Synthetic Neurobiology Confocal Microscope Protocol

V3.0 beta

NOTE: Do not use the confocal from these instructions alone! Be trained extensively first. Ask an experienced person to show you how to use it, and work with them for your first 3-4 times. A lot can go wrong using the confocal.

NOTE 2: To get familiar with the scope, **i**t's recommended that you get good at taking pictures on the camera on the confocal, before using it in confocal mode. This is beyond the scope of this document.

NOTE 3: Be sure you have access to E15-016, where the confocal is kept. Write Ed if you need to get access.

NOTE 4: Be sure to sign up on the Google Calendar, both to reserve the time, and so that we know who has been using it. Write to Ed to be added to the Google Calendar.

1. **Power Up**
   a. Follow instructions on the wall to turn on the mercury lamp and laser. These instructions are reprinted below, in case the sign gets lost. (compare to the steps below)
      i. Turn on the lasers
         1. Turn on Mercury Lamp (ebq 100) for fluorescence (on/off switch on white box, green lights will turn on). (There is another lamp box there, which is for the lower trans-illumination halogen lamp on the scope; you will likely not use that one.) Once turned on, you should not turn off the mercury arc lamp for at least 30 minutes, or risk burning out the bulb.
         2. Turn on main system power strip, cable tied to the frame supporting the air table.
         3. Turn on the two HeNe lasers (633, 543), by turning the key.
         4. For Argon Laser – BE CAREFUL! Use a flashlight if you can't see the knobs.
            a. Make sure toggle (silver toggle switch) is Standby (down position)
               i. Power knob (black knob) counter clockwise all the way
            b. Power On (black plastic switch)
            c. Key to I position (should start out in O position)
            d. Wait 3 minutes for warm up – VERY IMPORTANT
            e. Put silver toggle switch in run position (up position)
            f. Slowly turn power knob to 6.1 (i.e., small black triangle points to the sharpie-indicated dash)
   b. If needed (should turn on with the power strip), turn the microscope on – the green switch on the right of the scope
      i. The LCD screen display on top of the scope will turn on
   c. Computer login
      i. Username: neuro
      ii. Password: abacus35
      iii. Our data is on d:\AIM_DATA\neuromedia
   d. Launch Pascal 4.0 SP2
      i. Online Mode, Start

2. **Getting Ready**
   a. VIS - mercury arclamp mode
   b. LSM – laser mode
   c. Open four menus – position on left screen (keep right screen for scanning)
      i. Lasers – lasers that are on
      ii. Microscope Control – objectives, mercury arc lamp filters
      iii. Scan Control – zoom, resolution, z-stack
      iv. Configuration Control – laser filters, laser cubes, channel gains and offsets
   d. Click File -> New
      i. Make a database for your images
      ii. In D:\Neuromedia\yourUserID
      iii. Fill in properties if you want and minimize

Laser Control

Lasers

Laser Unit	Wavelength	Power
Argon	458, 488, 514 nm	25 mW
HeNe1	543 nm	1 mW
HeNe2	633 nm	5 mW

Microscope Control

Microscope Settings

Apply Store Delete Assign Button

n.n n.n n.n
n.n n.n n.n
n.n n.n n.n

Objective
EC Epiplan-Neofluar 20x/0.50 HD DIC M27

Reflector
None

Tube Lens
Lens LSM

Reflected Light
Off 0.0 %

Transmitted Light

Scan Control

Close New Find Fast XY Start Stop XY Scan XY cont Line Sel

Channel Settings

Mode Spot Line Frame Use ROI Z Settings Stack

Channels Ch2

Pinhole 158 Pinhole Ø = 1.89 Airy Units 1 Max

Optical slice < 6.8 µm

Detector Gain 844
Amplifier Offset 0.1
Amplifier Gain 1

Excitation

Laser

Line active Transmission [%]

458 nm	0.1	
488 nm	76.2	
514 nm	0.1	
543 nm	0.1	
633 nm	0.1	

Configuration Control

Channel Mode Lambda Mode Online Fingerprinting

Single Track Multi Track Ratio

List of Tracks

Name	Channels	Light (nm)
☑ 488 bandp...	Ch2	488

Add Track Remove Store/Apply Single Track

Beam Path and Channel Assignment

LSM 510 Camera

NFT 545 HFT 488 None

Specimen

BP 505-530 Ch2 Excitation

None Ch1

Spectra Close Config

LSM 5 PASCAL

File Acquire Process 3D View Macro Options Maintain Window Help

New Open Save Save As Import Export Multi Print

File Acquire Process 3D View Macro Options Maintain Exit

3. **Configuration**
   a. **See screen shot above for good screen layout. (Ed prefers to swap the scan control and configuration control from what is shown above.) You should use the right monitor to store new pictures.**
   b. Microscope Control
      i. Click on Objective – pick desired objective. DO NOT TRUST THE LABELS ON THE MENU – inspect the objective on each setting first. People swap the objectives a lot on this confocal, and the labels are not correct, most of the time.
      ii. Bio versus Material lenses
         1. Make sure you are not using Material lenses – look for the indent / rim / reflector in the lens – **epiplan = a phase lens, not a fluorescence lens**
         2. To remove, unscrew it, place in the holder, and replace with the Bio lens (our 20x or 63x lens)
         3. We have at least four bio lenses – 2.5x, 20x, 40x, 63x. The first three are air lenses, the last is an oil lens. Do not use air lenses with oil!
         4. If you need to place the slide holder in the stage – remember, the red dot goes with with spring in the back right corner. Do not force the slide holder into the stage!
      iii. Click on Reflector – pick FITC for GFP/Alexa 488, TxRed for Alexa 568/rhodamine, FSet46HE for Texas Red/mCherry.
         1. Check the filters, make sure the correct light is visible coming up from the lens
      iv. Push all black metal filter blocks on the right side of the microscope in until they click, and raise the apertures to full open. Make sure the light mirror in the back, that toggles between halogen and mercury, points to mercury lamp.
      v. The button on the front of the scope, with the triangle on it, increases or decreases the light power of the halogen lamp on the top.

   c. Configuration Control
      i. Click on Multi Track, if you want to scan different colors. (This next section is easiest to explain in person)
         1. Add Track – should be only one track, if you have one color to see
         2. Store/Apply Single Track – select desired configuration. Apply – loads. Store – overwrites (or saves the configuration to a new name).
         3. Example: 488 with bandpass
            a. Excitation laser – 488, for GFP
            b. Mirror – HFT 488, which bounces 488 towards the specimen
            c. Second Mirror – NFT 545, bounces light bluer than 545 towards Ch. 2 PMT
            d. Filter before Ch2 – BP 505-530
            e. Set Ch1 to green color for ease of visualization
         4. Only use Ch1 if you need to filter by long pass (e.g., if you have a second color, or the bandpasses don't work) – **ADVANCED TOPIC**
            a. In general, Band Pass (Ch. 2) takes cleaner images than Long Pass (Ch. 1), but Long Pass takes brighter images than Band Pass
   d. Scan Control
      i. Click on Mode
         1. Set Frame Size for 512x512 (typical), 1024x1024 or higher (publication quality)
         2. Make sure it's on 8 Bit
         3. Scan in One Direction, to the right
         4. Best to use a 63x oil and zoom out if you can
      ii. Click Channels
         1. Lets you set gain, offset, etc.
         2. Amplifier gain should be 1, amplifier offset should stay at 0.1 or so

3. You will change the detector gain, and the laser power transmittance, chiefly
4. For each channel, click "1" for the pinhole, and remember the minimum across all the pinholes. Then set all of the pinholes to this minimum.

4. Capturing Images
   a. VIS mode
      i. Switch to VIS (Acquire -> VIS)
         1. To see brightfield, set Reflector = None, Transmitted light = 10% (you can control with the mouse or with the button on front of the confocal), Eye/camera switches set to Eye.
      ii. Turn on a reflector – FITC for GFP, for example. NOTE: the colors may be mislabeled. For example, "Texas Red" may point to rhodamine, whereas "FSet46" could point to Texas Red. Look at the color for each before using. BLUE = FITC/GFP/Alexa488, GREEN = rhodamine/Alexa568/Cy3, GREEN-YELLOW = mCherry/TexasRed, INVISIBLE = DAPI (UV!).
      iii. Find area of interest (use 20x before 63x – 63x is oil, see below, and will get oil on your sample, and is also harder to see)
      iv. Turn off Reflector the instant you are done looking through the eyepiece, as you are continuously bleaching your sample with light.
      v. Turn off the halogen lamp too (using the front-microscope knob or mouse), to avoid bleaching, and also during fluorescence monitoring.
   b. LSM mode
      i. Turn off Reflector
      ii. Scan Control -> Fast X/Y to capture. Use FastX/Y (to avoid going to slow, use just one channel – the most informative channel!) while you turn the focus to find the focal plane. Turn the detector up, too. Once you find the layer you want by turning the focus, you can click "Find" if you like.
         1. Use the Histogram to set just a few % of the pixels at saturation, manuaklly adjusting Detector Gain and Laser Transmission, to achieve the right image.
         2. Note that confocal focus doesn't always equal eye focus.
         3. You can adjust the pinhole to collect more light, but use this as a very last resort. Else, use the method in 3.d.ii.4, above.
      iii. Click Single to get final image
      iv. SAVE YOUR IMAGE. (Later you can re-open the file, and click the green "Reuse" button to reuse all the old software-settable settings!! Important for being quantitative, and for scanning similar ways over time.)
   c. Z Stack mode – **ADVANCED TOPIC**
      i. if needed, select "mark first/last.
      ii. Click Fast X/Y, find the brightest layer using the focus, and adjust detector gain/laser transmittance to make that layer look good, as described above.
      iii. Focus with knob until you get to one edge of the focus – click Mark First
      iv. Focus with knob until you get to another edge – click Mark Last
      v. Stop
      vi. Adjust Interval based upon the optimal slice (e.g., optical slice, as determined by the pinhole setting, may be 6.8, hence Z Stack Interval should be able the same – but use fewer to go faster, or get a cleaner picture, or bleach less). (Widely-spaced z-stacks can make for very clean, very 3-D looking, images.)
         1. This is why Z-stacks looks really good at hi mag, if you want closely-spaced optical slices.
      vii. Click Start
      viii. SAVE YOUR IMAGE
      ix. Click Slice in Image window, scroll through slices
      x. Now you can make 3-D rotating movies/image sequences using the "3D" menu.
   d. Using the Oil Lens – **ADVANCED TOPIC**
      i. Rotate to 63x oil lens

  ii. Place a drop of Immersol 518F oil (no other!) on the lens using the included safe spatual in the oil bottle. Use a small amount. See how this is done by an expert.

  iii. Switch to VIS mode (see above)

  iv. Find the right area to image

  v. You have very limited time before the slice becomes bleached – 63x is bright!

    1. Practice on reliable dummy samples

  vi. Switch to LSM mode (see above)

  vii. Click Fast X/Y

  viii. Adjust stage location, focus, and channel parameters to find the cells. In particular, you may need to adjust pinhole as your optical slice thickness will be smaller.

    1. Cheating: Make Pin Hole bigger and Gain smaller to get a low noise picture – but note, this will blur in the z-axis greatly. A good last resort.

  ix. When done, wipe off the oil with the lens paper, using lens cleaning solution – do not use anything else (KimWipes, etc.) as they will permanently damage the lens.

  x. DO NOT USE OIL ON ANY OTHER LENS than the 63x.

  xi. Clean your slide (NOT THE LENS) with a kimwipe or lens paper and some windex, wiping very very gently so as not to disturb the coverslip.

 e. Saving

  i. Always click Save As in Image Window

  ii. Try opening the Image to ensure it was saved properly – Click File / Open

  iii. Back up your files onto a jump drive

    1. Sometimes, rarely, files are corrupted

5. Shutting down

 a. Follow the steps on the wall (compare to the steps below)

  i. Exit Pascal software

  ii. Shut down the computer using the Ctrl-Alt-Del + shutdown or the start menu.

  iii. For Argon laser

    1. Turn power knob counter clockwise (off)

    2. Toggle (silver switch) to Standby

    3. Key to 0 position

    4. Wait 3 minutes

    5. Power off

  iv. Turn off other lasers

  v. Turn off main system power strip (under air table)

  vi. Turn off mercury lamp

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6. Viewing confocal images

 a. Install the program File\neuro\archive\Inst_ib.exe

General comments from Brian:

You can capture fluorescence images from the camera too but the Lsm confocal software must be opened first. If you open the axio vision software first, then you will get port errors requiring a reboot. If the Lsm is open first, it will say there is a port error but it works fine.

Follow the directions for the argon laser very carefully. Do not skip the proper waiting time because you will destroy the laser. For the hbo lamp (and any high power xenon or mercury lamp), do not turn off the lamp within 30 minutes of turning it on and vice versa. You will stress the bulb and ruin its lifetime. The replacement is over $1k. Note that I am NOT referring to the halogen bulb on the transilluminator that most will use. You can turn that on and off at will for the most part.

Although we have extra filters, do not change the filters without talking to me or Manu first. However if you can't find one of us, then make sure the scope is off and all software is shutdown. If you change it while the scope or software is on, you can destroy the filter cube holder. I will spare you the details.

Unless you will take it upon yourself to maintain the security weekly, don't ask to put the computer online. It has been compromised too many times. Once the computer goes down so does the scope for about one month because that is how long it takes Zeiss to fix it.

Do not leave a mess! It is not our machine and it is one of the bread winners when we give tours to CBA sponsors. No CBA means no money for the shop, fab equipment, SEM, etc.; pretty much everything useful in the basement that is not in 044.

The 40X will not collect any fluorescence, and may give you funny images even if you are imaging by eye or with the CCD camera instead of the PMT in confocal mode. It is highly specialized and expensive lens designed to image through multiple semi-thick stacks of materials with different indices of refraction. It is very useful for materials characterization and micro-fabricated devices, but not much else. So, you will have to jump between the 20X air and 63X oil lens. If you want something in between, you can use the zoom function as a kluge. Note that if you zoom in from the 20x, you will bleach your dyes much faster.

The algorithm on the autofind function is only so smart, and mostly changes the detector gain given a fixed pinhole and laser power. If you change the offset or amplifier gain, it may give you different parms if you autofind again.

The camera on the scope has a massive field of view compared to most CCDs. So, if you need semi-macro images, it is not a bad option. If you need true macro images, the Manalis Lab has a Nikon Coolpix with an eyepiece mount that works very well up to maybe 1cm [Note: out of date information]. Unfortunately, the camera is less sensitive to the fluorescence than most. Also, light can come through the eye piece and effect your imaging with the camera (confocal PMT is unaffected), so you may have to close the curtains, or flip the switch directly above the toggle between eyepiece and camera. That switch changes the focal distance of the tube for other Zeiss eyepiece parts, and by doing so, the light coming through the eyepiece will interact with your sample less.

Note, this final item is a matter of personal preference, but I advise that if the image is a "money shot" you capture it twice. Once with the optimal settings for the three main parameters (detector gain, offset, and amp gain), as well as with only the detector gain altered (i.e. offset and amp gain are set to 0 and 1, respectively). The effect of altering the latter two parameters can effectively be done in software, either with the LSM package or Photoshop. By changing them during acquisition, you can lose a lot of information from your image that is irrecoverable, most notably if you change the offset too much. The reason for suggesting acquiring twice is as follows. Some families of journals like Nature and ACS don't allow you to alter things in software like Photoshop without full disclosure of exactly what you have done, so you want the image altered during acquisition (those of you who have ever submitted images of gels probably know annoying this is). However, some journals like PNAS do allow you to Photoshop images as long as it doesn't affect your quantitative analysis or the integrity of the image. In that case, you want to Photoshop it later.