ABRF-LMRG - 3D Microsphere Standard Sample Study #3
Imaging Protocol FV1000 – 3D Signal-to-Noise (Slide 2)

1) Turn on and warm up the lasers for at least one hour. Take the DIC elements OUT of the lightpath.
2) Put a 0.5 NA or higher objective lens in place. If necessary, put immersion media on the lens. (Recommend one using the Olympus 60x oil objective, if available, for one of your tests).
3) **Load Alexa 488 and Alexa 546** in the **Dye List** to facilitate setting up the lightpath quickly. (Double check the settings in the **Light Path** window (the icon with a wrench and a rainbow –located under the dyelist button). Use the DM 405/488/559/635 nm Main Beam Splitter to reflect the two laser excitation lines. Using the VBF (Variable Barrier Filter), adjust the channels to emission bandwidths of **~495-540 nm** and **~560-650 nm**.
4) In the "Image Acquisition Control" menu, set the sequential mode to "**Line", choose a Kalman of 4.** Set **up 'Harddisk recording'**. In the "Acquisition Setting" menu, moving from the top to bottom of the menu: Choose one-way imaging (left to right), choose a pixel dwell of **2 us/pixel**, choose a **Frame Size** of **512x512 pixels**, choose a **Zoom factor of 1.5 to 2.0** to achieve a final pixel size between 0.20 and 0.30 um (see 'i' window under Image Acquisition Control menu for pixel size information). Choose a **z step size of 1.0** (for SNR tests).
5) Set the **Pinhole** to 1 Airy unit (AU).
6) Set each detection channel photomultiplier tube (PMT) - indicated by **HV** under the Image Acquisition control menu - for each laser to 700-800 units (preferably, 700 units if signal strong enough). Use **Ctrl H** (Hi-Lo) to check the saturation levels. An average intensity of ~3500 units for each colour channel is ideal but it is important to ensure that no pixels are saturated with these gain settings.
7) The **Digital Offset** should be set to zero – so that no pixels read zero intensity units. -Double check there are no zero intensity (blue) pixels using the CTRL H (Hi-Lo) feature.
8) Set the **Digital Gain** to 1.
9) Suggested laser intensities are: Green–25 mW Argon Ion-488 nm laser = >1% and Red –20 mW-559 nm laser = >1%. Recheck the images for each channel and ensure that intensity values of ~3500 grey levels are maintained with these specific laser settings and that no pixels measure saturated intensities.
10) Press the **XY button** (no depth) 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 CTRL H for the plane of focus. 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 (not the HV setting!).**
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 1.0 μm** between images. Use the **Live** scanning mode and set the **Z-Stack** using the **Start/End** menu. 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 **XYZ** button to perform the **Z-Stack** acquisition.
14) Save the image stacks as .oib files and also as export files. Names the files as follows:

    <u>IMPORTANT:</u>

    "Lastname_Firstname_Microscope_Platform_MagnificationX_NAY_ImmersionMedia_SNR" e.g. Brown_Claire_Zeiss710_63X_NA1.4_Oil_SNR)

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15) Please follow this link to submit a sample information form for each dataset you submit https://tinyurl.com/LMRG-vial2 (takes a few seconds to load correct link if you click on it).