ABRF-LMRG - 3D Microsphere Standard Sample Study #3
Imaging Protocol Zeiss 710 – Point Spread Function (Slide 1)

1) Turn on and warm up the lasers for at least one hour.
2) Put a 1.0 NA or higher objective lens in place. If necessary, put immersion media on the lens.
3) Under the **Light Path** window set up the confocal for imaging the 0.1 µm green-fluorescent microspheres. For example under **Visible Light,** set up the light path using the 488 nm laser and use the **488** nm Main Beam Splitter (**MBS 488**). Set up the one channel with an emission bandwidth of **~495-540 nm**.
4) In the **Acquisition Mode** window set the scan mode to **Frame**. Choose a **Frame Size** of 512x512 pixels, **Line Step** of **1, Scan Speed** of **9** (pixel dwell time of **~1.27 µs/pixel**), **Mean Line Averaging** of **4** to reduce pixel noise, **Bit Depth** of **12 Bit** and adjust the **Zoom** factor to achieve a **Pixel Size** of **0.07 µm**. Under **Direction** set for unidirectional scanning (indicated by right pointing arrow).
5) Set the **Pinhole** for each channel to **1 Airy unit (AU)**.
6) Set each detection channel photomultiplier tube (PMT) or **Gain (Master)** to 700-800 units. An average intensity of ~3500 units is ideal for each colour channel but it is important to ensure that no pixels are saturated with these gain settings. **Note:** there may be microsphere aggregates larger than 0.1 µm in your sample; do not include these when setting the intensity thresholding.
7) The **Digital Offset** must be set above zero (~12) so that no pixels read zero intensity units.
8) Set the **Digital Gain** to 1.
9) Suggested laser intensity is: Green–25 mW Argon Ion-488 nm laser = 1%. Recheck the images for each channel and ensure that intensity values of ~3500 grey levels or maintained with the specific laser settings and that no pixels measure saturated intensities.
10) Press the **Snap** button to take an image of the microspheres in the plane of focus.
11) Perform a final verification of the image acquisition settings for each channel using the **Range Indicator** Look-Up Table (LUT). Click on the colour bar for each track in the image to change the colour. Under the **Dimensions** tab at the bottom of the image select the palette and choose the **Range Indicator** LUT. Zero intensity pixels will be displayed as blue and saturated pixels as red. If there are blue pixels increase the **Digital Offset**, if there are red pixels reduce the **laser power**.
12) Choose the **Z-Stack** option and set up the Z-axis scanning with a total z-stack size of 100 µm and a step size of 0.2 µm between images. Use the **Live** scanning mode and set the **Z-Stack** using the **First/Last** window. Focus below the sample and mark the **first plane** after the sample goes out of focus. Set the **last plane** at 100 µm above the **first plane**.
13) Press the **Start Experiment** button to perform the **Z-Stack** acquisition.
14) Save the image stacks as .lsm files and also as .tif files. Names the files as follows:

<u>IMPROTANT:</u>
"Lastname_Firstname_Microscope_Platform_MagnificationX_NAY_ImmersionMedia_PSF"
e.g. Brown_Claire_Zeiss710_63X_NA1.4_Oil_PSF)