

# Work no 5: Confocal microscopy

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## 1 Purpose of the work

Your goal is to learn the basics of confocal microscopy in practice.

## 2 Background

### 2.1 Confocal microscopy

The main idea behind confocality is that it suppresses the signal outside the field-of-focus. It means that the signal is acquired only at a defined distance from the lens, without the troubling background. More theory behind confocal microscopy is presented at the lecture.

## 3 Instruments/software used

### 3.1 Microscope

Zeiss LSM 510 Meta confocal microscope. The frame of the microscope is the same with the ones used earlier in fluorescence practice but as an addition there is a confocal scanhead connected to the frame. The light source is also different. Whereas ordinary fluorescence microscope uses a light source that covers a wide spectral range, confocal microscope uses narrow bandwidth lasers. The detector is not a camera but a photomultiplier tube (PMT).

### 3.2 Software

The software used is Zeiss LSM 510 3.2 control program. A free viewer is available from Zeiss<sup>1</sup>.

## 4 Information on sample

The sample is the same mouse ear as earlier in wide-field imaging. The stainings used are:

- AlexaFluor 594-conjugated antibody (red) staining is used to show lymphatic vessels.
- AlexaFluor 488-conjugated antibodies (green) are used to show cell nuclei as they bind to a factor located in the nucleus.

The absorption and emission spectra of the antibodies are shown in Fig. 1. Since the confocal microscope uses narrow bandwidth lasers to excite the fluorophores, you have to choose carefully which lasers match your choice of dyes.

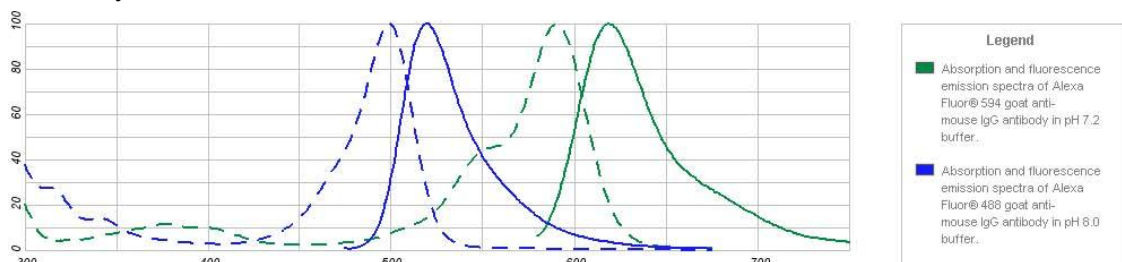


Figure 1: AlexaFluor 488 and AlexaFluor 594 spectra..Dotted line: absorption spectrum; solid line: emission spectrum.(<http://www.invitrogen.com/>)

## 5 Your task

- Build up a configuration that is used for scanning confocal images of a sample with two dyes.
- Take a Z-stack if there is time.

## 6 Practical instructions

### 6.1 Choose lasers

Go to ACQUIRE / LASER in the main menu bar. You will need to use lasers whose emitted wavelengths match as closely as possible the maximum excitation wavelengths of the sample fluorochromes (488 and 594 nanometers). The lasers needed are already on. Increase the output power of the argon laser to 50%.

### 6.2 Viewing the sample

To view your sample through oculars without laser scanning, click VIS-button on the right (Fig. 2). This will allow only the light from halogen or mercury bulb to pass through the oculars.

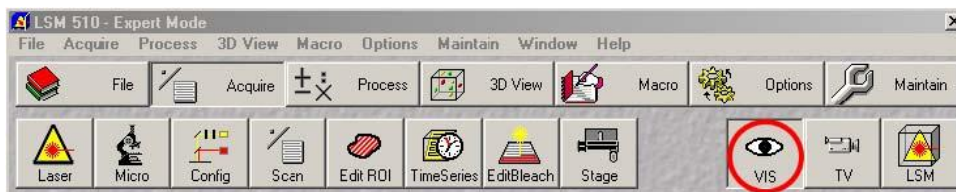


Figure 2: Button to enable ocular viewing

Next go to ACQUIRE / MICRO for microscope settings (Fig. 3).

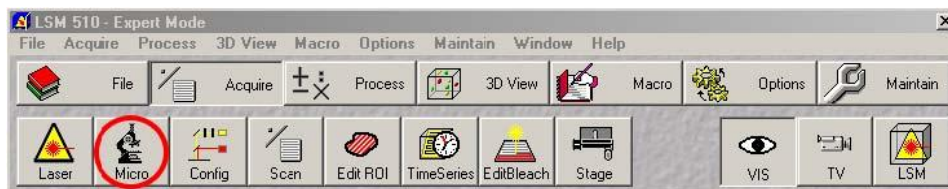


Figure 3: Button for opening microscope settings

All microscope manipulation is done through this panel (Fig. 4); do NOT change anything through the microscope itself except focusing and the stage position through the joystick. Use the joystick for large movements and the black round wheel for fine movements. Select first if you want to move along X or Y axis by pressing the corresponding X/Y button.

Choose the 40x/1.3NA objective by clicking the objective button. Apply a drop of oil on the lens of the objective and put your specimen on the stage so that it fits snugly the indentions -remember inverted position (coverslip down)! Use the metal clips to secure the slide.

To view your sample with mercury light source (HBO), select from REFLECTOR the filter appropriate for your staining and click REFLECTED LIGHT on. The proper filter for

- Alexa 594 is F SET 00.
- Alexa 488 is F SET 16.

Find an interesting place in your sample.

When you end viewing your sample through oculars, choose NONE for the REFLECTOR and click again on REFLECTED LIGHT to turn it off. Also check that TRANSMITTED LIGHT is off.

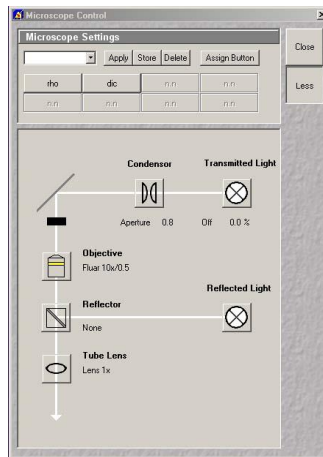


Figure 4: Microscope settings panel

### 6.3 Creating configurations

To view your sample using LSM 510 META, click LSM on the right (Fig. 5). This will stop any light from passing through the oculars.

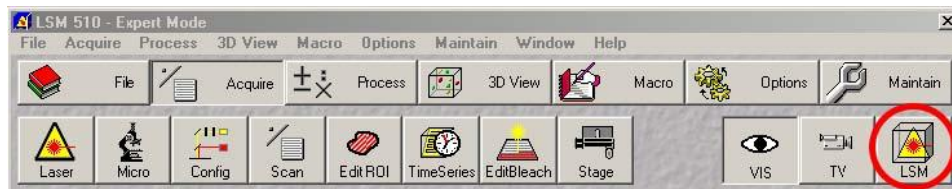


Figure 5: LSM button

To set the beam path configuration go to ACQUIRE / CONFIG (Fig. 7).

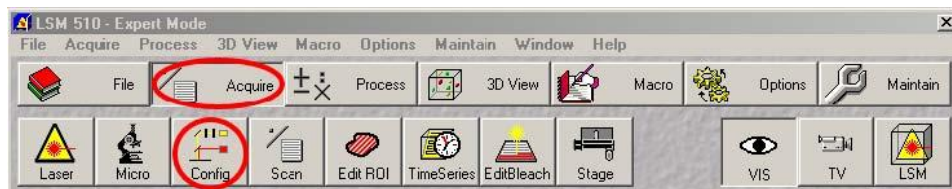


Figure 6: Config button

Since our sample uses multiple fluorochromes choose CHANNEL MODE / MULTI TRACK in the configuration control window (Fig. 8). Add a track so that you have two tracks in the configuration. Enable them both with the tick on the left side of the name of the track. Name the tracks Alexa 488 and Alexa 594 (overwrite the default names).

Create the following configuration to acquire the first track:

1. Excitation:
  - Click excitation button (1) and select Argon laser (488 nm) with 10% transmission rate.
2. Main dichroic beam splitter (HFT):
  - Choose excitation wavelengths that will pass to the specimen (these should match with the laserline you use). Use as few wavelengths as possible and do not use wavelengths that are within the emission spectrum as they are cut off.
3. Secondary dichroic beam splitter (NFT):
  - Choose mirror to divert all wavelengths to the detector Ch2. This setting could be used to split different wavelengths in two separate detectors.

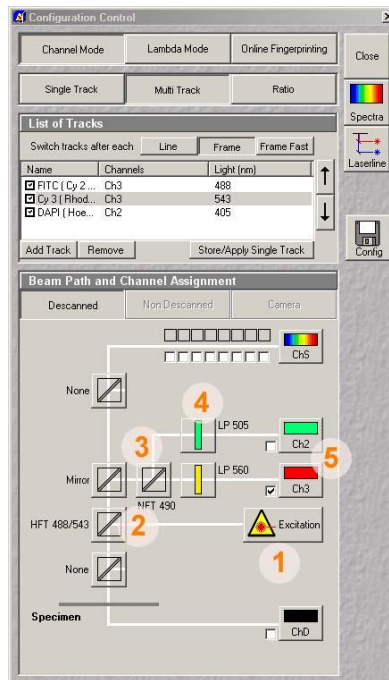


Figure 7: Configuration control window

#### 4. Emission filter:

- Choose a long pass (LP) filter that passes the emission wavelengths of the sample but stops the excitation wavelengths. Excitation is at 488 nm, so choose a long pass above that.

#### 5. Detector:

- Enable detector Ch2 by ticking the box next to its button. To change pseudocolor used for the signal, click the Ch2 button. Alexa 488 is green and Alexa 594 is red.

Make the configuration for the second track. First you need to highlight the name of the track. Then select HeNe1 laser (543 nm) with 75% transmission, and choose beam splitters and filters accordingly. You can check the configuration by clicking the Spectra button in the upper right corner of the configuration window. It shows the excitation wavelength and the wavelengths to be detected. Make sure you do not detect the excitation.

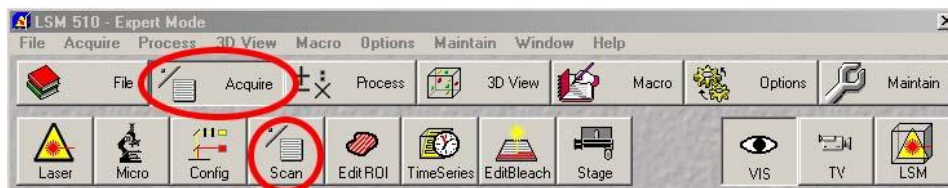


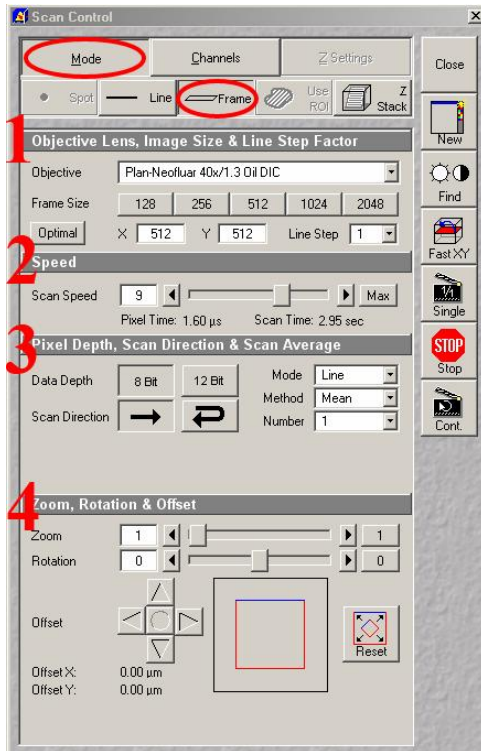
Figure 8: Scan button

### 6.4 Scanning images

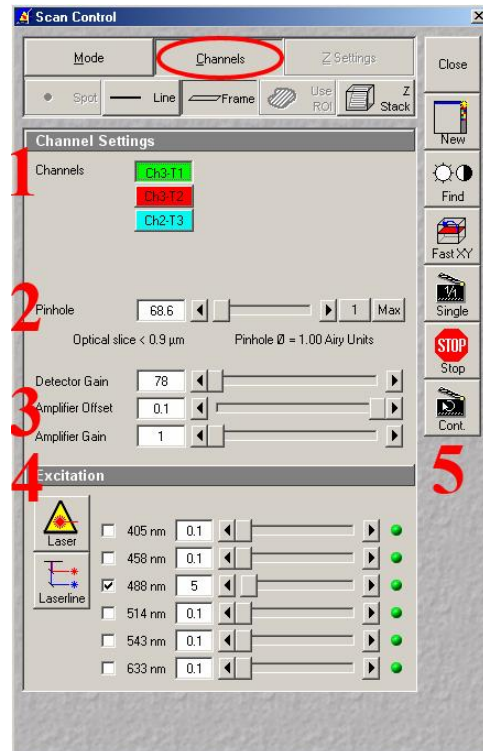
Go to ACQUIRE / SCAN in the main menu bar (Fig. 8). In the MODE / FRAME section of the scan control window (Fig. 10(a)), to speed up final adjustments, make sure that:

- frame size is relatively small (512 is good).
- scan speed is fast (9).
- data depth is 8 bits.
- zoom & rotation controls are reset.

Next go to CHANNELS tab in the scan control window (Fig. 9(b)). First select the Alexa 488 channel by clicking on its button, then click the “1” button. This sets the pinhole to 1 Airy unit. Write down the optical slice thickness shown below the pinhole size. Set pinhole size for the Alexa 594 channel to match the optical slice thickness of the first channel.



(a) Frame settings



(b) Channel settings

Figure 9: Scan control

Next go to CHANNELS tab in the scan control window (Fig. 9(b)). First select the Alexa 488 channel by clicking on its button, then select pinhole size to '1' by clicking the "1" button. This sets the pinhole to 1 Airy unit. Write down the optical slice thickness shown below the pinhole size. Set pinhole size for the Alexa 594 channel to match the optical slice thickness of the first channel.

Detector gain and offset are best set automatically with the Find button (brightness and contrast symbols on the right side of the window). Excitation (4) was already set earlier, but check that a correct laser is used with the correct channel. Adjust both channels for pinhole and gain/offset settings.

The Fast XY button on the side panel scans the sample in fast mode, so you can fine-tune the scan area and focus. Stop button interrupts scanning. The New button will create a new image window for the next scan. Scan your sample by using the Single button. For better image quality, you may increase frame size and/or slow down scan speed.

## 6.5 Saving images

When you save your images in LSM file format, all information about scanning parameters will be saved as well. LSM files can also be opened with the free LSM Browser (only for PC) that can be downloaded from Zeiss web page.

When you save your images in LSM file format, they have to be saved in a database. If you want to save your image in a new database, first click SAVE AS. Then, select NEW MDB to create a database. Give a name to the database. After creating a database, you can add images in it by using SAVE. A database is just a directory with LSM files inside.

To save the image in TIFF format, use the EXPORT function.

## 7 Problems

Think for later:

- How would you compare wide-field and confocal images?



## 8 Z-stacks

This part is an additional task. You can perform it if you have time after finishing with 2D images.

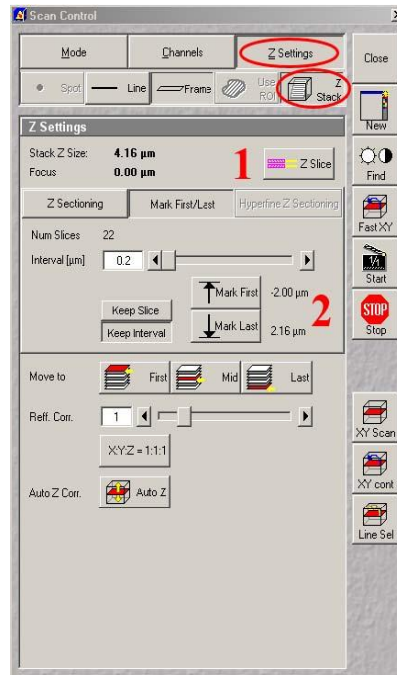


Figure 10: Z-Stack Controls

Start by going to Z SETTINGS / Z STACK in scan control window (Fig. 10).

For optical slice settings, click Z SLICE (Fig. 10, 1). OPTICAL SLICE window pops up. Click OPTIMAL INTERVAL. Optimal interval will set the interval so that adjacent slices overlap by 50 %.

Define first and last slices by clicking on MARK FIRST/LAST button (Below 1 in Fig. 10). Press XY cont on the panel on the right. During scanning, use the manual focusing knobs of the microscope to set the boundaries of your z-stack (rotate away from you = up, towards you = down). Do not let your Z-stack span more than 50 microns, and keep the slice number below 20, otherwise it will take a long time to scan the stack. Select the range so that you can see signal in all optical slices. When you have reached the top, click the MARK FIRST button. For bottom, click MARK LAST button. Click STOP to stop the XY cont scan, and click START to acquire the Z-stack.

By clicking GALLERY in the image window you can see all slices, and by clicking the DATA button on the right corner you will see the depth of each slice. ORTHO: orthogonal view (= xy, xz, yz planes for stacks), measurements in 3D.

1) <http://www.zeiss.com/C12567BE0045ACF1/Contents-Frame/CAA2EF638EC5F0D3C1256ADF0050E2F1>