

# ABRF-LMRG - 3D Microsphere Standard Sample Study

## Imaging Protocol Leica SP5 – Vial 1: “PSF 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 1 Properties and Imaging Set Up for PSF analysis**

Thermo-Fisher Cat. #	Size	Intensity	Excitation/Emission	LASER	Detector
F8803	0.1µm	100%	505/514	488	495-540
F8820	1µm	100%	540/560	NA	Data NOT collected

Scan Configuration:

0.1µm beads: excitation laser 488nm @ 10% – pmt-2 (495-540nm)

### **Protocol**

- 1) Make sure the Argon/488 laser is turned on and fully warmed up. Allow 20-30 min. .
- 2) Go to the **Configuration** Tab and verify the bit depth is set to 8-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 1 sample slide on the stage and focus on the larger 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 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 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 0.07µm for X,Y.
- 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.

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- 9) In the Image window, 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 (254).
- 10) Select your active PMT 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 just 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** just above where green pixels start to appear. Setting the offset properly may require setting the dial sensitivity to 0.1% (see step 2).
- 11) Verify the scan settings by hitting the **Start** button. Make sure you are still not saturating when you scan at a non-“live” scan speed.
- 12) 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 ~100µm deep, when no more beads are visible.
- 13) Verify that no beads are saturated while focusing through your z-stack.
- 14) Stop the **Live** scan.
- 15) Choose the “z-step size” radio button in the Z-stack window and type in 0.2µm.
- 16) Click on Start to collect the entire z-stack. This will result in  $\sim 100\mu\text{m}/0.2\mu\text{m} = 500$  slices. This could take >30min, depending on your frame size.
- 17) When the stack is finished save the .lif file according to the following convention:  
“Lastname\_Firstname\_Microscope\_Platform\_MagnificationX\_NAY\_ImmersionMedia\_PSF”  
e.g. Brown\_Claire\_LeicaSP5\_63X\_NA1.4\_Oil\_PSF)
- 18) Please follow this link to submit a sample information form for each dataset you submit <https://tinyurl.com/LMRG-vial1> (takes a few seconds to load correct link if you click on it).