ABRF-LMRG - 3D Microsphere Standard Sample Study
Imaging Protocol Leica SP5 – Vial 2: "SNR Analysis"

There are two different vials of beads used in this study:

Vial 1 has two different size beads, 0.1μm green beads, which will be used for the PSF analysis, and 1μm red beads that will not be imaged, but are there to assist in finding the sample plane.

Vial 2 is a mix of different colors and intensities, which will be used for analyzing 1) intensity and signal to noise ratio (SNR) as a function of depth and 2) microscope sensitivity as a function of imaging depth.

Vial 2 Properties and Imaging Set Up for SNR , Intensity and Sensitivity Analysis

Cat. #	Size	Intensity		Excitation/ Emission	LASER	Detector
I-7219	2.5μm	3%	Component e	505/515	488	500-540
	2.5μm	30%	Component f	505/515	488	500-540
I-7224	2.5μm	3%	Component e	580/605	543	590-630
	2.5μm	30%	Component f	580/605	543	590-630

Sequential Scan Configuration:

Scan 1: excitation laser 543nm @ 20% – pmt-3 (590-630nm)

Scan 2: excitation laser 488nm @ 15% – pmt-2 (500-540nm)

Protocol

1) Make sure the Argon/488 and 543 lasers are turned on and are fully warmed up. Allow 20-30 min. .
2) Go to the **Configuration** Tab and change the bit depth to 12-bit under the **Settings** menu. Then, still under the **Configuration** Tab, go to **Ctrl Panel,** select the **Offset** dial and change it from 40% per turn to 0.1% per turn.
3) Place the vial 2 sample slide on the stage and focus on the red beads in the sample using a TXRed, Cy3 or similar epi-fluorescence filter. Switch to your "GFP" filter and verify the presence of the green beads as well. Try to select an area that has a good distribution of the different color beads across the field of view at different depths.
4) Put a 0.5 NA or higher objective lens in place. Put immersion media on the lens, as necessary.
5) Set up your sequential scan configuration as indicated above.
6) Set the **Acquisition Mode** to **xyz**. In the **XY Scan Control** window adjust the **Format** and **Zoom factor** to achieve a pixel size of **0.25 μm or as close as possible to it.** The necessary format and

zoom settings will change depending on the objective selected and the field of view required to get a good sample of each color bead. (e.g. a 40x/1.25 n.a. objective using a 1024 x 1024 frame size and a **1.5x** zoom gives a pixel size of ~252 nm in xy. Where as a 63x/1.4 n.a. objective using a 1024 x 1024 frame size with a **1x** zoom gives a pixel size of ~ 240nm in xy).

7) Set the **Pinhole** to **1 Airy Unit. Scan Speed** to **600 Hz.** Make sure **Bi-directional X** is **NOT** active.
8) Use 4x LINE averaging for both sequential scans.
9) Make sure to select the range indicator mode (second option on LUT control) using the icon in the upper left corner of the image window. In this mode saturated pixels appear blue and zero intensity pixels appear green, pixels in between go from orange (1) to white (4094).
10) Select one of the PMTs and start a **Live** scan. Turn up the PMT **Gain** until you see some blue pixels on your beads and then turn it back down until the blue <u>just</u> goes away completely. If this requires raising the **Gain** above 825 units, turn up the laser. Ideally you want to be lower. Make sure you are in the middle/brightest part of your bead along the z-axis when you do this so that you are **not saturating** any part of the bead (i.e. over a range of focal planes). Set the **Offset** <u>just</u> above where green pixels start to appear. Setting the offset properly may require setting the dial sensitivity to 0.1% (see step 2).
11) Stop the **Live** scan. Turn off the first laser and PMT.
12) Repeat steps 8-10 for the second PMT /laser combo.
13) Turn on both lasers and PMTs. Verify the scan settings by hitting the **Start** button. Make sure you are still not saturating when you scan with both lasers at a non-"live" scan speed. You may want to toggle the LUT button to "pseudo-color" to see all the different color beads, but then toggle it back to range-indicator before setting up the Z-stack.
14) Open up the Z-Stack interface. Start a **Live** scan and turn the z-position dial on the control panel counter-clockwise to move the focus position down - to the top/outside of the coverslip - until no more beads are visible. Set the **Begin** position. Then turn the focus knob clockwise to focus deep into the sample and set the **End** position at ~100um deep, when no more beads are visible.
15) Verify that no beads are saturated while focusing through your z-stack.
16) Stop the **Live** scan.
17) Choose the "z-step size" radio button in the Z-stack window and type in 1μm.
18) Click on Start to collect the entire z-stack. This will result in ~ 100μm/1μm = 100 slices. This could take >30min, depending on your frame size.
19) When the stack is finished save the .lif file according to the following convention:
"Lastname_Firstname_Microscope_Platform_MagnificationX_NAY_ImmersionMedia_SNR"
e.g. Brown_Claire_LeicaSP5_63X_NA1.4_Oil_SNR)