Confocal - 2

Instructions for Leica SP2 Confocal Equipted with Visible Laser Lines

By Jon Ekman

Basic operation instructions as of 3/9/2010
**Start-up**

1) Turn ON the scanner switch on the console. The scanner must warm up for 2 minutes prior to starting the Leica LCS software. This will also turn ON the green (543-nm) and red (633-nm) helium/neon lasers. Note: you do not need to turn the keys labeled HeNe to start these lasers. These keys are generally left in the ON position.

3) Turn ON the red rocker switch labeled Ar/ArKr on the left-hand side of the console. This starts the cooling fan for the Ar Laser. Turn the key above the switch to start the laser. The switch works just like a car ignition; turn the switch to the start position and release it to the on position. At this point, the three orange lights at the top of the panel should be on, and the three rocker switches should also be lit up.

4) After 1 minute adjust the laser power dial to the tape arrow. This may already be set. For best stability, the laser will need to warm up for 15-20 minutes.

5) If you will be needing the use of epifluorescence microscopy, turn ON the mercury lamp using the rocker switch on the small box next to the microscope.

6) Turn ON the power to the microscope stand with the outside rocker switch located at the base of the large box on the right side of the microscope.

7) Log on to the computer.

9) Launch the LCS program with the icon on the desktop. Your session will automatically be logged and databased through ITG’s logging software.

For **widefield fluorescence viewing:**

Adjust Fluorescent filter changer keys to desired filter (1-DAPI, 2-FITC, 3-Rhod) open shutter and set port to VIS to find focus on your sample with your eyes. LED will light next to VIS on ports area.

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**Start-up**

1. Turn ON Scanhead
2. Turn ON Laser Cooling
3. Turn ON Light Source(s)
4. Turn ON Microscope Stand
5. Log on to Computer
6. Load Software

**Align Optical Components**

1. Fluorescent Visualization
2. Transmitted Light Visualization
3. Koehler Illumination

**Acquire Image**

1. Basic Image Acquisition
2. Volumetric Data Sets
3. Sequential scanning
4. Time Lapse

**Save Data**

1. Save to Network
2. Save to Local Machine

**Shut-down**

1. Check Field Rotation = 0
2. Quit Software
3. Turn OFF Lasers
4. Turn OFF Laser Cooling
5. Turn OFF Scanhead
6. Turn OFF Light Source(s)
7. Clean Oil Objectives
8. Turn OFF Microscope Stand
9. Log out of Computer

**Tip for focusing:** Bring objective as close as possible to cover slip. Use Coarse Focus Key (down) or fine focus handwheel (rotate CCW) to lower objective while viewing through eyepieces. You will pass through focus.
Align Optical Components

**Visualization:**

<table>
<thead>
<tr>
<th>Fluorescence</th>
<th>Transmitted Light</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>To set up the optics for fluorescence:</strong></td>
<td><strong>To set up the optics for bright field viewing:</strong></td>
</tr>
<tr>
<td>- The switch for the mercury arc is on the small power supply box next to the scope stand; it should be turned ON.</td>
<td>- Using the Fluorescent filter cube changer keys, choose the position marked with the number ‘4’ (Laser or Transmitted light).</td>
</tr>
<tr>
<td>- For fluorescence, turn OFF the transmitted light by turning the black dial away from you on the front, left, bottom of microscope. Turn away from you until the display panel on front of the microscope reads 0*V.</td>
<td>- Check the tube lens module is set to “1x” position.</td>
</tr>
<tr>
<td>- The upper row of buttons on the front of the microscope controls filters and dichroic mirrors (filter cube turret).</td>
<td>- Check the shutter before the lamp field stop is in the Vis position.</td>
</tr>
<tr>
<td>Choose From Position 1, 2 or 3:</td>
<td>- Intensity of bright-field illumination is increased by turning black dial, left, bottom of microscope, toward you.</td>
</tr>
</tbody>
</table>
  1= DAPI, Hoechst | - Place your sample on the stage and then carefully raise the objective using the upper black button from the pair of square black buttons (coarse focus) on the right side of the microscope. If using an oil lens, raise the objective until it just spreads out the oil as it contacts your slide. Then observe the specimen through the oculars. You can use the focus knob now to gradually bring your specimen into focus. Check your slide periodically to make sure you are not over-focusing and pushing up the specimen with the lens. This can damage the lens. |
  2= FITC, GFP | - Press the Shutter button on the front of the microscope to send excitation light up through the objective. |
  3= Rhodamine, Texas Red | - Select the VIS port (only the LED for VIS should light) |
  4= Laser, or Transmitted Light | - You should see excitation light coming from the lens and you should be able to view focus through the oculars. |

**Transmitted Light Parts**

Almost always, the condenser needed for transmitted light imaging is sitting next to the microscope. There is a mark on the microscope indicating the proper height for the condenser. Use a 3mm allen to snug it into place. Please do not over tighten. The S23 condenser is the one in this room. A higher numerical aperture condenser can be found in the multi-photon confocal room (confocal-1).

**Koehler Illumination**

For optimum results in light microscopy, precise control of the light path should start before the light reaches the specimen. Prof. A. Koehler of Carl Zeiss was the first to apply exact control of the light path in the illuminating beam, a method known as “Koehler illumination.” Koehler illumination centers and adjusts the condenser lens to match the numerical aperture of the objective being used.

**PROCEDURE**

1. Focus on specimen with the objective to be used for data collection.
2. Close down lamp field stop [diaphragm at top (1)] while viewing.
3. Lower condenser slightly (2) until diaphragm image is in focus.
4. Center image using condenser centering screws (3).
5. Open diaphragm (1) to edge of field, fine focus and open further to just clear field.
6. Adjust contrast using condenser diaphragm (4).
7. Insert Bertrand Lens (5) and check to see that 75%-90% of visible aperture is filled with light (more light = better resolution but less contrast).

**BENEFITS**

- Evenly illuminated image.
- Brilliant image without reflection or glare.
- Minimum heating of specimen.
Preparing the microscope optics for confocal scanning:
• If widefield fluorescence illumination has been used, press the shutter control key on the front of the microscope stand block extraneous light from the mercury arc excitation source.

• Set fluorescent filter cube changer to the “4-LASER” position.

• Ensure the transmitted light illumination column is pulled forward to its normal upright position, this is the laser safety switch.

Initial software settings for confocal scanning:
1. Choose Beam
Click on the ‘Beam’ button.

2. Load Beam Path Settings
Choose the settings to be used in your experiment.
Basic settings include the laser wavelength and power, dichroic beamsplitter, and the detectors to be used along with corresponding detection range.

3. Select Mode
XYZ is the system default. Time series and Wavelength (Lambda) scan options are also available.

4. Select Format
Click in the ‘Format’ panel and select the xy sampling resolution of your image.

Quick Notes:

Caution:
Zoom changes when you change scan Speed

The scope default settings are:
mode: xyz
format: 512x512
Zoom: 1
Expan.: 3 or 6 depending on objective selected
Speed: 400Hz
Z-Galvo is controlled by Position knob (Focus)
5. Check Zoom
As a general rule, it is good to have at least two sampling intervals (pixels) per resolvable unit. To achieve this, the table below may be of use.

Useful guidelines for objectives are:

<table>
<thead>
<tr>
<th>Objective</th>
<th>Zoom</th>
<th>Format</th>
</tr>
</thead>
<tbody>
<tr>
<td>63x</td>
<td>x 3</td>
<td>1024x1024</td>
</tr>
<tr>
<td>63x</td>
<td>x 6</td>
<td>512x512</td>
</tr>
<tr>
<td>40x</td>
<td>x 6</td>
<td>1024x1024</td>
</tr>
<tr>
<td>40x</td>
<td>x 10</td>
<td>512x512</td>
</tr>
<tr>
<td>20x</td>
<td>x 8</td>
<td>1024x1024</td>
</tr>
<tr>
<td>20x</td>
<td>x 10</td>
<td>512x512</td>
</tr>
</tbody>
</table>

Keep in mind that zooming exposes the sample to more laser fluence. Therefore, to avoid bleaching or otherwise damaging the sample, it is often best to be conservative when using the scanning zoom. Using a lower sampling resolution reduces the amount of laser energy required to image at a given brightness for a given scan speed.

6. Check Beam Expander
The software selects a beam expander for the objective used, it is not advisable to change this setting.

7. Check Field Rotation
Select Field button to adjust field rotation. — Remember to set Field Rotation back to Zero (0) before logging out of software. If you forget to do this the scanhead may not initialize correctly for the next user.

8. Set Pinhole to 1 Airy Unit (Airy Disc)
Check the pinhole size on the panel describing confocal settings. If necessary, change pinhole size to 1 Airy Disc (traditional setting used for confocal imaging). The 'Pinhole' button will give you the information about the absolute value of the pinhole.

9. Select Speed
For “Speed” the default frequency is 400Hz. This means that the system will scan 400 lines in one second. Using a lower scan speed you get better signal-to-noise ratio (at the cost of potentially increasing photobleaching). For “Scan” most people use the default (unidirectional scan). Bi-directional scan doubles the scan speed, however, recording images in bidirectional scan mode can result in a phase shift between the forward sweep and flyback of the scanning beam. To address this problem when using bi-directional scanning mode, you should click the “Phase” button to open a dialog window, which you can use to correct the shift.

10. Adjust Zoom
Use the ‘Zoom’ button to go to appropriate Zoom level. Also, you may use ‘Zoom In’ button both during scan or between scans to select a region of interest on the live image. Click on the ‘Zoom In’ button, point to the appropriate area, hold down the left mouse button and stretch the rectangle. There is also a knob for zooming on the panel box.

11. & 12. Select Continuous then Adjust Smart Gain & Smart Offset
To start the scan, Hit the ‘Continuous’ scan button and adjust the PMT Smart Gain and Smart Offset (first two knobs on panel box) while observing the specimen in the experiment window.

13. Use Q-LUT to set Over/Under (adjust dynamic range of PMT)
The special ‘Glow Over/Under’ type of pseudocoloring provided by pressing the ‘Q LUT’ button next to the active image is useful to adjust the brightness so that the maximum dynamic range of the PMT is made available. In this color scheme, pixels which are saturating the PMT are colored blue and pixels which have a value of 0 are colored green. The idea is to set the PMT gain so that the brightest pixels are just slightly under being saturated, and the offset such that the darkest pixels are just above a value of zero.

14. Stop continuous Scanning
Clicking on the ‘Continuous’ button again will stop the continuous imaging. Now you may change the color of the image to the appropriate hue (green, blue or red) after you adjusted everything. This can be done by selecting the color listing associated with a particular PMT. However, it is often useful to image everything in “Q-LUT” mode, watching for saturation. Also, monochromatic images, especially those colored blue, will often appear dark to the human eye. This can be remedied through the use of contrast expansion algorithms in postprocessing of the images. You may change the colors later, after imaging using the ‘Leica LCS Lite’ software.

15. Save Global Confocal Config
After you have adjusted the brightness, pinhole etc, and you are satisfied with the quality of image, use the ‘Save’ button on the window with the detector settings if you wish to save your global confocal configuration. Your settings can now be loaded from the User area of the Beam Path Settings window.

16. Choose Line Averaging
Image averaging is a method used to improve the signal-to-noise ratio of an image. To use image averaging, first click either “Aver” (average by frame) or “Li.A.” (average by line) button and select the number of images to be averaged. This will reduce the appearance of noise in each image. Averaging by line permits averaging during a continuous scan; averaging by frame permits the automatic switching of settings between channels during a sequential scan (see the Leica LCS documentation for more in depth information).

17. Scan Image
If you want to obtain single focal plane image, use the ‘Single Scan’ button.

Remember save after you acquire an image and save often.
Acquiring Volumetric Datasets (Z-Series)

Acquiring volumetric datasets:

1. Set Pinhole to 1 Airy Unit
2. Check Mode
3. Check Format
4. Check Zoom
5. Check Speed

6. Select Continuous
   The top and bottom of the volume of interest are identified interactively using the 'Z pos' control (the 6th dial on the panel box). Turn this clockwise and then counter-clockwise until you find the top and bottom of your region of interest.

7. Set Begin Point
   Move to the top of your sample turning the Z-position control dial clockwise. Click on the “Begin” button (it should turn white). This marks the beginning of your 3D image.

8. Set End point
   Turn z-control counter-clockwise until you find the bottom end of your region of interest. Then click on the “End” button (it should turn white as well). This marks the end of your 3D volume.

9. Stop Continuous

10. Check Section Thickness
    Select the ‘Sect’ button, and choose the desired number of focal planes. If you click instead on “Other”, you may enter the focal plane spacing, and then the program will calculate the number of focal planes required to cover the distance between your endpoints marked by “Begin” and “End”. By default the software determines the number of sections which will yield the maximum optical z-resolution for an ideal sample.

11. Start Continuous
12. Adjust Smart Gain & Smart Offset
    Use Q-LUT to check that there are now points of over saturation in your region of interest throughout your series that you are to acquire. Make adjustments to areas of over/under saturation.
13. Stop Continuous

14. Choose Line Averaging
    If you plan to line average set it now.

15. Scan Series
    To begin data collection, click on large button labeled ‘Series’. The Gall. button on Image Display Window can be selected to view series progress.

Z-Galvo vs. Z-Wide: To use the stage focus motor to acquire Spatial Image Series instead of Z-galvo just select Z-Wide (button next to Continuous) and then select Z Wide Position for focus control knob. This will change the default range of movement from 166.68 microns to 8085.01 microns and allow control of Z-depth with remote focus knob, instead of the fine focus on the microscope stand.
Sequential Scanning
(temporal separation of lasers)

Sequential scanning or multi-tracking:
Sequential scanning is a method used to decrease the cross-contamination of signal readings from multiple probes with overlapping emission spectra. In sequential scanning, the images of the individual channels are acquired separately, first with one excitation wavelength, then with the other.

To set up a sequential scan you have to set all parameters for the first recording method and save the settings as an instrument parameter setting. Next, all instrument parameters for the second recording method are set and also saved as an instrument parameter setting.

In the ‘Beam Path Setting’ dialog window, click on the button labeled ‘Sequential Scan’ in the bottom left corner. Copy the instrument parameter settings into the ‘Sequential scan settings’ list box. In the ‘Mode’ list box, select one of the three sequential scan modes. This selection defines when the sequential recording methods are alternated; ‘between lines’ can only be used if the detector ranges do not change between the instrument parameter settings used in the sequential scan. The ‘between lines’ mode is the only mode which can be used for a continuous scan. In the ‘Parameter’ list box, check the parameters which will be used for the recording of all sequential recording methods.

Next, adjust all other instrument parameters which differ depending on the type of recording: spatial image series, time image series or spectral image series.

Click on the ‘Series’ button or on the ‘Single Scan’ button to start the sequential image recording.

Time-lapse Series

Time-lapse series:

Click on the ‘Mode’ button to select the ‘xt’, ‘xyt’, ‘xzt’ or ‘xyzt’ scan mode. For the many modes available you still need to set the dynamic range of the PMT.

Next, click on the ‘Time’ button and set all parameters required for the time image Set number of z-planes series.

Lastly, click on the ‘Series’ button to start recording the time image series.
Save Data

1. Save to remote network share. —Default (zeus.itg.uiuc.edu)

You may also save files to Workspace (D: or E:\Workspace\"username"). A folder was created there at login for each user. This is a useful option if the network is down.

Single images, z-stacks, multi-channel images will be saved as a series of TIFF images that can be viewed in other programs (Adobe Photoshop, Fiji, ImageJ, Imaris, Image Pro Plus etc...).

Remember to always save files and never delete them until after you have a backup copy.

**Single Laser Scan Notes:**

<table>
<thead>
<tr>
<th>Acquire Image (X-Y slice)</th>
<th>Acquire Image (Z-Series)</th>
<th>Acquire Image (X-Y-T slice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Check Beam</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Load Beam Path Settings</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>Select Mode</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>Set Pinhole to 1 Airy Unit</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>Check Field Rotation = 0</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>Set Pinhole to 1 Airy Unit</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>Select Speed</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>Select Continuous</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>Adjacent Smart Gain &amp; Smart Offset</td>
</tr>
<tr>
<td>11</td>
<td>11</td>
<td>Set Begin Point</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>Stop Continuous</td>
</tr>
<tr>
<td>13</td>
<td>13</td>
<td>Check Section Thickness</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>Adjust Smart Gain &amp; Smart Offset</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>Choose Line Averaging</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>Scan Series</td>
</tr>
<tr>
<td>17</td>
<td>17</td>
<td>Scan Image</td>
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</tbody>
</table>

**Sequential Scan Notes:**

<table>
<thead>
<tr>
<th>Sequential Scan Notes:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequential Slice (X-Y slice)</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>Acquire Image</td>
</tr>
<tr>
<td>Load Beam Path Settings</td>
</tr>
<tr>
<td>Select Format</td>
</tr>
<tr>
<td>Select Speed</td>
</tr>
<tr>
<td>Select Continuous</td>
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<tr>
<td>Adjacent Smart Gain &amp; Smart Offset</td>
</tr>
<tr>
<td>Set End point</td>
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<tr>
<td>Check Beam</td>
</tr>
<tr>
<td>Stop Continuous</td>
</tr>
<tr>
<td>Check Series</td>
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<tr>
<td>Stop Continuous</td>
</tr>
<tr>
<td>Check Series</td>
</tr>
</tbody>
</table>

By far the most effective way to reduce channel cross talk is to do a sequential frame scan.

If you plane to acquire more than three channels or wish to use a different primary dichroic or move any optical hardware between channels then sequential frame scan should be used.

17 channels max can be collected using the sequential frame scan.
**Shut Down**

1) Clean any immersion oil from the objectives.

2) If the scan rotation feature was used, please remember to reset the scan rotation through the software interface. If you forget to do this the scanhead may not initialize correctly for the next user.

3) Switch to the 10x objective, save your files and exit the LCS program.

4) Turn the red Scanner switch on the console off.

5) Turn the Ar/ArKr laser key to the off position.

6) Log off of the computer—don’t forget to check the electronic logbook for accurate recording of instrument usage time (notebook icon on desktop).

7) Turn off the argon cooling fan using the red rocker switch.

8) Turn off the microscope base and mercury lamp (under the computer) with the appropriate switches.

always allow the Argon laser to cool down for 5 minutes.

### Objective Quick Notes:

<table>
<thead>
<tr>
<th>Part #</th>
<th>Resolution Z (488nm)</th>
<th>Resolution XY (488nm)</th>
<th>DIC Obj Prism</th>
<th>DIC S1 Condenser</th>
<th>Coverslip</th>
<th>WD(mm)</th>
<th>Phase Ring</th>
<th>NA</th>
<th>Objective</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x . . . . .</td>
<td>HC PL FLUOTAR</td>
<td>.30</td>
<td>.0.0</td>
<td>--</td>
<td>--</td>
<td>11.0</td>
<td>PH2</td>
<td>NA</td>
<td>5x-100x</td>
</tr>
<tr>
<td>20x . . . . .</td>
<td>HCX PLAN APO</td>
<td>.70</td>
<td>.17</td>
<td>--</td>
<td>--</td>
<td>0.59</td>
<td>PH2</td>
<td>NA</td>
<td>10x-100x</td>
</tr>
<tr>
<td>40x OIL . . .</td>
<td>HCX PL APO CS</td>
<td>.125</td>
<td>.17</td>
<td>--</td>
<td>--</td>
<td>.1</td>
<td>PH3</td>
<td>NA</td>
<td>5x-100x</td>
</tr>
</tbody>
</table>

All require 0.17 mm coverslip unless noted

<table>
<thead>
<tr>
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<th>Phase Ring</th>
<th>NA</th>
<th>Objective</th>
</tr>
</thead>
<tbody>
<tr>
<td>63x AIR . . .</td>
<td>PL FLUORTAR CORR</td>
<td>.7</td>
<td>.0.0</td>
<td>--</td>
<td>--</td>
<td>.07</td>
<td>PH2</td>
<td>NA</td>
<td>10x-100x</td>
</tr>
<tr>
<td>63x OIL . . .</td>
<td>HCX PL APO CS</td>
<td>.14</td>
<td>.0.1</td>
<td>--</td>
<td>--</td>
<td>.17</td>
<td>PH3</td>
<td>NA</td>
<td>10x-100x</td>
</tr>
<tr>
<td>63x WATER . .</td>
<td>HCX PL APO CS</td>
<td>.12</td>
<td>.0.0</td>
<td>--</td>
<td>--</td>
<td>.18</td>
<td>PH3</td>
<td>NA</td>
<td>10x-100x</td>
</tr>
</tbody>
</table>

Condensers available:

- [S1 (DIC & Phase & BF)] 0.900NA located on Confocal #1
  - Objective range: 10x-100x
  - Condenser prisms: K2-KS+K11 only with condenser top 0.90 S1
  - Objective prisms: A-E
  - Prisms D wide shearing = higher contrast
  - Coverglass specification: 0.17 = for use with a 0.17 mm coverglass (DIN/ISO) No. 1 or No. 1.5

- [S23 (Phase & BF)] 0.530NA located on Confocal #2
  - PH0, PH1, PH2, PH3
  - Objective range: 5x-100x

More on Objectives:

- Tube length oo, reference focal length of tube lens fB = 200 mm, parfocalizing distance 45 mm
- Numerical aperture is adjustable for darkfield applications on these objectives
- Immersions: AIR = No immersion media-bridging/mountant required for optimal resolution
- OIL = DIN/ISO standard immersion oil R = 1.51
- Technique: LSCM = Laser Scanning Confocal Microscopy
- DIC = Differential Interference Phase Contrast
Refractive Index & Specimen Preparation

It is vitally important to keep in mind that the specimen is effectively part of the optical train; in order for optical sectioning technologies such as confocal and multiphoton microscopy to deliver results that accurately reflect the sample, the refractive index of the specimen must match the refractive index for which the objective optics were designed. Serious aberrations will result when this is not the case. Measurements in the z-axis will be grievously inaccurate when refractive index mis-match between the sample and the objective design is present. Furthermore, the presence of interfaces between boundaries of differing refractive index in the imaging path will hamper high-fidelity data collection. In short, the results obtained from samples which are not of the correct refractive index, or are heterogeneous in the refractive indices present in the optical path, will be questionable and in some cases, entirely indefensible. Dry (air-immersion) objectives function correctly at one z-position only: the interface between a 170-micron thick coverslip of refractive index=1.514 and the sample. Any penetration into the sample changes the ratio of air to sample in the beam focus, and this induces spherical aberration. Thus, measurements in the z-axis when using dry objectives are not accurate.

Mounting specimens in glycerol/buffer is an approach popularized in the 1970’s and 80’s for widefield fluorescence microscopy and is not suitable for high-quality confocal microscopy. Thick, turbid biological specimens are generally best dehydrated, cleared and mounted in oil of wintergreen. It is not always advisable to dehydrate specimens. Optically clear aqueous specimens should be mounted in a modern poly-vinyl alcohol based mountant such as ProLong Gold (Invitrogen).

Fluorescein is an ancient and poorly optimized fluorophore and is not generally used in modern fluorescence microscopy except when very rapid photobleaching is a desired quality. FITC and TRITC are not the best either. The more modern Alexa dyes and cyanine derivative probes are much more robust and generally they are brighter as well.

Instrument Settings and Limitations

The use of beam expanding optics other than those for which the objective optics are designed will severely degrade image quality and will change the focusing properties of the objective. Centration of the image will also be shifted from the centration for which the scanning mechanism is calibrated. Laser microscopes are designed to image microscopic samples. Microscopic features are too small to perceived with the human eye. The working distance of lenses is limited by the numerical aperture (N.A.) of the lens; in other words, high N.A. lenses have short working distances. The working distance of a 10x N.A. 0.4 lens is about 2.2 mm. The working distance of a N.A. 1.32 63x lens is only about 70 microns. The confocal microscope is designed to perform best under high numerical aperture conditions using immersion optics. For this reason, samples over about 150 microns in any dimension may only be marginally appropriate for viewing with a conventional laser scanning microscope. Resolution and contrast are interrelated. Features which approach the theoretical resolution limit for a particular wavelength and lens combination will have inherently low contrast. To achieve near-theoretical resolution performance from a laser scanning microscope, it is usually necessary that the sample is highly reflective and backscattered imaging mode is used. The smaller a fluorescent feature of interest is, the more brightly stained it needs to be in order to resolve it from neighboring features.

The laser scanning microscope is not designed to be used as a profilometer in a non-immersion (air) environment. Profilometers and scanning probe microscopes may be more suitable for use under such conditions. The position of sub-resolution features can be accurately determined in three-dimensional space only if the sample is rigorously index matched to the refractive index for which a given lens has been designed. Air-immersion (dry) objectives will never yield accurate measurements with respect to z because they are designed to image thin histological samples and they are not designed for 3-D microscopy. It is frequently tempting to make excessive use of the zoom capabilities that laser scanning microscopes provide. Keep in mind that the data acquired can be digitally resized in post-processing software; the important concern is to have sufficient sampling resolution to resolve the smallest structures of interest. The laser energy exposure to the sample increases as the square of the zoom factor, thus it is easy to bleach or otherwise destroy a sample through the use of excessive zoom without a concurrent reduction in the amount of laser power being used. Imaging at as low a sampling resolution as possible will permit the most efficient collection of signal from the sample. This is because the integration time for the photomultiplier tubes will be longer for lower resolutions.

At greater zoom levels, lower sampling resolutions may be used to yield the same effective resolution. For instance, if 1024 x 1024 pixels are required to image a sample at the full optical resolution with a zoom of 4, then 512 x 512 pixels should be adequate.
References

Calibration Standards
Calibration Grid Slide from Micro Brightfield:
http://www.mbfbioscience.com
Fluorescent Reference Slides from Microscopy Education:
http://www.microscopyeducation.com

Leica SP2
Leica Microsystems Inc.
2345 Waukegan Road
Bannockburn IL 60015
www.leica-microsystems.com

Commercial anti-fade mounting media
• ProLong Gold Antifade Mountant:
• Vectashield Antifade Mounting Medium:
http://www.vectorlabs.com/VECTASHIELD/VECTASHIELD.html
• VECTASHIELD HardSet Mounting Medium:

Fluorescence Microscopy Books/Papers

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