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EMBL-CMCI course I

Basics of Image Processing and Analysis

ver 2.0.1

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Abstract

Aim: students acquire basic knowledge and techniques for handling digital image data by interactively using ImageJ.

NOTE: this textbook was written using ImageJ ver 1.44g (JRE 1.6.0_15). Exercises are recommended to be done using Fiji since most of plugins used in exercises are pre-installed in Fiji.

Many texts, images and exercises especially for chapter 1.3 and 1.4 were converted from the textbook of Matlab Image Processing Course in MBL, Woods Hall (and many of these are originally from a book *Digital Image Processing using Matlab*, Gonzalez et al, Prentice Hall). I thank Ivan Yudushkin for providing me with the manual he used there.

This text is progressively edited. Please ask me when you want to distribute.

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1.1 Basics of Basics

Handling of digital images in scientific research requires knowledge on the characteristics of digital image. Signals we see in images are quantitative information. We are taking pictures, but we are also measuring signal by numbers. In this section, we learn the very basics of the numerical nature of digital images. Inappropriate handling not only lowers the quality of your analysis, but it could also be possible that your processing is considered as the "manipulation of data". For this latter point, please also refer to [Rossner and Yamada \(2004\)](#). There are some limits on image processing to maintain the scientific validity. Standards on scientific image processing could be found in "Digital Imaging: Ethics" by [Cromey \(2007\)](#).

1.1.1 Digital image is a matrix of numbers

Digital image we see on computer screen is made up of pixels. We can see individual pixel by zooming up the image using magnifying tool¹. Width and height of the image are defined by the number of pixels in x and y direction. Each pixel has brightness, or intensity (or more strictly, amplitude) between black and white. Within image file saved in computer hard disk, intensity of each pixel is written. The number is converted to the grayness of that pixel on monitor screen. We usually do not see these numbers in the image displayed on monitor, but we could access these numbers in the image file by converting the image file to a text file (It's also possible in a limited way by putting mouse pointer over the image).

Exercise 1.1.1-1

Conversion of image to a text file Make a new image by [File > New > Image...]. In dialog window, make a new image with the following parameters:

- name = test.txt
- type = 8bit
- Fill with Black

¹ Zooming in / out of the image does not change the content of the image.

- 10 pixel width
- 15 pixel height
- Slices = 1

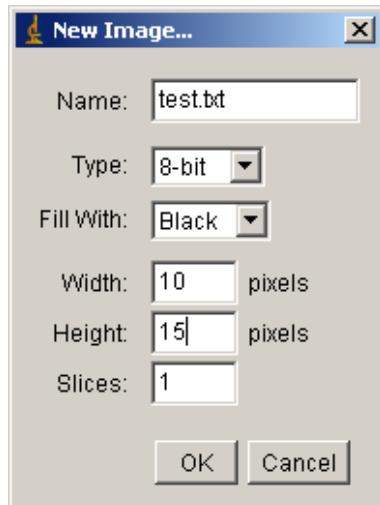


Figure 1.1: New Image Dialog

Clicking "OK", you will see a new window showing a black image. At the top of the window you could see the file dimension ("10 x 15"), bit-depth and the file size (I explain these values later. Fig. 1.2). Take the pen tool and draw some shape (what ever you want. If you do not see anything drawn, then you need to change the color of the pen to white by [Edit Option Colors...] and set Foreground Color to white). Then do [File > Save as > Text image] and save the file.

You will find that the name of the file ends with ".txt". Open File Explorer (Win) or Finder (Mac) and double click the file. The file will be opened in text editor.

What you see now is a "text image", a 2D matrix of tab-delimited numbers. At the left most column in the example (Fig. 1.3), there are only zeros. This corresponds to the left column pixels in the image, where the color is black. In the middle in the example image, there

are several "255". These are the white part of the image. In the text image, edit one of the numbers (either 0 or 255) and change to 100. Then save the file with a different name, such as "temp.txt". Then from ImageJ, [File > Import > Text Image...]. Now, you see some difference. You might easily find out that the image now has a dark gray dot.



Figure 1.2: A digital image (a) is a matrix of numbers (b).



Figure 1.3: White line (a) corresponds to non-zero numbers (b).

Note: In hard disk, pixel values are not written as a 2D matrix but as single array. Matrix is reproduced after loading according to the width and height of the image. Pixel values of image stacks (3D or 4D), are also in 1D array in hard disk. 3D or 4D matrix is reproduced according to additional infor-

mation such as slice and channel number. Such information is written in the "header" region of the file, which precedes the "data" region of the file where the 1D array of pixel array is contained. Header structure is various depending on the file format, such as TIFF or BMP (we will see more in "file formats and header" section).

1.1.2 Image Bit-Depth

Image file has a defined bit depth. You might have already heard terms like "8-bit" or "16-bit" and these are the bit depth. 8-bit means that the gray-scale of the image has $2^8 = 256$ steps: in other words, the grayness between black and white is divided into 256 steps. 16-bit means $2^{16} = 65536$ steps. The difference is that with 16-bit, one can assign gray-level much more precise for each pixel; in other words "grayness resolution" is higher. Microscope images are generated mostly by CCD camera (or something similar, with matrix of photo-sensor). CCD chip is a matrix of sensors. Each sensor receives photons and converts the number received to the grayness number for a pixel at the corresponding position within the image. Larger bit-depth enables larger dynamic range with more precise conversion results. For this reason, larger bit-depth is normally recommended for quantitative imaging. Draw back is that it takes longer time for data transferring as the bit-depth becomes larger. This may intern limits the time resolution of image sequences.

Why do we use " 2^n "? This is because computer uses binary numbers. Minimum units are then 0 or 1. 8-bit means for example a number to be represented as 8-digits binary number, something like "00001010" (= 10 in decimal). Then the minimum value is = "00000000" ("0" in decimal) and the maximum is 11111111 ("255" in decimal). 8-bit image allows 256 scales for the grayness (using calculator application in your computer, you could easily convert binary number into normal decimals and vice versa). In case of 16-bit image, the scale is 2^{16} so there are 65536 steps.

We must keep in our mind that the nature is continuous. In conventional mathematics you learn in school, decimal point enables you to represent infinite steps between 0 and 1. But digitization of the nature loses the infi-

nite steps such that 0.44 will be rounded to 0 and 0.56 will be 1. Thus, the bit-depth limits the resolution of the analogue to digital conversion (AD conversion). Higher bit depth generally allows higher resolution.

ImageJ has a high-bit-depth format called signed 32-bit floating point image. In all above cases with 8-bit and 16-bit, the pixel value is represented in integer but floating-point type enables decimal points (real number) for the pixel value such as "15.445". Though 32-bit floating point image can be used for image calculation, many functions in ImageJ do not work properly so cares should be taken to use this image type. If you want to know more about the 32-bit format, read the following box (a bit complicated; you could just pass through)

32 bit FLOATING POINT images utilizes efficient use of the 32 bits. Instead of using 32 bits to describe 4,294,967,296 integer numbers, 23 bits are allocated to a fraction, 8 bits to an exponent, and 1 bit to a sign, as follows:

$$V = (-1)S * 1.F * 2^{(E - 127)},$$

whereby:

S = Sign, uses 1 bit and can have 2 possible values

F = Fraction, uses 23 bits and can have 8,388,608 possible values

E = Exponent, uses 8 bits and can have 256 possible values

Practically speaking, this allows for an almost infinite number of tones between level "0" and "1", more than 8 million tones between level "1" and "2" and 128 tones between level "65,534" and "65,535", much more in line with our human vision than a 32 bit integer image. Because of the infinitesimally small numbers that can be stored, the 32 bit floating point format allows to store a virtually unlimited dynamic range. In other words, 32 bit floating point images can store a virtually unlimited dynamic range in a relatively compact way with more detail in the shadows than in the highlights and take up only twice the size of 16 bits per channel images, saving memory and processing power. A higher accuracy format allows for smoother dynamic

and tonal range compression.

(Quote from <http://www.dpreview.com/learn/?key=bits>)

1.1.3 Converting the bit-depth of image

In many occasions, you might want to decrease the bit-depth of image simply to reduce the file size (16-bit file becomes half the file size when it is converted to 8-bit), or you might need to use certain algorithm that is available only with 8-bit images (there are many such cases), or so on. In any case, this will be a good experience for you to see the limitation of bit-depth.

Here, we focus on the conversion of a 16-bit image to an 8-bit image to study its effect and associated possible errors.

Exercise 1.1.3-1

Let's first open a 16-bit image from the sample. [File > Open > m51.tif]. Choose line selection tool and draw a vertical line (should be yellow by default: called line ROI). Then do [Analyze > Plot Profile...]. A window pops up. See figures 1.4 and 1.5.

Figure 1.4 is the profile of the pixel values along the line ROI you just have marked on the image (Fig. 1.5). X-axis is the distance from the starting point (in pixel) and the y axis is the pixel value along the line ROI. The peak corresponds to the bright spot at the center of the image.

Let's convert the image to 8-bit. First check the state of "Conversion" option by [Edit > Option > Conversion]. Be sure that the checkbox "scale when converting" is off.

Do [Image > Type > 8-bit]. The line ROI is still there after the conversion. Do [Analyze > Plot Profile...] again. You will find another graph pops up. Compare the previous profile (16-bit) and the new profile (8-bit).

Conversion causes changes in the y-value. Shapes of the profile look

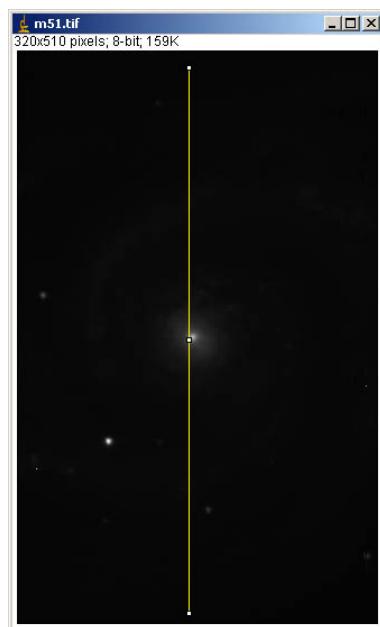


Figure 1.4: Setting a vertical line Roi.

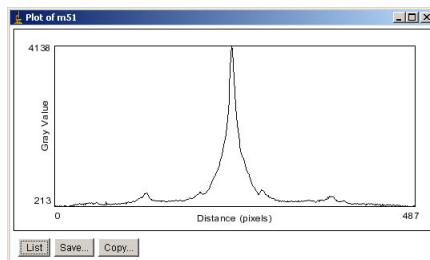


Figure 1.5: profile of that ROI



Figure 1.6: Conversion Option. Scaling is turned off in this case.

mostly similar, so if you normalize two images, the curve may overlap. This is because the image is scaled according to the following formula.

$$I_8(x, y) = \frac{I_{16}(x, y) - \min(I_{16}(x, y))}{\max(I_{16}(x, y)) - \min(I_{16}(x, y))} * 255$$

where

$I_{16}(x, y)$: 16-bit image

$\min(I_{16}(x, y))$: the minimum value of 16-bit image

$\max(I_{16}(x, y))$: the maximum value of 16-bit image

$I_8(x, y)$: 8-bit image

Save the line ROI you created by [Analyze Tools ROI manager...]. A small dialog window pops up, so click "Add" button in the right side. Number appears in the left side, which indicates the name for the ROI you made. ROI manager stores coordinates of the start / end points of the line ROI.

Now, change the option [Edit > Option > Conversion] that the checkbox "scale when converting" is OFF. Open the 16-bit image again by [File > Open > m51.tif]. Then again, do [Image > Type > 8-bit]. Apparent difference you see is that now the picture looks like a overexpose image. Find the ROI manager window and click the ROI number you stored in above. Same line ROI will appear in the new window. Then do [Analyze > Plot Profile...]. Third profile shows very different shape compared to previous ones. This is because the values above 255 is now considered as "saturated", which means that whatever the value is, numbers larger than 255 becomes 255.

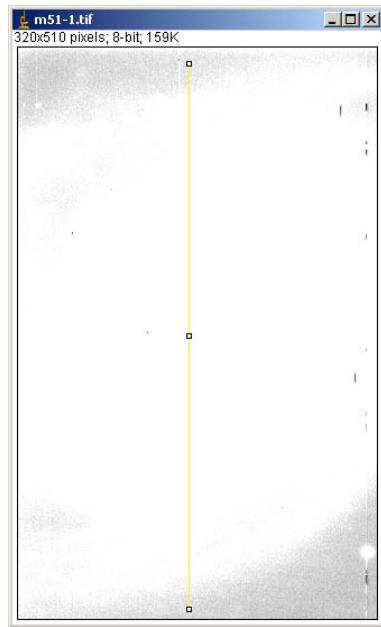


Figure 1.7: m51 image converted to 8-bit without scaling.

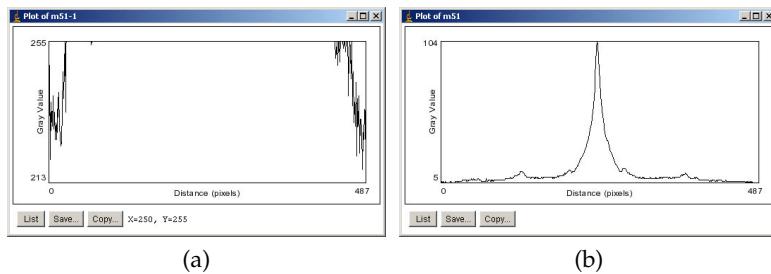


Figure 1.8: (a) Intensity profile of 1.3b. If the conversion was done with scaling, then the profile would look like (b).

When you do conversion, very different results could appear depending on how you scale, like we have just seen. But in many cases, you do not recognize such changes just by looking at the image: for this reason, one should keep in mind that the conversion may "saturate" or cause artifacts in the image - screwing up scientific images to non-scientific ones.

1.1.4 Math functions

Digital image is a matrix of numbers. We can calculate images like usual math. If there is a flat image with pixel value of 10, and if you add 1 to the image, then all pixel values become 11. We think about a pixel at (5, 10), and we write down the calculation as follows:

$$f(5, 10) = 10 \quad (1.1)$$

$$g(5, 10) = f(5, 10) + 1 = 11 \quad (1.2)$$

We generalize this. x and y are the coordinates within the image.

$$g(x, y) = f(x, y) + 1 \quad (1.3)$$

The original image is $f(x, y)$ and the result after the addition is $g(x, y)$.

Likewise images could also be subtracted, multiplied and divided by number.

Exercise 1.1.4-1

Simple math on 8-bit image: Prepare a new image following the initial part of the exercise 1.1.1. Now, bring the mouse pointer over the image and check the "value" that appears in the status bar in the ImageJ window². All pixel values in the image should be "value = 0". "x=..., y=..." in the status bar.

Commands for mathematical operations in ImageJ are as follows.

```
[Process > Math > Add...]
[Process > Math > Subtract...]
[Process > Math > Multiply...]
[Process > Math > Divide...]
```

² In the exercise 1.1.1, we converted the image to a text file and check the pixel values, but it is also possible to check the value pixel by pixel using this method.

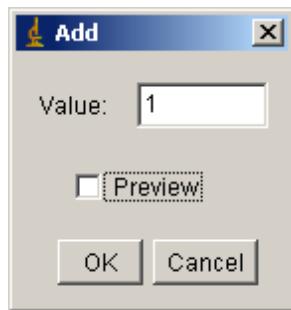


Figure 1.9: Add dialog.

Add 10 to the image: Do [Process > Math > Add...]. A dialog window pops up so you could input 10. Press OK button. Now, place the mouse pointer over the image to check that the pixel values actually became 10. Then select the pen tool from the tool bar, and draw a diagonal line in the window. Check again the pixel value. The line you just drew has pixel value 255. Then add 10 again to the image by [Process > Math > Add...]. Check the pixels by placing the pointer. The black part is now 20, but what happened to the white line?

Since the image bit-depth is 8, the available number is only between 0 and 255. When you add 10 to a pixel with its value 255, the calculation returns 255 because the number "265" does not exist in 8-bit world. The similar limitation applies for other calculations also. If you multiply a pixel value 100 by 3, the answer in normal mathematics is 300. But in 8-bit world, that pixel becomes 255.

How about division? Close the currently working test image, and prepare a new image as you did in 1.1.1. Zoom up the image, and add 100 ([Process > Math > Add...]); you should see the image turns to gray from black). Check pixel values by placing the pointer above the image. Now, Divide the image by 3 ([Process > Math > Divide...]). Check the result by placing the mouse over the image. The pixel value is now 33. Since there is no decimal places in 8-bit, the division returns the rounded value of ($100 / 3 = 33$). One could also divide image by any real number, such as 3.1415. The answer will be in integer in all cases in 8-bit and 16-bit. In case of floating

point 32-bit image, the calculation results are different. We study this in the next exercise.

Exercise 1.1.4-2

Simple Math on 32-bit Image: prepare a new 32-bit image (in the [New > Image...] dialog, select 32-bit from the "type" drop-down menu). Then add 100 to the image. Check that the image pixel values are all 100. Then divide the image by 3. Check the answer. This time, answer has decimal places.

Bit-depth limitation of digital image is very important for you to know, in terms of quantitative measurements. Any measurement must be done knowing the dynamic range of the detection system prior to the measurement. *If you are trying to measure the amount of protein, and if some of the pixels are saturated, then your measurement is invalid.*

1.1.5 Image Math

In the previous section we calculated using single image. Likewise, we can do calculation using two images. For example, if there are two images with a same dimension, and

$$f(5, 10) = 100 \quad (1.4)$$

$$g(5, 10) = 50 \quad (1.5)$$

... meaning that the pixel value at the position (5, 10) in the first image f is 100 and in the second image g is 50, we can add these values and have a third image

$$h(5, 10) = f(5, 10) + g(5, 10) = 100 + 50 = 150 \quad (1.6)$$

General form is then as follows that applies to all pixels in the image:

$$h(x,y) = f(x,y) + g(x,y) \quad (1.7)$$

Note that this only works when the image width and height are identical. Above is an example of addition. More numerical assignments are available in ImageJ:

Add	$\text{img1} = \text{img1} + \text{img2}$
Subtract	$\text{img1} = \text{img1} - \text{img2}$
Multiply	$\text{img1} = \text{img1} * \text{img2}$
Divide	$\text{img1} = \text{img1} / \text{img2}$
AND	$\text{img1} = \text{img1 AND img2}$
OR	$\text{img1} = \text{img1 OR img2}$
XOR	$\text{img1} = \text{img1 XOR img2}$
Min	$\text{img1} = \min(\text{img1}, \text{img2})$
Max	$\text{img1} = \max(\text{img1}, \text{img2})$
Average	$\text{img1} = (\text{img1} + \text{img2}) / 2$
Difference	$\text{img1} = \text{img1} - \text{img2} $
Copy	$\text{img1} = \text{img2}$

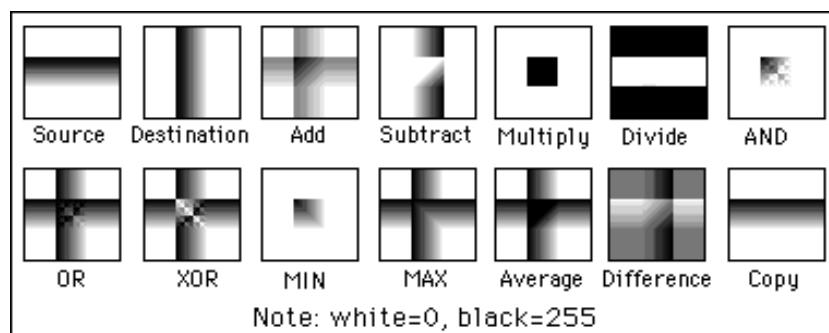


Figure 1.10: Taken from ImageJ web site. <http://rsb.info.nih.gov/ij/docs/menus/process.html>

Exercise 1.1.5-1

Image Math – subtraction:

Open Images **cells_ActinDNA.tif** and **cells_Actin.tif**. The first image is containing images from two channels. One is actin labeled, and the other is DNA labeled. We isolate the DNA signal out of the first image by image subtraction. Do [Process > Image > Calculator...]. In the pop-up window, choose the appropriate combination to subtract **cells_Actin.tif** from **cells_ActinDNA.tif**. Don't forget to turn on the checkbox "Create New Window".

1.1.6 RGB image

Color images are in RGB format (could also be so pseudo-color image, or 8-bit color, but this is just because of LUT. See section 1.1.7). Another popular format is "CMKY" but this format is optimized for printing purpose (you may have heard it already when you want to print something in Photolab). RGB stands for three primary colors. Red, Green and Blue. If all of them are bright at the same intensity, then the color is white (or gray). If only red is bright, then the color is red, and so on. A single RGB image thus has three different channels. In other words, three layers of different images are overlaid in a single RGB image. Each channel (layer) has a bit depth of 8-bit. So a single RGB image is 24-bit image. For this, the file size of color pics becomes three times larger than a grayscale 8-bit image. Don't save 16bit image in RGB format, since you lose a lot of information, for automatic conversion from 16 to 8 bit takes place.

Exercise 1.1.6-1

Working with RGB image:

- (a) Open the image **RGB_cell.tif** by [File > Open]. Then split the color image to 3 different frames [Image > Color > Split Channels].
- (b) Merge three frames by [Image Color > Merge Channels...].

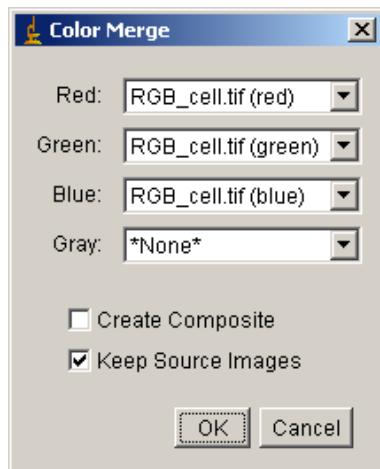


Figure 1.11: Color Merge Dialog

In the dialog window, choose an image name for each channel. Uncheck "Create Composite" and check "keep source images". Then try swapping color assignments to see the effect.

(c) Working on each channel separately: Close all windows and open the "RGB_cell.tif". Do [Image > Color > Channel Tools...]. Then click button "More" and select "Create Composite".

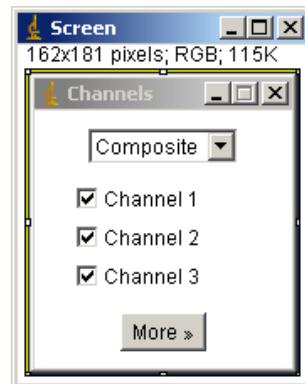


Figure 1.12: Channel Tool

Resulting image is a three-layer stack and each layer corresponds to one of R, G or B. Each layer can be processed individually.

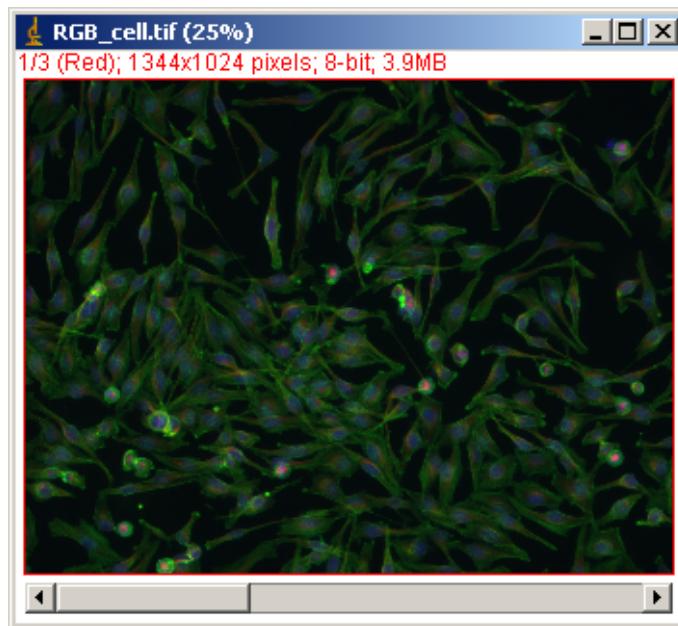


Figure 1.13: Composite image. Note slider at the bottom for switching between three channels.

Using Channel Tools again,



Figure 1.14: Channel Tool, now only selected for Channel 2.

Choose "color" from the pull-down tab, instead of "Composite". Select channel 2 (in this image, this will be Green channel). Select a part of the image using Rectangular ROI.



Figure 1.15: ROI selection, in channel 2. Note the position of slider.

Then do [Edit > Clear]. This will pop up a window.



Figure 1.16: Asking you whether you want to process all channels.

Click No, because you want to process only one channel.

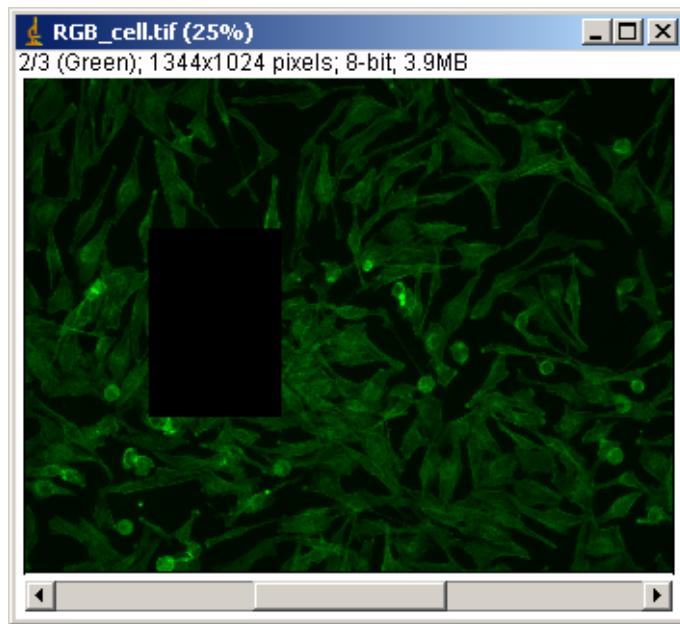


Figure 1.17: Channel 2 pixel values inside selected ROI becomes 0.

Troubleshooting: If the ROI is not cleared (becomes bright), then you should change the background color setting. Do [Edit > Option > Colors...] and you will see a pop-up window like this.

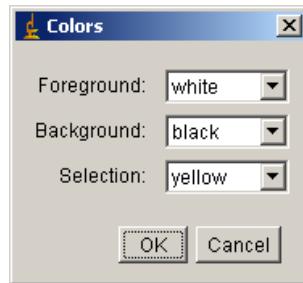


Figure 1.18: Color selection dialog.

Make sure that the background is "black". Do the ROI clearing again.

Select "Composite" in the pull-down tab of channel tool.



Figure 1.19: Choosing Composite, all channels visual.

Resulting image should look like below.

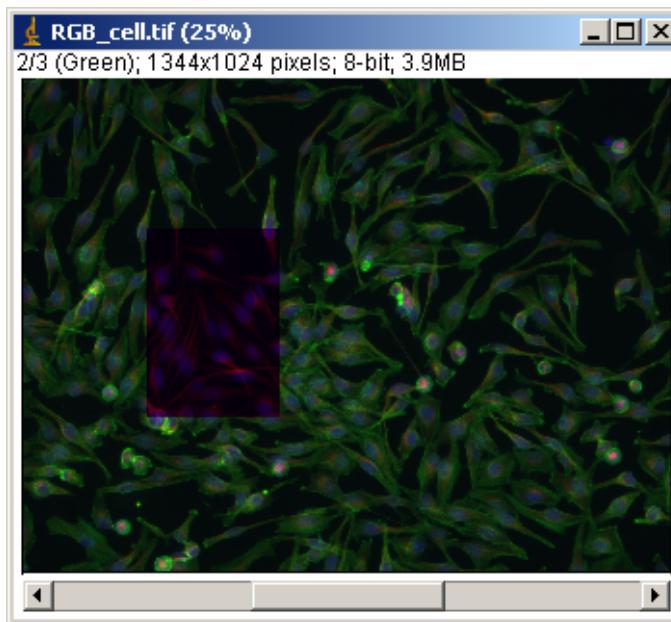


Figure 1.20: Only channel2 is devoid of image within the selected ROI.

In this case, green channel intensity in green channel ROI is now set to 0 ("clear"). You could do such processing of single channel by simply selecting a channel by horizontal scroll bar and do the processing directly, such as drawing square ROI and deleting that part in that channel in "composite" view.

1.1.7 Look-Up Table

We now look at how matrix of numbers is converted to actual image. Let's think about a row of pixels with increasing pixel values from 0 to 255 (so there are 256 pixels in this row). Computer monitor will show a gradient of intensity that is linearly increasing its brightness from black to white. This is because the software is giving a command to the monitor, such that "this pixel (x, y) is 158 so the corresponding voltage required for this position (x, y) in the screen should be **mV". For this command to be composed, software needs a so called "look-up table" (LUT).

This is just like a situation when you start checking a menu in a restaurant with limited amount of money in your pocket. Say you want to eat a pizza. You have only €10 in your pocket. Looking at the pizza menu, you will not try to find what you want from names of pizza and what are the toppings, but instead you will check the prices listed in the right side of the menu trying to figure out which pizza is less than €10. When you find €7.5 in the list, then you slide your sight to the left side of the menu, and find out that the pizza is "Margherita". Similar to this, software first checks the pixel value and then goes to the look-up table (menu) to find out which brightness should be passed to the monitor as a command (= find a convincing price in the menu, then sliding your sight to the left and find out which pizza to order).

Exercise 1.1.7-1

For 8-bit images, there is a default LUT normally called "grayscale". To see the LUT, open the image **Cell_Colony.jpg** and then do [`Image > Color > Show LUT`]. LUT window pops up showing the relationship between pixel value and pixel intensity. Try change the LUT by [`Image > Lookup Tables > Spectrum`]. Pixel value does not change by this operation, but the pixel intensity changes that the image appears differently now. Check the LUT again by do [`Image > Color > Show LUT`].

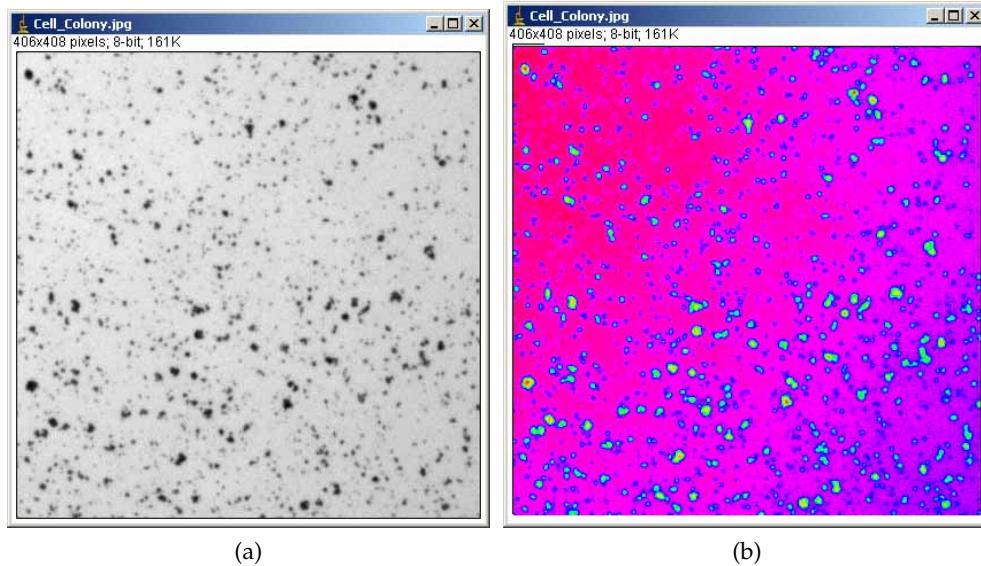


Figure 1.21: Grayscale LUT (a) converted to spectrum LUT (b).

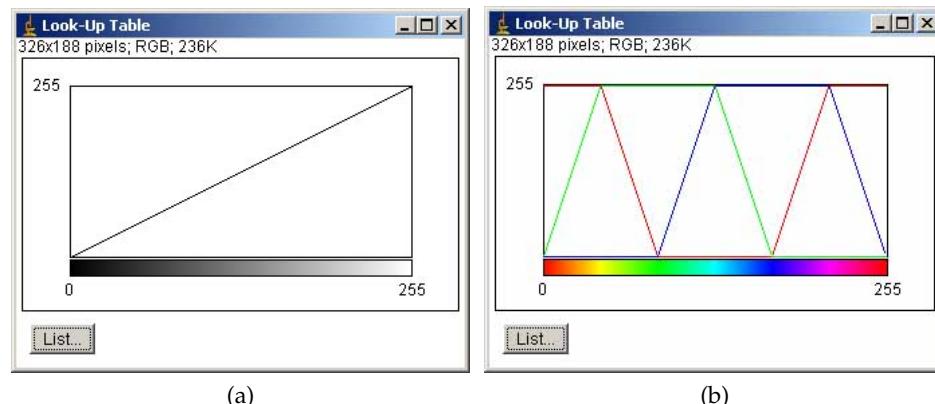


Figure 1.22: (a) Grayscale LUT and (b) spectrum LUT

1.1.8 Image File Formats: Header and Data, Stacks, Multi-dimensional images

Image file contains two types of information. One is the image data, the matrix of numerical values. Another is called "header", which contains the information about the architecture of image. Since software must know information such as bit-depth, width and height, and the size of the header

before opening the image, the information is written in the "header". The total file size of an image file is then

$$\text{Total filesize} = \text{headersize} + \text{datasize}$$

There are many different types of image formats, such as TIFF, BMP, PICT and so on. Each format has differently sized header, and also the location of the information. In biology, microscope companies create their own formats to include more parameters about the image, such as the type of microscope used, used objectives, binning, shutter speed, time intervals, user name and so on.

Presence of company-specific formats complicates us because that image could only be openable from software provided by that company. For this reason, there are an excellent Plug-in which enable importing of specific format to ImageJ³

You do not have to know all the details about the architecture of various image formats (thanks to the bioformats plugin), but it is important for you to know that the difference resides mainly in the header. The data part is in most cases same, something like what we have seen already using text image (for more details on header, refer to the appendix [1.6.1](#)).

Multidimensional data: When you take time-lapse images or z-stack, then there will be a series of images with same prefix and serial number like image0001.tif, image0002.tif, image0003.tif... and so on. These files could be imported by ImageJ and watch the sequence like a movie. Such an image series is called "stack" in ImageJ and can be saved as a single file. File extension is typically ".tif", which is same as the single frame file. In Metamorph, popular software used in biology, uses .stk as the file extension. These files can be loaded directly in ImageJ. We will study more on the actual use of the image stacks in section [1.5](#).

Compression: Another important point that we must keep in our mind is that we should not compress data, if you want to get some numbers out of them. Popular compression formats are like JPEG and GIF. Compression of images deals with throwing away redundant part or mathematically in-

³

LOCI Bioformat Plugin:

<http://www.loci.wisc.edu/ome/formats.html>

terpolating some parts of the image. This causes artifacts in the image and it could even be "manipulation" of data. We must avoid using compression for the images we use for measurements. These compression formats are also called "lossy" formats, as the process of compression loses data. PNG is a compression format that does not alter the original pixel values.

Exercise 1.1.8-1

Open the example image **wt1.tif**. Do [Image > Show Info...]. Scale (pixels/inch) is listed in the information window, which was read out from the header of the image. Then do [Image > Properties], also showing the scale.

1.1.9 Resizing images. (Shrinking and Enlarging)

When we say "resizing" images, this does not mean zooming in or out the image⁴. Resizing changes the original data. If we have an image of size 10 pixels by 10 pixels and resize it to 200%, the image becomes 20 x 20. If we resize it by 50%, then the image becomes 5 x 5. Resizing is a simple task that could be done by [Image > Adjust > Size...]. This is a simple operation but one must take care about how pixels will be produced while enlarging and reduced while shrinking. If the enlarging is simply two times larger, we could imagine that each pixel will be copied three times to produce a block of four pixels to complete the task. The pixel values of the newly inserted pixels will then be identical to the source pixel.

But what happens if we want to enlarge the image by 150 %? To simplify the situation, think about an image with 2 x 2 pixels. Then the resulting image becomes 3 x 3. To understand the effect, do the following exercise.

Exercise 1.1.9-1

Open the example image **4pixelimage_sample.tif**. The image is ultra small, so zoom it up to the maximum (as much as you can, you must click on or *Ctrl - +*). You now see four pixels in the window. Duplicate the image by [Image > Duplicate]. Magnify again. "Select all"

⁴ Zooming is done by the magnification tool (icon of magnification glass) and this simply enlarges or shrinks the pixels, not modifying the original data.

by [Edit > Selection > Select All]. Then [Image > Adjust > Size...]. In the dialog window, input the width 4 and height 4 (corresponds to 200% enlargement). Turn on the check box "aspect ratio" on and "Interpolation" off. Then click OK. Check the pixel values in original image, and the enlarged image.

Exercise 1.1.9-2

Do the similar resizing, but this time enlarge the image by 150%. Check the pixel values.

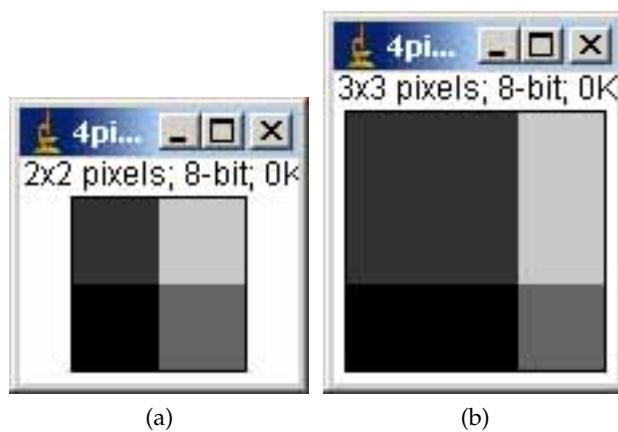


Figure 1.23: Artifacts produced by resizing. (a) Four pixel image and (b) nine pixel image after resizing.

The resizing in the exercise was without interpolation – the check box was OFF. Interpolation is similar to the one dimensional interpolation we do with graphs. In case of images, the gradient is also two dimensional so the situation is a bit more complex. There are various methods for interpolating image. The interpolation method used in ImageJ is the *bilinear interpolation*. Briefly, the bilinear interpolation algorithm samples pixel values in the surrounding of the insertion point, and calculates the pixel value for that position⁵. One must keep in mind that the result of enlarging or shrinking of image depends on the interpolation method – and scientific results could be altered depending on the method you use.

⁵ For more details on bilinear interpolation, refer to <http://www.cambridgeincolour.com/tutorials/image-interpolation.htm>.

1.1.10 ASSIGNMENTS

Assignment 1-1-1: Digital image = matrix of numbers

Edit a text image using text editor. Be sure to insert space between numbers as separator. Save the text file and open it as an image in ImageJ by importing text image function.

Assignments 1-1-2: bit depth

1. How many gray scale steps does a 12-bit image have?
2. Describe in text how a 1-bit image looks like.

Assignment 1-1-3: bit depth conversion

Use m51.tif (16-bit!) sample image to draw a plot profile, as we did in the course. In the profile plot window, a "list" button is at the left-bottom corner. Click the button. You will then see a new window containing a column on numbers. These numbers can be copy & pasted to spread sheet software such as Openoffice Calc or MS Excel. Overlay three curves in a graph, and discuss the difference in two different ways of bit-conversions.

Assignments 1-1-4: Simple math with Image

1. Try subtracting certain values from the image you created in the Assignment 1-1-1 and check that the values cannot be less than 0.
2. Prepare an 8-bit image with pixel value 200. Divide the image by 3, and check the answer.
3. Prepare a 16-bit image. In the [File > New > Image...] dialog, select 16-bit from the "type" drop-down menu. Try adding certain value to check the maximum pixel value.
4. Discuss why measurement of fluorescence intensity using digital image is invalid when some pixels are saturated.

Assignments 1-1-5: LUT

Open "Cell_Colony.tif". Use LUT edit function and design your own LUT to highlight the black dots in Green and the background in Black. "LUT editor" can be activated by [Images > Color > Edit LUT...]. Instruction for the LUT editor is at

<http://rsb.info.nih.gov/ij/plugins/lut-editor.html>

You might be able to manage using it without reading the web instruction; just try!). LUT (.lut file) could also be edited using Excel.

Assignments 1-1-6: File size and image bit depth, image size

If there is an image with width = 100 pixels and height = 200 pixels, what would be the expected size of the image file in bytes? 1 byte = 8-bit.

Create a new image with the dimension as above, and save the image in "bitmap (.bmp)" format and check the file size. Is it same as you expected, or different? Save the same image in text file format and check the file size again.

Assignment 1-1-7 Resizing

1. Enlarge the sample image 4pixelimage_sample.tif by 150% while the "Interpolation" check box in the size adjustment window is ON. Study the pixel values before and after the enlargement. What happened? Describe the result.
2. Change "canvas size" by [Image > Adjust > Canvas Size] for any image. What's the difference to "Resize"?

1.2 Intensity

An image has two types of information: intensity and shapes (though they are both "intensity", shapes more like a meaningful distribution of intensity). In scientific fluorescence images, intensity of signal is very important because the signal intensity is directly related to the density of the substance. We now study how to interpret signals, and how to extract numerical values out of intensity distribution.

1.2.1 Histogram

If there is an 8-bit image with 100 pixel width and 100 pixel height, there are 10,000 pixels. Each of these pixels has certain pixel value. Intensity histogram of an image is a plot that shows distribution of pixel counts over a range of pixel values, typically its bit depth or between the minimum and the maximum pixel value within the image. Histogram is useful for examining the signal and background pixel values for determining a threshold value to do segmentation. We will study image threshold in [1.4](#).

Exercise 1.2.1-1

Open **Cell-Colony.tif**. Do [Analyze > Histogram]. A new window appears. The x-axis of the graph is pixel value. Since the image bit-depth is 8-bit, the pixel values range from 0 to 255. The y-axis is the number of pixels (so the unit is [count]). Since 255 = white and 0 = black, the histogram has long tail towards the lower pixel value. This reflects the fact that the image has white background and black dots.

Check pixel values in the histogram by placing the mouse pointer over the plot and move it along the x axis. Pixel count appears at the bottom of the histogram window. Switch to the cell colony image, and place the pointer over dark spot. Read the pixel value there, and then try finding out the number of pixels with the same value in the histogram. What is the range of pixel values which corresponds to the spot signal?

Histogram could also be used for enhancing contrast of image. Several different algorithms are available for this: histogram normalization, his-

togram equalization and local histogram normalization.

If the histogram is occupying only part of the available dynamic range of image bit depth (8-bit: 0 – 255, 16-bit: 0 – 65535), we could adjust pixel values of image to increase its range so that contrast become more enhanced. There are two ways to do this: normalization and equalization.

Normalization

With normalization, pixel values are normalized according to the minimum and the maximum pixel values in the image and bit-depth. If the minimum pixel value is p_{min} and the maximum is p_{max} in an 8-bit image, then normalization is done as follows:

$$\text{NewPixelValue} = \frac{(OriginalPixelValue - p_{min})}{(p_{max} - p_{min})} * 255$$

Equalization

Equalization converts pixel values so that the values are distributed evenly within the dynamic range. Instead of describing this in detail using math formula, I explain with a simple example below (Fig. 1.24). We consider an image with its pixel value ranging between 0 and 9. We plot histogram from this image and the result looks like black bars in the figure below (Fig. 1.24a). For equalization, a cumulative plot is first prepared from such histogram (adding up histogram values: Fig. 1.24b, black curve). To equalize the presence of pixel values within range between 0-9, we need to have a straight diagonal cumulative plot (in other words, probability density of the presence of pixel value should be near-flat). To have such plot, we shift histogram bars at pixel values 5 and 7 to 7 and 9, respectively (now, bars are in red after shifting). With this conversion cumulative plot now looks more straight and diagonal (Fig. 1.24b right, red curve).

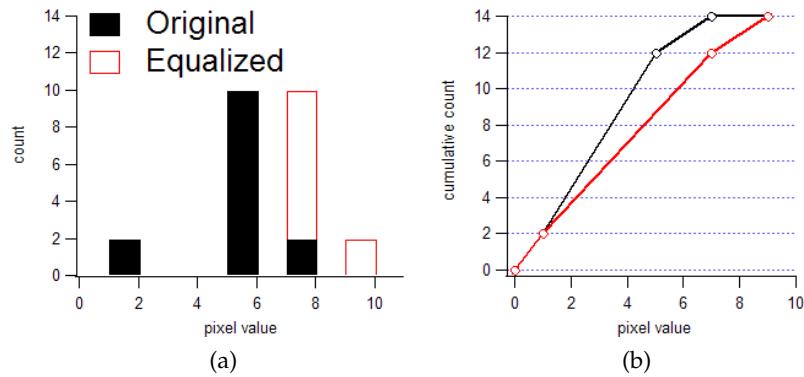


Figure 1.24: (a) Histogram Equalization: Very simple case. The actual calculation uses cumulative plots (b) of the histogram of original image, and uses it as a look up table to convert original pixel value, applied point-by-point.

Exercise 1.2.1-2

Histogram Normalization and Equalization:

Open sample image **g1.tif**, and then duplicate the image by [Image > Duplicate]. Click the duplicate and then [Image > Process > Enhance Contrast]. A dialog window pops up. Set "Saturated Pixels" to 0.0% and check "Normalize", while uncheck "Equalize Histogram", then click OK. Compare histogram of original and normalized images .

Duplicate **g1.tif** again by [Image > Duplicate]. Click the duplicate and then [Image > Process > Enhance Contrast]. A dialog window pops up. Set "Saturated Pixels" to 0.0% and uncheck "Normalize", while check "Equalize Histogram", then click OK. Compare histogram of original, normalized and equalized images ([Analyze > Histogram]).

Exercise 1.2.1-3

Local Histogram Equalization (Optional):

Histogram equalization could be done on local bases. This becomes powerful, as more local details could be contrast enhanced. You could try this with the same image **g1.tif**, by [Plugins > CMCI Course Modules

> CLAHE] (if you have installed course plugin in ImageJ) or if you are using Fiji, [Process > Enhance Local Contrast (CLAHE)]

1.2.2 Region of Interest (ROI)

To apply certain operation to a specific part of the image, you can select a region by "region of interest (ROI)" tools. The shape of the ROI could be various, such as rectangular, elliptical, polygon, free hand or a line (straight, segmented or free hand). There are several functions that will be used often in association with ROI tools.

Exercise 1.2.2-1

Cropping. Open any image. Select a region by rectangular ROI. Then [image > Crop]. This will remove the unnecessary part of the image, to reduce calculation time.

Exercise 1.2.2-2

Masking. Open any image. Select a region by rectangular ROI. Then [Edit > clear]. [Edit > Clear Outside]. After checking what happened, do [Edit Fill]. (same operation could be done by [Edit > Selection > Create Mask])

Exercise 1.2.2-3

Invert ROI. Open any image. Select a region by rectangular ROI. Then [Edit > Selection > Make Inverse]. In this way, you can select region excluding the region you initially selected.

Exercise 1.2.2-4

Redirecting ROI. Open any two images. In one of the image, select a region by rectangular ROI. Then activate the other image by clicking that window, and do [Edit > Selection > Restore Selection]. ROI with same size and position will be reproduced in the window.

Exercise 1.2.2-5

ROI manager. You can store the position and size of the ROI in the memory. Select a region by rectangular ROI. Then [Analysis >

Tools > Roi Manager]. Click "Add" button to store ROI information. Stored ROI can be saved as a file, and could be loaded again when you restart the ImageJ.

1.2.3 Intensity Measurement

As you place the mouse pointer over individual pixel, value of that pixel is indicated in the ImageJ menu bar. This is the easiest way to know pixel values (intensity), but it only works for each single pixels. Here we learn a way to get statistical information of a group of pixels within ROI. This has more practical usages for research.

To measure pixel values of a ROI, ImageJ has a function [Analyze > Measure]. Before using this function, you could specify the parameters you want to measure by [Analyze > Set measurements]. There are many parameters in the "Set measurement" window. Details on these parameters are listed in the Appendix 1.6.4. For intensity measurements following parameters are important.

- **Mean Gray Value** - Average gray value within the selection. This is the sum of the gray values of all the pixels in the selection divided by the number of pixels. Reported in calibrated units (e.g., optical density) if Analyze/Calibrate was used to calibrate the image. For RGB images, the mean is calculated by converting each pixel to grayscale using the formula $gray = 0.299red + 0.587green + 0.114blue$ or the formula $gray = (red + green + blue)/3$ if "Unweighted RGB to Grayscale Conversion" is checked in Edit/Options/Conversions.
- **Standard Deviation**- Standard deviation of the gray values used to generate the mean gray value.
- **Min & Max Gray Level** - Minimum and maximum gray values within the selection.
- **Integrated Density** - The sum of the values of the pixels in the image or selection. This is equivalent to the product of Area and Mean Gray Value. The Dot Blot Analysis example demonstrates how to use this option to analyze a dot blot assay.

A short note on image-based fluorometry: In biochemical experiments, scientists measure protein concentration by measuring absorbance of UV light or by labeling proteins with dyes or fluorescence and measure the intensity of emission. This is because light absorbance or the intensity of fluorescence intensity is proportional to the density of protein in the irradiated volume within cuvett. Very similar to this when fluorescence image of cells are taken, pixel values (which is the fluorescence intensity) are proportional to the density of the labeled protein at each pixel positions. For this reason, measurement of fluorescence intensity using digital imaging could be considered as the two dimensional version of the conventional fluorometry.

Exercise 1.2.3-1

Open a sample image **cells_Actin.tif**. Before actually executing the measurement, do [Analyze > Set Measurements]. A dialog window opens. There are many parameters listed in the upper-half of the window.

The parameters we select now are:

- Area
- Integrated Density
- Mean Gray Value

Check the box of these three parameters. Integrated density is the sum of all the pixel values and Mean Gray Value is the average of all the pixel values within ROI. So

$$\text{IntegratedDensity} = \text{Area} * \text{MeanGrayValue}$$

Select one of the cells in **cell_Actin.tif** image and zoom it up using magnifying tool. Switch the tool to "Polygon" tool. Draw polygon ROI around the cell. Then do [Analyze > Measure]. A window titled "Results" pops-up, listing the measured values. Check that the integrated density is the multiplication of area and the mean gray value.

This value is not the actual intensity of the fluorescence within the cell. Since this value also contains the background intensity (offset).

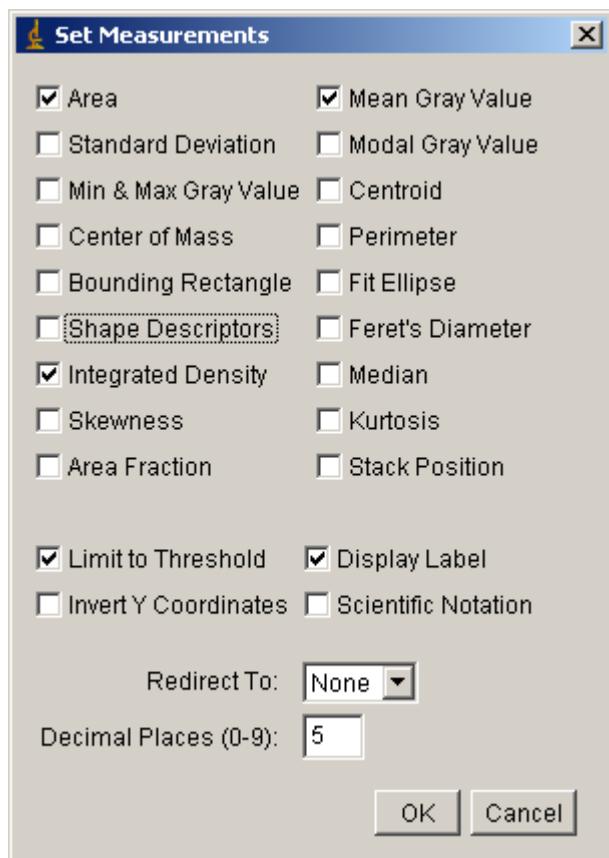


Figure 1.25: Set Measurement Window

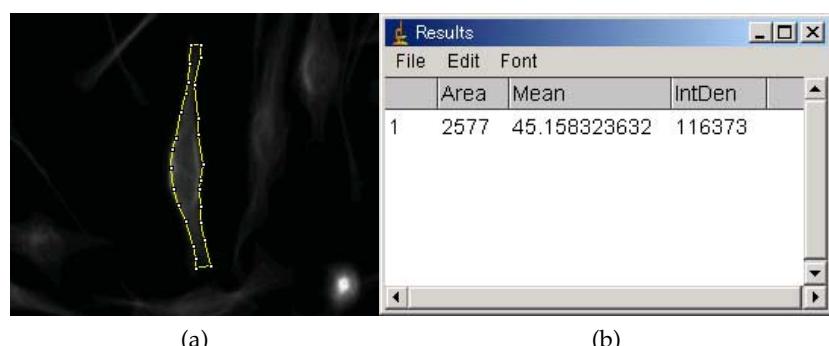


Figure 1.26: (a) Tracing Cell Edge by Segmented ROI and Measuring the Intensity within selected area.

Measure the background intensity by creating a new ROI in the area where there is no cell.

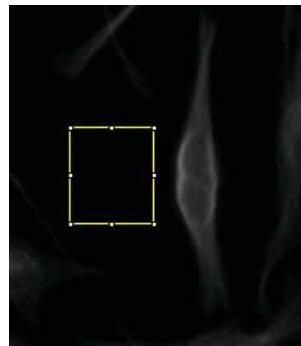


Figure 1.27: Measurement of Background

NOTE: When ROI is not assigned (no yellow bounding rectangle or so), then the measurement of full image field will be measured.

1.2.4 Image transformation: Enhancing Contrast

Some of you might already have experience with the contrast enhancing of digital images, since in most of imaging software like the ones that come with digital camera usually have this function. Low contrast image has small difference in the tones that the object is difficult to see. If the contrast is too high, then the tone difference is too much that the picture is "over exposed". Adjustment of contrast controls the tone difference to optimize the visual resolution. The contrast enhancement primarily changes the LUT, so that the original image data is unaffected (you will see in the following exercise). The original data is changed only when you click "Apply" button. Then the pixel values are re-scaled according to the LUT you set.

Care must be taken for contrast enhancement since pixel values are altered. This could be regarded as "fraud" or "manipulation" in science, especially if you are measuring image intensity. If all images that you are trying to compare were contrast enhanced according to same numerical parameter, then the images could be compared. Even then, there will be another pitfall if you artificially saturate the image: this happens often especially in

the case of low bit-depth images.

Exercise 1.2.4-1

Open image `gel_inv.tif`. Do [Image > Adjust > Brightness/Contrast].

Pop-up window appears which looks like the figure below (left). The graph shown in the upper part of the window is histogram of the image just like you have seen in the section 1.2.1 Histogram. Since the image is 8-bit, scale in the x axis is 0 to 255. There is also a black diagonal line. This corresponds to the LUT of the image on the screen: pixel values in x is translated into the gray value on the screen (brightness on the screen is y-axis).

The slope and position of the LUT can be altered by click and dragging four sliding bars under the graph, each with the name minimum, maximum, brightness and contrast. Try changing these values and studying the effect on the image.

QUESTION: What is the problem with the adjustment shown in the right side of the figure below?

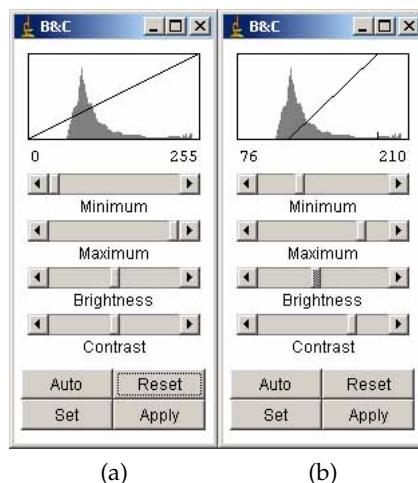


Figure 1.28: (a) Histogram of `gel_inv.tif` before enhancing contrast. (b) Some bad adjustments example.

Exercise 1.2.4-2

Continued from above: the original image file is not changed at this stage. When you push "Apply" button at the bottom-right corner, the

original image is altered numerically. Try set the LUT as you like, and push the Apply: what happened to the Histogram and the LUT indicator? (you can always "Undo" the "Apply" by [Edit > Undo], or revert to the original file by [File > Revert]).

Exercise 1.2.4-3

With RGB image, it is possible to adjust the brightness and contrast for individual color channel. Open the image **RGB_cell.tif**, then do [Image > Adjust > Color Balance]. There is a pull-down menu to specify the channel you want to change. Try changing different channels to optimize the image.

1.2.5 Image correlation between two images: co-localization plot

In many experiments we encounter situation where we need to compare the localization of two or more proteins and examine whether those proteins are co-localized. In many cases this has been evaluated by visual inspections. But with digital images, it is possible to evaluate the degree of co-localization more quantitatively. This is done by plotting a so called "co-localization plot". To do this in ImageJ one could download a plugin **Colocalization_Finder**⁶ and install it.

Degree of colocalization could be then parametrized by using statistical values such as Pearson's coefficient and Mander's coefficient. These values have advantages and disadvantages depending on the image properties. For detailed description on these issues, refer to [Bolte and Cordelières \(2006\)](#).

Additional insights, pitfalls and tips on localizing spot signals could be found in [Waters \(2009\)](#). This paper also provides detailed examination of the precision of dot detections.

⁶download from
[colocalization-finder.html](http://rsb.info.nih.gov/ij/plugins/colocalization-finder.html)

1.2.6 ASSIGNMENTS

Assignment 1-2-1:

Suppose that you had an 8-bit grayscale image of three objects of different distinct intensities against a bright background. What would the corresponding histogram look like?

Assignment 1-2-2:

With image **cell_Actin.tif**, do the measurement with 4 cells and one background in image as we did in the exercise. This time, store ROI in ROI manager (refer to [1.2.2 Roi](#)) and do the measurement at once. First, you store 5 different ROI one by one ("Add" button). Then click "Show all" button in the ROI manager. Activate all ROI by clicking the ROI name in the list while pushing down the SHIFT key. Then click "Measure" button. All five ROI will be measured at once. Average the values and describe the results in text.

Assignment 1-2-3:

1. Optimize the contrast of image **m51_8bit.tif**. Be careful not to saturate the pixels so you don't throw away important information in the lower pixel values. Check the histogram. Don't close the histogram window
2. After applying the adjustment above (clicking "Apply" button), check the histogram again. Compare the histogram before and after. What happened? Discuss the reason.

1.3 Filtering

Filtering improves the appearance of digital image. Then recognition of shapes becomes easier by noise removing and enhancement of the structural edge. Not only for the appearance, filtering improve the efficiency of "segmentation" which we will study in the next section. Segmented image could be used as a mask to specify regions to be measured. Note that the filtering alters the image so one must realize that in most cases, processed images cannot be used for quantitative intensity measurement without precise knowledge on what would happen to the numerical values after the processing.

There are two different types of filtering: one involves the frequency domain of images (Fourier transformed image), while the others deals with spatial domain. We first study the spatial domain filtering and its central concept "convolution", and walk through various types of convolutions. We then study the frequency domain filtering (FFT). In FFT world, convolution of an image with filter kernel could be done simply by multiplication between two images.

1.3.1 Convolution

In the [Process] menu, we see a long list of filters. An important concept for understanding what these commands will do to your image is "convolution". A small mask (called kernel) is moved along the image pixel by pixel and apply operations using the surrounding pixel values (see Fig. 1.29). The result of operation is over-written to that pixel position as the new pixel value.

To understand how the convolution is done, we take a one-dimensional example (Fig. 1.30).

We have a small 1D array f , and we want to convolve a kernel w (i). We first rotate the kernel by 180 degrees that the order is now reversed. Then we align f and w to match the position of the last element of kernel to the first element of f . Since we want to have all the kernel elements to have

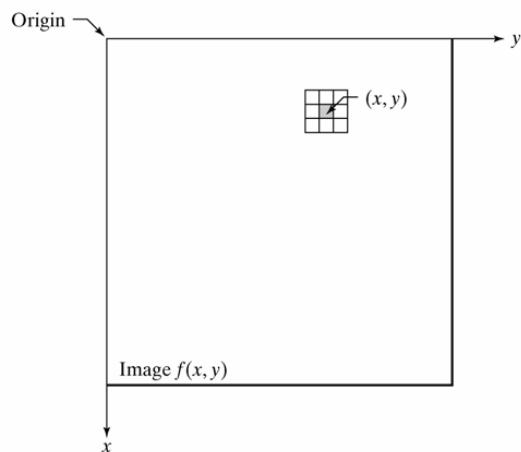


Figure 1.29: An image and a Kernel (figure taken from DIP)

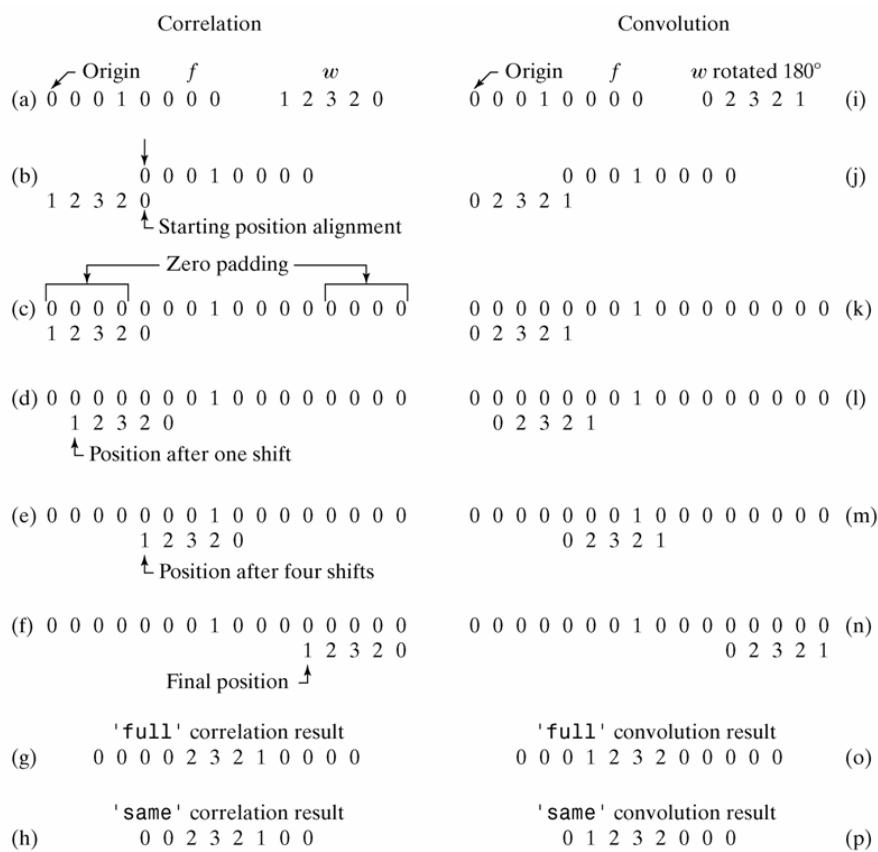


Figure 1.30: 1-dimensional convolution and correlation (figure taken from DIP)

corresponding partner, we "pad" f by 0. This is just for the convenience of calculation (k). Then you multiply each element pairs (5 pairs in this case) and sum up the results. since all partners in f are 0, the sum of multiplication is 0. We note this as the first element of "full convolution result" (o). We then slide w to the left by one element, do the multiplications and summing up again. Note the result as the second element of "full convolution result" (o). Like wise, we do such calculation step by step until last element of w matches the last element of f (n). After then, we throw away padded elements from the output 1D array to have a resulting array with same length as the original f (p).

Convolution and correlation

Two closely-related bilinear operations that are especially important for information processing are *convolution* and *correlation*.

In the simplest case, correlation can be described as a comparison of two fields at all possible relative positions. More specifically, if χ is the correlation of two one-dimensional fields ϕ and ψ , $\chi = \phi * \psi$, then $\chi(r)$ reflects how well ϕ and ψ match (in an inner-product sense) when relatively displaced by r . Mathematically,

$$\chi(r) = \int_{\Omega} \phi(s - r)\psi(s)ds$$

Higher dimensional correlations are the same, except that r is a relative displacement *vector* rather than a scalar.

Convolution, $\chi = \phi \otimes \psi$, is essentially the same as correlation, except that the field ϕ is reflected before the comparison takes place:

$$\chi(r) = \int_{\Omega} \phi(r - s)\psi(s)ds$$

Convolution is useful because: (1) its algebraic properties are more like multiplication, and thus more familiar, than correlation; and (2) many physical processes (e.g. linear systems, such as dendritic nets) perform convolutions.

Quote from: <http://www.cs.utk.edu/~mclennan/anon-ftp/FCMC-tr/node14.html>

In two dimensional matrix, see the example in Fig. 1.31.

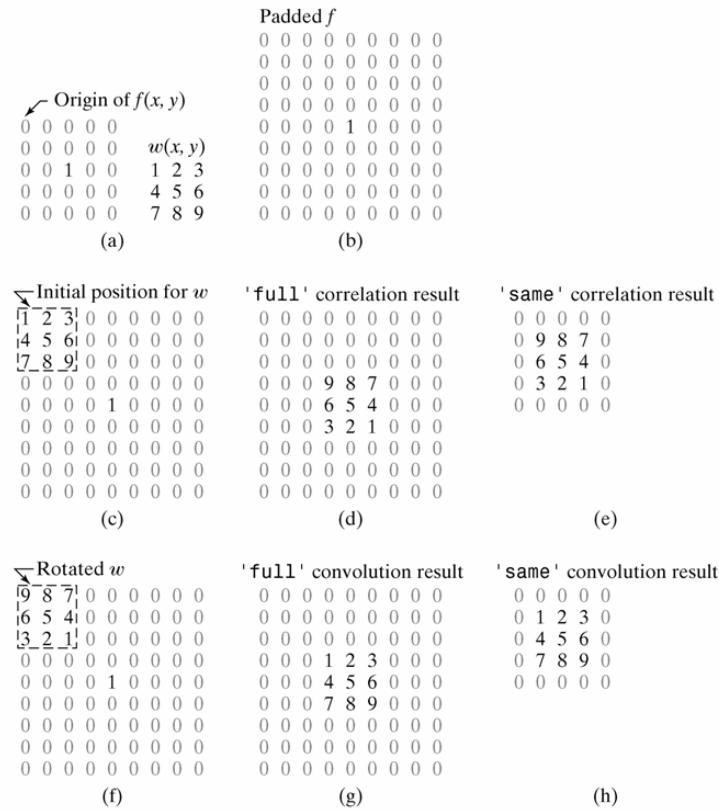


Figure 1.31: Two-dimensional convolution (figure taken from DIP)

The matrix (a) is first padded (b), then starting from the top-left corner (f) the matrix is convoluted (g) and then the padded rows and columns are removed to return an output matrix with the same dimension as the original (h).

1.3.2 Kernels

In [Process] menu, we have many operators such as smooth, sharpen, find edges... and so on. Many of them are called "linear filters" because the result of filtering is a linear combination of the original image pixel values. We study various kernels in this section.

Smoothening

"Smoothening" operation, which is used for attenuating noise (Median kernel is better for shot-noise removal but for learning purpose we stick to the smoothing), is done by applying the following kernel to the image.

$$\begin{array}{ccc} 1 & 1 & 1 \\ 1 & 1 & 1 \\ 1 & 1 & 1 \end{array}$$

Let's take an example of an image with a vertical line in the middle.

$$\begin{array}{ccccc} 0 & 0 & 10 & 0 & 0 \\ 0 & 0 & 10 & 0 & 0 \\ 0 & 0 & 10 & 0 & 0 \\ 0 & 0 & 10 & 0 & 0 \\ 0 & 0 & 10 & 0 & 0 \end{array}$$

Just for now, we forget about the padding for explanation, and first apply the kernel to the top-left corner for calculating convolved value at (1, 1) pixel position (note: top-left element position is (0, 0)). Then the calculation is

$$\begin{aligned} output(1,1) &= (0 \times 1 + 0 \times 1 + 0 \times 1 + \\ &\quad 0 \times 1 + 0 \times 1 + 0 \times 1 + \\ &\quad 10 \times 1 + 10 \times 1 + 10 \times 1) / 9 \\ &= 30 / 9 \\ &= 3 \end{aligned}$$

The sum of multiplication is divided by 9, which is the sum of all elements in the kernel. This is to normalize the convolution, so that the output value will not be too large compared to the original. We then shift the kernel one step in x-direction, and apply the kernel for calculating (2, 1) position.

$$\begin{aligned} output(2,1) &= (0 \times 1 + 0 \times 1 + 0 \times 1 + \\ &\quad 10 \times 1 + 10 \times 1 + 10 \times 1 + \end{aligned}$$

$$\begin{aligned}
 & 0 \times 1 + 0 \times 1 + 0 \times 1) / 9 \\
 & = 30 / 9 \\
 & = 3
 \end{aligned}$$

You might have now understood that the "smooth" operation is actually averaging the values in the surrounding. Applying the kernel through the image, the new image will be:

$$\begin{array}{ccccc}
 0 & 2 & 2 & 2 & 0 \\
 0 & 3 & 3 & 3 & 0 \\
 0 & 3 & 3 & 3 & 0 \\
 0 & 3 & 3 & 3 & 0 \\
 0 & 2 & 2 & 2 & 0
 \end{array}$$

The vertical line is then now broader and darker – the smoothing effect. Note that the pixels in the first and the 5th rows were calculated with zero padding so that values are 2 rather than 3. This is the boundary effect unavoidable with any filtering by convolution. We could attenuate this effect if we do padding by duplicating neighboring pixels. Then the result of convolution then becomes

$$\begin{array}{ccccc}
 0 & 3 & 3 & 3 & 0 \\
 0 & 3 & 3 & 3 & 0 \\
 0 & 3 & 3 & 3 & 0 \\
 0 & 3 & 3 & 3 & 0 \\
 0 & 3 & 3 & 3 & 0
 \end{array}$$

Exercise 1.3.2-1

Working with kernel: in ImageJ, one could design original kernel and do convolution for images. Open **microtubule.tif** image and zoom up so you can see individual pixels. Do [Process > Filter > Convolve]. A pop-up window appears (see the image below). One could edit the kernel. Be sure to make spaces between numbers. By clicking OK, the kernel will be applied to the image.

Try replacing the default kernel with the smoothening kernel we studied above. Apply the kernel to sample image **microtubule.tif**. In-

crease the dimension to 5×5 , 9×9 and do the smoothening.

Question: what happened when the size of the kernel became larger?

Check the preview option, so that the change in the kernel could be visualized directly while editing.

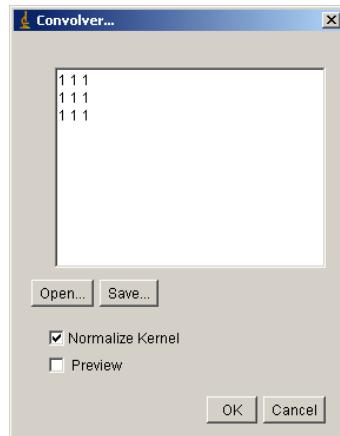


Figure 1.32: Convolver Window.

Sharpen

$$\begin{array}{ccc} -1 & -1 & -1 \\ -1 & 12 & -1 \\ -1 & -1 & -1 \end{array}$$

This kernel sharpens the image, also known as Laplacian. Side effect: noise is also enhanced.

Find Edge (gradient)

Following two kernels are applied independently. Square root of the sum of the square of two result images will be calculated (called "Sobel Filter": for more details, see Appendix [1.6.5](#))

$$\begin{array}{ccc} 1 & 2 & 1 \\ 0 & 0 & 0 \\ -1 & -2 & -1 \end{array}$$

and

$$\begin{array}{ccc} 1 & 0 & -1 \\ 2 & 0 & -2 \\ 1 & 0 & -1 \end{array}$$

Gaussian Blur

This kernel blurs (in positive sense, we call it "smooth") the image by convolution using a square Gaussian (bell-shaped) kernel. The width of the kernel, in pixels, is $2\sigma + 1$, where σ is entered into a dialog box (ImageJ documentation). Following three kernels are with different sigma 2 (5x5), 3 (7x7) and 7 (15x15).

Gauss 5 x 5 (Sigma = 2)

$$\begin{array}{cccccc} 1 & 1 & 2 & 1 & 1 \\ 1 & 2 & 4 & 2 & 1 \\ 2 & 4 & 8 & 4 & 2 \\ 1 & 2 & 4 & 2 & 1 \\ 1 & 1 & 2 & 1 & 1 \end{array}$$

Gauss 7 x 7 (Sigma = 3)

$$\begin{array}{cccccccc} 1 & 1 & 1 & 2 & 1 & 1 & 1 \\ 1 & 2 & 2 & 4 & 2 & 2 & 1 \\ 2 & 2 & 4 & 8 & 4 & 2 & 2 \\ 2 & 4 & 8 & 16 & 8 & 4 & 2 \\ 2 & 2 & 4 & 8 & 4 & 2 & 2 \\ 1 & 2 & 2 & 4 & 2 & 2 & 1 \\ 1 & 1 & 1 & 2 & 1 & 1 & 1 \end{array}$$

Gauss 15 x 15 (Sigma = 7)

2	2	3	4	5	5	6	6	6	5	5	4	3	2	2
2	3	4	5	7	7	8	8	8	7	7	5	4	3	2
3	4	6	7	9	10	10	11	10	10	9	7	6	4	3
4	5	7	9	10	12	13	13	13	12	10	9	7	5	4
5	7	9	11	13	14	15	16	15	14	13	11	9	7	5
5	7	10	12	14	16	17	18	17	16	14	12	10	7	5
6	8	10	13	15	17	19	19	19	17	15	13	10	8	6
6	8	11	13	16	18	19	20	19	18	16	13	11	8	6
6	8	10	13	15	17	19	19	19	17	15	13	10	8	6
5	7	10	12	14	16	17	18	17	16	14	12	10	7	5
5	7	9	11	13	14	15	16	15	14	13	11	9	7	5
4	5	7	9	10	12	13	13	13	12	10	9	7	5	4
3	4	6	7	9	10	10	11	10	10	9	7	6	4	3
2	3	4	5	7	7	8	8	8	7	7	5	4	3	2
2	2	3	4	5	5	6	6	6	5	5	4	3	2	2

Median

None-linear filters are so called because the result of applying the filter is non-linear. Median filter used for the removal of noise is one of such filters. In ImageJ, the command will be [Process > Filter > Median]. Following is the principle of how median filter works. When we apply median filter with a 3×3 size, ImageJ samples 3×3 neighbors surrounding the target pixel. Graphically, if the sampled region looks like below (target position contains 2 now)

1	7	9
4	2	8
6	5	3

Then we align these numbers in the ascending order.

1 2 3 4 5 6 7 8 9

We take the median of this sequence (= 5) and replace the value 2 with 5.

1.3.3 Morphological Image Processing

Mathematical morphology is a powerful tool that can be used to extract features and components from an image. It is often used to pre-process or post-process of images to facilitate analysis. In this process a small shape (structuring element, not necessarily square like we did in the previous section) is translated across the image during the course of processing. Certain mathematical logic operations are performed on the image using the structuring element to generate the processed image. In this section, we first introduce dilation and erosion, two fundamental operations in mathematical morphology. We then describe morphological operations obtained by combining erosion and dilation.

Dilation

Dilation is an operation that grows objects in a binary image. The thickening is controlled by a small structuring element. In Fig. 1.33 you can see the structuring element on the right and the result after applying dilation on a rectangle.

Erosion

Erosion shrinks or thins objects in a binary image. After erosion the only pixels that survive are those where the structuring element fits entirely in the foreground (Fig. 1.34).

In above examples structuring elements are asymmetrically shaped. In ImageJ, structuring elements are squares so the dilation: erosion effects are even along both axes.

Exercise 1.3.3-1

Load noisy-fingerprint.tif and broken-text.tif. Apply dilations or erosions with different iterations.

For setting iterations, do [Process > Binary > Options]. Binary option window opens and you can set several parameters. "Count"

The structuring element translated to these locations does not overlap any 1-valued pixels in the original image.

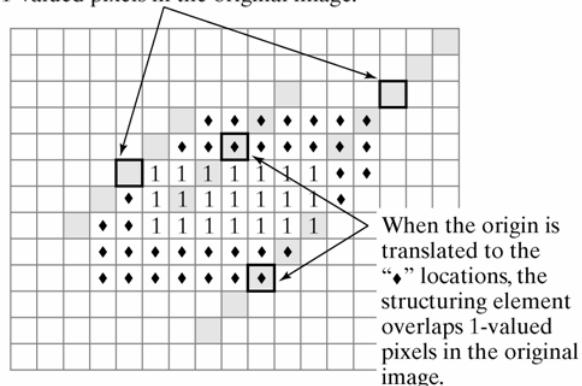


Figure 1.33: Dilation (figure taken from DIP).

Output is zero in these locations because the structuring element overlaps the background.

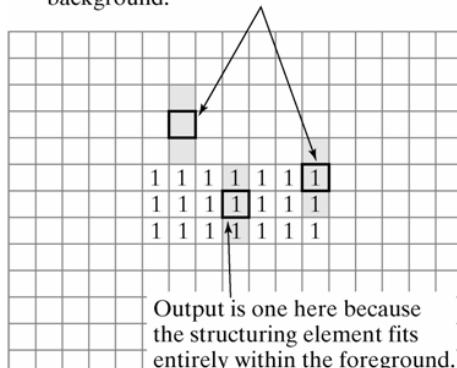


Figure 1.34: Erosion (figure taken from DIP)

matters with setting the number of overlapping pixels between structuring element and the object that determines output 0 or 1. Larger count causes less degree of erosion or dilation.

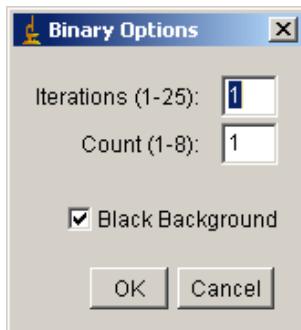


Figure 1.35: Setting Iteration.

From ImageJ manual: Iterations specifies the number of times erosion, dilation, opening, and closing are performed. Count specifies the number of adjacent background pixels necessary before a pixel is removed from the edge of an object during erosion and the number of adjacent foreground pixels necessary before a pixel is added to the edge of an object during dilation. Check Black Background if the image has white objects on a black background.

1.3.4 Morphological processing: Opening and Closing

Combinations of morphological operations can be very useful in removing many artifacts present in images. This will become very useful after segmenting an image. The first operation we will see is opening, which is an erosion followed by dilation. Opening smooths object contours, breaks thin connections and removes thin protrusions. After opening, all objects smaller than the structuring element will disappear. Closing is a dilation followed by erosion. Closing smooths object contours, joins narrow breaks, fills long thin gulfs and fills holes smaller than the structuring element.

Exercise 1.3.4-1

Load noisy-fingerprint.tif and broken-text.tif. Apply opening and closing to the images by [Process > Binary > Open] and [Process > Binary > Close].

Exercise 1.3.4-2

(Optional) Next, we do morphological processing using anisotropic structuring element.

Invoke [Plugins > CMCI Course Modules > Morphology] and design vertical structuring element first with (1) diameter = 3. This is simply by inputting "3" in the Diameter field. Click tiles to activate/deactivate positions. Click "Apply" button to do the actual processing. Then you could try with a larger diameter: (2) diameter = 9. Apply these two different structuring elements to dilate noisy-fingerprint. Discuss the difference in outputs.

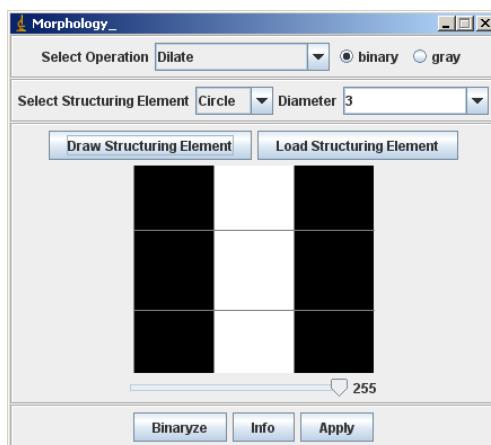


Figure 1.36: Morphological Image Processing dialog, to design structuring element.

1.3.5 Minimum and Maximum

Morphological transformation on grayscale image is very useful for subtracting the background, such as to eliminating the shading of the image such as shown in fig below. Opening and closing we studied above works only with binary images. For gray scale images, one can use Minimum (like Open) and Maximum (like Close) under [Process Filters]. One could

remove all features smaller than the structuring element by "Minimum" operation of gray images. In the following example we will experience this.

Exercise 1.3.5-1

Open rice.tif. Then [Process > Filters > Minimum]. In the dialog window, input the radius of the structuring element (structuring element is circular). Find a radius that removes rice from the image. Successfully removed image is the background image. Then remove the background image from the original image.

Note: For bright field images, background actually should be removed by division rather than subtraction such as:

$$\text{Corrected_Image} = \frac{\text{Specimen} - \text{Darkfield}}{\text{Brightfield} - \text{Darkfield}} * 255$$

1.3.6 Background Subtraction

Besides the background subtraction we studied above using Minimum and Maximum filtering, there are special function designed for the background subtraction (extension of morphological processing). ImageJ has a direct background subtraction by Rolling ball algorithm ⁷ In the dialog window when you execute this operation, you will be asked for the Rolling ball radius. This should be at least as large as the radius of the largest object in the image that is not part of the background.

Exercise 1.3.6-1

Open rice.tif. Do background subtraction by [Process > Subtract > Background]. Change the Rolling ball radius and study the effect.
Question: what happens when the rolling ball radius is smaller?

Background subtraction explained so far uses morphological processing. There are several other methods including

1. High-pass filter

⁷Stanley Sternberg's article, "Biomedical Image Processing", IEEE Computer, January 1983).

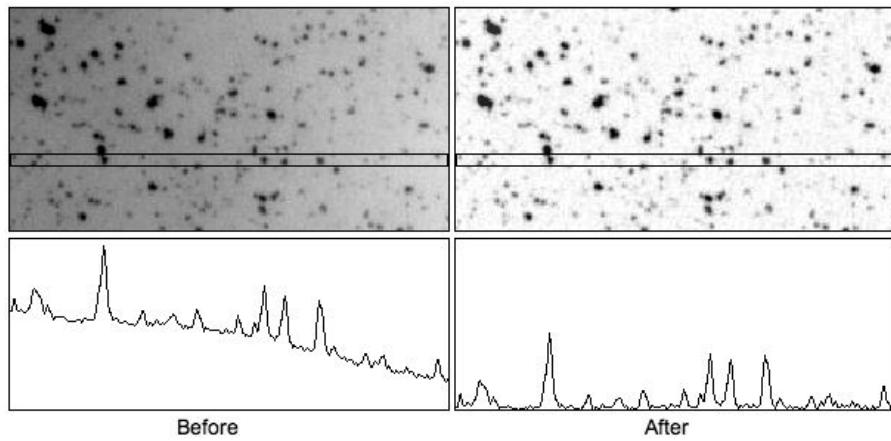


Figure 1.37: Background Subtraction (from ImageJ site).

2. Flat-field correction by polynomial fitting
3. Deconvolution

Pseudo high-pass filtering could be done by subtracting largely blurred image by Gaussian blurr (such as with sigma of 20) from the original image. Otherwise, band-pass function under [Process > FFT] could be used for high-pass filtering.

For polynomial fitting, ImageJ plugin "Polynomial_Fit" written by Bob Dougherty could be used to estimate the background ⁸.

Practical information on background subtraction for bright field images is available in ImageJ wiki⁹. For flat field correction protocol, see "Optical Microscopy Primer" site¹⁰.

Protocol for the background subtraction for fluorescence images using calibration slide and ImageJ could be found in [Miura and Rietdorf \(2006\)](#).

For the background removal of fluorescence time series, bleaching of fluorescence should be considered. See recent article by [Schwarzfischer et al. \(2011\)](#).

⁸http://www.optinav.com/Polynomial_Fit.htm

⁹<http://imagejdocu.tudor.lu/imagej-documentation-wiki/how-to-how-to-correct-background-illumination-in-brightfield-microscopy>

¹⁰<http://micro.magnet.fsu.edu/primer/digitalimaging/imageprocessingintro.html>

Deconvolution removes out-of-focus emission signal. See appendix 8 for an extensive tutorial using ImageJ plugin. You could also refer to a classic review ([Wallace et al., 2001](#)).

1.3.7 Other Functions: Fill holes, Skeltonize, Outline

Frequently, after some morphological operation we need to fill the holes in a binary image. For example, we detect the boundary of a cell and want to obtain an object which is filled and covers the cell. In this example we will see its effect.

Exercise 1.3.7-1

Open **book-text.tif**. Then fill holes by [Process > Binary > Fill Holes].

1.3.8 Batch Processing Files

Once you established a processing protocol, then you could create a pipeline to process many files automatically one-by-one. Such task is called *batch processing*. Prerequisite for using batch processing function in ImageJ is that all files that you want to process are stored in a single folder. Another preparation for batch processing is that you should *record* the processing command, in the following way shown in the exercise.

Exercise 1.3.8-1

Here, we use numbered-tiff file series as an example to do batch processing. Open `/sample_images/spindle-frames/eg5_spindle_500016.tif`. In order to record the processing command, do [Plugins Macros Recorder...]. A recorder window starts up:



Figure 1.38: Macro Recorder Window

Then go back to the spindle image (activate the image window by clicking the title bar) and then do [Process > Subtract > Background]. Just put some values in the subtract background dialog, and click OK, and check that the image is background subtracted (Fig. 1.39).

Check the Recorder window again. You will see that a new text line is added. This text should be like it is shown in Fig. 1.40.

```
run("Subtract background...", "rolling=5 disable");
```

number after `rolling=` could be various according to your input, and

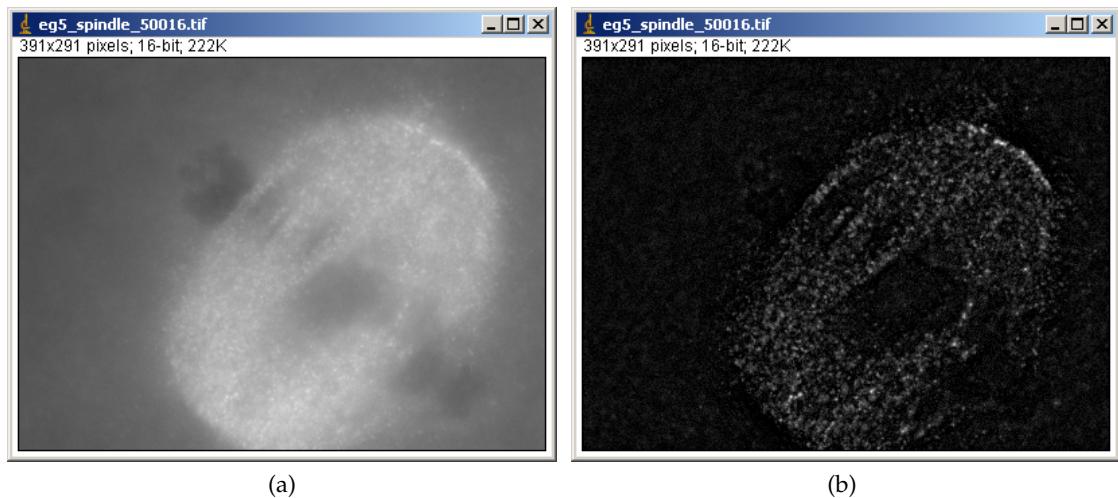


Figure 1.39: Spindle images (a) before and (b) after the background subtraction.

also other arguments might be present after that depending on the checks you did during the Subtract Background dialog.



Figure 1.40: Recorder window after Subtract Background command.

Copy and paste this text command, and paste it somewhere to keep it. Then do [Process > Batch > Macro...]. This will create a new window titled "batch Process". Paste the text command you prepared above in the text field of this batch Process window (Fig. 1.41).

To set the input folder (where files to be processed are stored), click "Input..." button and select the folder. Then set the output folder (where processed files will be stored, choose one that is empty), click

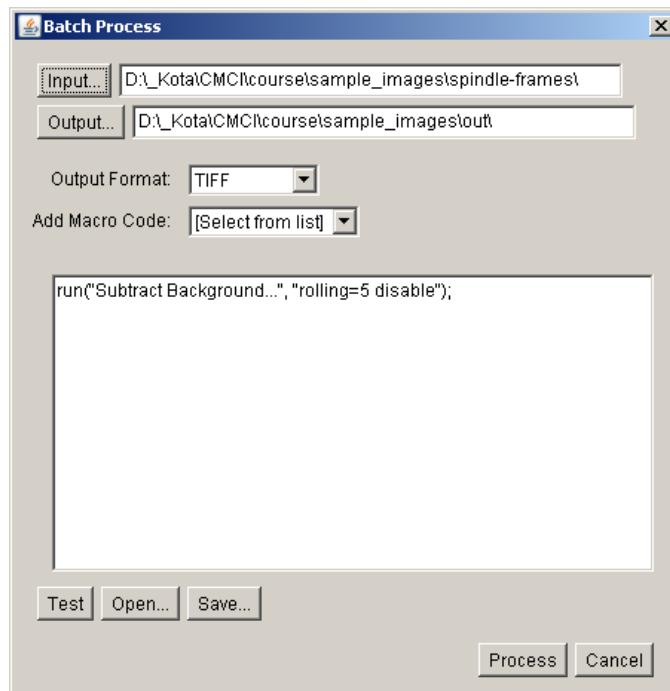


Figure 1.41: Batch Process window.

"Output" button and select a folder. Check the options so that they look like above, then clicking "Process" button will start the batch processing of all files in the input folder. Check the images created in the output folder to see images are actually the processed version of input folder.

In above exercise, we had only one processing command, but you could add many more text commands, which you could extract by using "Recorder".

1.3.9 Fast Fourier Transform (FFT) of Image

FFT converts spatial-domain image data (what you are normally seeing image) to frequency domain data. FFT is used because

1. in some occasion, calculation could be done much faster in frequency domain than in spatial domain. *i.e.* convolution.
2. Some image-processing techniques could only be done in frequency

domain.

Exercise 1.3.9-1

Reversibility of FFT

Open **microtubule.tif** by [File > Open]. Then apply FFT by [Process > FFT > FFT]. A new window showing frequency-domain image (2D power spectrum, log display) appears. To check that FFT is reversible, apply [Process > FFT > Inverse FFT] (Fig. 1.42).

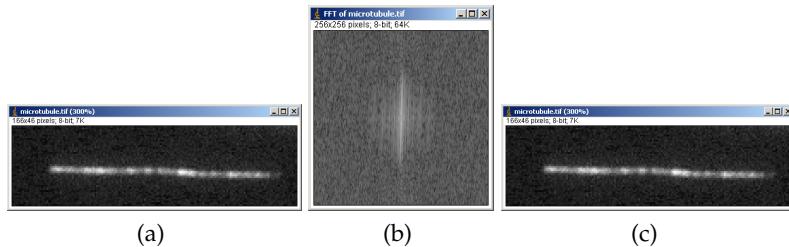


Figure 1.42: Reversibility of FFT. (a) Original, (b) FFT, (c) Inverse FFT.

Here is an intuitive explanation of what frequency domain image is: Orientation of patterns in spatial domain image has a clear relationship with the resulting FFT image. We take example four images with stripes differently oriented, vertical, diagonal (right to left or left to right) and horizontal (Fig. 1.43)¹¹. When these images are transformed by FFT, resulting images show high intensity peaks (which means higher value) that reflect the direction of stripe pattern (Fig. 1.44).

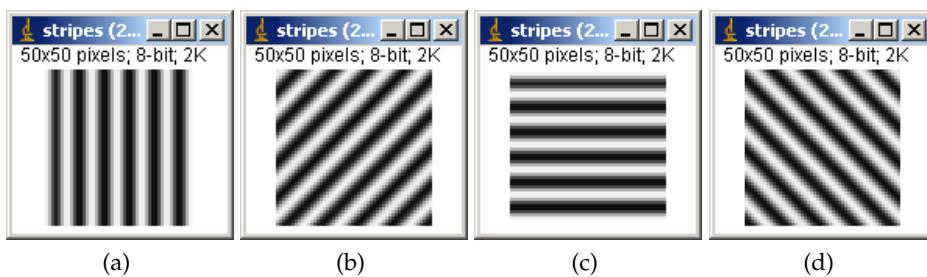


Figure 1.43: Original images (Spatial-domain images).

¹¹ To generate such stripe images for studying FFT, use macro code in Appendix 1.6.9.

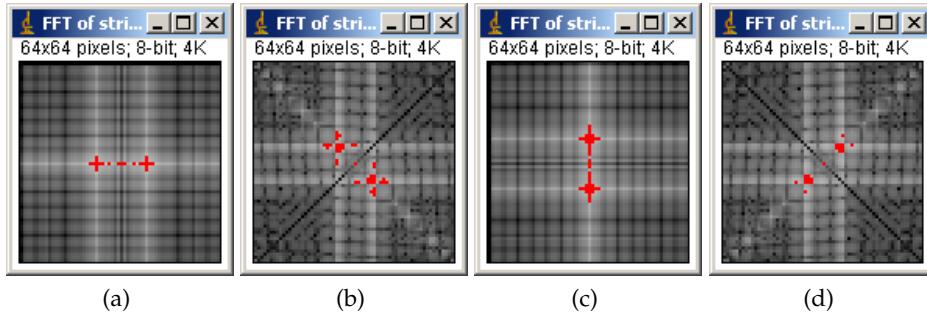


Figure 1.44: FFT images (Frequency-domain images). High intensity values are highlighted in red.

For example, FFT image of stripes in horizontal direction (Fig. 1.43a) shows high intensity peaks that are horizontally aligned (Fig. 1.44a). Stripes in vertical direction (Fig. 1.43c) become vertically aligned peaks in FFT image (Fig. 1.44c). In general, values in FFT image would show peaks aligned in the direction of the repetitive pattern appeared in original spatial-domain image. If the pattern appears in all direction (isotropic pattern, such as concentric rings) would then end up in circular signal in FFT image.

Frequency of patterns in spatial-domain image also has a clear relationship with the resulting FFT image. See spatial-domain images shown in below. Stripe frequency decreases from left to right. In corresponding FFT images shown in the second row, high-intensity pixels (high-lighted in red) become closer to the center of FFT image as the frequency of pattern in the original spatial-domain image decreases.

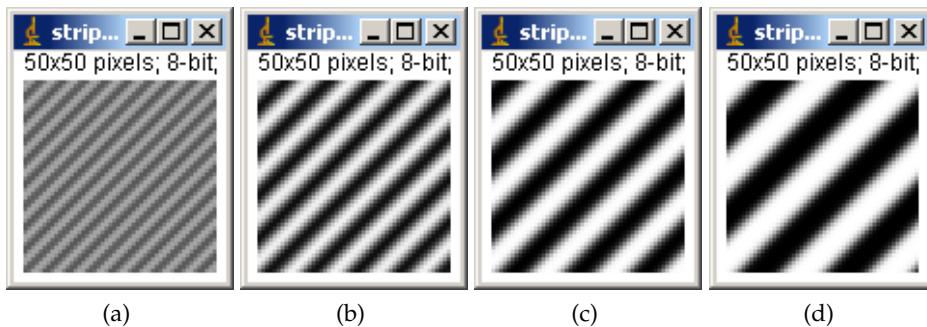


Figure 1.45: Original images (Spatial-domain images) with various frequency.

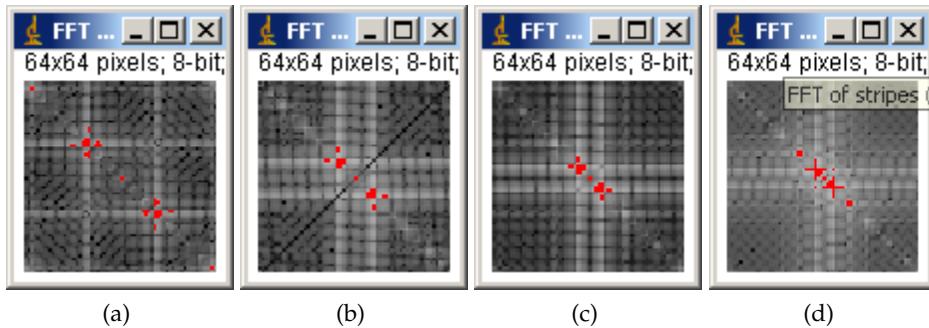


Figure 1.46: FFT images (Frequency-domain images) of images with various frequency.

Frequency-domain image is a plot with vertical frequency in vertical axis and horizontal frequency in horizontal axis. Two axis crosses at the centre of the image. Schematic drawing of FFT image in below shows how FFT signal would be distributed in terms of original spatial-domain image.

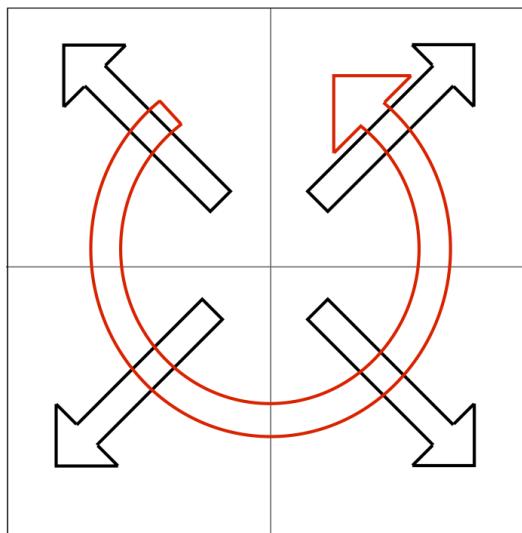


Figure 1.47: Distribution of Signals in 2D Power Spectrum. Frequency of pattern increases from center towards periphery (black arrows). Direction of pattern is reflected in the alignment of signal in 2D power spectrum.

Signals with lower frequency, such as objects, will be placed close to the origin (center of the 2D power spectrum image), while higher frequency signals such as noise will be placed further from the origin. Noise in general has no spatial bias, so the signal of noise will predominantly appear in all

over the peripheral region of 2D power spectrum (FFT image). Anisotropic patterns in original image will result in anisotropic signal in 2D power spectrum *e.g.* horizontal pattern will cause horizontally aligned high intensity peaks.

1.3.10 Frequency-domain Convolution

Convolution we studied in section 1.3.1 was a iterative procedure. Convolution kernel was applied every time you slide the position of the kernel by one pixel in x or y direction. This processing could be much simpler by using the FFT image, just by multiplying FFT kernel to it. We experience this in the exercise below.

Exercise 1.3.10-1

Convolution in Frequency-domain.

Edit Laplacian filter kernel using the convolution interface ([Process > Filter > Convolution]). 3x3 Laplacian kernel looks like this:

$$\begin{matrix} 0 & -1 & 0 \\ -1 & 4 & -1 \\ 0 & -1 & 0 \end{matrix}$$

Save the kernel somewhere in your computer as `Laplacian3x3.txt`.

Open sample image `blobs.tif` by [File > Open]. and then convert

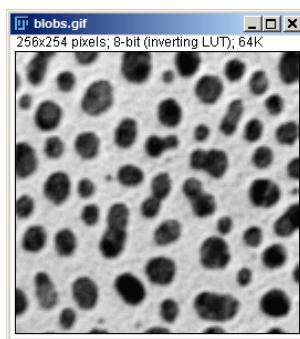


Figure 1.48: blobs.gif

the image to 32-bit.

[Image > Type > 32-bit]

then increase the canvas size to 256 x 256. “Position” should be “center”.

[Image > Adjust > Canvas Size].

Duplicate the image, so that we can do both spatial-domain convolution and frequency domain convolution.

We first do the convolution in spatial-domain, similar to what we have done already in the section 1.3.1 but we do this for comparison with convolution in frequency domain.

Spatial-Domain Convolution

[Process > Filter > Convolution]

In the convolution panel, click “open” and choose the Laplacian you created in above. Be sure to check “Check Normalized”. Result should look like

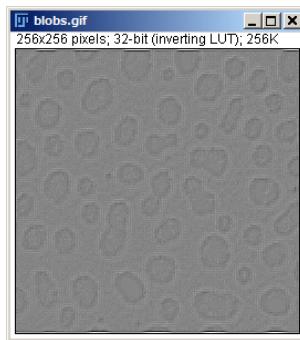


Figure 1.49: blobs.gif, Laplacian kernel applied

Frequency-Domain Convolution

We first prepare the the Laplacian kernel image. Open the kernel `laplacian3x3.txt` by:

[Image Import Text Image]

It should be very small, but if you zoom up the image is should look like:

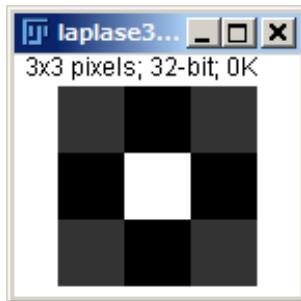


Figure 1.50: Laplacian3x3.txt, text image zoomed up

Then adjust the image size to 256 x 256.

[Image > Adjust > Canvas Size...]

Be sure to set “position” to “center” and check “zero fill”.

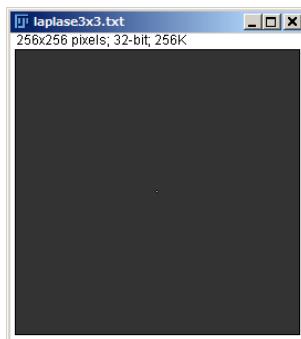


Figure 1.51: Laplacian3x3.txt, canvas size adjusted

Then we do the convolution by:

[Process > FFT > FD math...]

choose blob.gif and laplace3x3.txt for image1 and image2 respectively. Operation should be “Convolve”. Uncheck “Do Inverse transform”, and OK. Image named “Result” appears.

Then do the inverse FFT by:

[Process > FFT > Inverse FFT]

Check that you could get the original image by “deconvolve” operation using FD math.

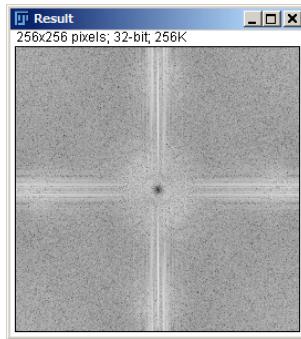


Figure 1.52: Frequency domain convolution of Blob image

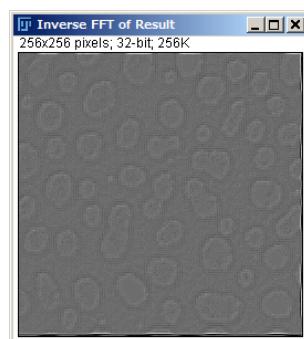


Figure 1.53: Frequency domain convolution of Blob image, now in Spatial domain

1.3.11 Frequency-domain Filtering

Filtering using FFT image (2D power spectrum) is a way of improving image quality and also for efficient segmentation. One typical example would be noise reduction. As we have seen in Fig. 1.47, noise is high frequency isotropic signal that will be in the periphery of FFT image. We could then remove noise by simply throwing away peripheral signals in FFT image to reduce noise.

Exercise 1.3.11-1

Removing noise using FFT image.

Open **microtubule.tif** by [File > Open]. Then apply FFT by [Process > FFT > FFT]. A new window showing microtubule converted to a frequency-domain image (2D power spectrum) appears. Make a rectangular ROI covering vertical streak at the center of the FFT image, and then [Image > Clear > Outside] to convert peripheral signals to 0 (black). If Clear Outside produced white periphery, check [Edit > Options > Colors] to see if the background color is black). [Process > FFT > Invert FFT] to see the spatial-domain image after filtering.

Similar to this exercise, we could separate a spatial domain image to high-frequency part and low-frequency part to isolate two overlapping signals to each, such as shown with an example below.

Adding high-frequency (Fig. 1.54a) and low-frequency (Fig. 1.54b) stripe images results in an overlapped image of two frequencies (Fig. 1.54c). This image math could be done easily using [Process > Image Math]. It is not easy to isolate each of two original images from this overlapped image by spatial domain filtering, but it could be done in a pretty simple way if you FFT Fig. 1.54c image and use the frequency-domain image (Fig. 1.55a). FFT image could be separated to two images, one from the center (called "low-pass", Fig. 1.55b) and the other from peripheral (called "high-pass", Fig. 1.55c). [Process > FFT > Invert FFT] of each FFT image would result low-frequency stripe image (Fig. 1.56b) and high-frequency stripe

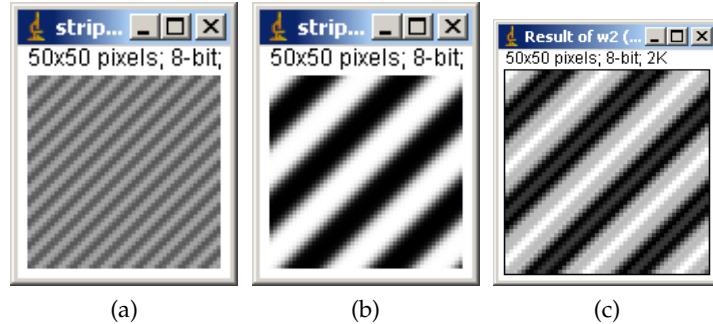


Figure 1.54: Images of (a) high frequency pattern, (b) low frequency pattern, (c) a and b combined.

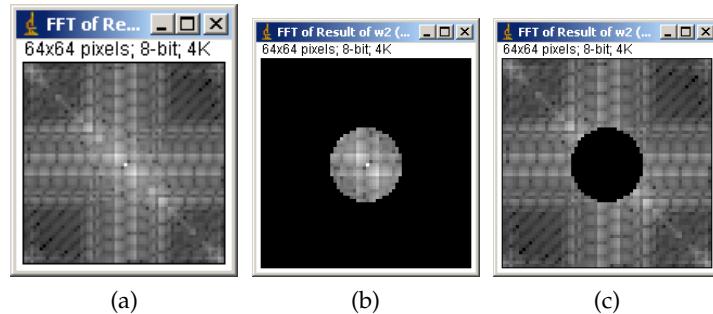


Figure 1.55: (a) FFT image (2D power spectrum) of Fig. 1.54c could be separated to (b) low frequency part near the origin and (c) high frequency part in the periphery.

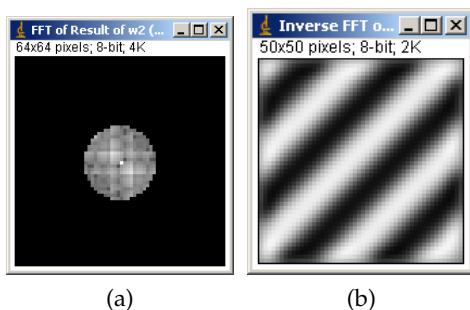


Figure 1.56: (a) Lower frequency part could then be invert-FFT to visualize (b) only the low-frequency pattern.

image (Fig. 1.57b).

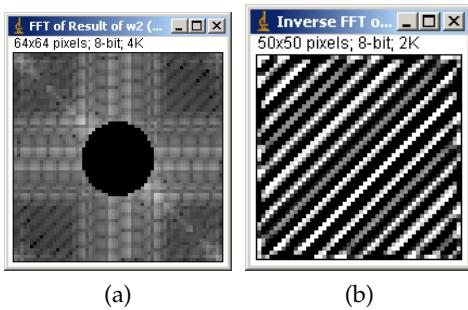


Figure 1.57: (a) Higher frequency part could then be invert-FFT to visualize (b) only the high-frequency pattern.

Above was an example of low-pass filtering (for isolating low frequency stripes) and high-pass filtering (for isolating high frequency signal). We could utilize more complex filters to isolated more specific frequency signals. Such filter is called *band pass filter*. Band pass filtering is available in [Process > FFT > Band Pass Filter...].

1.3.12 ASSIGNMENTS

Assignment 1-3-1: Convolution and Kernels

1. Design your own kernel, apply it to an image of your choice and discuss what it does.
2. Gaussian kernel: Open the Gaussian kernels (in sample image folder, Gss5x5.txt, Gss7x7.txt and Gss15x15.txt) by [File > Import > Text Image]. Then try getting the line profile of 2D Gaussian, crossing the peak of the curve. The line profile across the 2D Gaussian should be 1-D Gaussian curve. Save the resulting graphs as image files.
3. Visualize the Gaussian kernel using the "surface plot" [Analyze > Surface Plots...].

1.4 Segmentation

Segmentation refers to the process in which an image is subdivided into constituent regions or objects. These objects can be further processed or analyzed for the extraction of quantitative information. Biological image data is usually messy and noisy, and as a result difficult to segment properly. Multiple image processing steps are often required for segmentation of such. We often combine segmentation with various morphological processing and filtering techniques such as described in the previous section to achieve accurate and robust segmentation of an image. Image segmentation algorithms are generally based on one of two basic properties of intensity values: discontinuity and similarity. In the first category, the approach is to partition an image based on abrupt changes in intensity. Edge-detection algorithms we will study below is in this category. In the second approach, an image is partitioned into regions that are similar according to set of predefined criteria. Thresholding and watershed segmentation are in this category.

1.4.1 Thresholding

Many biological images comprise of light objects over a constant dark background (especially those obtained using fluorescence microscopy), in such a way that object and background pixels have gray levels grouped into two dominant modes. One obvious way to extract the objects from its background is to select a threshold T that separates these modes:

$$g(x,y) = \begin{cases} 1 & \text{if } f(x,y) > T \\ 0 & \text{otherwise} \end{cases} \quad (1.8)$$

where $g(x,y)$ is the thresholded image of $f(x,y)$.

In a sense, thresholding is an extreme case of contrast enhancement we studied already (1.2.4). By setting a threshold value T and converting the image, pixels with values above T becomes white and otherwise black (color could be in inverse).

Image thresholding operation turns the image into black and white image which is called *binary image*¹².

Exercise 1.4.1-1

Although there is a function specialized for the thresholding, we first try thresholding images using brightness / contrast function we used in the previous section. Open the image **2D_Gel.jpg**. Then [Image > Adjust > Brightness/Contrast]. In "B&C" contrast control window, set the "minimum" and "maximum" to a same value. You then should see the LUT curve is vertical, like shown in the figure below. Then change the brightness: this will change the x-position of the vertical LUT, and at the same time, you will see the ratio of black and white area changes. This corresponds to the changing of thresholding value.

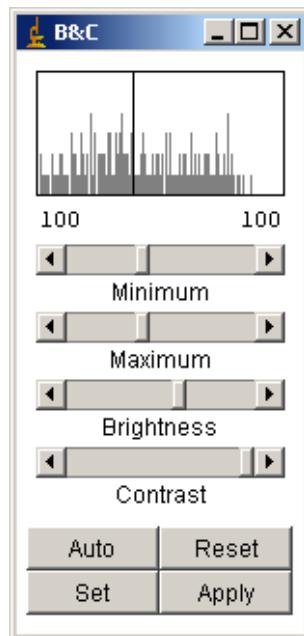


Figure 1.58: Thresholding by using Brightness/Contrast Control Dialog.

Threshold could be also done by setting two threshold values, lower limit and upper limit: which means that pixels with a certain range of values

¹²Some of [Process...] operations work only with binary images, so thresholding is a prerequisite for those filters

could be selected. This operation is sometimes called "density slice". We then have a new rule as follows, with lower threshold value T_1 and upper threshold value T_2 .

$$g(x,y) = \begin{cases} 1 & \text{if } T_2 > f(x,y) > T_1 \\ 0 & \text{otherwise} \end{cases} \quad (1.9)$$

Exercise 1.4.1-2

Open the image **2D_Gel.jpg** (or revert the image to the original by [File > Revert] if you still have the image used in the previous exercise). Then do [Image > Adjust > Threshold...]. You will see that the Gel image is automatically thresholded. The area highlighted in red is where pixels with value lower than 0 and higher than 149. In the "Threshold" window, the range of values that is highlighted is shown by red rectangular frame over the histogram. Try changing the upper and lower threshold value using the sliding bar below and study the effects on highlighted area in image.

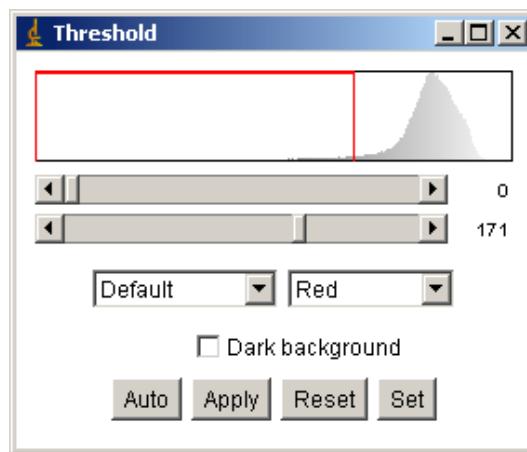


Figure 1.59: Thresholding Dialog.

Set button in the threshold window enables to you input the lower and upper threshold numerically. The original image file is not altered until you push the button **Apply** at the bottom of the threshold window. Click **Apply**, and check the result of conversion by saving it

as a text file, or simply by checking the pixel values using the value indicator in the status bar.

Instead of manually setting the threshold level, many algorithms for automatically setting the threshold level exist. `Auto` button in the threshold window is one of these automatic thresholding algorithms. Various algorithms for automatic determination of threshold value are available (such as Otsu, Maximum Entropy and so on) and you could choose one of them by drop-down menu on the left side. Following is a list of available Algorithms:

- IsoData
- Maximum Entropy
- Otsu
- Mixture Modeling
- Huang
- Intermodes
- Li
- Mean
- MinError
- Minimum
- Moments
- Percentile
- RenyiEntropy
- Shanbhag
- Triangle
- Yen

For choosing an algorithm, one way is to look for original papers describing the algorithm and think which one would work best for your purpose, but in practice, you could try one by one and just choose one that fits your demand.

In batch processing, command for automatic threshold would be like this:

```
setAutoThreshold("Huang dark");
```

The first argument is the name of the algorithm and could be any of the ones listed above.

1.4.2 Edge Detection

Edge Detection technique explained in [1.3.2 Find Edge](#) is useful for segmenting edge of object. We take an example of microtubule filament image.

Exercise 1.4.2-1

Open image microtubule.tif. We expect that the values will exceed 255 (8-bit) so convert the image to 32-bit by [Image > Type > 32-bit].

To apply Sobel kernel in x and y direction, duplicate the image by [Image Duplicate...]. To the original image apply convolution in x-axis. Do [Process > Filter > Convolve] and input the following kernel:

$$\begin{matrix} 1 & 0 & -1 \\ 2 & 0 & -2 \\ 1 & 0 & -1 \end{matrix}$$

Be sure to make space between elements. Then to the duplicated image, apply convolution with the following kernel:

$$\begin{matrix} 1 & 2 & 1 \\ 0 & 0 & 0 \\ -1 & -2 & -1 \end{matrix}$$

Now we must get the root square of the sum of squared of each image, which is

$$\text{Result} = (\text{microtubule.tif}^2 + \text{microtubule-1.tif}^2)^{0.5}$$

For this calculation, one could use ImageMath function in ImageJ or ImageExpression Parser in Fiji. Depending on your setup, please do one of the following two ways.

ImageJ, by ImageMath

Each image can be squared by [Process > Math > Square].

Then to squared images, do [Process > Image Calculator] for the addition of two squared images. This command pops up a window like below.

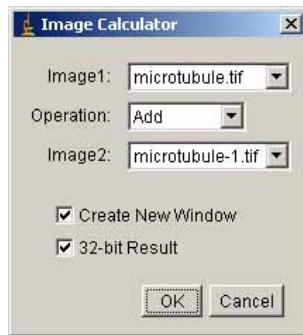


Figure 1.60: Image Calculation

From the pull down menu, select **microtubule.tif** and **microtubule-1.tif**. Don't forget to check **Create new window** and **32-bit results**. Then click **OK**. There will be a new window. To this new image titled **result of microtubule** do [Process > Math > Square Root] for the final calculation. You probably would see only black frame, this is because 32-bit image is not properly scaled and you need to adjust LUT. To do so, do [Image Adjust Brightness and Contrast] and in the Brightness & Contrast window, click **Auto** to auto-scale the LUT.

Fiji, by Image Expression Parser

To do complex calculation between two images, Image Expression parser could be used. Select [Process > Image Expression Parser]. Input following in the Expression field:

$\text{sqrt}(A^2+B^2)$

Then Choose **microtubule.tif** for "A" and **microtubule-1.tif** (duplicate) for "B". B might not be shown at the start up. To show B selection field, click "..." button at bottom-left corner.

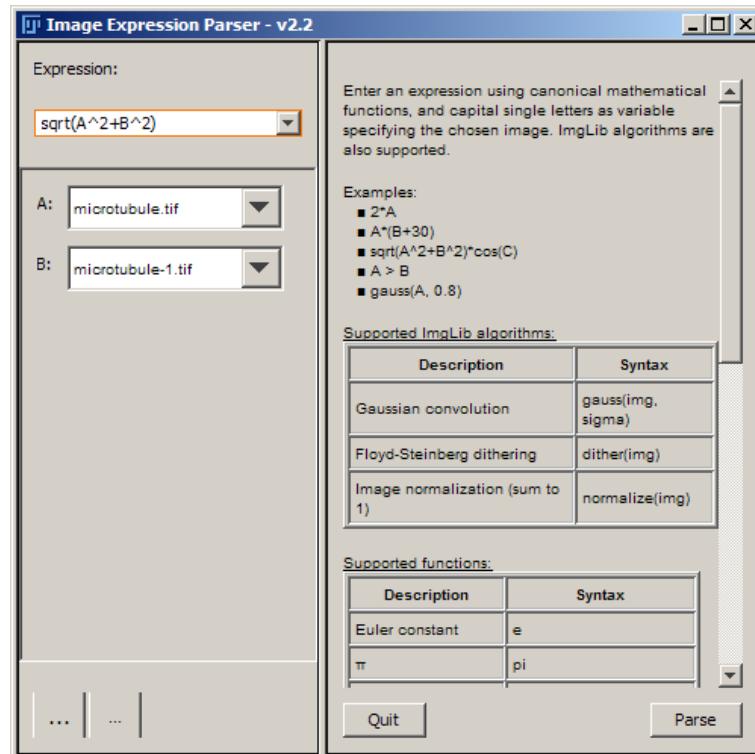


Figure 1.61: Image Expression Parser (Fiji)

For comparing the original and the edge detected images, go back to the image **microtubule** and do [File > Revert]. Then with "Result of Microtubule" do [Image > Type > 8-bit] to scale down the bit-depth. Merge the images by [Image > Color > RGB Merge...]. In the following dialog window, choose appropriate color for each image such as shown below.

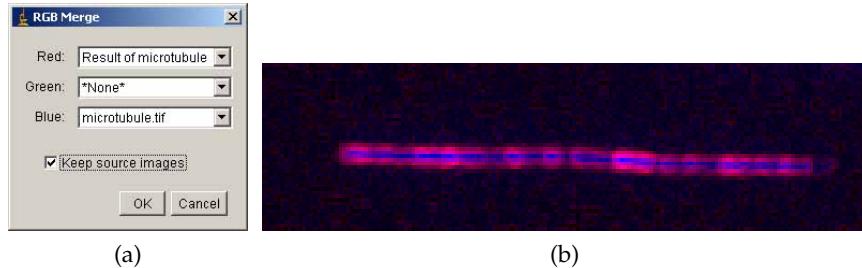


Figure 1.62: RGB merge of detected edge over the original image. (a) Dialog for assigning channels. (b) Example of merged color image, with original image in blue and edge-detected image in red

1.4.3 Morphological Watershed

In the previous sections, we discussed segmentation based on thresholding and edge detection. Morphological watersheds provide another way to segmentation of objects. One advantage of watersheds is that it produces continuous segmentation boundaries and often tends to produce stable segmentation results. In ImageJ, watershed algorithm could be found at [Process > Binary > Watershed].

To understand the watershed transform we view a grayscale image as a topological surface, where the values of $f(x,y)$ correspond to heights:

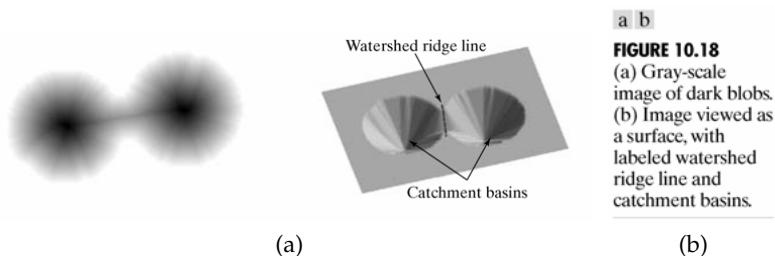


Figure 1.63: Watershed Principle

Consider the topographic surface on the right. Water would collect in one of the two catchment basins. Water falling on the watershed ridge line separating the two basins would be equally likely to collect into either of the two catchment basins. Watershed algorithms then find the catchment basins and the ridge lines in an image (Fig. 1.63, 1.65).

The algorithm works as follows: Suppose a hole is punched at each regional local minimum and the entire topography is flooded from below by letting the water rise through the holes at a uniform rate. Pixels below the water level at a given time are marked as flooded. When we raise the water level incrementally, the flooded regions will grow in size. Eventually, the water will rise to a level where two flooded regions from separate catchment basins will merge. When this occurs, the algorithm constructs a one-pixel thick dam that separates the two regions. The flooding continues until the entire image is segmented into separate catchment basins divided by watershed ridge lines.

Exercise 1.4.3-1

Open binary image **Circles.tif**. You see two circles fused. Such situation often occurs with cells or organelle, and you might want to simply separate them as different objects.



Figure 1.64: Circles.tif

Do [Process > Binary > Watershed]. You now see that two circles are separated at the constricted part of the fused circles.

If you want to know a bit more details, read the following quote how ImageJ does this.

Watershed segmentation is a way of automatically separating or cutting apart particles that touch. It first calculates the Euclidean distance map (EDM) and finds the ultimate eroded points (UEPs). It then dilates each of the UEPs (the peaks or local maxima of the EDM) as far as possible - either until the edge of the particle is reached, or the edge of the region of another (growing) UEP. Watershed segmentation works best for smooth convex objects that don't overlap too much. (quote from ImageJ

web site)

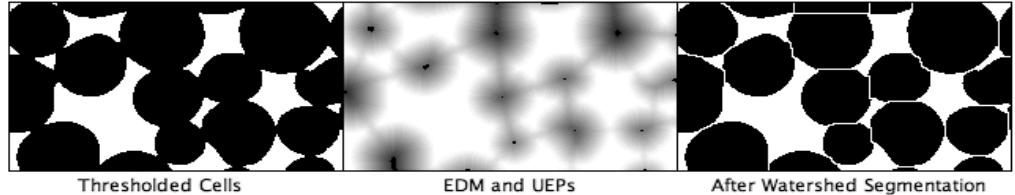


Figure 1.65: Watershed Segmentation

ImageJ first computes the distance transform on binary image. The distance transform of a binary image is the distance from every pixel to the nearest nonzero-valued pixel, as example in Fig. 1.66 shows.

1	1	0	0	0	0.00	0.00	1.00	2.00	3.00
1	1	0	0	0	0.00	0.00	1.00	2.00	3.00
0	0	0	0	0	1.00	1.00	1.41	2.00	2.24
0	0	0	0	0	1.41	1.00	1.00	1.00	1.41
0	1	1	1	0	1.00	0.00	0.00	0.00	1.00

Figure 1.66: A small binary image (left) and its distance transform (right)

1.4.4 Particle Analysis

After the segmentation, objects could be analyzed for extracting various parameters. Powerful function in ImageJ for this purpose is particle analysis function. It counts the number of objects (particles) in the image, extract morphological parameters, measures intensity...and so on. For details on extractable parameters, refer to Appendix 1.6.4 of this textbook. Here, we take an example to learn the basic use of this function.

Exercise 1.4.4-1

Single Particle Detection Load **Circles.tif** and segment two circles by watershed operation as we did in the previous section. Then select Wand Tool  from the tool bar. Click one of the circles. Check that a ROI is automatically created at the edge of the circle.

Automatic detection is done by pixel similarity principle. In the above case using the wand tool, computer searches for pixels in the surrounding of the clicked pixel for similarity in the pixel value. Similar pixels will be labeled. Then in the next round, computer searches for each of the labeled pixel for similar surrounding. This continues until searching hits the boundary. Particle analysis uses the same strategy, but there will be specific labeling for each particle.

Exercise 1.4.4-2

Multiple Particle Analysis: Open **rice.tif**. Before applying particle analysis, target particles must be segmented by image threshold. Try thresholding the image... What you would find out immediately is that there is shading in the image that rice can not be uniformly selected by thresholded. For this reason, do the background subtraction as we already did in [1.3.5](#). We then work with the subtracted image in the following.

Set Threshold again, and then click "Apply" in the Threshold dialog window. The image then should be black and white. This binarized (segmented) image will be used as a reference for setting boundary of each rice particle.

Open file **rice.tif** again. Since there is already a window with same title, this second one should have a title "rice-1.tif". By having this original image, boundary that will be set using binarized image will be "redirected" when measurement is actually taking place¹³.

Before doing particle analysis, you must specify what you want to measure for each particle through [Analysis > Set Measurements].

¹³It is also possible to do the measurement without redirecting: for example, you could simply image-threshold, and while the image is highlighted with red, (do not click Apply button) and do "Analyze particle". This will then detect particles which are highlighted.

Select Area, Circularity, Centroid and Perimeter (details on these parameters are written in Appendix 1.6.4). In addition, set “redirect to” to the original image (rice-1.tif) so that the measurement will be done with the original image and not with the thresholded image.

Activate the threshold the image, and then do [Analyze Analyze Particles...]. A dialog window pops up. Input following parameters:

- Size: "0-200" size above 200 pixel area will be excluded from the measurement
- Circularity: use default "0-1.0"
- Show: Outline
- Checkbox:
 - Check "Display Results" to display the result table.
 - Check "Clear Results" to refresh the measurement recordings.
 - Check "Exclude on Edges" to exclude particles touching the edge of the image.

Then Click "OK". Two windows pop up. One is the outline image of the measured particles, with number labels. These numbers correspond to the numbers you see at the left most column in the result window, which also popped up. Examine the outline image. At the bottom, there seems to be some particles which are counted but only part of the particles are included.

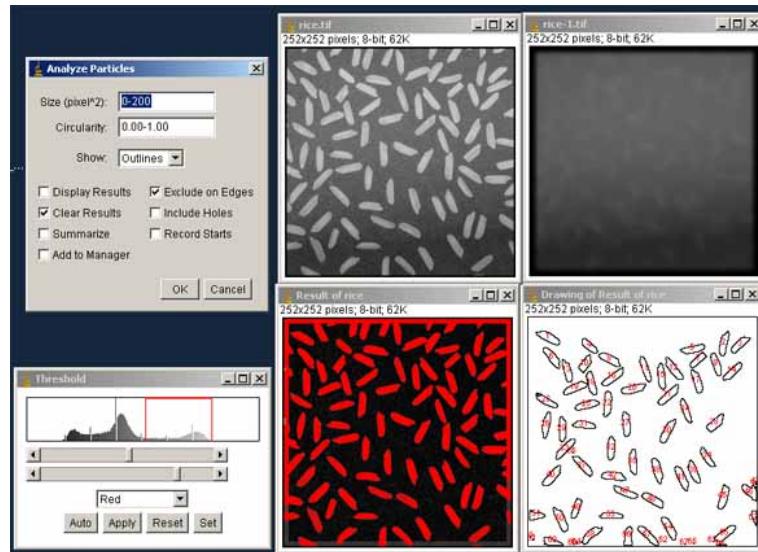


Figure 1.67: Multiple particle Analysis

QUESTION: How can we eliminate edge-touching particles from the analysis? Which parameter in the "Analyze Particle" dialog should we change? Try again with different parameter values.

1.4.5 Machine Learning: Trainable Segmentation

Segmentation could also be done by letting computer to learn what you think as signal and background. To do so, one could use machine learning technology with your instruction. We use **Trainable Segmentation plugin**. In brief, you mark what you decide as signal and background, and then let the algorithm to "Train" the classifier of the image. Classifier is a flexible virtual machine that studies your markings and it comes up with a protocol to categorize each pixel into classes. When you start the plugin, you will see a window with image you want to segment, and buttons in the surrounding. Panel in left side is for commands, and the panel in right side is for assigning your markings either signal or background. There are only two classes on start up but you could add more classes (e.g. signal 1, signal 2, background) by "create new class" in the left panel (Fig. 1.68).

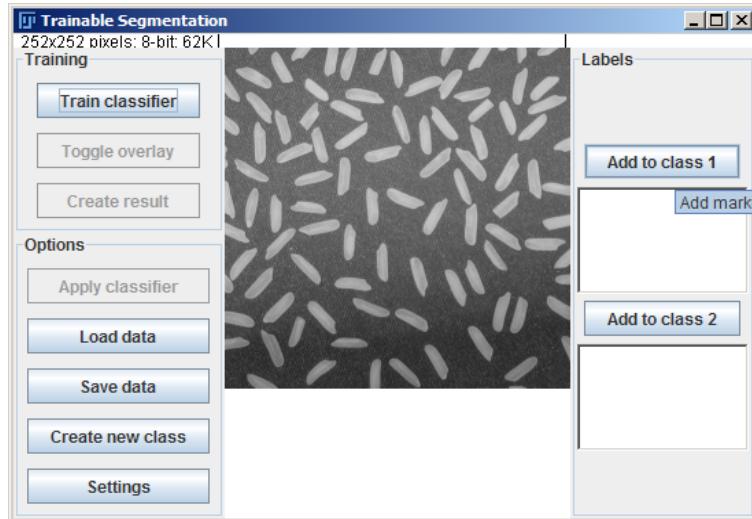


Figure 1.68: Trainable Segmentation Window

Exercise 1.4.5-1

(This exercise is only available with Fiji) Open **rice.tif** and then select [Plugins > Segmentation > Trainable Segmentation]. Your task here is to segment rice grains using trainable segmentation. Zoom up the image using magnifying tool as usual image. Then choose free hand ROI tool, start marking a rice grain. If you are satisfied with ROI, then click **Add to Class1**. Then again using freehand ROI tool, mark background. Then add this another class by **Add to Class2**. Your trainable segmentation window should look something like Fig. 1.69.

Click **Train Classifier** in the left panel, then calculation starts that takes for a while. When calculation finishes, you will see that most of rice overlaid red and background in green (Fig. 1.70). Zoom out the image and check again. You might see that some of rice grains are not segmented well – so we should train more. Use freehand ROI tool to mark that was unfortunately categorized as background, and add it to class 1 (**Add to Class 1**). Then **Train classifier**, check the segmentation results... You could iterate such marking and training until you get a satisfactory segmentation result. TIP: You could delete

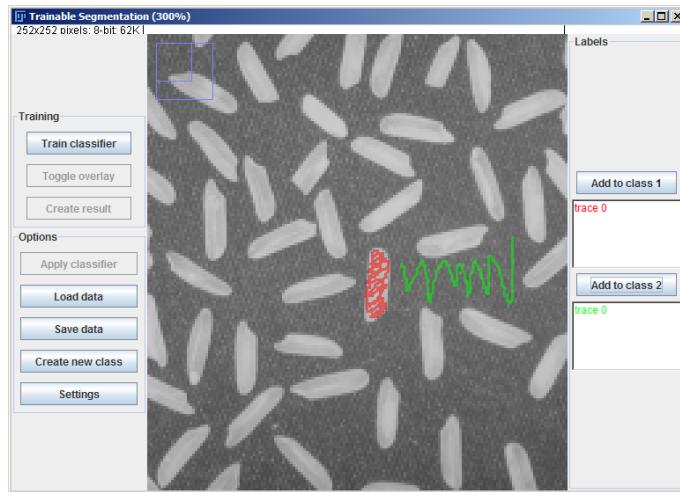


Figure 1.69: Marking signal and background

specific trace already listed in Class listing by double clicking.

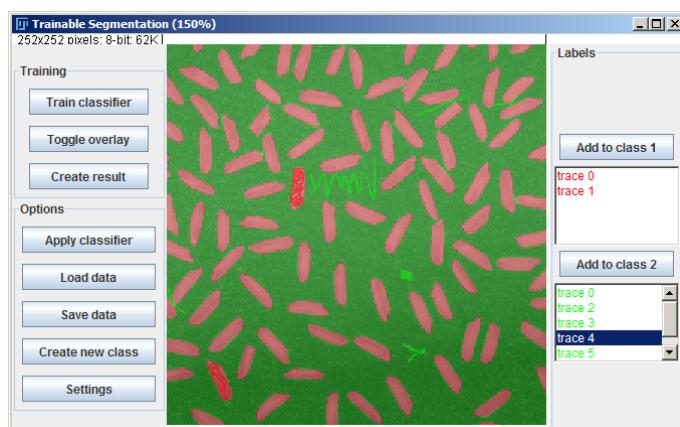


Figure 1.70: After first round of trainable segmentation.

To create segmented image, click `Create result`. A separate window with segmented image will open (Fig. 1.71).

To save the training data you did, click `save data`. Saved *.arff file could be loaded later to segment another image (should be rice in this case, of course!) using `Load data` button.

OPTIONAL : Segmented image could be used as a mask to ana-

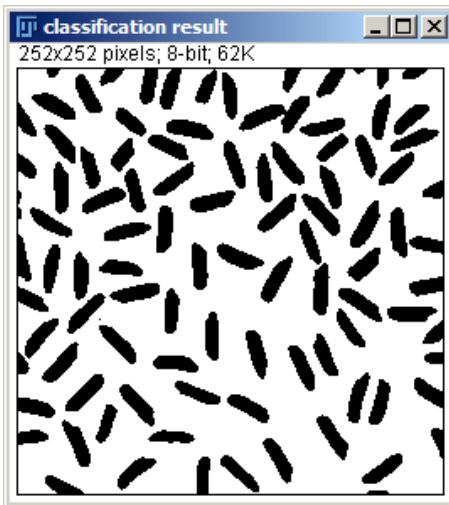


Figure 1.71: Segmentation Result of Trainable Segmentation Plugin

lyze original image. To do so, open both the original **rice.tif** image and binary image (segmented rice.tif image). Then [Analyze > Set Measurement], and set redirect to drop down menu to original image (rice.tif). Then activate the segmented image window, threshold the image (but do not "apply"), and do particle analysis. Detection of particles are done in the segmented image, and measurements will be redirected to corresponding particle areas in the original image.

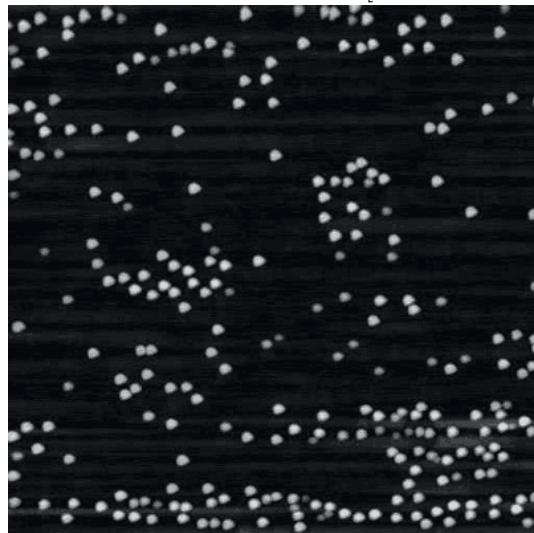
1.4.6 ASSIGNMENTS

Assignment 1-4-1 Quantum dots.

Devise a segmentation strategy to segment the following image of quantum dots (quantumdots.tif). Extract the following parameters from the image:

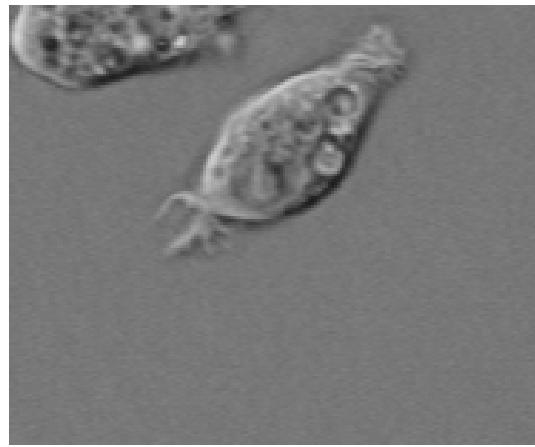
1. number of dots
2. histogram of areas
3. histogram of integrated densities

You can draw histogram of the results in the "Results" window by using a function associated with the Result window [Edit > Distribution...].



Assignment 1-4-2 DIC images of cells.

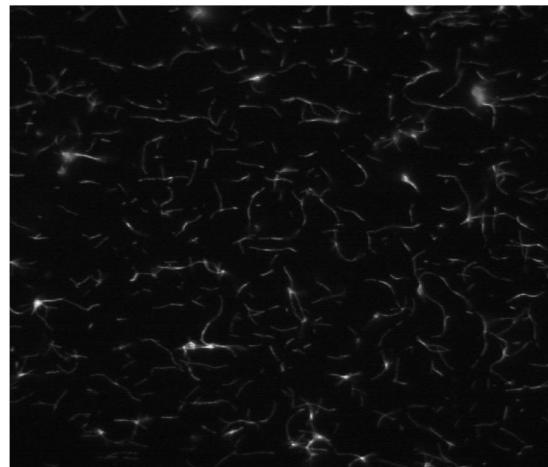
Find the boundary of this cell (dic_cell.tif) using any of your favorite image segmentation techniques.

**Assignment 1-4-3 Actin filaments.**

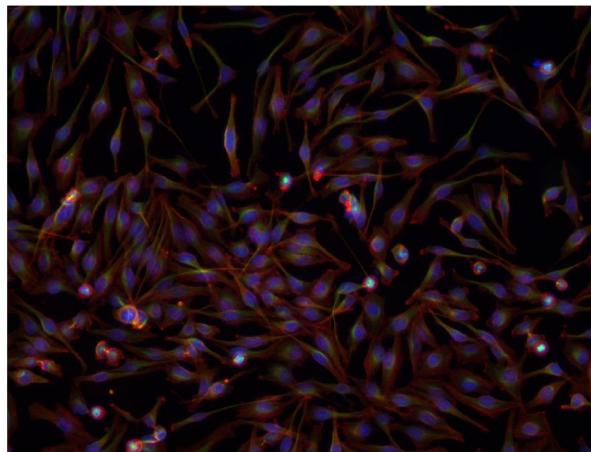
Devise an image processing strategy to obtain the distribution of filaments from this image (actin.tif), and subsequently calculate

1. the mean filament length
2. the variance in filament length
3. the number of filaments.

Note: image segmentation may be complicated by the low light levels in the image.

**Assignment 1-4-4 Fixed cells.**

Here are some cells that are fixed and stained for actin (red), tubulin (green) DNA (blue), and a histone marker (not shown, 4color_cells). Devise an image processing strategy to segment the cells. You may operate on any of the color channels in the image (or a multiple of them). This problem is especially tricky because many of the cells are touching.



1.5 Analysis of Time Series

In this section, we learn how to analyze dynamics using image sequences. A time series of digital images, usually called "stack", contains temporal dynamics of position and intensity, and kinetics can be obtained from these data. In general there are three types of dynamics.

1. Position does not change but intensity changes over time.
2. Position changes but the intensity does not change.
3. Both Position and Intensity change over time.

An example of type (1) is the measurement of cargo transport dynamics in vesicle trafficking ([Hirschberg et al., 1998](#)). Transition of protein localization from ER to Golgi then to the plasma membrane was measured over time by measuring the signal intensity in each statically positioned compartment. This type of technique has evolved to various sophisticated methods based on the same principle -measurement of signal intensity at a constant position. Type (2) corresponds to the measurement of movement, or object tracking, and an example is the single particle tracking of membrane surface proteins ([Murase et al., 2004](#)). An example of type (3) measurement is the measurement of chemotaxis-related protein accumulation dynamics during the *Dictyostelium* cell migration ([Dormann et al., 2002](#)). Analysis of type (3) dynamics is more specific and advanced, so refer to other literature ([Miura, 2005](#)).

1.5.1 Importing Image Sequence

Image sequences captured through microscopes in most cases consists of multiple of single image frames with numbering (e.g. image0001.tif, image0002.tif, image0003.tif...). By importing such files, we can create a window called "stack". Image stacks are said to be "3D" because there are three dimensions, x and y axes, and either z axis or time (in the following, we abbreviate the former as "x-y-z" and the latter as "x-y-t").

Stacks could be saved as a single file. The file header will contain the information on number of frames that the image contains. This number will be

automatically detected when the stack is opened next time, and reproduces the stack again. When the sequence is a time series of three-dimensional stacks, the dimension size of the file is four, and there are two different ways on how the files are stacked: (1) xy – z – t or (2) xy – t – z. The order we see common is (1) but in some cases you might find out that the order is in (2). If you need to change the order from one to the other, you could use "HyperVolume Shuffler" plugin to change the order. In dimensions above three, there no information is kept within the file about how the file is ordered. One must know it by keeping a note separately, or decide the order by going through the sequence.

HyperVolumeShuffler Plugin

<http://rsb.info.nih.gov/ij/plugins/hypervolume-shuffler.html>

Dimension could be even higher. When there are multiple channels with 4D image, say channel 1 (ch1) and 2 (ch2) each for different labels within a same cell, then the file is 5D. There could be many possibilities in the order of the file but this could also be sorted using the HyperVolumeShuffler PlugIn.

Exercise 1.5.1-1

Import multiple files as an image stack. [File > Import > Image Sequence...] will open a dialog window and you must specify the first file of the image series. Select sample sequence **eg5_spindle_50000.tif...** Then another dialog window pops up (Fig. 1.72).

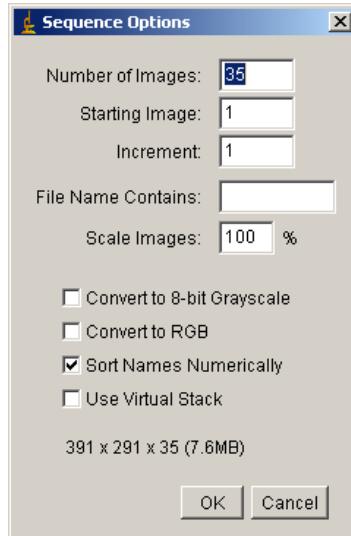


Figure 1.72: Importing multiple frames as a Stack

ImageJ automatically detects the number of files in the folder, and then another window opens to ask for the number of images you want to import, which number you want to start with, increment between the numbering of the file, and also part of the name that is unchanged through out all the files you want to import. There are other options such as scaling and conversion of the image but-depth, but these operations could be also done afterwards. The imported image sequence is within one window, or a stack.

Don't close the stack, exercise continues.

Exercise 1.5.1-2

In the ImageJ tool bar, there is an icon » at the right most position. Click and from drop down menu, select "Stack tools". Video player-like interface appears in the tool bar (1.73). Try different buttons. In addition to playback icon button, [Image > Stacks > Start Animation] will also play back the movie. Try changing the playback speed by [Image > Stack > Animation options] (Fig. 1.74).

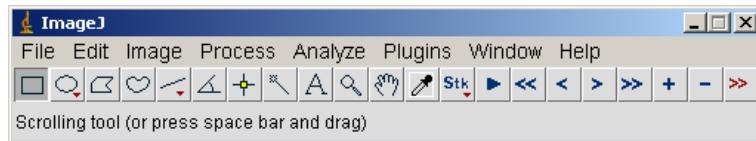


Figure 1.73: ImageJ tool bar in Stack Tool mode.



Figure 1.74: Animation Option Window

1.5.2 Difference Images

In many cases, mathematical manipulation on image sequences is effective in visualizing dynamics. "Difference images" (also called "subtraction images") is one of such techniques. A difference image of successive frames in a sequence is given by:

$$D_j(x, y) = I_{j+1}(x, y) - I_j(x, y) \quad (1.10)$$

where D is the difference image, and j is a given plane in the stack. Difference images highlight features of the image that change rapidly over time, much in the same way that a spatial gradient enhances contrast around an edge. The change is usually brought about by movement of an object in the image, or by a kinetic process that has not reached steady-state (like photobleaching).

Exercise 1.5.2-1

Open image stack **1703-2(3s-20s).stk**. Then duplicate the stack [Edit > Duplicate]. Don't forget checking the "Duplicate entire stack".

Go back to the original stack, delete the last frame (frame 31) [`Image > Stacks > Delete Slice`]. Alternatively, you could simply click "-" button in the stack tools at the last frame. Activate the duplicated stack and delete the first frame. Then do subtraction [`Process > Image > Calculator`]:

$$\text{OriginalStack} - \text{DuplicatedStack}$$

This will then subtract frame 1 – frame2, frame2 – frame 3 ... and so on. Do you see Frapped Region in the difference image stack?

1.5.3 Projections

A maximum intensity projection of an entire image stack is given by

$$M(x, y) = \max_j(I_j(x, y)) \quad (1.11)$$

The maximum on the right hand side is the maximum in intensity value at a given pixel position over all stacks in an image. Maximum projections collapse the entire dynamics of the stack onto a single plane. This function is especially useful for visualizing entire trajectories of moving particles on a single plane.

Exercise 1.5.3-1

Open image `listeriacells.stk`. Then do all types of projections you could choose by [`Image > Stack > Z projection...`].

Question: Which is the best method for leaving the bacteria tracks?

1.5.4 Measurement of Intensity dynamics

Temporal changes in fluorescence level matters a lot in biological experiments since the change is directly related to the molecular mobility and production. Here, we study how to obtain the intensity dynamics out of image sequences.

Exercise 1.5.4-1

Load image stack **1703-2(3s-20s).stk**. This is a sequence of FRAP experiment. Draw a ROI (could be any closed ROI) surrounding the area where the photobleaching takes place. Then do [`Image > Stacks > Plot z-axis Profile`].

There will be two windows: "Results" window and a graph of intensity dynamics.

Try adding Second ROI to measure the background, simply by making another ROI. So the intensity measurement again with this new ROI.

OPTIONAL: Numerical values in the table can be copy and pasted in spread sheet software like Excel or OpenOffice Calc. If you know how to use R, then you could save the results table as a CSV file and read it from R. Try draw a graph in your favorite plotting software.

When you measure the fluorescence level, it is very important to measure the background intensity and subtract the value from the measured fluorescence. This is because the baseline level adds offset to the measured value, so you could not quantify the true value.

1.5.5 Measurement of Movement Dynamics

Movement is an essential component of biological system. To quantify the movement dynamics, various methods has been developed. In case of image sequences, particle tracking is a popular way to quantify movement ([1.5.7](#), [1.5.8](#)). In any tracking methods, the ultimate goal is to obtain the position coordinate of a target object in each image frame, so that the movement of the target object can be represented as changes in the position coordinate. Velocity and movement direction can then be calculated from the resulting coordinate series. Since tracking deals with segmentation and position linking, the process is rather complex. Besides tracking, there is a easier way to represent and quantify movements occurring in sequences. The technique is called "kymographs ([1.5.6](#): see below)". This method loses positional information but for measuring velocity, the method is easy and fast.

The process of tracking has two major steps. In the first step the object must be segmented. This then enables us to calculate the coordinate of the object

position, such as its centroid. There are various ways to do segmentation (see 1.4). In the second step the successive positions of the object must be linked to obtain a series of coordinates of that object. We call this process "position linking". Position linking becomes difficult when there are too many similar objects. In this case it would become impossible to identify the object in the next time point among multiple candidates. Position linking also becomes difficult when the successive positions of the object are too far apart. This happens when the time interval between frames is too long. In this case, multiple candidates may appear in the next time point even though similar objects are only sparsely present in the image frame. If the target object is single and unique, linking of coordinates to successive time points has none of these problems.

1.5.6 Kymographs

Kymographs are a two-dimensional time traces, where time t is in Y axis and space along a one-dimensional contour is in X, and the dynamical variable $F(x,t)$ is visualized as an image. Kymographs provide a fast and convenient way to visualize motion and dynamics in microscopy images.

Exercise 1.5.6-1

We try measuring the speed of tubulin flux using Kymographs. For doing this exercise, you need "Slice Remover" PlugIn¹⁴.

- (1) Load the image stack of a spindle labeled with speckle amounts of tubulin. Name of the file is **control_1.stk**. Observe the movie. Note that the tubulin speckles flux towards the both spindle poles. One way to measure the rate of flux is to create a kymograph along a straight line that runs from one spindle pole to the other.
- (2) Remove initial 28 slices by [plugins > course > Slice Removal] (Fig. 1.75).

¹⁴To install the plugin refer to 1.6.2. If you are using Fiji, Slice Remover is already installed. Access the command by [Image > Stack > Manipulation > Slice Remover]



Figure 1.75: Slice Removal dialog

(3) Contrast enhance, and then convert to 8-bit. Then do maximum intensity Z-projection [Image > Stacks > Z-projection].

Choose segmented line ROI tool (should right click to choose). Then trace one of the tracks in the projection image (Fig. 1.76).



Figure 1.76: Tracing Projected Image. Note Small Yellow Segmented ROI in the middle of the image.

Go back to the stack, [Edit > Selection > Restore Selection]. Then [Image > Stacks > Reslice...]. Don't change parameters in the dialog window, simply OK. In the kymograph you just now generated, use straight line ROI tool to make a selection along diagonal bright signal (Fig. 1.77).

Install macro "K_read_kymoLineROI.txt" by [PlugIns > Macros > Install...]

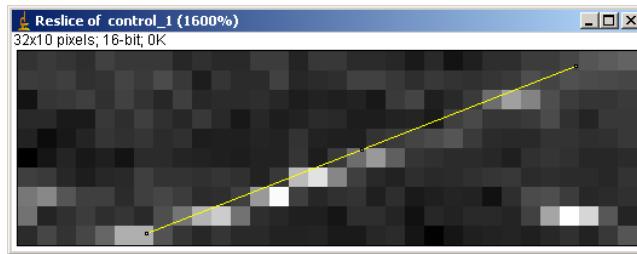


Figure 1.77: Measurement of Kymograph

and then select the macro file in file chooser, and click OK. Do [PlugIns > Macros > Show Line Coordinates and Speed]. Results will appear in the Log window.

Note: Jens Rietdorf and Arne Seitz made a Kymograph plug-in for ImageJ. It enables multiple ROI selections, to make your life easier.

<http://www.embl.de/eamnet/html/kymograph.html>

1.5.7 Manual Tracking

In the simplest case, tracking can be done manually. The user can read out the coordinate position of the target object directly from the imaging software. In ImageJ, user can read out position coordinate indicated in the status bar by placing the cross-hair pointer over the object. Then coordinates can be listed in standard spreadsheet software such as Microsoft Excel for further analysis.

An ImageJ plug-in is also freely available to assist such simple way of tracking. An obvious disadvantage of the manual tracking is that the mouse-clicking by the user could be erroneous, as we are still human who gets tired after thousands of clicking. For such errors, measurement errors can be estimated by tracking the same object several times and this error could then be indicated together with the results. Otherwise, automated tracking is more objective, but if you have only limited number of tracks to analyze, manual tracking is a best choice before start to explore complex parameter space of automated tracking setting.

The manual tracking can be assisted by an ImageJ plug-in "Manual Tracking". This plug-in enables the user to record x-y coordinates of the posi-

tion where the user clicked using mouse in each frame within a stack. The download site has a detailed instruction on how to use.

<http://rsb.info.nih.gov/ij/plugins/track/track.html>

Exercise 1.5.7-1

Manual Tracking: In a spindle, microtubules attach to chromosomes through structures called kinetochores. In the stack **kin.stk**, kinetochores are labeled with a fluorescent marker. Your task now is to track the movement of individual kinetochores using the ManualTracker PlugIn. Enhance contrast and convert the image stack from 16-bit to 8-bit (just to decrease the memory load. If your computer is powerful enough, you don't need to downgrade the stack). Then do [Plugins > Course > Manual Tracking]: a window pops up (Fig. 1.78).

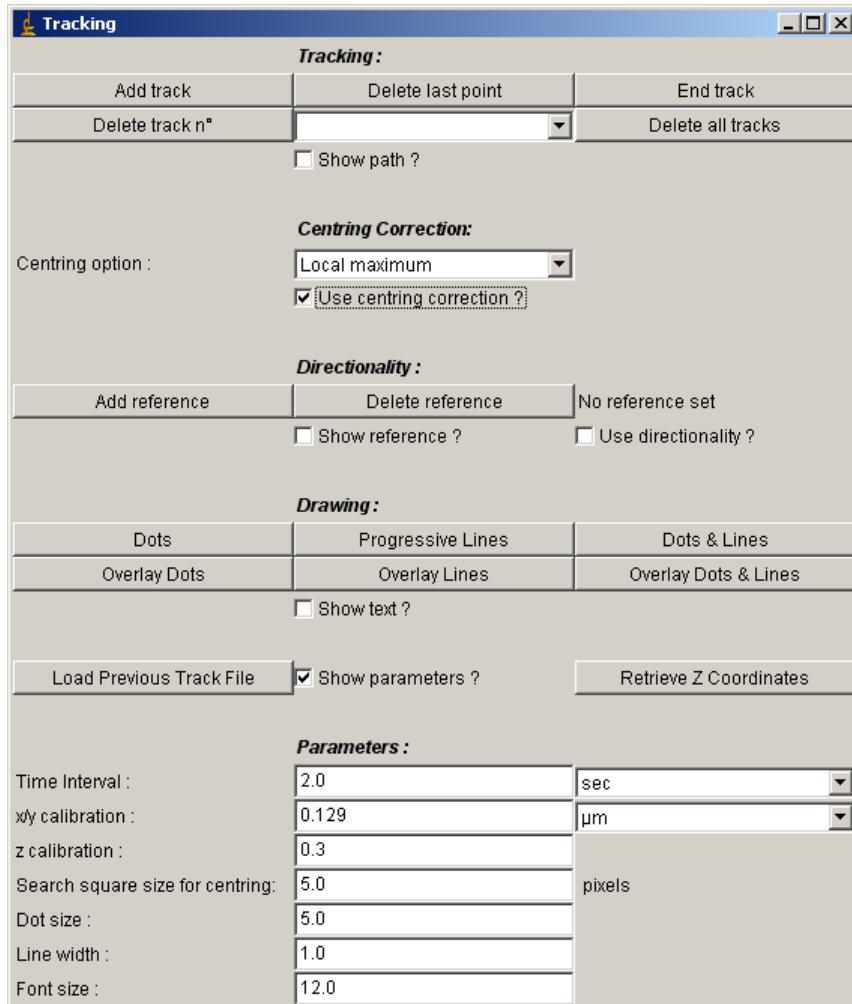


Figure 1.78: Manual Tracker Interface

Then

1. Check "centering correction", use Local Maximum.
2. Start manual tracking by clicking "Add track".
3. End tracking by "End Track"
4. Show tracks by "Drawing" Functions"

You could track different particles by repeating steps between 2 and 3. Results window will list the measured positions for particles (Fig. 1.79a), and step 4 will show a track overlay image stack (Fig. 1.79b).

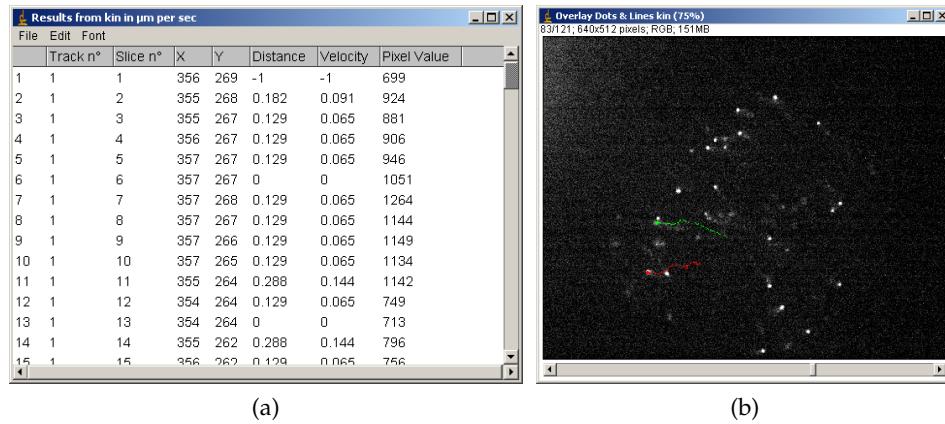


Figure 1.79: Manual Tracking Results (a) Results table and (b) Track Overlay view.

Tracked data can be saved by activating "Result" window and [File > Save as...]. **OPTIONAL:** Copy and paste the result table and plot the track in Excel.

1.5.8 Automatic Tracking

Automatic tracking reduces the work loads of the researcher, enabling them to deal with a huge amount of data¹⁵. Statistical treatments can then be more reliable. In addition, the results can be regarded more objective than manual tracking. Automatic tracking is an optional function that can be added onto some imaging software including ImageJ. However, these readily available functions are not adaptable for all analyses since the characteristics of target objects in biological research vary greatly. Especially when the target object changes shape over time, further difficulty arises. We try an automatic tracking plugin in this section, but keep in mind that the algorithm for automated tracking has large variety so that knowing the algorithm well and examination of whether a certain algorithm matches to your object is inevitable for successful tracking.

In the following, I will list some of the standard methods for the automatic segmentation of objects, which is the first part of the automated tracking. Detected objects are then linked from a frame to the other.

¹⁵Texts of this section is mostly from ([Miura and Rietdorf, 2006](#)).

CentroidMethod

The centroid is the average of all pixel coordinates inside the segmented object and is the most commonly used feature for representing the object position. The centroid coordinate $p_c(x, y)$ can be calculated as

$$p_c(x, y) = \left(\frac{\sum x_i}{n_x}, \frac{\sum y_i}{n_y} \right) |_{x, y \in \mathfrak{R}} \quad (1.12)$$

where \mathfrak{R} is the region surrounded by the contour, and n is the total number of pixels within that region. In other words, all x coordinates of the pixels inside the object are added and averaged. The same happens for all y coordinates. The derived averages for x and y coordinates represent the centroid coordinates of the object. The position of the object can be measured for every frame of the sequence manually. In ImageJ, centroid calculation is available in the "Particle Analysis" function.

Gaussian Fitting Method

For spherical signals such as fluorescence beads or fluorescently labeled sub-resolution particle, the signal intensity distribution can be fitted to a standard two-dimensional Gaussian curve ([Anderson et al., 1992](#); [Schütz et al., 1997](#); [Tardin et al., 2003](#)):

$$I(x, y) = z_0 + z_n \exp\left\{-\frac{(x - X_n)^2 + (y - Y_n)^2}{W_n^2}\right\} \quad (1.13)$$

where $I(x, y)$ is the intensity distribution of an image, z_0 is the background intensity, and z_n is the height of the peak. W_n is the width of the curve that peaks at (X_n, Y_n) . This peak position is the signal position. Although this fitting method is restricted to spherical or oval objects, it yields the most precise measurements even with a very low signal-to-noise ratio ([Cheezum et al., 2001](#)). Another advantage of the Gaussian fitting method is that the results are in sub-pixel resolution. Such high resolution can for example enable the analysis of molecular diffusion in the nm resolution. More discussion on the localization accuracy in the position measurements can be found in the literature ([Ober et al., 2004](#); [Martin et al., 2002](#); [Thompson et al.,](#)

2002).

Pattern Matching Method

In this method, a kernel containing a template pattern of the object is compared to different positions within the image in order to find a position with the highest similarity to the kernel. A cross-correlation function $C(x, y)$ is usually used to evaluate the resemblance of the template with different parts of the image, (Gilles et al. 1988):

$$C(x, y) = \sum_{i=0}^{n-1} \sum_{j=0}^{m-1} I(x+i)(y+j)\{K(i, j) - \bar{K}\} \quad (1.14)$$

where $I(x, y)$ is the intensity distribution of the image frame and $K(i, j)$ is a $n \times m$ pixels kernel that contains the template image pattern. \bar{K} is the mean intensity of the kernel. $C(x, y)$ will be maximal at the position where the pattern best matches. In actual application, the template pattern is sampled in the k -th image frame $I_k(x, y)$ by the user manually or by semi-automatic segmentation. Then the cross-correlation value $C(x, y)$ between this template kernel and the consecutive $k + 1$ -th image frame $I_{k+1}(x, y)$ can be calculated. [Gelles et al. \(1988\)](#) introduced the following formula for obtaining the peak position (x_c, y_c) :

$$x_c = \frac{\sum x\{C(x, y) - T\}}{\sum \{C(x, y) - T\}} \quad (1.15)$$

$$y_c = \frac{\sum y\{C(x, y) - T\}}{\sum \{C(x, y) - T\}} \quad (1.16)$$

where T is the threshold value and negative values are discarded. (x_c, y_c) corresponds to the centroid of the magnitude of correlation that is thresholded by T .

Since the cross-correlation function 1.14 tends to give higher values at brighter regions rather than at regions of similar shapes, a normalized form of cross-

correlation can also be used:

$$C_n(x, y) = \frac{\sum_{i=0}^{n-1} \sum_{j=0}^{m-1} [I(x+i)(y+j) - \bar{I}] \{K(i,j) - \bar{K}\}}{M_{I_{x,y}} \cdot M_k} \quad (1.17)$$

$$M_{I_{x,y}} = \sqrt{\sum_{i=0}^{n-1} \sum_{j=0}^{m-1} [I_{x+i,y+j}]^2} \quad (1.18)$$

$$M_k = \sqrt{\sum_{i=0}^{n-1} \sum_{j=0}^{m-1} [K_{i,j}]^2} \quad (1.19)$$

where \bar{I} is the mean intensity of a portion of the image overlapping the kernel, and $M_{I_{x,y}}$ and M_k are the root mean square values of the kernel and the corresponding portion of the image, respectively. The precision of the measurement is high ([Cheezum et al., 2001](#)), but object tracking will fail when the shape of object changes radically between frames.

The cross-correlation method has been used extensively in single particle tracking (SPT). SPT is a technique developed for measuring the mobility of membrane bound proteins and the movement of motor proteins with a nanometer precision ([Gelles et al., 1988](#); [Geerts et al., 1987](#); [Schnapp et al., 1988](#); [Sheetz et al., 1989](#)) and was recently reviewed ([Ritchie and Kusumi, 2003](#)). In these studies, a single protein was attached to a very small gold particle or labeled with a fluorophore and its movement was analyzed by video microscopy. Theoretical examinations showed that different modes of protein movement can be discriminated with nano-meter resolution by measuring the mean square displacement of the labeled proteins ([Qian et al., 1991](#)). Various types of membrane protein motions, such as immobile, directed, confined, tethered, normal diffusion and anomalous diffusion, were resolved revealing the kinetics of the membrane protein mobilities ([Kusumi et al., 1993](#); [Saxton, 1997](#)). An automatic tracking program for multiple proteins has been developed by [Ghosh and Webb \(1994\)](#) and was used for measuring the movement of actin patches in Yeast([Carlsson et al., 2002](#)).

ImageJ Tracking Plugins

Here is a list of object tracking plugins available in ImageJ (Nov. 2006).

- Particle Tracker

<http://www.mosaic.ethz.ch/Downloads/ParticleTracker>

Uses local maxima for estimating particle position, and custom criteria for discriminating non particles.

- MTrack2

<http://valelab.ucsf.edu/~nico/IJplugins/MTrack2.html>

- MTrackJ

<http://imagescience.bigr.nl//meijering/software/mtrackj/manual.html>

Initialization should be done manually.

- Spot Tracker

<http://bigwww.epfl.ch/sage/soft/spottracker/>

Exercise 1.5.8-1

Open image stack **TransportOfEndosomalVirus.tif**. We use "Particle Tracker" for experiencing the automatic tracking ([Sbalzarini and Koumoutsakos, 2005](#)). There is also a detailed tutorial available in the web site, "Particle Tracker Tutorials" also added to this textbook in the Appendix [1.6.10](#), so refer to it as well for the following exercise.

Set correct dimensions of the image by

[Image > Properties...]

...Image stacks are by default taken as a z-series and not t-series. Set Slices to 1, and Frames to appropriate size (number of frames).

Start the ParticleTracker plugin

Start the particleTracker by "[Plugins > Mosaic > ParticleTracker 2D/3D]".

[Study Dot Detection Parameter]

This tracking tool has two parts. First, all dots in each frame are detected, and then dots in successive frames are linked. The first task then is to determine three parameters for dot detection. There are three parameters.

Radius

Expected diameter of dot to be detected in pixels.

- **CutOff**

Cutoff level for the none-particle discrimination criteria, a value for each dot that is based on intensity moment order 0 and 2.

- **Percentile**

Larger the value, more particles with dark intensity will be detected. Corresponds to the area proportion below intensity histogram in the upper part of the histogram.

Try setting different numbers for these parameters and click "Preview Detected". Red circles appear in the image stack. You could change the frames using the slider below the button.

After some trials, set parameters to what you think is optimum.

Set Linking parameters

Two parameters for linking detected dots should be set.

Link Range

... could be more than 1, if you want to link dots that disappears and reappears. If not, set the value to 1.

- **Displacement**

... expected maximum distance that dots could move from one frame to the next. Unit is in pixels.

After parameters are set, click "OK". Tracking starts.

Inspect the Tracking Results

When tracking is done, a new window titled "Results" appears. At the bottom of the window, there are many buttons. Click "Visualize all trajectories", and then a duplicate of the image stack overlaid with trajectories will appear.

Select a region within the stack using rectangular ROI tool and then click "Focus on Area". This will create another image stack, with only

that region. Since this image is zoomed, you could carefully check if the tracking was successful or not.

If you think the tracking was not successful, then you should reset all the parameters and do the tracking again.

Export the tracking results

To analyze the results in other software, data should be saved as a file. To do so, first click "All Trajectories to Table". Results table will then be created. In this results table window, select [File > Save As...] and save the file on your desktop. By default, file type extension is ".xls", excel format, but change this to ".csv". CSV stands for "comma separated file", and this is more classic but general data format which you could easily import in many software such as R.

1.5.9 Summarizing the Tracking data

Tracking of a moving object results in a list of position coordinates. This list can be saved as a text file and imported to spreadsheet software such as Excel. The instantaneous velocity is derived by calculating the distance between consecutive time points. For example, if the position of an object at time point t is (x_t, y_t) and the object moves to a position (x_{t+1}, y_{t+1}) in the next time point $t + \Delta t$, then the instantaneous velocity v is

$$v = \frac{\sqrt{(x_{t+1} - x_t)^2 + (y_{t+1} - y_t)^2}}{\Delta t} \quad (1.20)$$

For each frame, the instantaneous velocity can be calculated. One way to summarize the data is to average the resulting instantaneous velocities and append its standard deviation. In most cases in biology, velocity changes with time, and this dynamics can be studied by plotting instantaneous velocity vs. time on a graph. Such plotting reveals characters of the movement, such as acceleration kinetics or periodicity. Movements within organisms can be both random and directed. To make a clear distinction between these different types of movements, mean square displacement (MSD) plotting is a powerful method. Although this method has been extensively used in studying molecular diffusion within the plasma membrane, it can also be used in other scales such as bacteria swimming or cell

movement within tissue (Berg, 1993; Saxton and Jacobson, 1997; Kusumi et al., 1993; Witt et al., 2005; Suh et al., 2005).

The most basic equation that describes the diffusion is¹⁶

$$\langle r^2 \rangle = 4D\tau \quad (1.21)$$

where r^2 is the mean square displacement, D is the diffusion coefficient, τ is the time scale. The MSD is calculated by squaring the net distance a molecule moved during the period of time τ . For example, if an object moved from $(0,0)$ to (x,y) during a period of τ , the square displacement will be

$$r^2 = x^2 + y^2 \quad (1.22)$$

or

$$r = \sqrt{x^2 + y^2} = \sqrt{4D\tau} \quad (1.23)$$

If we have many samples, then we can average them and the mean square displacement (MSD) will be

$$\langle r^2 \rangle = \langle x^2 + y^2 \rangle \quad (1.24)$$

The equation 1.24 tells us that the MSD (r^2) is proportional to τ , which means that when MSD is plotted against various τ , the plot will be a straight line with a slope $4D$ (Fig. 1.80 plot a). If the mobility of the target object is lower, then the slope becomes less steep (Fig. 1.80 plot d).

The mobility of molecules is not always a pure diffusion. In the case when there is constant background flow, such as laminar flow in the medium, molecules drift in a certain direction. This is called diffusion with drifts, also called "biased diffusion". Curve b in the Fig. 1.80 is a typical MSD curve of this type. Since the flow causes the movement vt , where v is the

¹⁶This equation is for two-dimensional mobility. In case of 3D, $\langle r^2 \rangle = 6D\tau$.

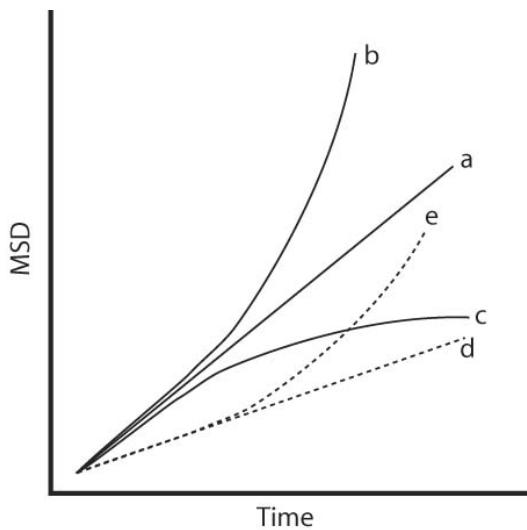


Figure 1.80: MSD plotting and different types of movement. (a) pure random walk (b) biased random walk (diffusion with drifts) (c) constrained random walk. (d) pure random walk, but with a lower diffusion coefficient. In the case of molecules, this could be due to larger molecule size. In the case of cells, this could be due to a less migration activity. (e) same as b, but with a lower diffusion coefficient.

flow rate, the displacement r will be

$$r = \sqrt{4D\tau} + v\tau \quad (1.25)$$

When $\text{MSD} < r^2 >$ is plotted against time, $v\tau$ causes an upward curvature of the graph. For example in case of chemotaxis, the direction of movement is biased towards the chemoattractant source, so that the curve becomes upward.

Another mode of movement is "constrained diffusion". This happens when the diffusion is limited within a space. Consider a molecule diffusing in a bounded space. The molecule can diffuse normally until it hits the boundary. In such a case, the MSD curve displays a plateau such that shown in curve c in Fig. 1.80. When τ is small, MSD is similar to the pure diffusion but as τ becomes larger, MSD becomes attenuated since the displacement is hindered at a defined distance. The constrained diffusion is often observed with membrane proteins ([Saxton, 1997](#)).

1.5.10 ASSIGNMENTS

Assignments 1-5-5-2:

Open tubulin dynamics image stack **kin_tubulin.stk** and do manual tracking like you did in the exercise [1.5.7](#). Track six points and plot the tracks in a same graph. Is there anything you can say about their dynamics?

Images: courtesy of Puck Ohi.

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1.6 Appendices

1.6.1 App.1 Header Structure and Image Files

For TIFF format, detailed description could be found at:

[http://www.ee.cooper.edu/courses/course_pages/past_courses/
EE458/TIFF/](http://www.ee.cooper.edu/courses/course_pages/past_courses/EE458/TIFF/)

1.6.2 App.1.5 Installing Plug-In

ImageJ

To install Plug-In, download the Plug-In file (*.class or *.jar) and put the file in the "plugin" folder within ImageJ folder. ImageJ must be restarted to see the plugin in the menu. By default, Plug-in appears under [Plugins]. If you want to change the location of the plugin in the menu, one can change the place by using [Plugins > Utilities > Control Panel].

If you have too many Plugins, there will be significant probability of so called "Class conflicts". Classes are the modules in ImageJ and Plugins. If there are two identical classes in ImageJ, then these classes causes the conflict in the process. To avoid this, one could have multiple ImageJ and install Plugin in each of them for different purposes, that there will be no Plugin overloads.

Fiji

Select [Plugins > Install Plugins...] then choose the plugin file (.jar or .class file).

1.6.3 App.1.75 List of accompanying PDF

- ImageJ_Manual.pdf

ImageJ Manual written by Tony Collins@Cell Imaging Core facility, Toronto Western Research Intsitute

- rossner_yamada.pdf

JCB paper about manipulation of image data.

- Time Series_Analyzer.pdf

Manual for the Time Series Analyzer PlugIn

- Manual Tracking plugin.pdf

Manual Tracker PlugIn manual

1.6.4 App.2 Measurement Options

Copied from

<http://rsb.info.nih.gov/ij/docs/guide/userguide-27.html#toc-Subsection-27.7>

Area

Area of selection in square pixels or in calibrated square units (e.g., mm^2 , μm^2 , etc.) if Analyze ▷ Set Scale... was used to spatially calibrate the image.

Mean Gray Value

Average gray value within the selection. This is the sum of the gray values of all the pixels in the selection divided by the number of pixels. Reported in calibrated units (e.g., optical density) if Analyze ▷ Calibrate... was used to calibrate the image. For RGB images, the mean is calculated by converting each pixel to grayscale using the formula

$$gray = (red + green + blue)/3$$

or

$$gray = 0.299red + 0.587green + 0.114blue$$

if Weighted RGB Conversions is checked in Edit ▷ Options ▷ Conversions...

Standard Deviation

Standard deviation of the gray values used to generate the mean gray value. Uses the Results table heading StdDev.

Modal Gray Value

Most frequently occurring gray value within the selection. Corresponds to the highest peak in the histogram. Uses the heading Mode.

Min & Max Gray Level

Minimum and maximum gray values within the selection.

Centroid

The center point of the selection. This is the average of the x and y coordinates of all of the pixels in the image or selection. Uses the X and Y headings.

Center of Mass

This is the brightness-weighted average of the x and y coordinates all pixels in the image or selection. Uses the XM and YM headings. These coordi-

nates are the first order spatial moments.

Perimeter

The length of the outside boundary of the selection. Uses the heading Perim.. With IJ1.44f and later, the perimeter of a composite selection is calculated by decomposing it into individual selections. Note that the composite perimeter and the sum of the individual perimeters may be different due to use of different calculation methods.

Bounding Rectangle

The smallest rectangle enclosing the selection. Uses the headings BX, BY, Width and Height, where BX and BY are the coordinates of the upper left corner of the rectangle.

Fit Ellipse

Fits an ellipse to the selection. Uses the headings Major, Minor and Angle. Major and Minor are the primary and secondary axis of the best fitting ellipse. Angle is the angle between the primary axis and a line parallel to the X-axis of the image. The coordinates of the center of the ellipse are displayed as X and Y if Centroid is checked. Note that ImageJ cannot calculate the major and minor axis lengths if Pixel Aspect Ratio in the Analyze > Set Scale... dialog is not 1.0. There are several ways to view the fitted ellipse:

1. The Edit > Selection > Fit Ellipse command replaces an area selection with the best fit ellipse.
2. The DrawEllipse macro draws (destructively) the best fit ellipse and the major and minor axis.
3. Select Ellipses from the Show: drop-down menu in the particle analyzer (Analyze > Analyze Particles...) and it will draw the ellipse for each particle in a separate window.

Shape Descriptors

Calculates and displays the following shape descriptors:

- **Circularity**

$4\pi \frac{\text{Area}}{\text{Perimeter}^2}$ with a value of 1.0 indicating a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated shape. Values may not be valid for very small particles. Uses the heading Circ.

- **Aspect Ratio**

The aspect ratio of the particle's fitted ellipse, i.e., $\frac{[\text{Major Axis}]}{[\text{Minor Axis}]}$. If Fit Ellipse is selected the Major and Minor axis are displayed. Uses the heading AR.

- **Roundness**

$4\frac{[\text{Area}]}{\pi[\text{Major axis}]^2}$ or the inverse of Aspect Ratio. Uses the heading Round.

- **Solidity**

$\frac{[\text{Area}]}{[\text{Convex area}]}$; Note that the Edit> Selection > Convex Hull command makes an area selection convex.

Feret's Diameter

The longest distance between any two points along the selection boundary, also known as maximum caliper. Uses the heading Feret. The angle (0 – 180 degrees) of the Feret's diameter is displayed as FeretAngle, as well as the minimum caliper diameter (MinFeret). The length of the object's projection in the X (FeretX) and Y (FeretY) direction is also displayed.

Integrated Density

The sum of the values of the pixels in the image or selection. This is equivalent to the product of Area and Mean Gray Value. With IJ1.44c and later, Raw integrated density (sum of pixel values) is displayed under the heading RawIntDen when Integrated density is enabled. The Dot Blot Analysis tutorial demonstrates how to use this option to analyze a dot blot assay.

Median

The median value of the pixels in the image or selection.

Skewness

The third order moment about the mean. The documentation for the Moment Calculator plugin explains how to interpret spatial moments. Uses the heading Skew.

Kurtosis

The fourth order moment about the mean. Uses the heading Kurt.

Area Fraction

For thresholded images is the percentage of pixels in the image or selection that have been highlighted in red using Image > Adjust > Threshold... [T].

For non-thresholded images is the percentage of non-zero pixels. Uses the heading %Area.

Stack Position

The position (slice, channel and frame) in the stack or hyperstack of the selection. Uses the headings Slice, Ch and Frame.

n.b.: For line selections the heading Length is created. For straight line selections, Angle is recorded even if Fit Ellipse is unchecked. Also, note that measurements that do not apply to certain selection types may be listed as *Nan*, *Infinity* or *-Infinity*.

The second part of the dialog controls measurement settings:

Limit to Threshold

If checked, only thresholded pixels are included in measurement calculations. Use **Image > Adjust > Threshold... [T]** to set the threshold limits. This setting affects only thresholded images (see **Settings and Preferences**).

Display Label

If checked, the image name and slice number (for stacks) are recorded in the first column of the results table, e.g., mri-stack.tif:9. For renamed selections (**Edit > Selection > Properties... [y]**) or selections measured via ROI Manager's measure command (see **ROI Manager...**), the selection label is appended, e.g., blobs.gif:0339-0163 or blobs.gif:mySelection.

Invert Y Coordinates

If checked, the XY origin is assumed to be the lower left corner of the image window instead of the upper left corner (see also **Image > Properties... [P]**).

Scientific Notation

If checked, measurements are displayed in scientific notation, e.g., 1.48E2.

Redirect To

The image selected from this pop-up menu will be used as the target for statistical calculations done by **Analyze > Measure... [m]** and **Analyze > Analyze Particles... commands**. This feature allows you to outline a structure on one image and measure the intensity of the corresponding region in another image.

Decimal Places

This is the number of digits to the right of the decimal point in real numbers displayed in the Results table and in Histogram windows.

1.6.5 App.3 Edge Detection Principle

Quote from MBL manual:

One way to find boundaries of objects is to detect discontinuities in intensity values at the edge of a region. These discontinuities can be found by calculating the first and/or second order derivatives of an image. The first derivative of choice in image processing is the gradient, defined as the vector:

$$\text{grad } f = [G_x \ G_y]$$

where $G_x = df/dx$ and $G_y = df/dy$ are the partial derivatives in the horizontal and vertical directions of the image. The magnitude of this vector is

$$|\text{grad } f| = (G_x^2 + G_y^2)^{1/2}$$

The gradient vector points in the direction of steepest ascent. The angle of steepest ascent is given by:

$$\alpha(x, y) = \tan^{-1}(G_x/G_y)$$

We can estimate the derivatives G_x and G_y digitally by linearly filtering the image with the following 3 by 3 kernels:

Image neighborhood

z_1	z_2	z_3
z_4	z_5	z_6
z_7	z_8	z_9

Sobel

-1	-2	-1
0	0	0
1	2	1

$$G_x = (z_7 + 2z_8 + z_9) - (z_1 + 2z_2 + z_3)$$

-1	0	1
-2	0	2
-1	0	1

$$G_y = (z_3 + 2z_6 + z_9) - (z_1 + 2z_4 + z_7)$$

Prewitt

-1	-1	-1
0	0	0
1	1	1

$$G_x = (z_7 + z_8 + z_9) - (z_1 + z_2 + z_3)$$

-1	0
0	1

$$G_y = (z_3 + z_6 + z_9) - (z_1 + z_4 + z_7)$$

Roberts

-1	0
0	1

$$G_x = z_9 - z_5$$

0	-1
1	0

$$G_y = z_8 - z_6$$

The Prewitt and Sobel operators are among the most used in practice for computing digital gradients. The Prewitt masks are simpler to implement than the Sobel masks, but the latter have slightly superior noise-suppression characteristics.

1.6.6 App.4 Particle Tracker manual

Tutorial from : <http://weeman.inf.ethz.ch/particletracker/tutorial.html>

PDF inserted from next page.

ImageJ

Image Processing and Analysis in Java

ParticleTracker - Tutorial

The ParticleTracker is an ImageJ Plugin for multiple particle detection and tracking from digital videos

Sample Data and Tutorial

This tutorial provides a basic introduction on how to select the right parameters for the algorithm of ParticleTracker and the right display and analysis options upon completion of it.

Written by [Guy Levy](#) at the [Computational Biophysics Lab, ETH Zurich](#)

To use this tutorial you should already be familiar with [ImageJ](#) and have the plugin [Installed](#).

It is also recommended to read the user manual before starting. In this tutorial we will guide you through an example using movies sample data:

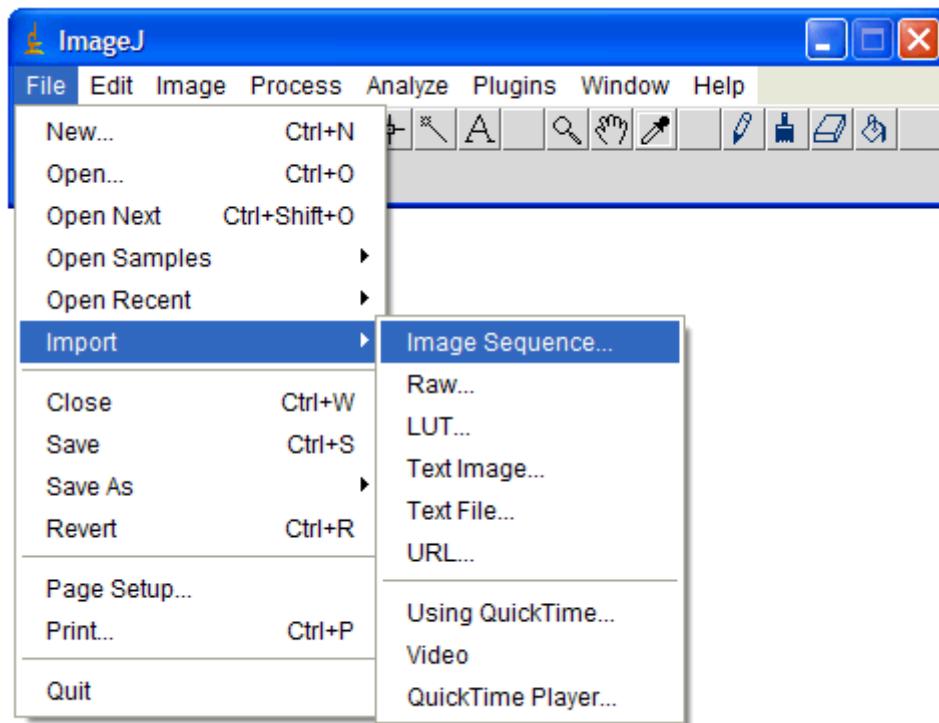
- [TransportOfEndosomalVirus.zip](#) - a real experimental movie (image sequence) from *Suomalainen et al., J. Cell Biol. 144 (4): 657-672* Kindly provided by [The Cell Biology group, Institute of Zoology, University of Zurich](#).
Fast minus and plus end-directed transport of endosomal ts1 virus labeled with Texas red. Virus was added to TC7/MAP4/MTB-GFP cells and 72 TR-images were recorded with intervals of 1.5 s starting 20 min p.i.. The GFP signal is indicated at the beginning. Bar = 10 µm
- [Artificial.zip](#) - Artificial image sequence, will be used later in the tutorial for a specific example
- [First Step - download sample movie and open it](#)
- [Second Step - select particle detection parameters and preview](#)
- [Third Step - viewing the results](#)
- [Fourth Step - re linking the particles](#)

First Step - download sample movie and open it

Download the [TransportOfEndosomalVirus.zip](#) file.

Both the movies are actually sequence of separate image files (.tif) all in a zipped folder.

After downloading the zip file, unzip it to your preferred folder and Start ImageJ. Load the image sequence by selecting the *Import -> Image Sequence...* from the *File* menu.



In the first shown dialog, select one of the images in the folder (the one you unzipped) and click "Open".

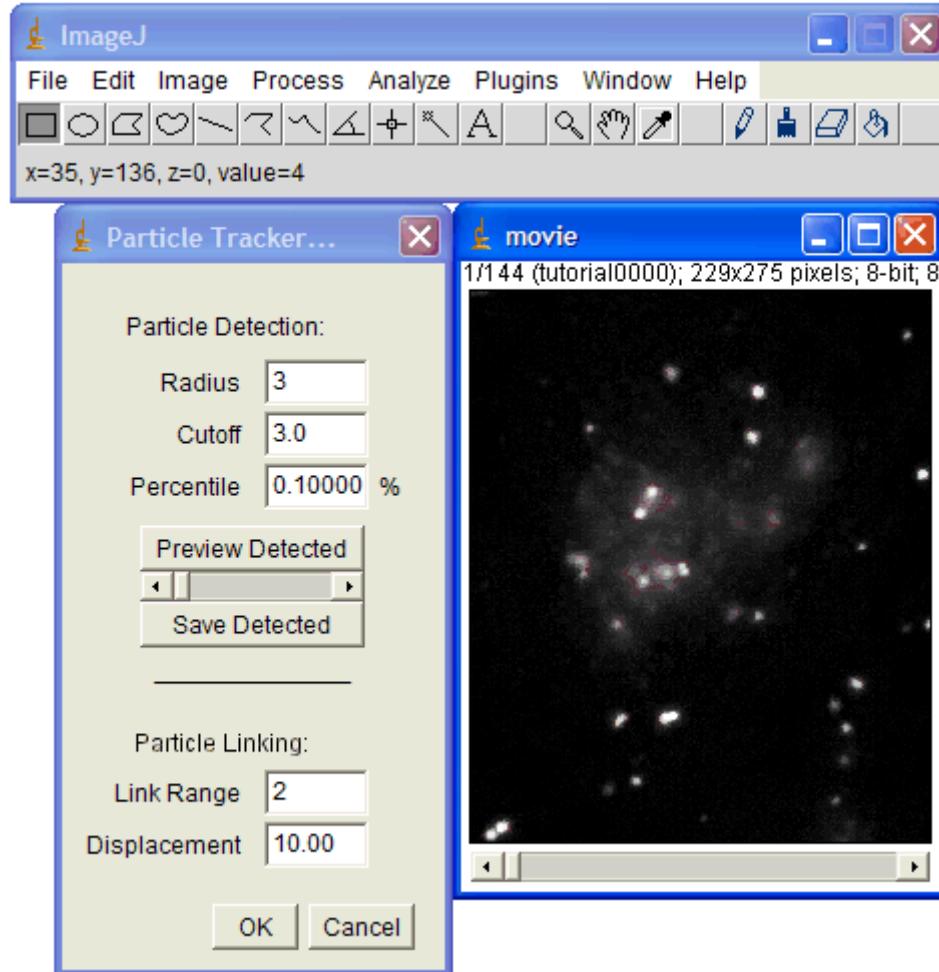
Use the second dialog to specify which images in the folder to open and/or to have the images converted to 8-bits.

When working with RGB images like the files of the experimental movie sample it is highly recommended to convert them to 8-bits. This way the memory consumption is reduced significantly.

Check the *Convert to 8-bit Grayscale* box and click OK.

Now, that the movie is open you can start the plugin by selecting ParticleTracker

from the *Plugins -> Particle Detector & Tracker* menu.



Second Step - select particle detection parameters and preview

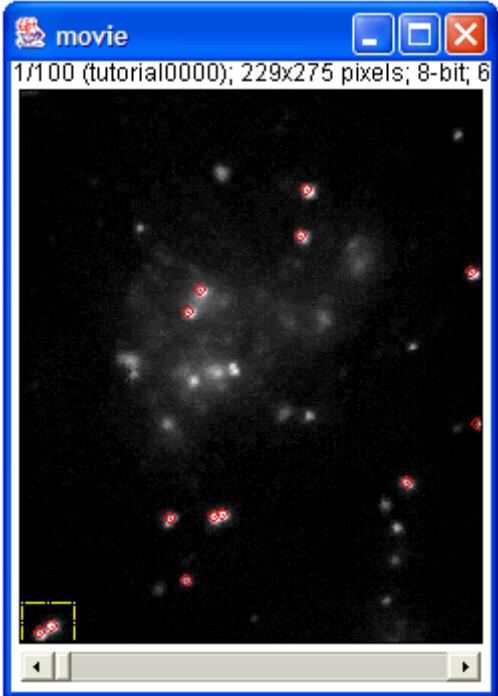
The dialog showing now has 2 parts: Particle Detection and Particle Linking.
The parameters relevant for detection are:

- Radius: Approximate radius of the particles in the images in units of pixels. The value should be slightly larger than the visible particle radius, but smaller than the smallest inter-particle separation.
- Cutoff: The score cut-off for the non-particle discrimination
- Percentile: The percentile (r) that determines which bright pixels are accepted as Particles. All local maxima in the upper r th percentile of the image intensity distribution are considered candidate Particles. Unit: percent (%)

In the particle detection part you can "play" with different parameter and check how well the particles are detected by clicking the preview detected button. There are no right and wrong parameters; it all depends on the movie, type of data and what is looked for.

Enter these parameters: radius = 3, cutoff = 0, percentile = 0.1 (default) - click on preview detected.

Notice the 2 very close particles marked in the image



Check the detected particles at the next frames by using the slider in the dialog menu (you cannot move the image itself while the dialog is open)

With radius of 3 they are rightly detected as 2 separate particles.

If you have any doubt they are 2 separate particles you can look at the 3rd frame. Change the radius to 6 and click the preview button.

Look at frame 1.

With this parameter, the algorithm wrongfully detects them as one particle since they are both within the radius of 6 pixels.

Try other values for the radius parameter.

Go back to these parameters: radius = 3, cutoff = 0, percentile = 0.1 (default) - click on preview detected.

It is obvious that there are more 'real' particles in the image that were not detected.

Notice, that the detected particles are much brighter than the ones not detected. Since the score cut-off is set to zero, we can rightfully assume that increasing the percentile of particle intensity taken will make the algorithm detect more particles (with lower intensity).

The higher the number in the percentile field - the more particles will be detected.

Try setting the percentile value to 2.

After clicking the preview button, you will see that much more particles are detected, in fact **too many particles** - you will need to find the right balance.

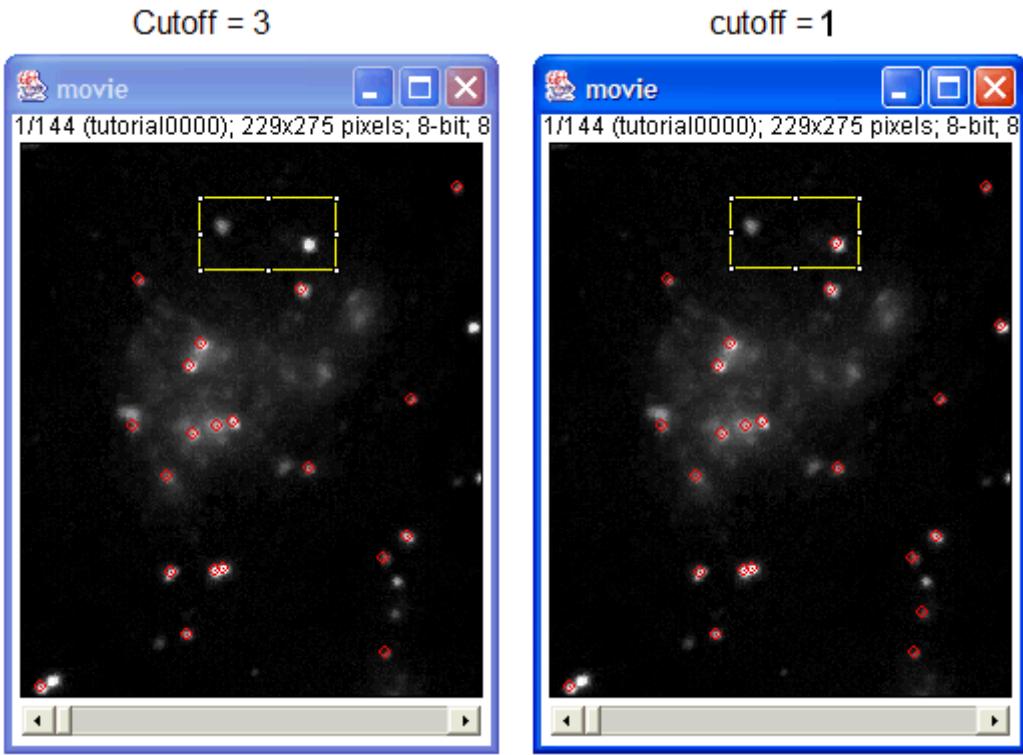
In this movie, percentile value of 0.6 will give nice results.

Remember! There is no right and wrong here - it is possible that the original percentile = 0.1 will be more suitable even with this film, if for example only very high intensity particles are of interest.

Set the parameters to radius = 3, cutoff = 1, percentile = 0.6 - click on preview detected.

Change the cutoff parameters back to its default (3) and click preview again.

Notice the particles marked in the two pictures:



With cutoff = 3 both particle are discriminated as non-particles and when cutoff = 1 only one gets discriminated.

The higher the number in the cutoff field the more suspicious the algorithm is of false particles.

This could be very helpful when one understand the method for non-particles discrimination as described in the [original algorithm](#).

It can also lead to real particles discrimination when the value is too high.

After setting the parameters for the detection (we will go with radius = 3, cutoff = 0, percentile = 0.6) you should set the particle linking parameters.

The parameters relevant for linking are:

- Displacement: The maximum number of pixels a particle is allowed to move between two succeeding frames.
- Link Range: The number of subsequent frames that is taken into account to determine the optimal correspondence matching.

These parameters can also be very different from one movie to the other and can also be modified after viewing the initial results.

Generally, in a movie where particles travel long distance from one frame to the other - a large link range should be entered.

In this movie a link range of ~20 is appropriate. Set it to 20.

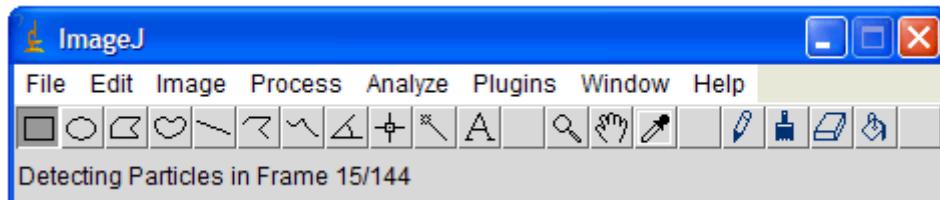
The linkrange value is harder to set before viewing some initial results since it is mainly designed to overcome temporary occlusion as well as particle appearance and disappearance from the image region and it is hard to notice such things at this stage.

Still an initial value has to be set, the default is 2 but we will continue with 3.

We will [return to these parameters](#) later with a different movie.

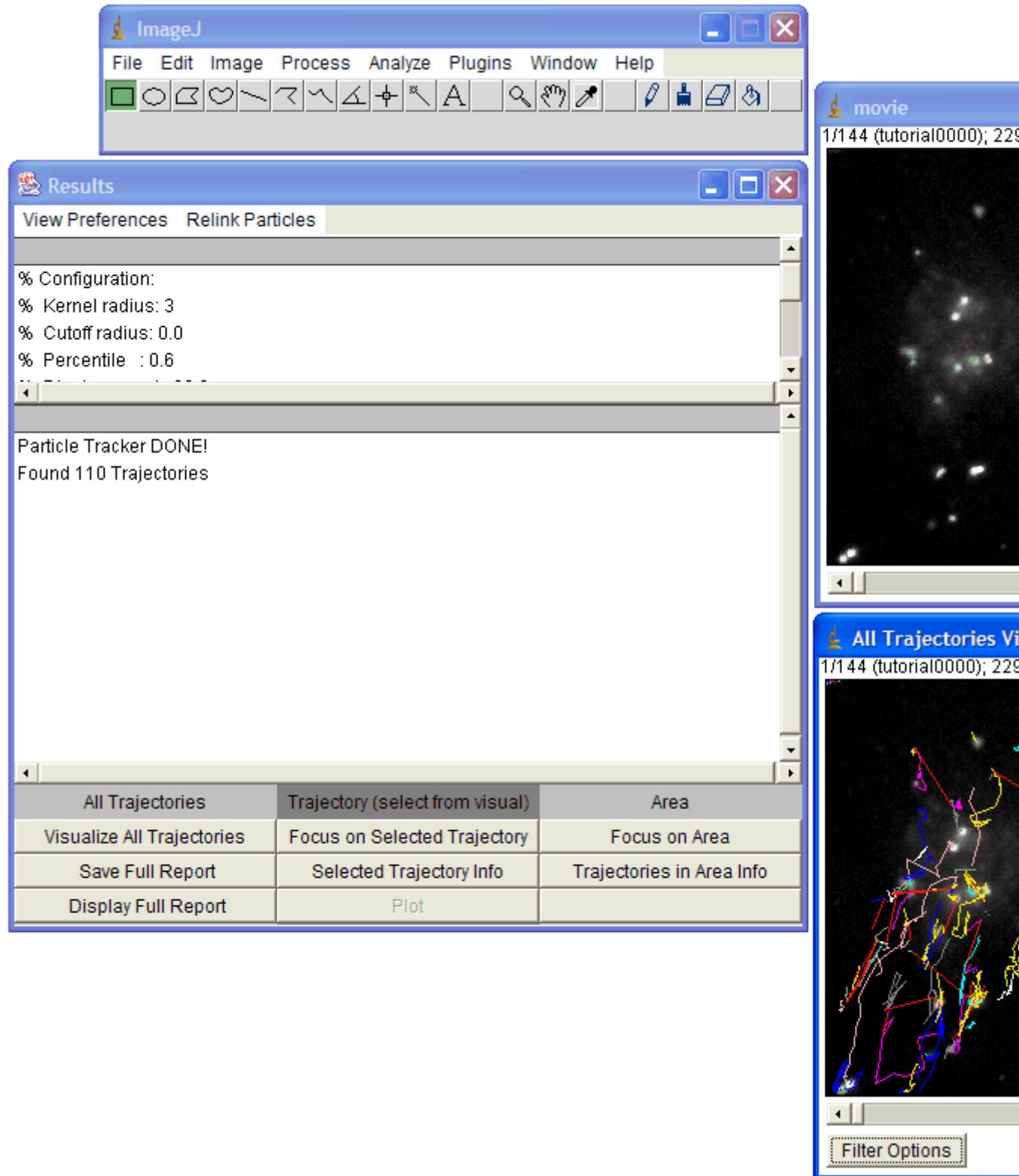
You can now go ahead with the linking by clicking OK.

The progress of the algorithm will be displayed in the main ImageJ Status Bar.



Third Step - viewing the results

After completing the particle tracking, the result window will be displayed.
Click the *Visualize all Trajectories* button to view all the found trajectories.



This window displays an overview of all 110 found trajectories - it cannot be saved!

It is hard to make sense of so much information.

One way to reduce the displayed trajectories is to filter short trajectories.

Click on the Filter Options button to filter out trajectories under a given length.

Enter 144 and click OK. All the trajectories will disappear - you can also see the message in the results window "*O trajectories remained after filter*".

Since the length of the movie is 144 frames there are no trajectories longer than 144 frames.

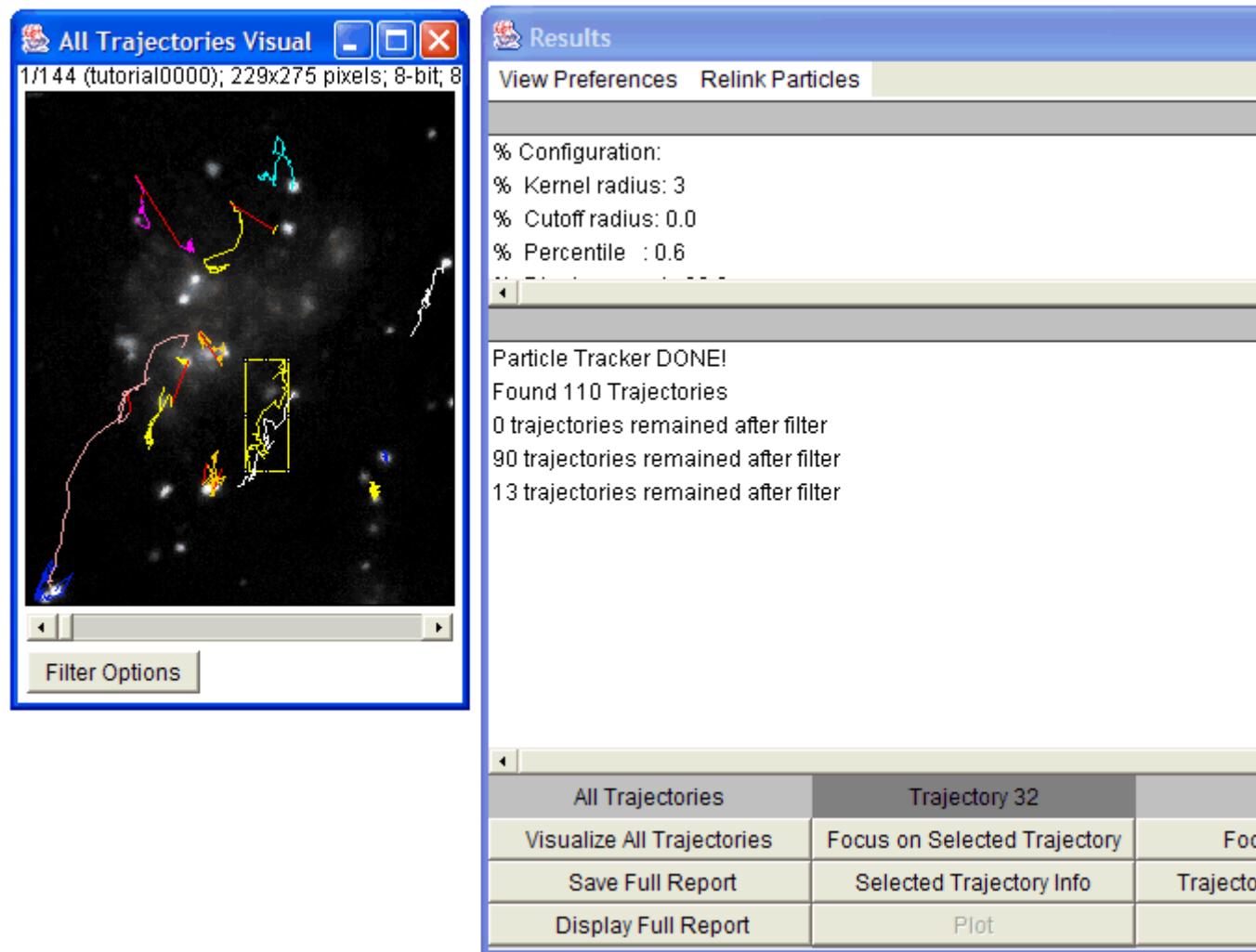
Filter again with 0 as input.

All trajectories are again displayed because by definition every trajectory length is at least 1 (spans over at least 2 frames).

Try other numbers for the filter option and notice the differences.

Set filter for 100, only 13 trajectories remained after filtering.

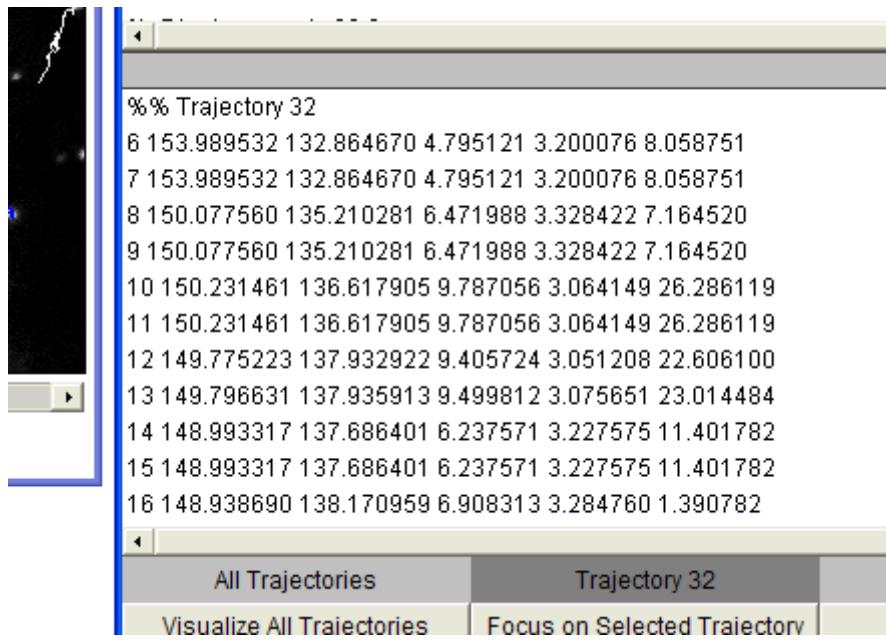
Select the yellow trajectory (the one shown here) by clicking it once with the mouse left button.



A rectangle surrounding the selected trajectory appears on the screen and on the trajectory column of the results window the number 32 is now displayed - it indicates the number of this trajectory (from the 110 found).

Now that a specific trajectory is selected you focus on it or get its information.

Click on *Selected Trajectory Info* button - the information about this trajectory will be displayed in the results window



The screenshot shows the ImageJ Trajectory viewer window. On the left is a grayscale image with a white trajectory line. The main window contains a text area with trajectory data and a bottom navigation bar.

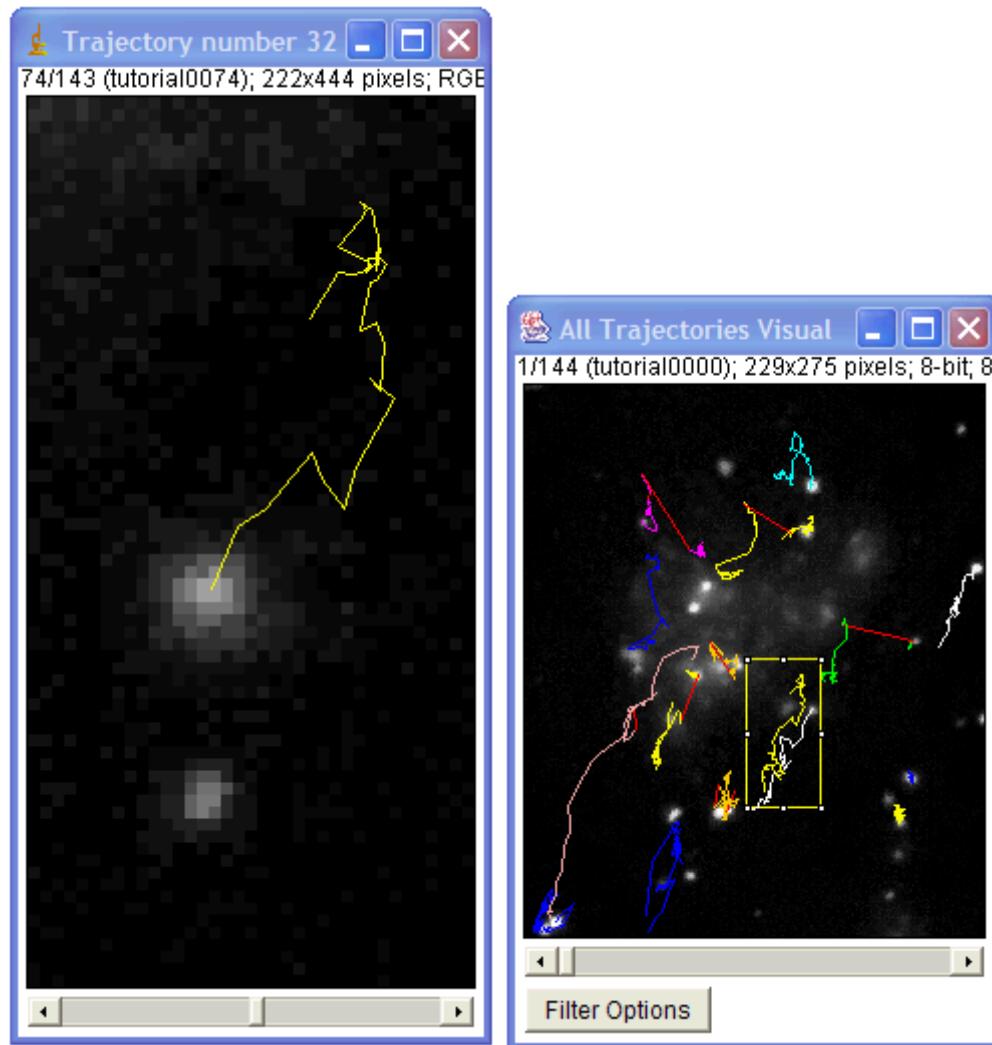
```
%% Trajectory 32
6 153.989532 132.864670 4.795121 3.200076 8.058751
7 153.989532 132.864670 4.795121 3.200076 8.058751
8 150.077560 135.210281 6.471988 3.328422 7.164520
9 150.077560 135.210281 6.471988 3.328422 7.164520
10 150.231461 136.617905 9.787056 3.064149 26.286119
11 150.231461 136.617905 9.787056 3.064149 26.286119
12 149.775223 137.932922 9.405724 3.051208 22.606100
13 149.796631 137.935913 9.499812 3.075651 23.014484
14 148.993317 137.686401 6.237571 3.227575 11.401782
15 148.993317 137.686401 6.237571 3.227575 11.401782
16 148.938690 138.170959 6.908313 3.284760 1.390782
```

All Trajectories Trajectory 32
Visualize All Trajectories Focus on Selected Trajectory

Click on the *Focus on Selected Trajectory* button - a new window with a focused view of this trajectory is displayed.

This view can be saved with the trajectory animation through the *File* menu of ImageJ.

Look at the focused view and compare it to the overview window - in the focused view the white trajectory that is close to the yellow is not displayed.



The particle is displayed but the white trajectory animation is not.

This is because we selected ***Focus on Selected Trajectory***.

Close this focus view.

Now we want to focus on area for number of trajectories view, we will focus on the area of the yellow and white trajectories as shown here.

Select a rectangle region of interest around these trajectories - click and drag the mouse on the overview to include them.

Click on the *Focus On Area* button - a new window with a focused view of these trajectories is displayed.

This time the animation of both trajectories is displayed.

Generally, any unfiltered trajectory part that is in the selected area will be shown. You may notice that some particles are showing but their trajectory is not animated, this is because they are filtered (remember we filtered for longer than 100).

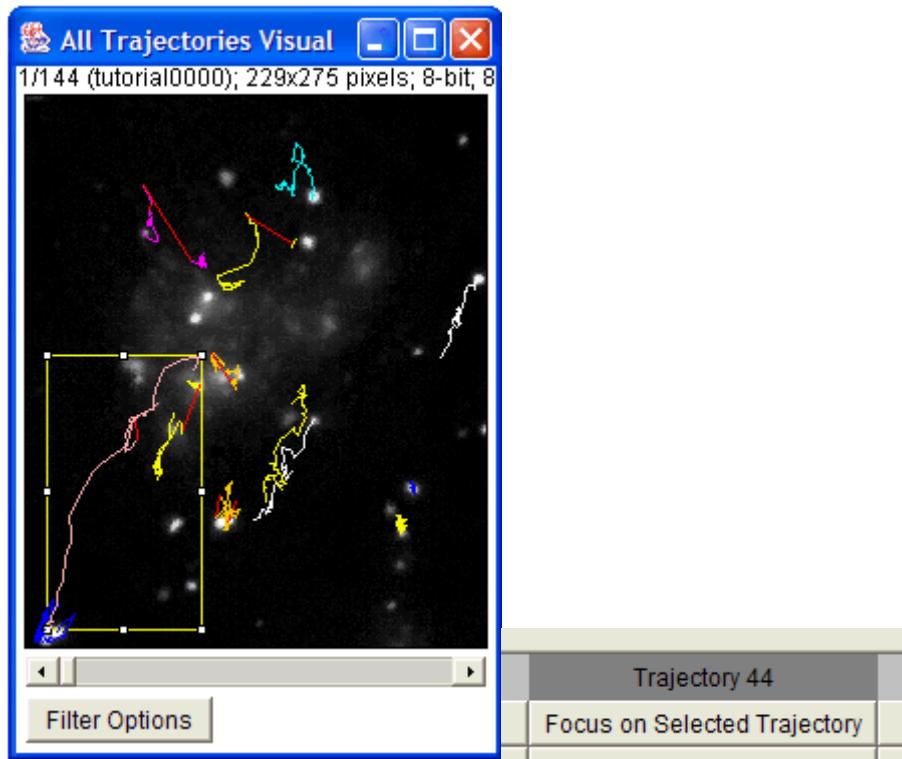
Close the focus window and reset the filter. You can do that by closing the overview window and reopening it by clicking the *Visualize all Trajectories* button or you can click the filter button and set the min length to 0 (default).

The last option is better since this way your area selection will stay.

Click again on the *Focus on Area* button - now all trajectories within the selection area is displayed.

The size of the focus window for specific trajectory and area focus is determined by the magnification factor relative to the original movie window size.

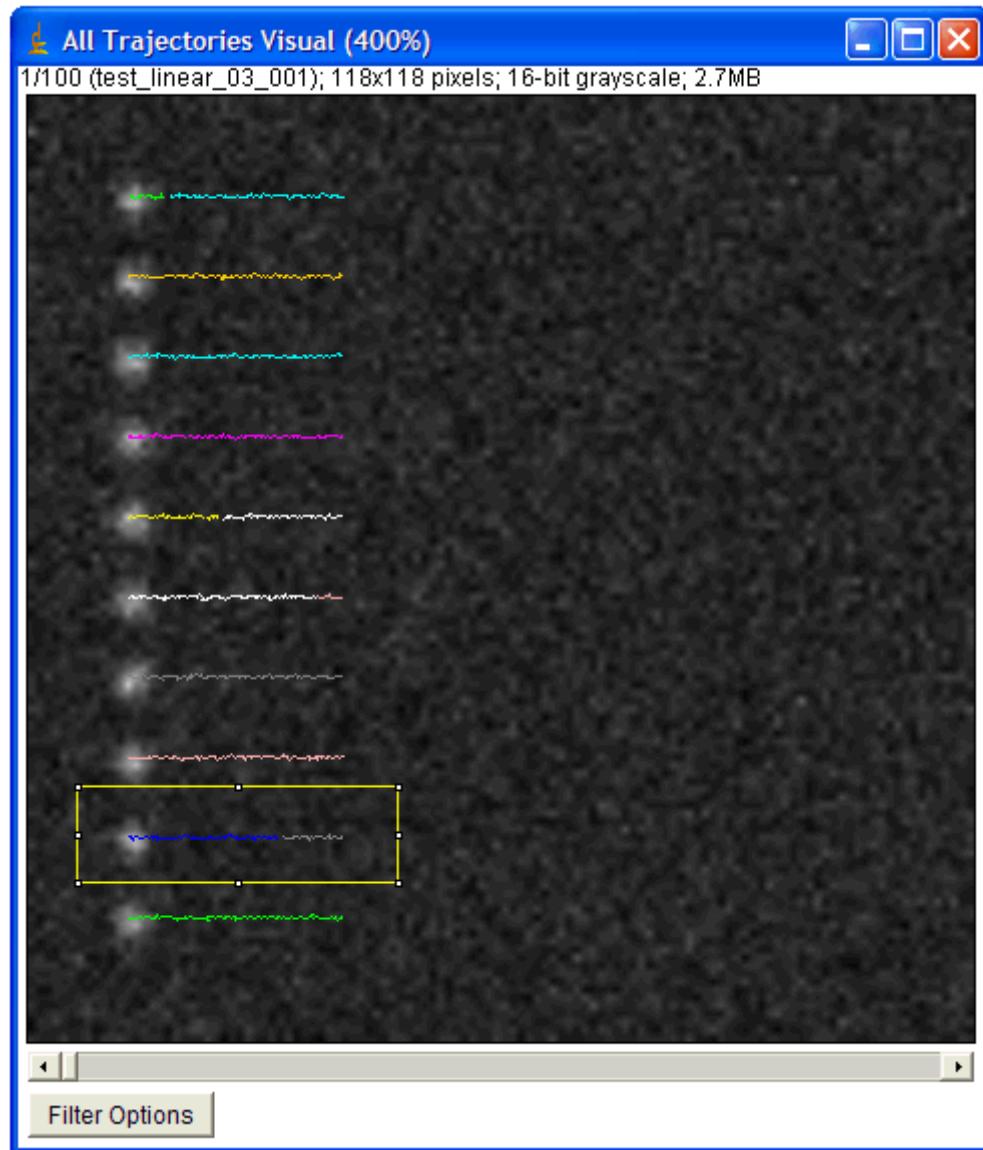
Select the pink trajectory (the one shown here). The trajectory number is 44.



Notice that the rectangle surrounding the selected trajectory is fairly big. If we focus on this trajectory with the default magnification factor (6) a large window will be created and may cause [memory problems](#) (especially in Mac Os). For this reason and others - you can change the magnification factor. Before clicking the *Focus on Selected Trajectory* button - go to *View Preferences* menu in the results window and select the *Magnification Factor* option. Select magnification of 2-4. Click on the *Focus on Selected Trajectory* button to see the size of the new window. Close the window.

Fourth Step - re linking the particles

To explain the re-linking option we will use a different data sample - Artificial.zip. Close all windows including the original movie window. Load the new image sequence from Artificial.zip and start the plugin. Set the particle detection parameters to: radius = 3, cutoff = 3.0, percentile = 0.9. Set the particle linking parameters to: link range = 1, displacement = 10. Start the algorithm and when it's done, click the View all Trajectories button. Zoom in on the overview window for better view. Select an area of interest to include the 2 adjacent blue and grey trajectories as shown here.



Increase the magnification factor to 8 and focus on that area.

Scroll through the focused view or view it with animation (*Image -> Stacks -> Start Animation*).

It seems that these two trajectories are actually 1 longer trajectory.

Why was it recognized as 2 trajectories? Scroll to slice/frame 71?

At this point, the detection algorithm, due to the set detection parameters and bad quality of the movie, did not detect the particle. This can also happen in real biological data.

Since the link range was set to 1 - the linking algorithm did not look ahead to frame 72 to check for a possible continuation to the trajectory.

Re-link the particle with link range = 2, go to the *Relink Particles* menu at the results window and select the set new parameters for linking.

In the dialog now opened - set the link range to 2 and click OK.

When the re linking is done, a message will be displayed in the results window:
Relinking DONE! Found 10 Trajectories



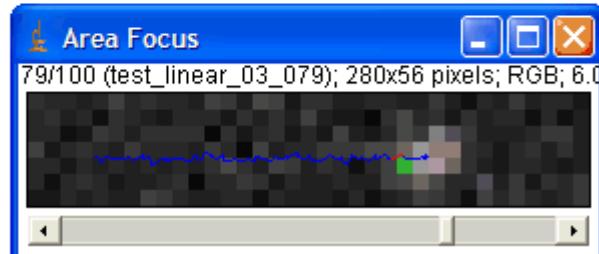
Results

View Preferences Relink Particles

% Width : 118 pixel
 % Height: 118 pixel
 % Global minimum: 0.0
 % Global maximum: 62.0

Relinking DONE!
 Found 10 Trajectories

All Trajectories	Trajectory (select f...
Visualize All Trajectories	Focus on Selected
Save Full Report	Selected Trajecto...
Display Full Report	Plot



You can already see that fewer trajectories were found (10 instead of 17). Click on the View all Trajectories button and compare the view to the one created with link range = 1.
 Focus on the blue trajectory.
 The previously 2 separate trajectories are now 1 and in frame 71, where the particle was not detected, a red line is drawn to indicate a "Gap" in the trajectory - meaning a part in the trajectory that was interpolated by the algorithm to handle occlusion, exit and entry of the particle.

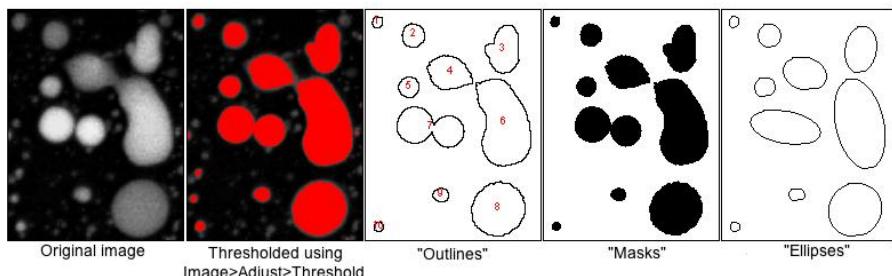
1.6.7 App.5 Particle Analysis

Copied from ImageJ website:

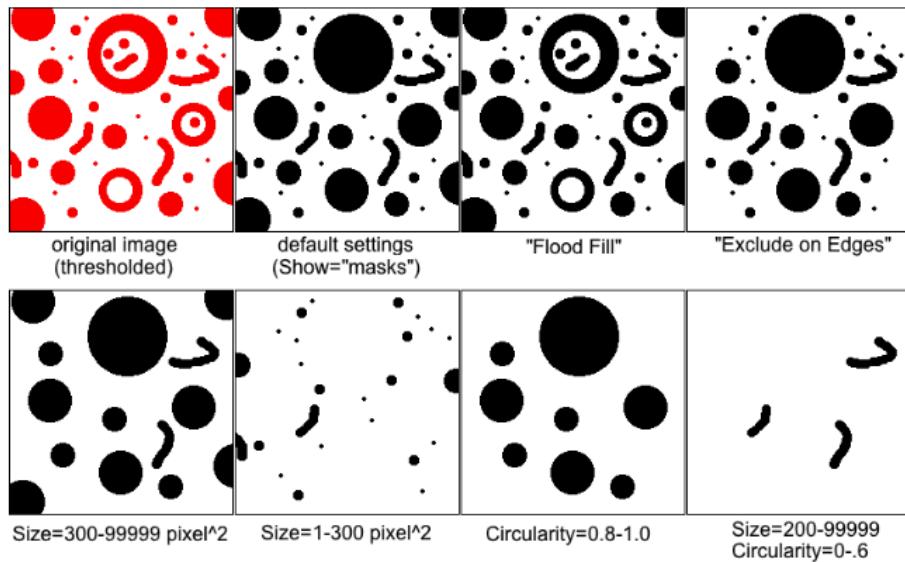
"Particle Analysis" counts and measures objects in binary or thresholded images. It works by scanning the image or selection until it finds the edge of an object. It then outlines the object using the wand tool, measures it using the Measure command, fills it to make it invisible, then resumes scanning until it reaches the end of the image or selection. Press the esc key to abort this process. Use Image/Adjust/Threshold to threshold an image.

Use the dialog box to configure the particle analyzer. Particles outside the range specified in the Size field are ignored. Enter a single value in Size and particles smaller than that value are ignored. Particles with circularity values outside the range specified in the Circularit field are also ignored. The formula for circularity is $4\pi(\text{area}/\text{perimeter}^2)$. A value of 1.0 indicates a perfect circle. Note that the Circularit field was added in ImageJ 1.35e.

Select Outlines from the "Show:" pop-up menu and ImageJ will open a window containing numbered outlines of the measured particles. Select Masks to display filled outlines of the measured particles or Ellipses to display the best fit ellipse of each measured particles.



Check Display results to have the measurements for each particle displayed in the "Results" window. Check Clear Results to erase any previous measurement results. Check Summarize to display, in a separate window, the particle count, total particle area, average particle size, and area fraction. Check Exclude on Edges to ignore particles touching the edge of the image or selection.



Check Flood Fill and ImageJ will define the extent of each particle by flood filling instead of by tracing the edge of the particle using the equivalent of the wand tool. Use this option to exclude interior holes and to measure particles enclosed by other particles. The following example image contains particles with holes and particles inside of other particles.

1.6.8 App.6 Image Processing and Analysis: software, scripting language

ImageJ is not only the tool for scientific image processing and analysis. I list some other software and tools used by EMBL researchers here and add some description about each of them. For more details, please refer to an article by [Walter et al. \(2010\)](#).

MatLab (Mathworks), \$

With Matlab, you could access images as numerical matrix to do processing and analysis of images. Programming is possible with Matlab scripting language. Scripts could be kept as files and execute them directly from the Matlab command line. These files are called "m-files". Many imaging related tools are publicly available via Internet download. Scripts could be exported as execution files, and could be distributed without Matlab itself, as these stand-alone execution files only require freely available Matlab library. A free alternative is Octave. I have never tried this yet, but this freeware is under extensive development and worth for some trial.

Imaris (Bitplane), \$

Imaris is also a commercial software, especially powerful in interactive 3D visualization. 3D time course (sometimes called "4D") with multiple channels (then this would be called 5D) could also be visualized and interactively adjusted with their appearance. Some analysis packages are optionally available. I sometimes use optional package "Imaris Track" for tracking spotty signals. Algorithm for linking particles is excellent (uses graph-theory based algorithm developed in Danuser lab). In EMBL, you could try using this software by so called "floating license", which enables you to use the software from any computer within EMBL domain. The number of simultaneous usage is limited to three (as of June 2010). Another module that gives additional power to Imaris is "Imaris XT", which enables accessing Imaris objects from Matlab, Java or ImageJ. I have some example Java codes, so if you are interested I could give you as an example. Imaris is pretty expensive, and apparent disadvantage.

Python, Free

Python is not a software package, but is a scripting language. There are many other scripting languages like Ruby, but the merit of Python is that there are numerous libraries for image processing and analysis. In terms of scripting, accessibility is similar or more powerful than Matlab, since its bridging capability to many computer languages such as C, C++ and Java. Considering that the trend of image processing and analysis is getting more and more towards cross-language library usage, Python is a good choice to learn for high-end processing and analysis.

Cell Profiler, Free

This free software is a bit less with available functions compared to ImageJ, but is easier and robust in constructing pipelines for image processing and analysis. One could interactively construct pipeline and do high-throughput processing and analysis for many data.

1.6.9 App.7 Macro for Generating Striped images

Below is an ImageJ macro code for generating stripes to do some experiments on FFT. You could copy & paste this in a new macro window and install to generate stripes with various frequencies and orientation.

```
var linewidth = 8;
var size =50;
macro "vertical stripes"{
    vertical(size);
}

function vertical(winsize){
    newImage("stripes", "8-bit black", winsize, winsize, 1);
    setForegroundColor(255, 255, 255);
    setLineWidth(linewidth);
    for(i=0; i<getWidth();i+=linewidth*2 ){
        drawLine(i, 0, i, getHeight());
    }
    run("Gaussian Blur...", "sigma=1");
}

macro "diagonal stripes"{
    vertical(size*2);
    run("Rotate...", "angle=45 grid=0 interpolation=Bilinear");
    run("Specify...", "width="+size+" height="+size+" x="+
        size/2+" y="+size/2+"");
    run("Crop");
}
```

MacrostripeGenerator.ijm

1.6.10 App.8 Deconvolution Exercise

Exercise starts from next page.

In this exercise, following plugins are used. If you do not have them in your ImageJ or Fiji, download them and install. Deconvolution lab cannot be installed by [Plugins > Install]. Unpack the downloaded zip folder and place all the contents under plugins directory.

1. PSFgenerator <http://bigwww.epfl.ch/algorithms/psfgenerator/>
2. DeconvolutionLab <http://bigwww.epfl.ch/algorithms/deconvolution/>

Excercises on deconvolution

Alessandra Griffa

September 1, 2010

Exercise 1: 3D widefield PSF

Start the PSFGenerator plugin in ImageJ. Generate a 3D PSF for a widefield microscope with following parameters:

- $n_i = 1.518$
- NA = 1.4
- W040 = 0
- $\lambda = 500\text{nm}$
- amplitude = 255
- background = 0
- SNR= 100
- $x_0 = y_0 = 32$
- $z_0 = 64$
- $\Delta r = \Delta z = 50\text{nm}$
- $N_x = N_y = 64$
- $N_z = 128$

You can discard the image called *PSF for deconvolution*. Browse through the PSF using the VolumeViewer, the 3D Viewer and the Orthogonal Views tools of ImageJ. (hint: change the lookup table of the optic PSF into Fire to better appreciate its shape)

Change the numerical aperture (e.g. NA = 0.8) and/or the wavelength (e.g. $\lambda = 400$ or 600 nm). Generate the corresponding PSFs and describe the influence of these parameters.

Go back to the initial set of parameters. W040 controls the amount of spherical aberrations; set its value to 500 nm. Describe the effect on the PSF. What are possible causes of spherical aberrations?

Exercise 2: 2D inverse filtering

Load the images *FluorescentCells.tif* and *PSFDefocus.tif*. Launch the Deconvolution plugin.

2.1 Noise-free inverse filtering

We will first create an artificially blurred image using the defocusing blur kernel.

In the PSF section of the plugin, select the image *PSFDefocus.tif*. Make sure that Normalize PSF is selected. In the Algorithm section, select Convolution. Make sure that Add noise is deselected. Click the Run button. (note: to apply the plugin's functionalities to a specific image, you have to make sure that it was the last active image before clicking Run)

Try to deconvolve the widefield image you simulated using the inverse filtering method. In the Algorithm section, select Direct inversion and click Run. Comment the result.

2.2 Noisy inverse and Wiener filtering

We will create now an artificially blurred and noisy image (more realistic simulation).

Go back to Convolution in the Algorithm section. Activate the Add noise checkbox. Play around with both noise models at various noise levels (e.g. between 10, 30 and 60 dB), to get a feeling for this functionality. What are the principal sources of Gaussian and Poisson noise in microscopy images?

Choose the Gaussian model with a noise level such that you cannot visually distinguish the image from the one obtained at point 2.1. Apply the inverse filtering to restore the image and comment the results.

Try now the inverse filtering with regularization (Wiener filtering) on the noisy and blurred image. Select Regularized Direct Inversion in the Algorithm section and run the deconvolution with different values of the regularization parameter Lambda. How does the regularization parameter influence the result?

Exercise 3: 2D iterative deconvolution

Choose the Poisson model with a noise level of 30 dB. Select the Richardson-Lucy deconvolution algorithm, which is one of the simplest deconvolution algorithms for shot-noise limited imaging. Try out the algorithm with different numbers of iterations (no more than 100). What is the optimal number of iterations?

Suggestion: you can obtain a quantitative assessment of your prediction as follows (keep in mind that this is a synthetic experiment!): under Log, choose Normal; under SER, choose

Process SER and select *new reference* as the reference image. The plugin will indicate the error (in dB) of the current estimate with respect to the original image.

What do you observe? How can you explain it?

Perform 50 iterations of the Richardson-Lucy algorithm with TV regularization on the image corrupted by Poisson noise, with $\lambda=0.0005$. Comment the result both qualitatively and quantitatively (using the same error measurement as before). Compare the Richardson-Lucy deconvolution results at 50 iterations, with and without the TV regularization (hint: subtract the two images to highlight the differences).

Exercise 4: 3D deconvolution

Close all images and the Deconvolution plugin. Load the file *Microtubules.tif* into ImageJ: this is a widefield stack of a biological sample that we will deconvolve. In this exercise, we generate a synthetic PSF; another option would be to use a PSF obtained from an experimental measurement (typically by imaging sub-resolution fluorescent beads). Launch the PSFGenerator plugin and generate a PSF using the following parameters (which correspond to the acquisition settings):

- $ni = 1.518$
- NA = 1.4
- W040 = 0
- $\lambda = 517 \text{ nm}$
- amplitude = 255
- background = 0

- SNR= 100
- $x_0 = y_0 = 144$
- $z_0 = 192$
- $\Delta r = 89 \text{ nm}$
- $\Delta z = 290 \text{ nm}$
- $N_x = 288$
- $N_y = 384$
- $N_z = 32$
-

Perform a visual verification of the *Optic PSF* stack; after that you can discard it.

Note that the voxel size of the acquired image is coherent with the resolution of an objective with 1.4 NA, for the used wavelength.

Open the Deconvolution plugin. In the PSF section, select *PSF for deconvolution*. In the Algorithm section, select the Richardson-Lucy algorithm with 20 iterations. Compute maximum intensity projections of the original and deconvolved stacks. Compare them.

If there is some time left: try to perform a deconvolution with another algorithm (e.g. the Tikhonov-Miller algorithm) and comment the results. You may want to activate Test the algorithm on a small rectangular selection. You could also study the influence of using a PSF with wrong parameters (for example, change the NA value or introduce spherical aberrations).