

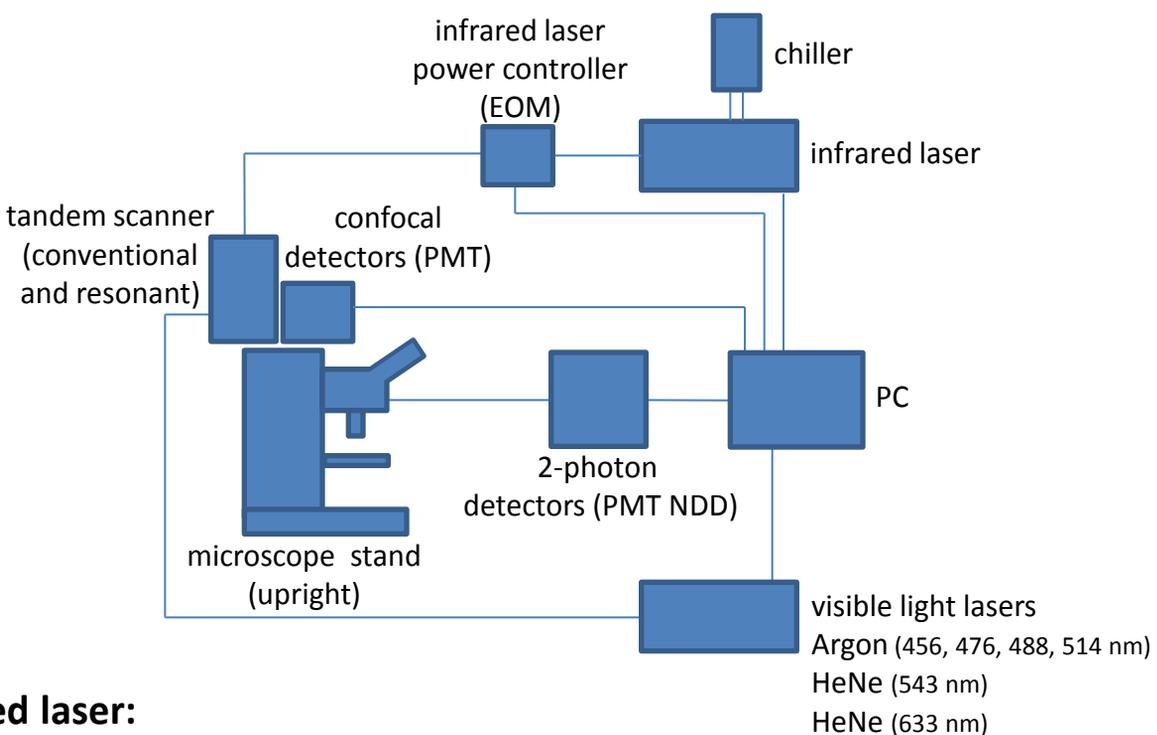
LEICA SP5 2-PHOTON/CONFOCAL SYSTEM

User manual



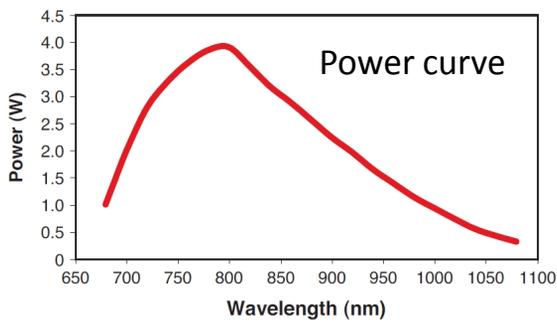
LA JOLLA INSTITUTE
for
ALLERGY & IMMUNOLOGY

COMPONENTS OF THE LEICA SP5 SYSTEM



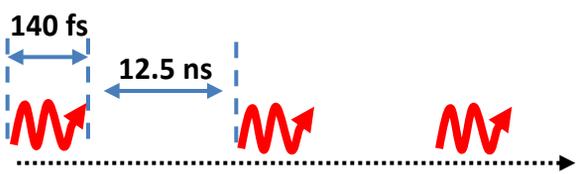
Infrared laser:

Chameleon Ultra II (Coherent)

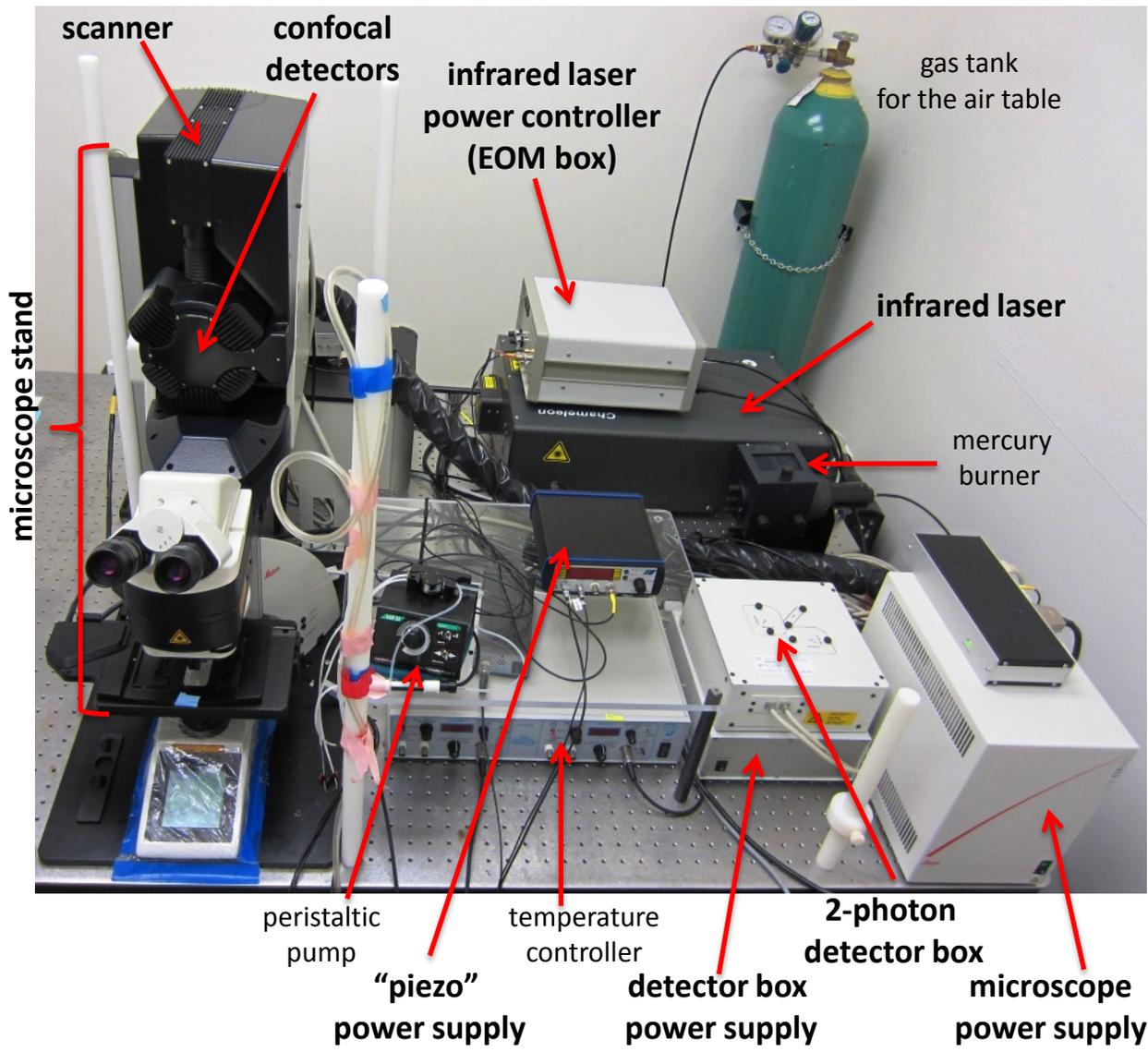


- Ti:Sapphire laser
- Tunable excitation range: 680-1080 nm
- Tuning speed = 40 nm/sec
- Pulse width = 140 fs
- Peak power > 3.3 W

Pulsed laser

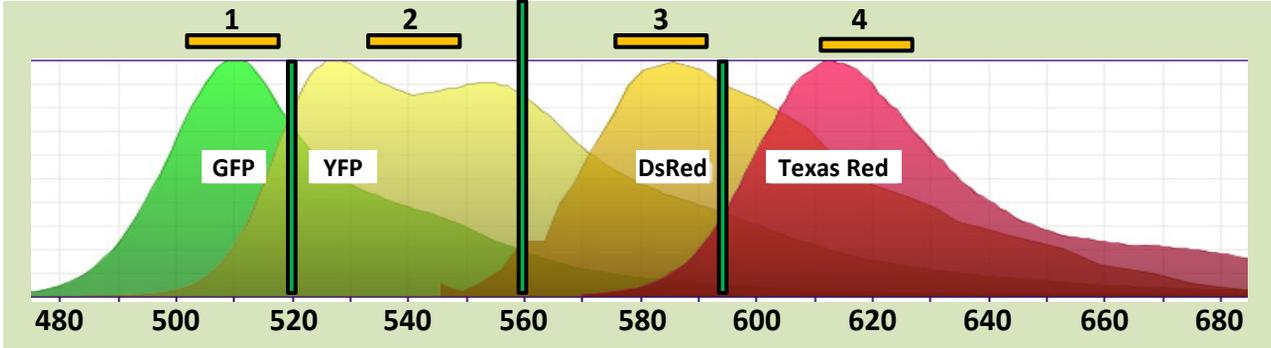


COMPONENTS OF THE LEICA SP5 SYSTEM

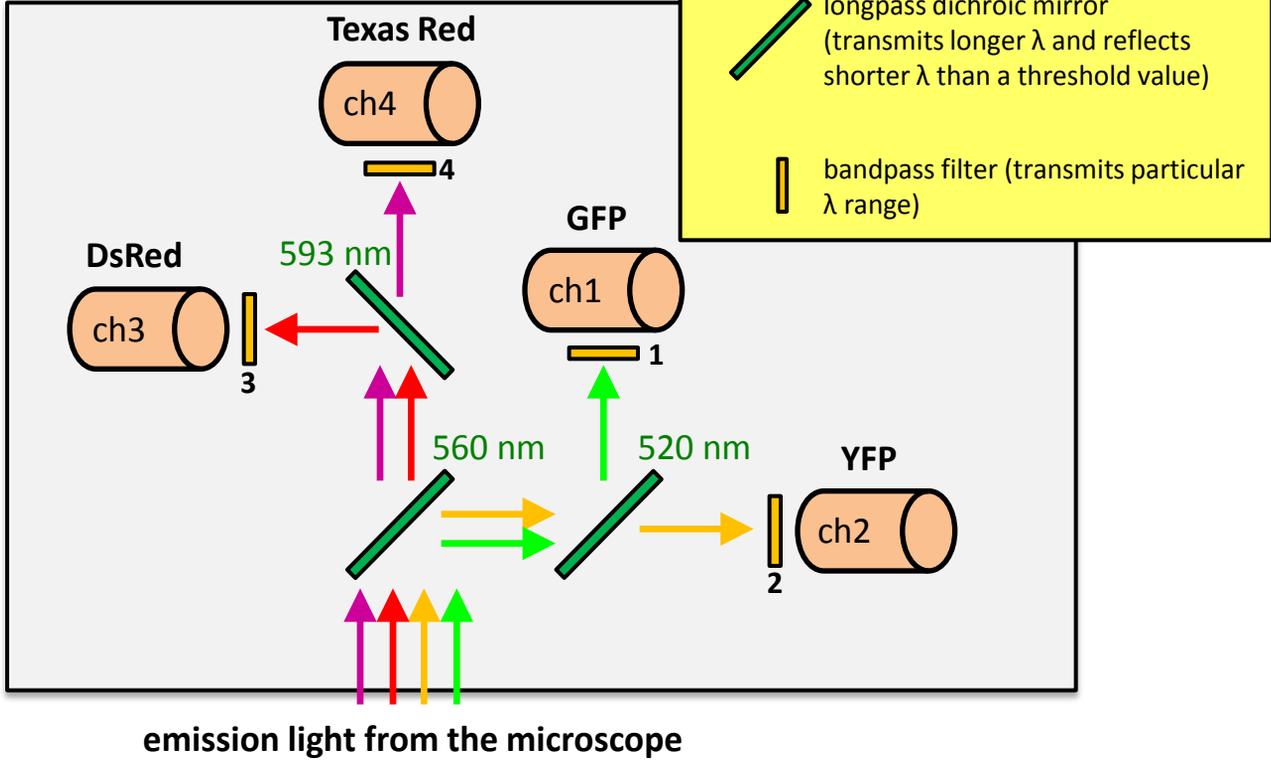


EMISSION LIGHT PATH IN NON-DESCANNED DETECTOR (NDD) BOX

example: separation of GFP, YFP, DsRed and Texas Red



Example setup of the detector box



Configuration of mirrors and filters is modifiable and can be changed to suit your needs.

**Available bandpass filters for the NDD box
(central wavelength/width of the band in nm)
for example: 460/50 filter transmits 460 ± 25 nm (435-485 nm range)**

- 460/50
 - 475/20
 - 513/17
 - 525/50
 - 535/22
 - 535/25
 - 536/40
 - 542/27
 - 576/10
 - 586/20
 - 585/40
 - 610/75
 - 624/40
 - 650/50
 - 655/12
 - 692/40
-
- 680 shortpass
 - 700 shortpass

**Available dichroic longpass mirrors for the NDD box
(longer wavelengths than the indicated threshold value pass through, shorter
wavelengths get reflected)**

- 495
 - 506
 - 514
 - 520
 - 560
 - 562
 - 593
 - 605
 - 620
 - 648
 - 660
-
- 495/605 (Transmission Bands: 505-550 and 620-675; Reflection Bands: 454-485 and 570-598 nm)
 - JP4 CFP-YFP (transmits both CFP and YFP emission)

Starting up the Leica SP5 2-photon/confocal system

Follow steps 1-4 if using the infrared laser for the 2-photon imaging.
To use the confocal mode with visible light lasers proceed to the step 5.



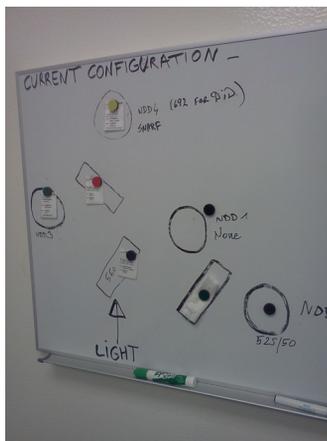
1
Check the chiller's temperature (should be ~20°C).



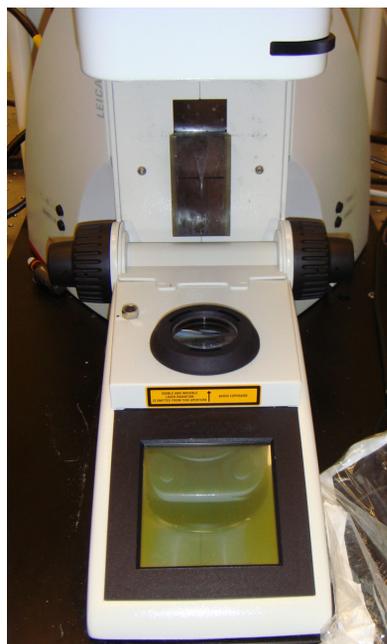
2
Turn on the EOM box (laser power controller) - the ORANGE switch.



3
Turn the key on the laser power supply to the ON position.

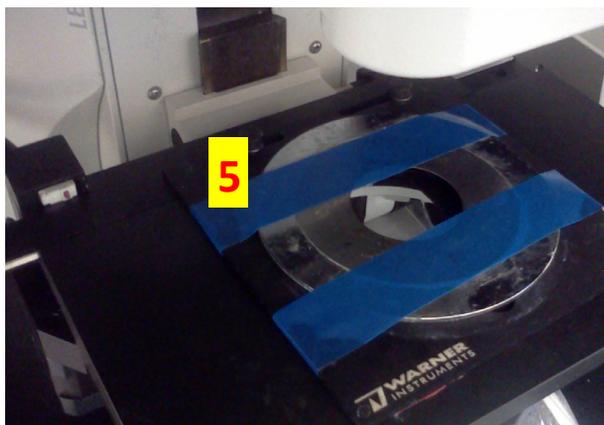
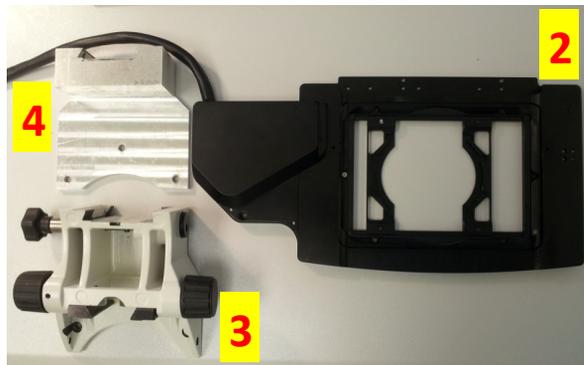


4
Check whether the filter configuration in the detector box is suitable for your experiment. If not, **ASK FOR ASSISTANCE IN CHANGING THE FILTERS/DICHOIC MIRRORS**. Do not open the box if the system is powered on (= the GREEN button #1 is ON on the main switch board).



5
Check the stage configuration. Choose a stage/objective adapter that is appropriate for your experiment. **ASK for assistance if not sure. DO NOT ADJUST OR CHANGE STAGE ADAPTERS WITH OBJECTIVES MOUNTED IN THE TURRET!!!**

Stage and objective adapters



1. Main “wide” stage.
2. SuperZ galvo stage - to be mounted onto the main stage, inner insert moves vertically independent of the main stage. **THIS STAGE IS USED ONLY FOR SLIDES AND DISHES.**
3. Main stage mounting adapter (by Leica).
4. Main stage mounting adapter (custom made) - enables larger working distance with bulky objects on the stage. **RECOMMENDED WHENEVER THE OLYMPUS 20X DIPPING OBJECTIVE IS USED.**
5. Stage accessory plate (by Warner Instruments) mountable onto the main stage - enables xy positioning.
6. Custom made stage accessory plate - alternative to “Warner” plate.
7. Objective adapter (“piezo” - galvanometric actuator) - moves the objective vertically independent of the main stage - max. travel distance 300µm. **RECOMMENDED FOR LIVE IMAGING.**
8. Power supply and controller for the “piezo”.
9. To use the “piezo” or the SuperZ galvo stage connect the adapter to the scan head by inserting a proper plug into “Z-Stage” position in the back of the scan head. Secure the plug with screws.

6

On the main switch board switch on the button #1 and #2 if you only want to use the infrared laser (**2-photon imaging**).

To use the visible light lasers (**confocal imaging**), additionally switch on the button #3 and turn the key #4 to the ON position.

Button #1 powers up: the computer, the microscope stand, the detector box.

Button #2 powers up the scan head.

Button #3 powers up the visible light lasers and a cooling fan for the Argon laser.

Key #4 initializes the visible light lasers.



7

Turn on the mercury burner power supply to be able to look at the sample through the eyepiece. The “lamp” diode should turn solid green. The bulb life time is ~200 hrs. If it starts flickering ask for assistance.



8

Log into your LIAI account and open LAS AF acquisition software.



9

If you plan to do live imaging with the highest acquisition speed check the box “Activate Resonant Scanner”. For static imaging leave this box unchecked and the system will be activated with a conventional scanner.

For the conventional scanner a default speed value is 400 Hz (400 lines per second). Higher speeds require a smaller size of the scanned field and automatically change the zoom value: zoom 1.7 at 700 Hz, zoom 3 at 1000 Hz, zoom 6 at 1400 Hz. Using the bidirectional mode doubles the speed.

For the resonant scanner only one speed is available: 8000 Hz (16000 Hz in bidirectional mode) and the minimal zoom is 1.7. In addition to high scanning speed the other advantage of the resonant scanner is decreased photobleaching due to shorter laser dwell time. This mode, however, generates noisier images which can be compensated with more line averaging during the acquisition.



Leica Application Suite

Advanced Fluorescence
2.6.0.7266

Init Hardware Server...
Started Server
Check configuration: -Piezo without condenser

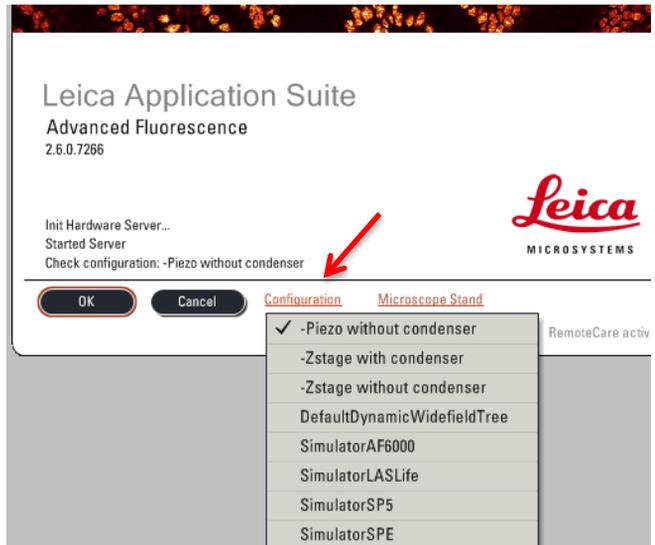


10

Click on the configuration button. A menu will appear with different options:

- Piezo without condenser - suitable for **LIVE** imaging using the “piezo” objective adapter,
- Zstage with condenser - imaging of **SLIDES** with transmitted light detection using the SuperZ galvo stage,
- Zstage without condenser - imaging of **SLIDES** without transmitted light detection using the SuperZ galvo stage.

Select the appropriate one and accept by clicking OK.



11

If you plan to do automated multi-area imaging, using joystick place the stage in the middle position (vertically) and then click YES once this window pops up. The stage will move around establishing the xyz limits. **MAKE SURE THAT OBJECTIVES (IF MOUNTED IN THE TURRET) WILL NOT BE DAMAGED BY ANY OBJECTS ON THE STAGE DURING THE INITIALIZATION.**



12 Default view of the Leica SP5 software

SYSTEM CONFIGURATION TAB

PROCESSING/ANALYSIS TABS

ACQUISITION TAB

objective selection

dichroic mirror selection (confocal imaging)

control panel of infrared laser power (2-photon imaging)

control panel of visible lasers power (confocal imaging)

3 internal detectors (confocal imaging)

4 external non descanned detectors (NDD) (2-photon imaging)

1 external detector for transmitted light (confocal imaging)

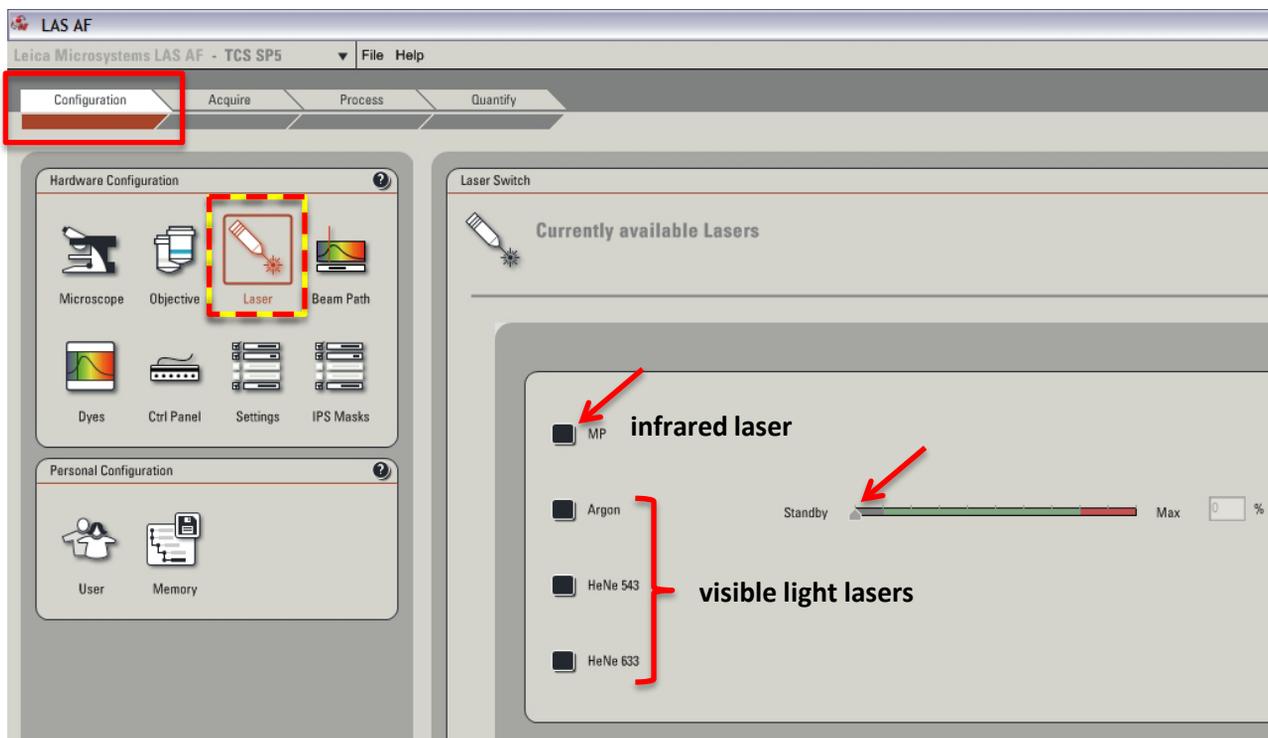
acquisition parameters control window

13

Configuration/Laser tab

If you use the infrared laser the MP (multiphoton) box in the “Configuration/Laser” tabs will be red provided the key on the laser power supply was turned to the ON position.

To activate the visible light lasers (**confocal imaging**) check the black boxes next to the laser names only for the lasers you need to use. When using the Argon laser move the slider to ~30%.

**Infrared laser:**

The infrared laser is tunable between 680 nm - 1080 nm. See example 2-photon excitation wavelengths of different fluorophores at the end of the manual.

Visible light lasers:

The Argon laser has 456, 476, 488, 514 nm excitation lines. The 456 and 476 nm lines are weak, whereas 488 and 514 nm lines are strong (avoid photobleaching of your fluorophores). HeNe 543 nm laser is weak, whereas HeNe 633 nm laser is strong.

Argon 456 nm suitable for CFP.

Argon 476 nm suitable for GFP.

Argon 488 nm suitable for green fluorophores: FITC, AF488, GFP.

Argon 514 nm suitable for yellow fluorophores: YFP.

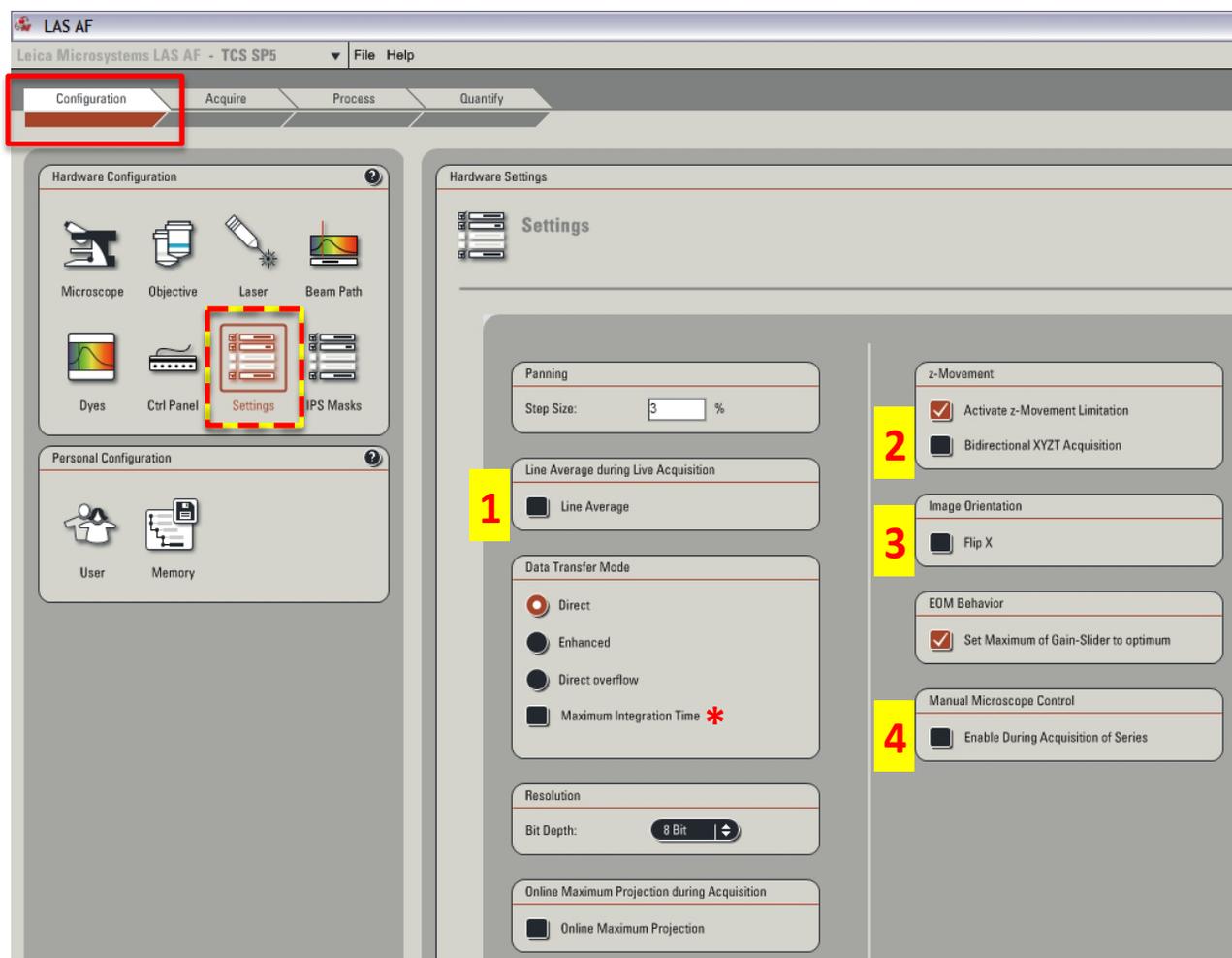
HeNe 543 nm suitable for orange/red fluorophores: PE, AF555, RFP, Cy3.

HeNe 633 nm suitable for far red fluorophores: APC, AF647, Cy5.

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Configuration/Settings tab

Default view



Things to check that may help (otherwise leave the default settings):

1. when scanning live it reduces image noise on the screen at a chosen value of line average (recommended when the resonant scanner is activated)
2. when acquiring a time lapse of a volume (xyzt mode) the scanning starts from the stack's begin to the end position and proceeds to the next time point starting from the previous end position - this option improves the accuracy and the speed of acquisition.
3. select to match the view of the sample through the eyepiece with the screen display - use also -90 degree rotation of the field of view in the Acquire tab.
4. when using the "piezo" adapter it enables additional manual control of the stage position to compensate for a tissue drift.

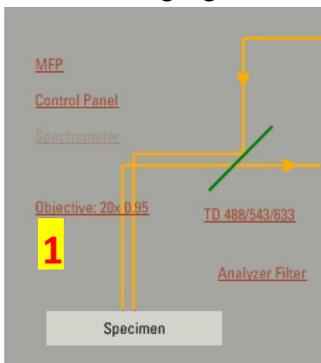
* checked by default in the resonant scanner mode, can stay unchecked in the conventional mode.

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Setting up the 2-photon imaging

- **Mount an objective into a proper position in the turret.**

Recommended objective for the 2-photon imaging is the Olympus 20x 0.95NA water dipping objective. To identify the position of an objective in the turret click the button 1 and expand a drop-down list of objectives (2) that are configured within the software. **ONLY IF THE STAGE IS FULLY LOWERED AND CLEAR OF ANY OBJECTS AND “PIEZO” IS NOT MOUNTED** you can click a particular objective from the list and the turret will automatically move to the position of the objective of choice. This objective will be then highlighted in the list.

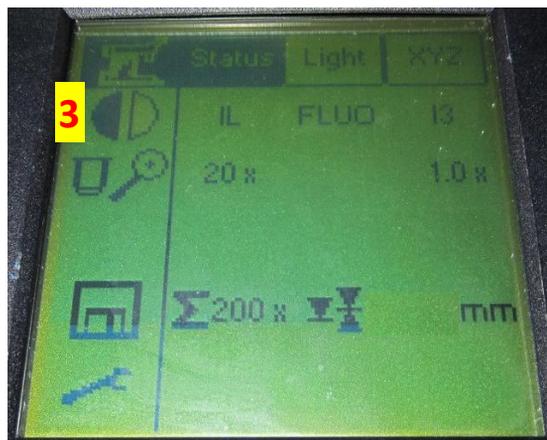


✓	HCX APO L 20x 0.95 IMM
	HCX PL APO lambda blue 20x 0.7 IMM
	HCX PL APO CS 40x 1.25 OIL 2
	HCX PL APO CS 63x 1.4 OIL
	HCX PL APO CS 63x 1.3 GLYC 21°C
	HCX PL APO CS 10x 0.4 DRY
	HCX APO L 20x 0.95 IMM

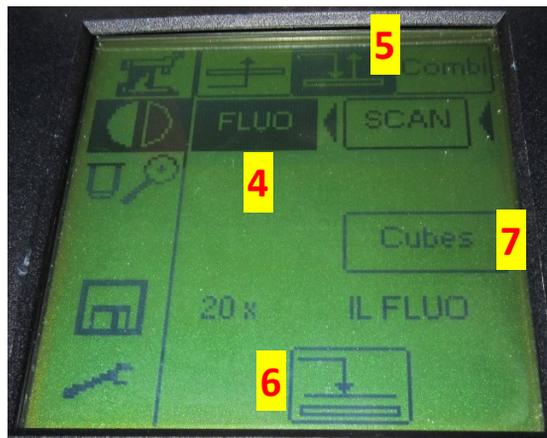
The Olympus 20x objective is named “HCX APO L 20x 0.95 IMM” and there are two positions assigned for it (“top” and “bottom” in the list). “Top” position is set up for the “piezo” adapter and has a metal ring for mounting the “piezo”. If you are going to use the “piezo”, mount it first on the turret and then screw the objective in but not “super” tight.

- **Bring your specimen into focus.**

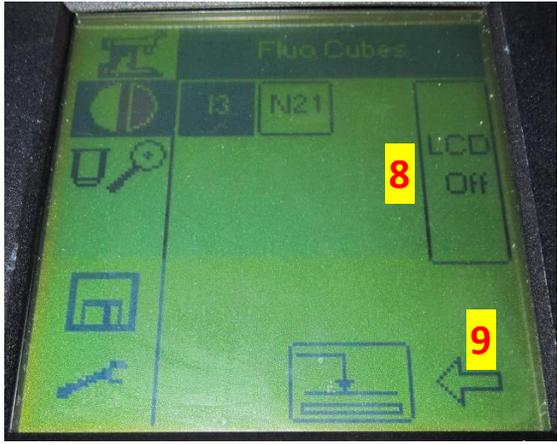
Default screen display after turning on the microscope



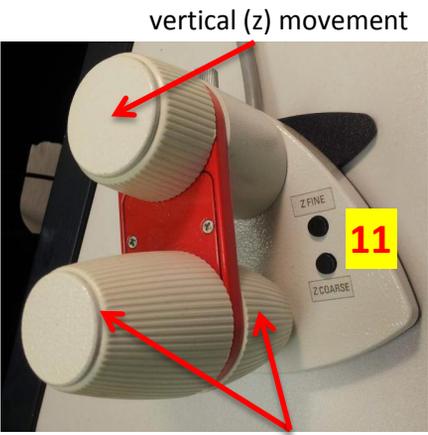
To observe the sample through the eyepiece open the shutter using the touchpad screen in the microscope stand. Press buttons 3, 4 and 5 to find the shutter button (6).



To switch between filter cubes use the button 7. Available filters: I3=GFP/FITC, N21=RFP/AF555. **SWITCH OFF THE DISPLAY BY PRESSING THE BUTTON 8 FOR THE 2-PHOTON IMAGING.** To go back to the previous screen press the button 9.

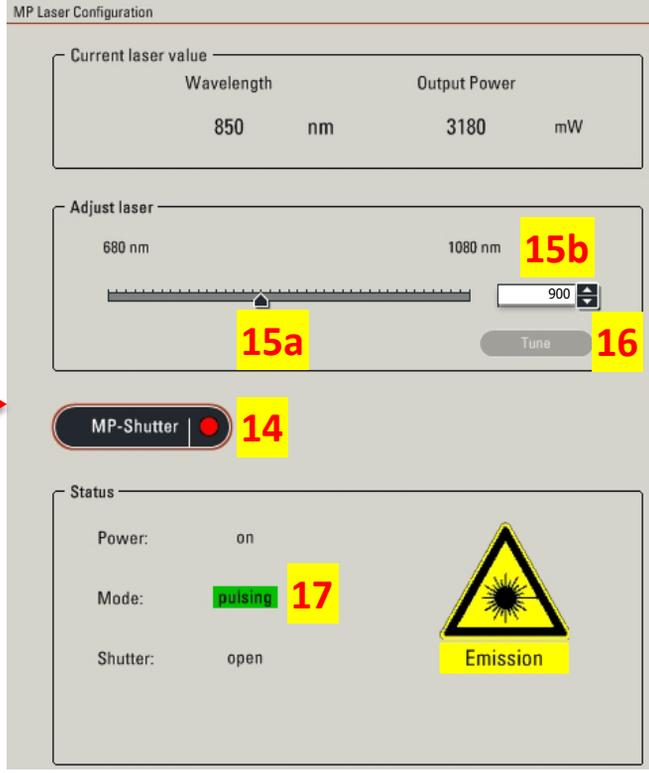
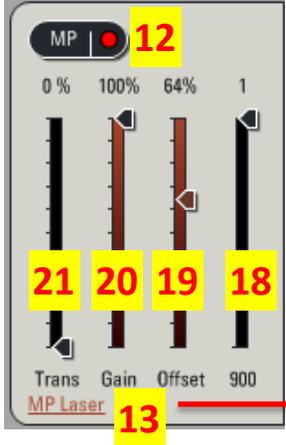


After you start scanning through the software, the system automatically switches to the “scan” position - button 10. To look at the sample through the eyepiece again press the button 4 and open the shutter - button 6.



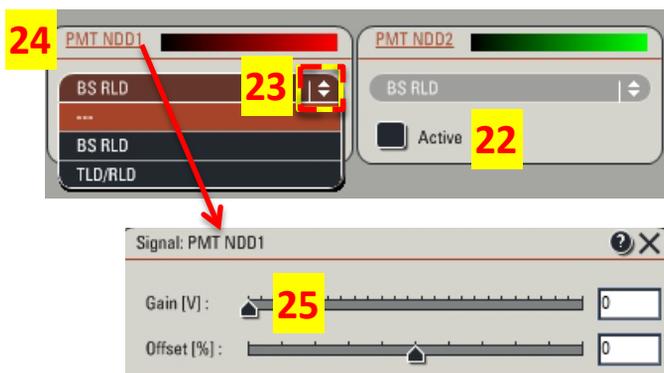
To position your sample on the stage use the joystick. Adjust the stage position with fine/coarse buttons (11) for xy and z movements on both sides of the joystick.

• Activate and adjust the infrared laser power.



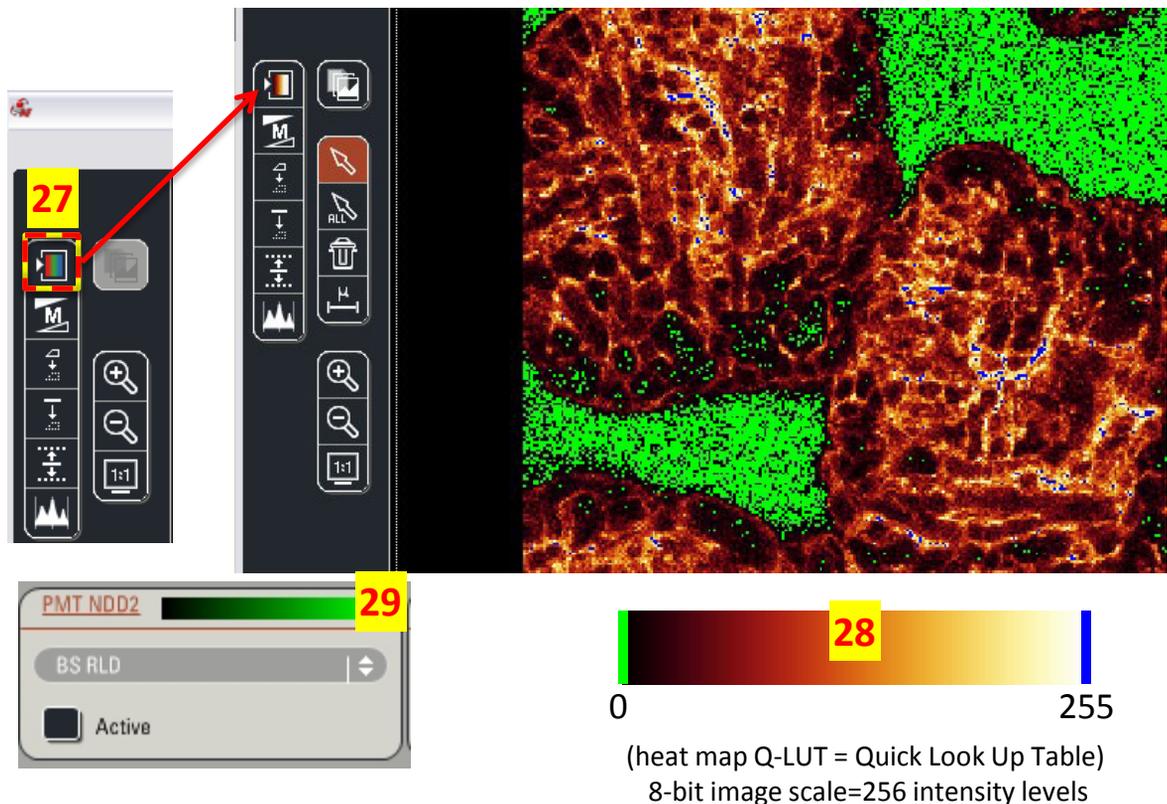
THE INFRARED LASER AT WAVELENGTHS ABOVE ~750NM IS NOT VISIBLE AND IF SET TO HIGH GAIN/TRANSMISSION CAN BURN THINGS. BE CAREFUL!!!

- To enable scanning with the infrared laser (MP mode) click the button 12.
- Click the “MP Laser” button (13) - a new window (“MP Laser Configuration”) will pop up.
- To open the shutter press and hold (2 sec) the button 14.
- Set a proper excitation wavelength by moving the slider (15a) or typing in a number (15b) and accepting with ENTER.
- Click the button 16 to tune the laser. After tuning the laser should be in a pulsing mode (17).
- Close the “MP Laser Configuration” window.
- Move the slider (18) up to position 1 (after tuning to a chosen wavelength, its value should be indicated below the slider).
- The offset slider (19) position is automatically linked to the laser wavelength and doesn’t have to be changed (provided that “EOM Behavior “option is checked in the “Configuration/Settings” tabs).
- Use the gain slider (20) to adjust the laser power. You can start with 50% gain, transmission (Trans) 0% (slider 21). **NEVER SET BOTH SLIDERS TO 100% AT THE SAME TIME AT WAVELENGTHS OF HIGH LASER POWER: 725-850nm** (see the laser power curve earlier).
- **Activate and adjust PMT NDD detectors**



To enable the detection of 2-photon excited signal activate external PMT NDD detectors - click the button 22 for each detector you are going to need. **FIRST SWITCH OFF THE MAIN LIGHT AND SWITCH ON THE DESKTOP LAMP. ALWAYS DEACTIVATE THE DETECTORS BEFORE SWITCHING THE MAIN LIGHT BACK ON!!!**

- Expand the menu (23) and choose “TLD/RLD”. It will automatically apply this setting to all active detectors.
- To set the gain on the detector click the PMT NDD button (24). Use the slider (25) or type in a value and accept with ENTER. You can start with gain values ~1000.
- Click “Live” button (26) to start live scanning. If you do not see any signal increase the laser’s gain. If it does not help check the gain values on detectors. If the values are already around 1200 (maximum 1250) then move up the transmission slider to bring more light into the sample.
- To have a full dynamic range of intensities in the image set the detector gain/laser power to values that give you a small number of oversaturated pixels, which can be viewed using a quick look-up table (LUT) (click once the button 27 - right monitor screen to get a heat map, clicking twice will give a gray scale image). The oversaturation (intensity of 255) is indicated with blue color and the intensity of 0 is green (28). To remove signal noise from the detector and obtain the 0 intensity value in the image you can adjust the detector offset if necessary - typical values: -0.15% to -1%.
- The gain and offset values on the detectors can be also adjusted through the control panel below the monitors. “Smart Gain/Offset” knobs regulate the values of a currently active channel, which is a channel that is selected/highlighted on the right monitor after clicking on it.
- Assign colors to channels - click the horizontal color bar (29) and choose a LUT of preference (can be changed after the acquisition).



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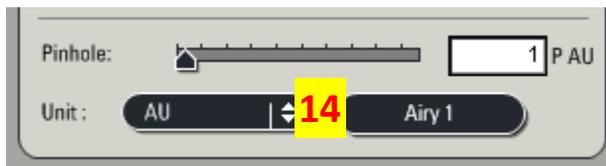
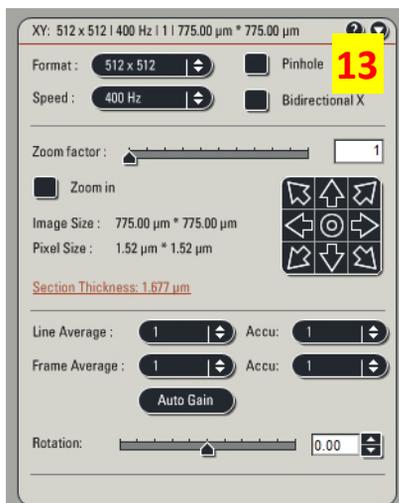
Setting up the confocal imaging

The screenshot shows the Leica confocal software interface with several key areas highlighted by numbered callouts:

- 1a**: Objective selection button.
- 1b**: List of objective lenses.
- 2**: Visible channel selection.
- 3**: Laser power sliders for different channels.
- 4**: Spectrometer wavelength scale.
- 5a**: Analyzer filter selection.
- 5b**: Filter wheel selection.
- 6**: Active channel checkboxes.
- 7**: Channel name dropdown.
- 8**: Spectrometer wavelength scale.
- 9a**: Spectrometer wavelength scale.
- 9b**: Spectral Range dialog box.
- 10**: PMT 1 gain control.
- 11**: PMT 3 gain control.
- 12**: Leica Settings dropdown.

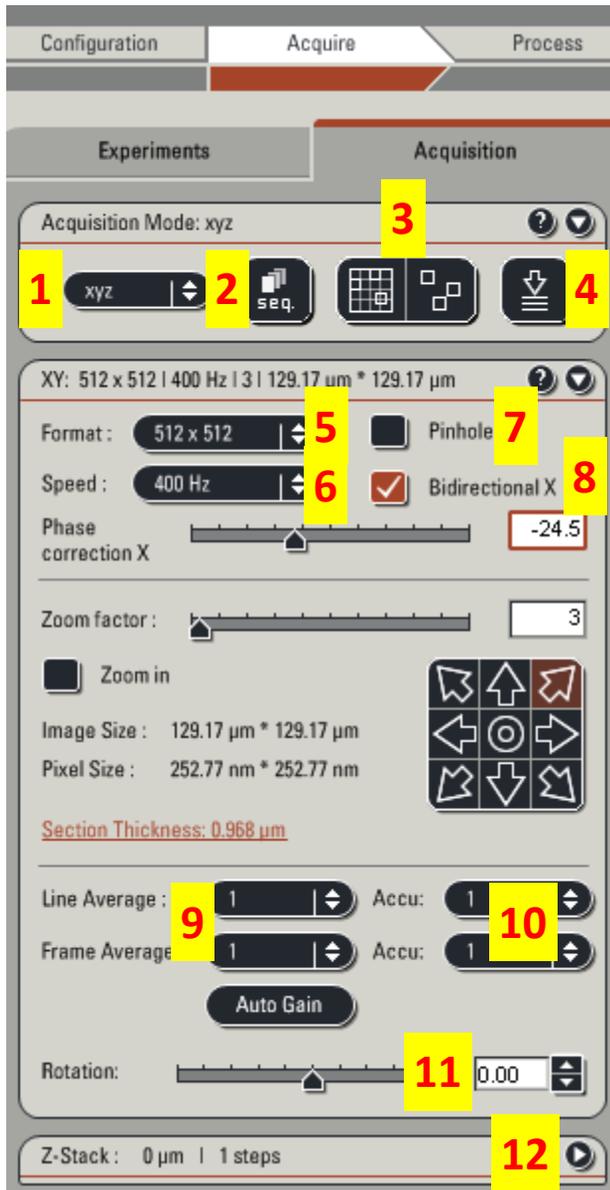
- Mount objective(s) into a proper position in the turret. To identify the position of an objective in the turret click the button 1a and expand a drop-down list of objectives (1b) that are configured within the software. **ONLY IF THE STAGE IS FULLY LOWERED AND CLEAR OF ANY OBJECTS AND “PIEZO” IS NOT MOUNTED** you can click a particular objective from the list and the turret will automatically move to the position of the objective of choice. This objective will be then marked in the list.
- Bring your specimen into focus. See instructions on the page 13.

- Click the button 2 to activate the visible light lasers.
- Use sliders (3) to set the laser power. As starting values you can set 488 nm line to 25%, 543 nm line to 75%, 633 nm line to 25%. The active laser lines will appear in the light spectrum display (4).
- Choose a dichroic mirror that is compatible with laser lines used (5a, 5b):
 - when using 488, 543 and 633 nm lines use a triple dichroic TD 488/543/633;
 - when using 488 and 543 nm you may choose DD 488/543 or TD 488/543/633;
 - when using 488 nm you may use RSP 500 or DD 488/543 or TD 488/543/633.
 In general, dichroic mirrors remove a reflection of the laser light that can be detected by the detector in the channel for a particular fluorophore causing increased background.
- Activate a PMT detector (6) that is the closest to the laser line used for excitation of your fluorophore.
- Expand the menu (7) and find your fluorophore to get a hint how its emission spectrum looks like - it will appear in the light spectrum display (8). If it is not on the list, use a spectra viewer on the internet available through major antibody providers (i.e. Invitrogen, Pharmingen, eBioscience).
- Set a spectrum range for the detection of your fluorophore based on its spectral properties. You can change the size of the bar (9a) - shrink or expand by pulling the edge of the bar or double-click on the bar and type in values for the chosen range (9b). **DO NOT PLACE THE BAR OF AN ACTIVE DETECTOR BELOW THE ACTIVE LASER LINE.**
- Set the gain on the detector by clicking the PMT button (10) and assign colors to channels (11) - follow the instructions described on page 16. Adjust your settings during live scanning.
- Leica's example settings for different fluorophores can be found and applied after expanding the list (12). You can also save your own settings and re-apply them in future experiments ("Save" button).
- Adjust the pinhole size if necessary. To do so activate "Pinhole" option (13) in the Acquire tab. Change the unit display (14) from "μm" (default) to "AU" - arbitrary units. The value 1 gives you the optimal resolution and rejection of out-of focus light. However, if your staining is dim and increasing the detector gain and laser power does not help set the pinhole to 1.5-2 AU to bring more light from the sample (image resolution will be compromised though).



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Setting up the image acquisition parameters (2-photon/confocal imaging)



1. Mode of acquisition: volume, time lapse, spectral. Typically: xyz - static volume stack; xyzt - volume stack over time; xyt - one plane over time.
2. Sequential acquisition with different settings (see below).
3. Tile mode and “mark and find” mode - automated multi-area acquisition (active when the stage was initialized). **Request training to use it.**
4. Autofocus - not functional in the system.
5. Image format - size of the image in pixels.
6. Scanning speed (default 400 Hz - conventional scanner, 8000 Hz - resonant scanner).
7. Pinhole size - only in the confocal mode (see earlier), not used for the 2-photon imaging.
8. Scanning mode - if “bidirectional X” activated the scanning speed doubles (=acquisition in both directions). Use the slider or type in a value accepted with ENTER to align the scanning phases for both directions - “Phase correction”. The value may need to be adjusted when changing the zoom.
9. Line/frame averaging - method of detector noise reduction. The selected number determines how many times a line or a frame is scanned to produce an image.
10. Line/frame accumulation - an image is produced by adding a signal from a chosen number of consecutive line/frame scans.
11. Field of view rotation. If set to -90 degree with “Flip X” option activated in the “Configuration/Settings” tab the image on the monitor screen will match the image seen through the eyepiece.
12. Expand this window to set up the imaging volume if in xyz or xyzt mode (see below).

Practical considerations - image quality vs. speed

The slower scanning speed the better image quality.

The higher scanning speed the smaller field of view imaged with a larger zoom (conventional scanner).

The larger image format the better image resolution but the longer acquisition.

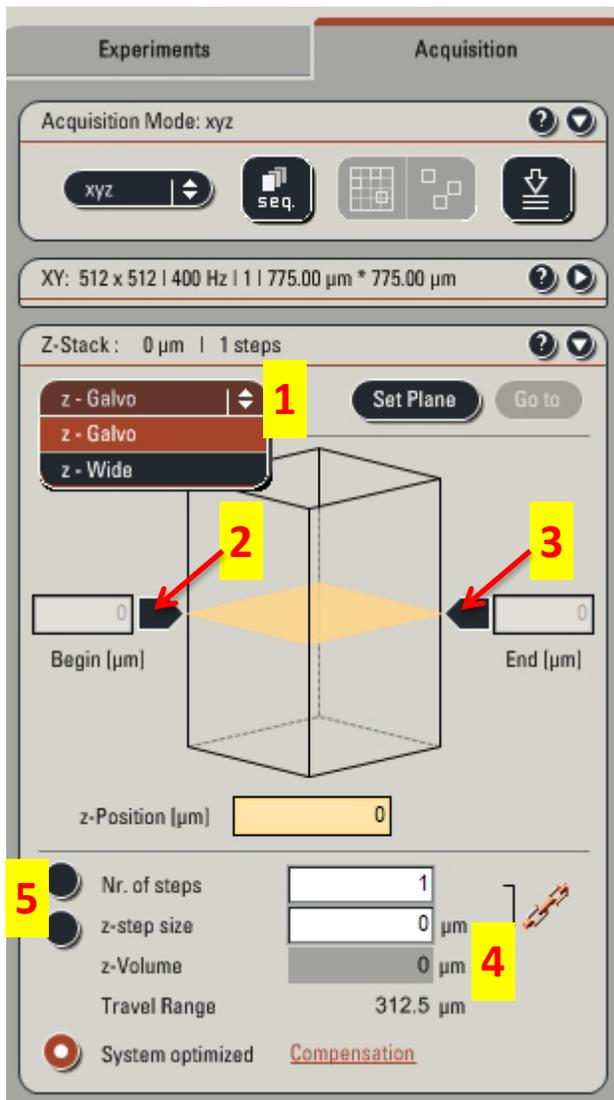
The larger value of line/frame averaging the better image quality but the longer acquisition.

The longer acquisition and the larger zoom the bigger chances of photobleaching and phototoxicity.

Suggested settings when recording a time lapse:

Conventional scanner: image format 512x512 pixels or smaller with 1-3 line averaging at 400-1000 Hz in the bidirectional mode;

Resonant scanner: image format 512x512 pixels or smaller with at least 8 line averaging in the bidirectional mode.



Setting up the imaging volume

- Choose which method of Z positioning (1) you will use. Z-Galvo stands for the “piezo” objective adapter or SuperZ galvo stage depending on which one is connected to the microscope stand. Z-Wide stands for the main stage. To change the scanning position in z-axis :
 - in the z-Galvo mode use a knob on the control panel below the monitors (last knob on the right - default setting)
 - in the z-Wide mode use the joystick.
- To set up the begin and the end of the imaging volume click (2) and (3), respectively (black button will turn brown). To overwrite previous positions unclick and click again the buttons at particular positions in z-axis. When the begin and the end are selected the scanning volume size will be displayed (4). When using the “piezo” the maximum travel distance is ~300 µm, for the SuperZ galvo stage ~1500 µm, the main stage - several centimeter range.
- Choose number of planes to image or z-step size (5) for the scanning of the chosen volume.

Setting up the sequential scanning

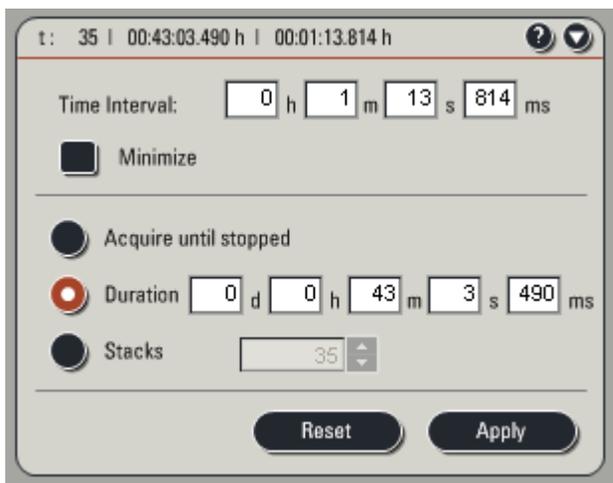
When the sequential mode is activated a new window pops up, where you can create different settings for each scan. Click “+” to add or “-” to remove a setting. When the infrared laser is activated two scanning options are possible:

- between frames - scanning proceeds to the next setting after acquiring a full frame
- between stacks - scanning proceeds to the next setting after acquiring a full stack, if in the xyz mode.

In the confocal mode there is an additional option of sequential scanning between lines - the requirement here is that the emission ranges of all used internal detectors have to be in an identical position for all created settings.



When doing the sequential scanning of a live sample there is always a time delay between consecutive scans (especially when scanning between stack) so the sample may look different in analyzed frames from two settings in the sequence (for example, if you image fast moving cells). Between lines (confocal) and between frames scanning gives a higher level of spatiotemporal accuracy between the sequential scans. Sequential settings can be saved and re-applied in future experiments (“Load” and “Save” buttons).

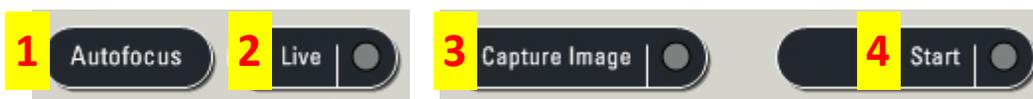


Setting up the time lapse scanning

- When the xyzt or xyt mode is activated a new window pops up, where you can create settings for the time lapse.
- Activate “Minimize” to determine what the minimal interval is between time frames with chosen acquisition parameters. You can type in the interval values of choice and accept with ENTER.
- Set the duration of acquisition or a number of time frames to be recorded.

18

Starting the acquisition

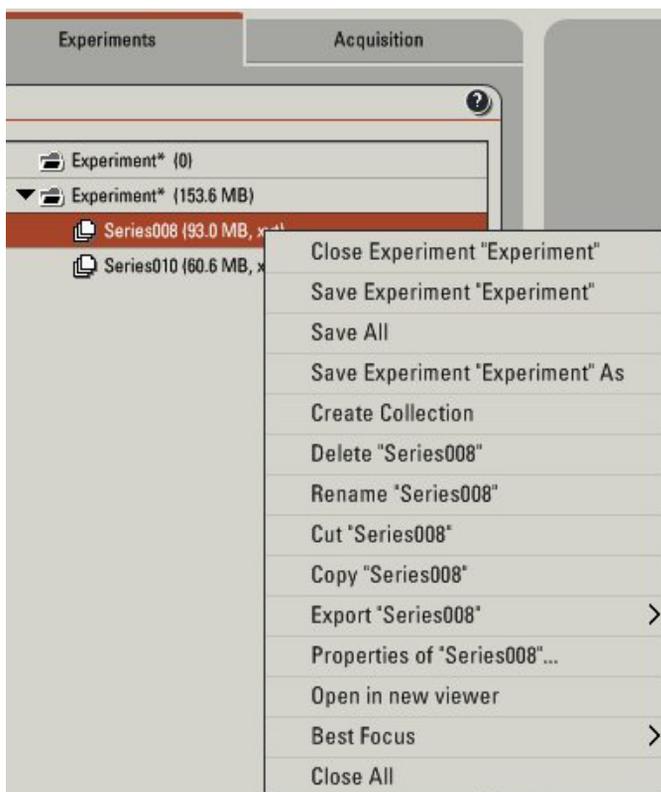


1. Autofocus option is not functional in the current system
2. Live scanning for adjustments of the sample and setting up the acquisition parameters.
3. Acquisition of a last previewed live image (one focal plane).
4. Full acquisition with chosen parameters - z-stack , time lapse or sequential acquisition.

19

Saving the experiments

- Click the Experiments tab (next to the Acquisition tab). You will see a list of images/series of images that you acquired. You can organize the images by renaming them or placing them in “collections”. To create a collection right-click anywhere in a field with a current experiment and choose “Create Collection” from a drop-down list. Then drag an image/series into the collection or select several images/series and CUT and PASTE into the collection.
- To save your data right-click anywhere in a field with a current experiment and choose “Save Experiment” or “Save Experiment As”.



- Save the experiment on the D drive in a folder with your name.
- Transfer your data to the public or your lab server as soon as possible.
- Leica format is *.lif which can be opened through:
 - Leica acquisition software (installed also on the work station in the Microscopy Suite) and free downloadable viewer : LAS AF Lite (viewing and exporting but no processing, PC only),
 - Imaris software on the work station in the Microscopy Suite (viewing, processing, exporting),
 - ImageJ with the LOCI/Bio-Formats plugin installed - free software (viewing, processing, exporting, not very user friendly).

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Shutting down the system

- Check the booking intranet webpage whether anybody is using the system after you.
- **IF SOMEONE IS USING THE SYSTEM AFTER YOU WITHIN NEXT 2H:**
- Deactivate all detectors.
- If using the infrared laser:
 - Close the laser's shutter.
 - Set the gain and transmission sliders of the infrared laser to 0%.
 - Deactivate the MP mode in the Acquire tab only.
- If using the visible light lasers:
 - Set the laser power control sliders to 0%.
 - Deactivate the visible lasers mode in the Acquire tab only.
- Close the software.
- Log off.
- Remove and clean objectives.
- Switch off the "piezo" power supply and remove the adapter from the turret.
- **IF NOBODY IS USING THE SYSTEM AFTER YOU:**
- All steps listed above.
- If using the infrared laser:
 - Switch off the EOM box.
 - Turn the key on the infrared laser power supply to the STAND BY position. Do not switch off the power supply.
- If using the visible light lasers:
 - In the Configuration/Lasers tab set the Argon laser power to 0% and deactivate all used lasers.
 - Turn the visible laser initialization key to the OFF position.
 - Wait 10 min (or until you hear the cooling fan switching off) and then switch off the visible laser power button (green button 3).
- Shut down the computer.
- Switch off the scanner button (green button 2).
- Switch off the microscope/computer button (green button 1).
- Switch off the mercury burner power supply.

QUICK GUIDE FOR THE 2-PHOTON LIVE IMAGING (resonant scanner):

1. Check the temperature on the chiller.
2. Turn on the EOM box.
3. Turn the key to the ON position on the infrared laser power supply.
4. Check the detector configuration - modify if necessary.
5. Check the stage configuration - modify if necessary.
6. Turn on the microscope and the computer (green button 1).
7. Turn on the scanner (green button 2).
8. Turn on the mercury burner power supply.
9. Log in to your LIAI account and start the Leica software.
10. Check the resonant scanner box.
11. Choose the "piezo without condenser" configuration.
12. Initialize the stage if automated multi-area acquisition is planned.
13. Mount the "piezo" adapter onto the turret and screw the objective in.
14. Switch on the "piezo" power supply.
15. Focus your sample.
16. Switch off the microscope display (LCD off).
17. Put the microscope cover on.
18. Activate the MP mode in the Acquire tab.
19. Open the MP shutter.
20. Set the excitation wavelength.
21. Move the first slider in the MP mode from position 0 to 1.
22. Set the MP gain slider to an initial value ~50%.
23. Deactivate internal PMT1 (active by default).
24. Switch off all the lights and dim the monitors.
25. Activate PMT NDD detectors.
26. Choose TLD/RLD mode from a drop-down list on active PMT NDD detectors.
27. Set PMT NDD gain to an initial value ~1000.
28. Click "Live" to scan.
29. Adjust the MP gain/transmission and/or PMT NDD gains to have a full dynamic range of intensities in the image - choose a heat map Q-LUT.
30. Choose an acquisition mode (xyz, xyzt...)
31. Choose an image size (512x512 or other).
32. Choose a scanning mode (unidirectional by default, bidirectional upon activation).
33. Adjust the phase if bidirectional scanning chosen.
34. Choose a line average value for noise reduction.
35. For xyz or xyzt acquisition set top and bottom of the scanning volume in Z-galvo ("piezo") mode and choose step size.
36. For xyzt or xyt acquisition set time interval and duration of the time lapse.
37. Click "Start " to acquire data.

QUICK GUIDE FOR THE CONFOCAL STATIC IMAGING OF SLIDES (MULTICOLOR SEQUENTIAL BETWEEN FRAMES SCANNING):

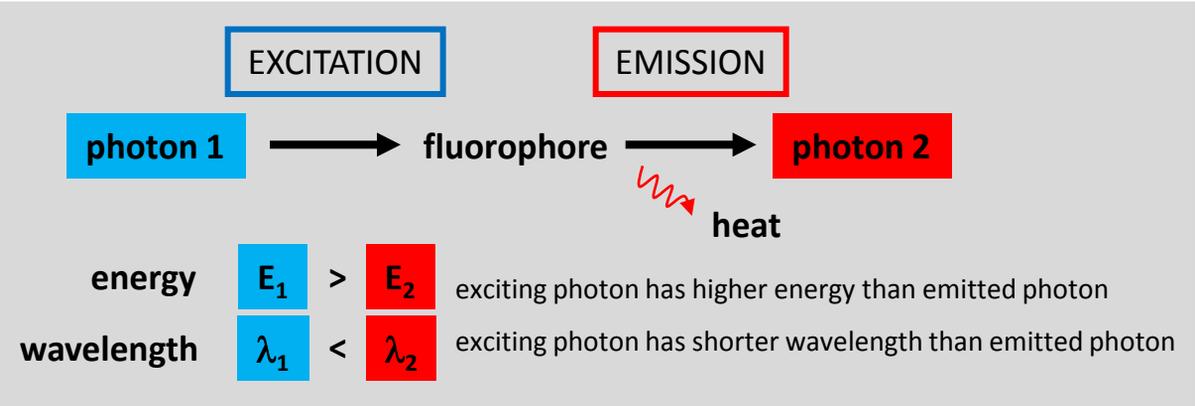
1. Check the stage configuration - modify if necessary.
2. Turn on the microscope and the computer (green button 1).
3. Turn on the scanner (green button 2).
4. Turn on the visible laser power supply (green button 3).
5. Turn the key (#4) to power up the visible light lasers.
6. Turn on the mercury burner power supply.
7. Log in to your LIAI account and start the Leica software.
8. Leave the resonant scanner box unchecked (will activate the conventional scanner).
9. Choose a proper configuration ("Z-stage with/without condenser" for SuperZ galvo adapter).
10. Initialize the stage if automated multi-area acquisition is planned.
11. Mount objective(s) into proper positions on the turret.
12. Focus your sample.
13. Activate necessary visible lasers in the Configuration/Lasers tab.
14. Activate the visible laser mode in the Acquire tab.
15. Activate sequential mode (if imaging more than 1 fluorophore).
16. Select a proper dichroic mirror compatible with activated laser lines.
17. Create settings for scan 1.
18. Select "between frames" scanning mode.
19. Set an initial value of laser power slider to ~30-50% (the Acquire tab).
20. Activate a PMT detector (closest to the chosen laser line).
21. Set PMT gain to an initial value ~800.
22. Adjust the width of the PMT bar to set the fluorophore's emission range.
23. Click "Live" to scan.
24. Adjust the laser power slider and/or PMT gain to have a full dynamic range of intensities in the image.
25. Activate pinhole and change its size if the signal is too low (resolution compromised).
26. Add additional scans to create setting for remaining fluorophores.
27. Repeat steps 19-25 (keep same pinhole size for all scans).
28. Choose an acquisition mode (xyz, xyzt...)
29. Choose an image size (512x512 or other).
30. Choose a scanning speed.
31. Choose a scanning mode (unidirectional by default, bidirectional upon activation).
32. Adjust phase if bidirectional scanning chosen.
33. Choose zoom (partially determined by the scanning speed).
34. Choose line average value for noise reduction.
35. For xyz acquisition set top and bottom of the scanning volume in Z-galvo (SuperZ galvo stage) or Z-Wide (main stage) mode and choose step size.
36. Click "Start " to acquire xyz data or "Capture Image" to acquire current focal plane.

Appendix

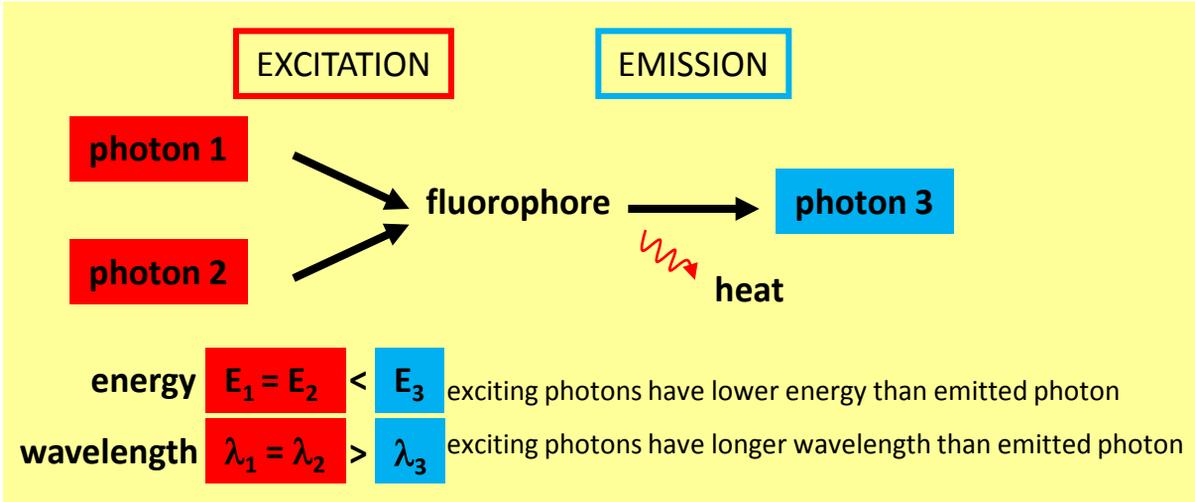
- **principles of the 2-photon imaging**
- **2-photon excitation spectra**

SINGLE vs. 2-PHOTON IMAGING

SINGLE-PHOTON EXCITATION

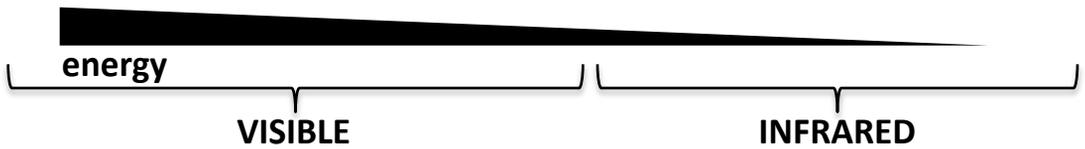
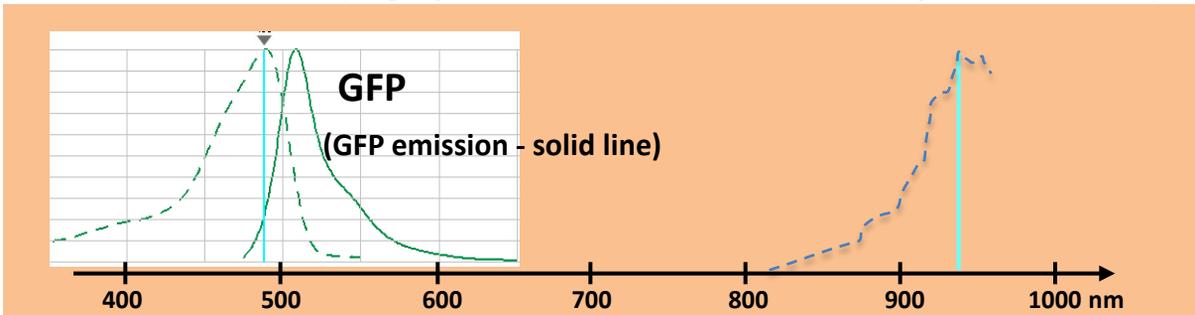


2-PHOTON EXCITATION



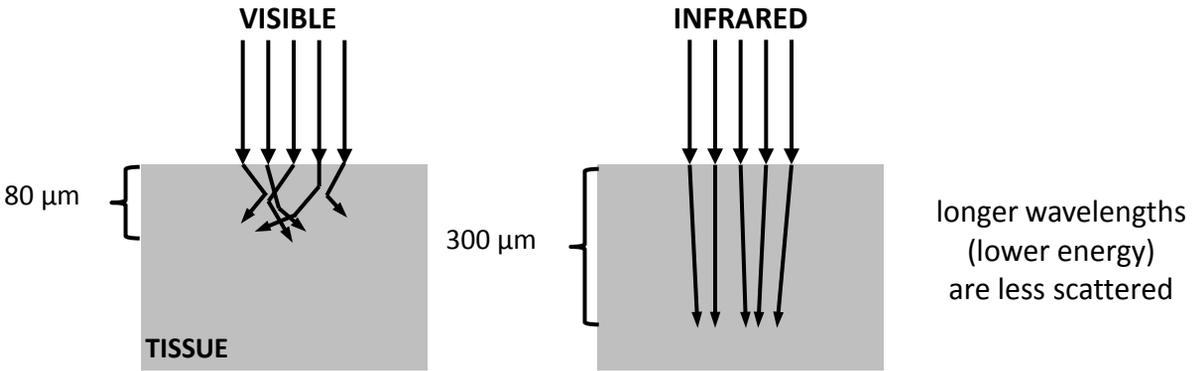
488 nm (single-photon excitation)

940 nm (2-photon excitation)

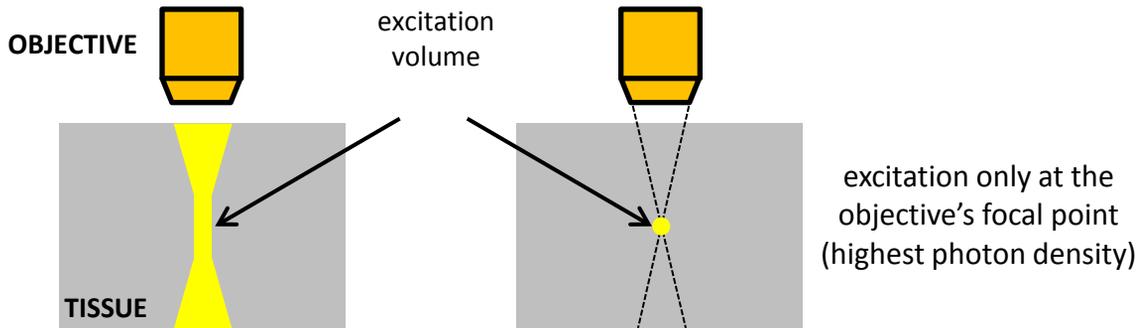


KEY FEATURES OF THE 2-PHOTON IMAGING:

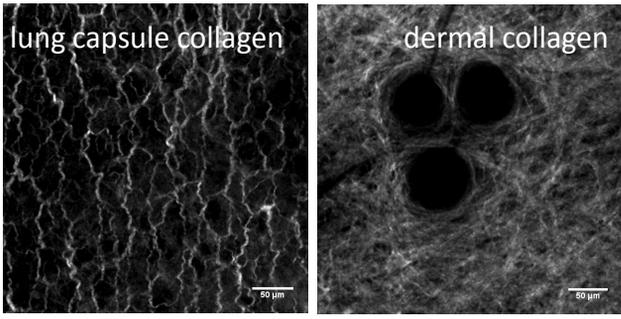
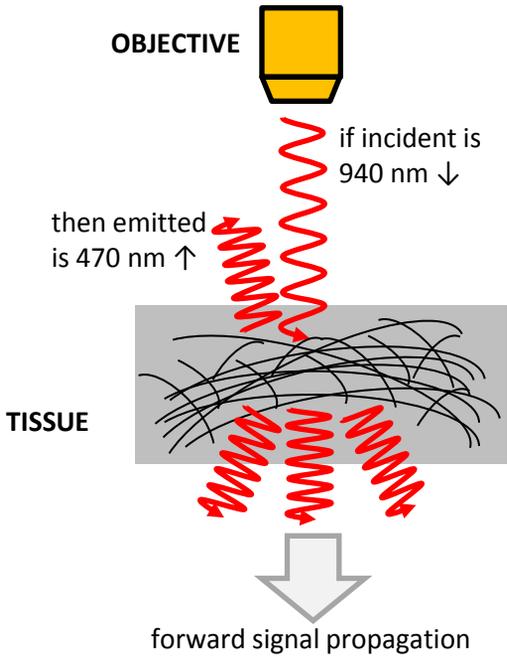
- DEEPER LIGHT PENETRATION



- LOWER PHOTOTOXICITY AND NO PHOTBLEACHING IN NON-FOCAL PLANES



- SECOND HARMONIC GENERATION



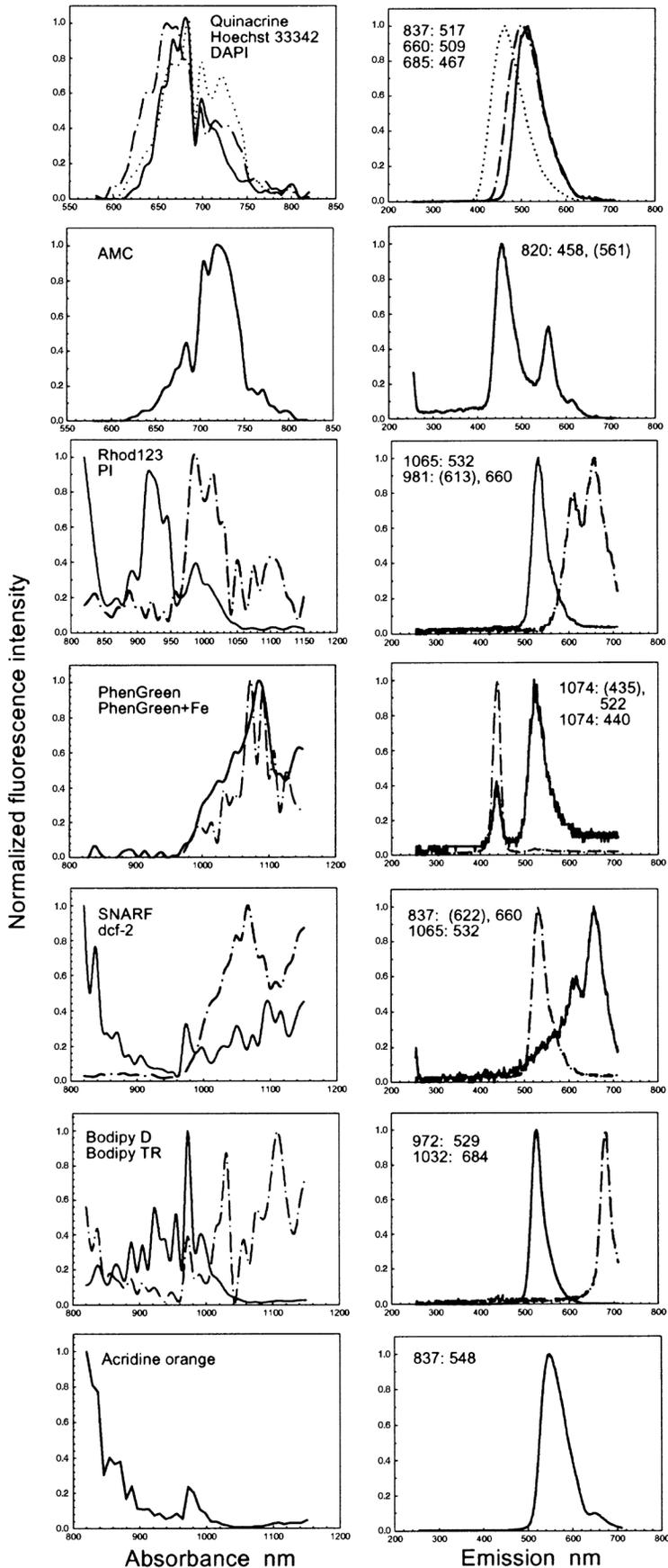
- the non-absorptive interaction of two photons with fibrillar structures without a center of symmetry (collagen, elastin, myosin),
- label-free method of imaging structures in tissue,
- emitted light wavelength is always a half of incident light wavelength.

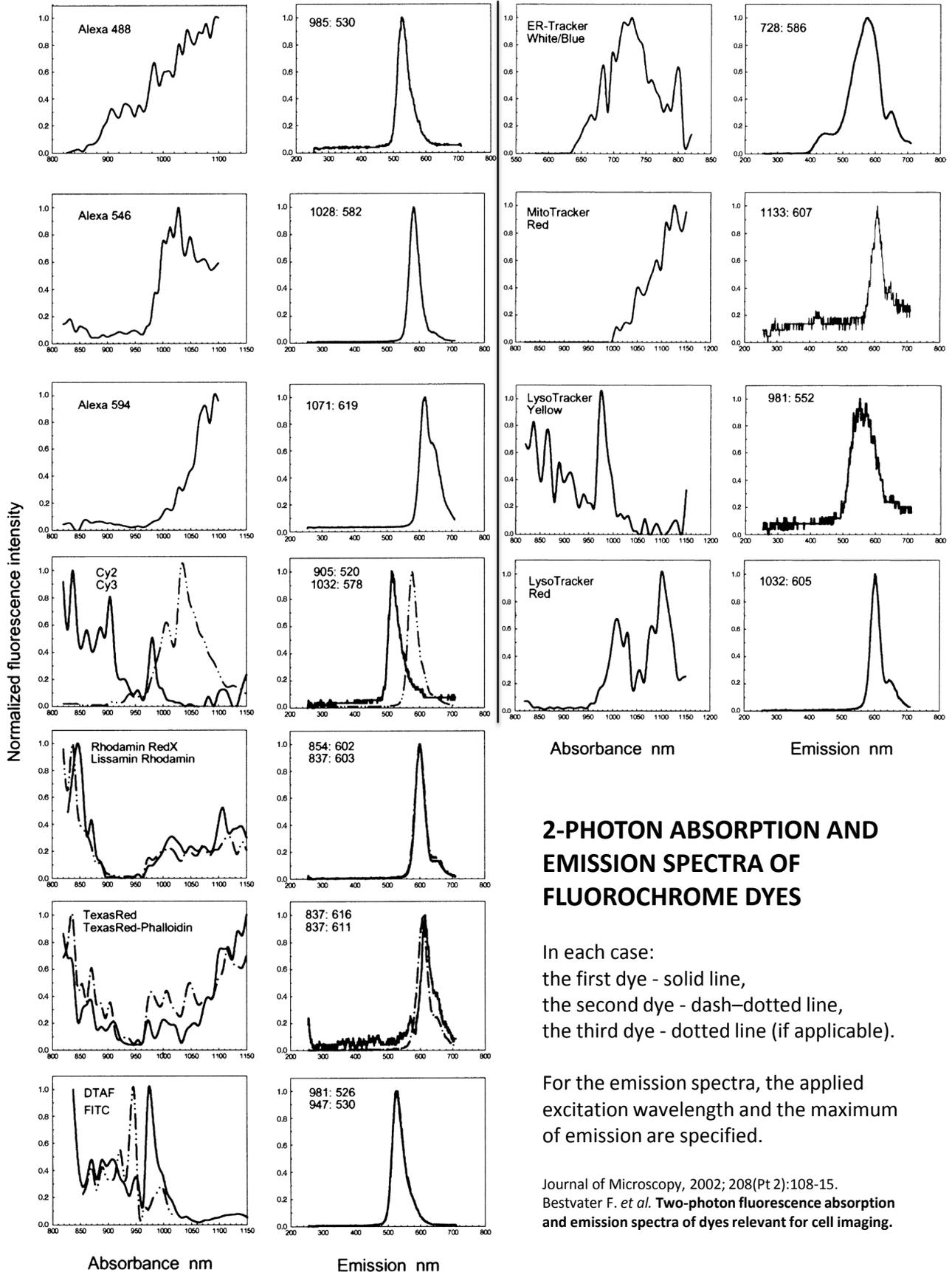
2-PHOTON ABSORPTION AND EMISSION SPECTRA OF FLUOROCHROME DYES

In each case:
 the first dye - solid line,
 the second dye - dash-dotted line,
 the third dye - dotted line (if applicable).

For the emission spectra, the applied excitation wavelength and the maximum of emission are specified.

Journal of Microscopy, 2002; 208(Pt 2):108-15.
 Bestvater F. *et al.* Two-photon fluorescence absorption and emission spectra of dyes relevant for cell imaging.





2-PHOTON ABSORPTION AND EMISSION SPECTRA OF FLUOROCHROME DYES

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Fluorochrome	TPE absorption maximum (nm)	Absorption shift TPE vs. $2 \times$ OPE (nm)	TPE emission maximum (at excitation) (nm)	Emission shift TPE vs. OPE (nm)
Dyes				
Acridine Orange	837 > 882 >> 981	–	548 (837)	–
aminomethylcoumarin methylcoumarin	703 < 722 >> 863	+4	458 (820)	+ 11
Bodipy FL ^a	920 << 972	–38	526 (972)	+15
Bodipy TRoad	1032 << 1108	–70	684 (1032)	+61
DAPI	685 > 697	–31	467 (685)	+6
DCF2 (H ₂ DCFDA) ^a	1065	+65	532 (1065)	±0
Hoechst 33342	660 > 715	–40	497 (660)	+10
lissamine rhodamine	837 >> 1116	–24	600 (837)	+5
PhenGreen-FL	1074	+90	435 << 522 (1074)	+5
PhenGreen-FL + Fe	1074		440 (1074)	
propidium iodide	989 > 1015 >> 1099	–13	612 << 657 (981)	–5, + 40
quinacrine	678 (697)	–168	517 (837)	+14
rhodamine 123	913 (1090)	–101	536 (947)	+7
SNARF-1	< 837	–200	622 << 660 (837)	+10
Organelle trackers				
ER-Tracker white/blue	728	–20	586 (728)	+11
LysoTracker Yellow	972	–104	435 << 552 (981)	±0
LysoTracker Red	1010 < 1100	–	605 (1032)	+15
MitoTracker Red	1133	–22	444 (1065) << 608 (1133)	+9
Antibody-conjugated				
Alexa Fluor 488	985	+3	530 (985)	+15
Alexa Fluor 546	1028	–78	582 (1028)	+13
Alexa Fluor 594	1074	–114	619 (1074)	+9
Cy2-IgG	837 < 905 > 981	–73	520 (905)	+14
Cy3-IgG	1032	–84	578 (1032)	+5
F-DTAF-IgG	< 820; 820 > 837 > 972	–8	527 (981)	+7
FITC-IgG	947	–39	530 (947)	+5
RedX-IgG	845 >> 1108	–	602 (854)	±0
TexasRed-IgG	1108 < (> 1150)	–	616 (837)	±0
TexasRed-Phalloidin	> 1150	–	611 (837)	+9

^aDissolved in DMSO.

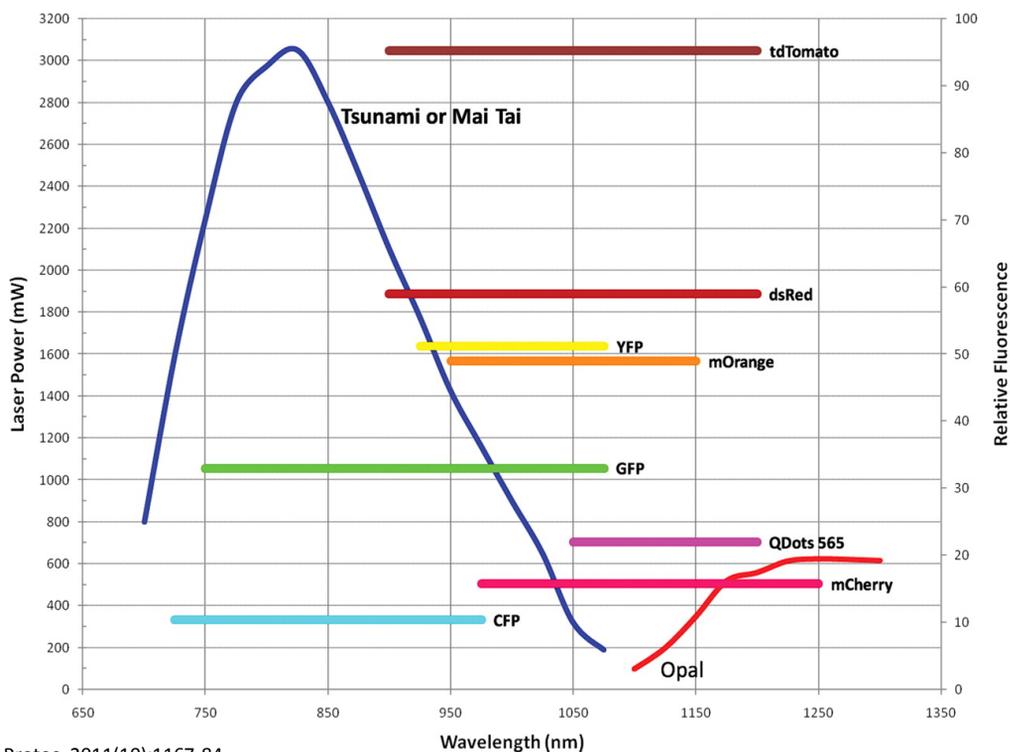
Fluorochrome excitation with one-photon and two-photon systems.

Some of the information provided (e.g. 2P exc with ">" and "<" marks) is from Leica. This information is from a Leica system with a SpectraPhysics laser. Other info is empirical on our Leica SP2 with a Coherent Mira 9000 system. If you have more fluorochromes to add or wish to modify the existing data, please contact us with the dye name and any synonyms, and other information that will make this chart more useful to all users.

shields@cb.uga.edu or farmer@cb.uga.edu

Application	Dye	Solvents	1P exc	2P exc	UV exc	Max em
Cell wall/ fibers	Aniline blue	water	500	790	607-610	
	Calcofluor	water	440/500-520	780>820	340-400	400-440
(Polysciences)	FungiFluor (cellufluor)	water	350-400	780	250-400	350-400
Nucleic acid stains	DAPI, Hoechst	Water	350/470	700, 780>820	345	455, 478
	Feulgen	Water, ethanol	480/560	780>820	460	550
	Propidium Iodide (PI)	Water	540		305, 536	617
	Acridine orange		467		503	530, 640
	Ethidium Bromide	Water	488		493	620
(Molecular Probes)	SYTO 13				488	509
	SYTO 85				579	599
	YOYO-1				491	508
Protein conjugates	FITC	Ethanol, Water	490/525	780>820	495	519
	TRITC (rhodamine)		541/572	800-840	547	572
	Rhodamine B			840		600
	Texas red		596/620	780	589	615
(Jackson Imm. Res.)	CY2	water	489/506	780>800	489	506
	CY3	water	550/570	780	512, 552	565, 615
	CY5	water	649/670	780<820	625-650	670
	AMCA (7 amino-4 methylcoumarin-3-acetate)		431/498	780	347	444
(Molecular Probes)	Alexa 488				495	519
	Alexa 532				531	554
	Alexa 546				556	573
	Alexa 568				578	603
	Alexa 594				590	617
Gene expression	BFP		395/509	780>820	382	448
(ClonTech)	CFP		434/477	780>840	430	476
	GFP		488/507	800-850	498	516
	E GFP		490	900-950	498	516
	YFP		514/527	860<900	520	532
	DsRed1 & 2 (redFP)				558	583
Mitochondria	Rhodamine 123		507/529	780-860	480	550
(Molecular Probes)	Mito Tracker red	DMSO			579	599
	MitoTracker red 594				598	630
	MitoTracker Green FM				490	516

Lipids	Nile Red	ethanol		810	559	640
Calcium	Calcium green		488/530	780>820	506	531
	Ca Green/ Texas Red		488/530, 596/620	780		
	Yellow Chameleon		464/527	780>820		
	Indo1-free			700		490
	Indo1-Ca ⁺⁺			590		405
	Fura-2 free			700		362
	Fura-2 Ca ⁺⁺			?		?
Viability, esterases (PolySciences)	Fluorescein Diacetate	acetone	495/520	780>820	490-512	515-535
	CTC (viab – bacteria)			720-740	450	630
Neuronal tracer	DID			780		
	Lucifer Yellow CH	EtOH (water)			488 (428)	531
Lysosomes	Lysotracker (Mol. Probes)	MeOH, DMSO			577	590
Membrane Probes	DiI			700	561	565
	Filipin				380	510
triple probe (on Leica/Coherent system)	Dapi, FITC, and Rhodamine			740		see above



Cold Spring Harb Protoc. 2011(10):1167-84.

Wyckoff J. *et al.* **High-resolution multiphoton imaging of tumors in vivo.**

The multiple fluorescent proteins and injectable agents that can be imaged using the combination of lasers (Tsunami, Mai Tai or Opal). The fluorophores are illustrated by comparing the power spectrum of the indicated lasers (left axis = laser power) with the 2-photon efficiency of excitation (right axis = relative fluorescence). This analysis indicates that Tomato > GFP > CFP and that all can be efficiently imaged.

Suitable dyes for Multi- Photon

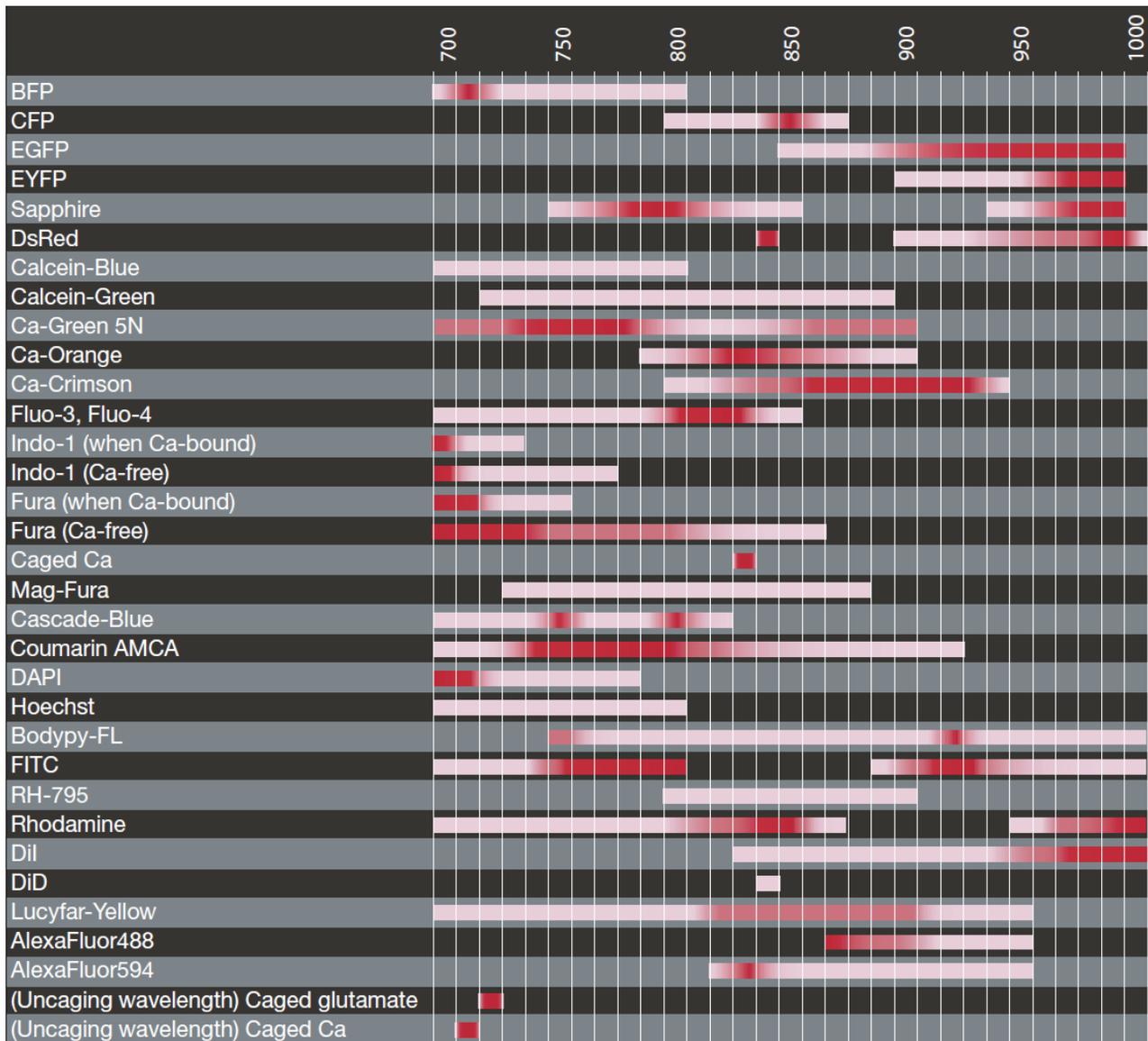
Conventional dyes have a broad excitation spectrum when excited with pulsed NIR lasers. The excitation spectra of different dyes may largely overlap.

This table provides an overview of the excitation spectra obtained with different dyes. The data are based on empirical measurements; they may slightly vary with specimen treatment. (See also: Xu, et al. (1996), PNAS, 93, 10763)

Fluorochrome	Absorption	Emission
Alexa Fluor 350	720-800	440
Alexa Fluor 488	720-800	515
Alexa Fluor 546	720-840	569
Alexa Fluor 568	720-840	596
Alexa Fluor 594	720-850	610
Alexa Fluor 633	720-900	647
AMCA	780-800	444
bis-MSB	680-750	420
Bodipy	900-950	512
Calcium Crimson	900	615
Calcium green	780-850	531
Cascade Blue	750-800	420
Coumarin 307	780-800	530
CY2	780-800	506
CY3	780	565, 615
CY5	780-820	670
Dansyl Hydrazine	700-750	440
DAPI, Hoechst	700-820	455, 478
DiA	800-860	580
DID	780-820	670
DiO	780-830	510
eCFP	800-900	476
eGFP	820-950	509
eYFP	860-950	532
Fluorescein	780 - 820	519
Indo-1 free	690-720	490
Indo-1 Ca ²⁺	690-720	400
Lucifer Yellow	860-890	533
Mito Tracker red	750-840	600
Nile Red	810	640
Oregon Green	780-860	526
Propidium Iodide (PI)	820-850	617
Rhodamin B	800-840	600
Rhodamine 123	780-860	550
Sytox Green	740-760 or 880-940	524
TRITC	800-840	572



We make it visible.



Websites with 2-photon excitation spectra:

http://www.drbio.cornell.edu/cross_sections.html

<http://www.spectra.arizona.edu/> (choose 2-Photon Cross-Sections in the Dye field)

Online tutorials on the 2-photon microscopy:

<http://micro.magnet.fsu.edu/primer/techniques/fluorescence/multiphoton/multiphotonhome.html>

<http://www.microscopyu.com/articles/fluorescence/multiphoton/multiphotonintro.html>

<http://www.olympusmicro.com/primer/techniques/fluorescence/multiphoton/multiphotonhome.html>

<http://zeiss-campus.magnet.fsu.edu/referencelibrary/multiphoton.html>