scale Bar**



- 4) LUT (Lookup Table): You can set an arbitrary color table for each channel of a given image. A lookup table is a map from each intensity value to another intensity value (either single or composite to display colors). Go through the menu **Image / Lookup Tables** and try some. Remember: the LUT is only used for visualization purposes, as it not changes the intensity value of each pixel. If you move the cursor (arrow) over the image, you should always be able to read the intensity value(s) in the bottom-left corner of the FIJI/ImageJ window.



D. 2D SEGMENTATION

1. On the open images, filters will be applied to generate black & white (binary) images, representing Regions Of Interest (ROIs, white) and background (black). Some indications for FIJI/ImageJ:
 - a. To binarize 8-bits images (256 levels of gray) you have the option **Image/Adjust/Threshold**. Remember to make cell membranes selection white, if otherwise you can invert it using **Edit /Invert**.
 - b. Try the histogram normalization and equalization algorithms **Process/Enhance contrast** as a pre-processing step before segmentation.
 - c. The binary filters: erode, dilate, and open/close can be found in the menu **Process/Binary**. At the same menu, you can indicate how many times to apply the filter by each selection. These operations will help removing unwanted speckles from the segmentation.
 - d. Save your partial result as **segmentation_ ImageName _YOUR INITIALS.tif**.
 - e. After obtaining the first segmentation, you can use the Watershed filter to separate fused structures with the option **Process/Binary/Watershed**.

E. 2D PARAMETERS

1. After segmentation, each connected segmented must be identified as a ROI by the software. To create ROIs use the option **Analyze/Analyze Particles** verifying to have the **Add to manager** option selected. Once ROIs are created, parameters like: area, perimeter, and center of mass can be computed. Some indications:
 - a. All parameters to be computed can be configured in **Analyze/Set Measurements**.
 - b. In the ROI manager window (available from **Analyze/Tools/ROI Manager**), you can obtain the parameters for the selected ROI list with the option **Measure**. For instance, the number of objects, and a table with parameters selected in the **Set Measurements** option.