ABRF-LMRG - 3D Microsphere Standard Sample Study #3
Imaging Protocol FV1000 – 3D Point Spread Function (Slide1)

1) Turn on and warm up the lasers for at least one hour. Take the DIC elements OUT of the lightpath.
2) Put a 1.0 NA or higher objective lens in place. If necessary, put immersion media on the lens. (Recommend using the Olympus 60x oil objective for one of your tests, if available).
3) Load Alexa 488 in the Dye List to facilitate setting up the lightpath quickly. The **DM 405/488** nm Main Beam Splitter will be selected. Using the VBF (Variable Barrier Filter), adjust the emission bandwidth of Channel 1 to **~495-540 nm**.
4) In the **Image Acquisition Control** window, 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 window: 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** to achieve a final pixel size of **0.07 um** (see 'i' button under the Image Acquisition Control window for pixel size information). Choose a z step size of **0.1** (for PSF tests).
5) Set the **Pinhole** to 1 Airy unit (AU) which is chosen when 'Auto' is selected under the Confocal Aperture.
6) Set each detection channel photomultiplier tube (PMT) - indicated by **HV** under the Image Acquisition control menu - to 700-800 units. 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. **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** 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 intensity is: Green–25 mW Argon Ion-488 nm laser = 1%. Recheck the image 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 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 0.1 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 as 0. Set the **last plane** at 100 µm above the **first plane**. The image should be automatically saved to harddisk recording since it will be a large stack.
13) Press the **XYZ** button to perform the **Z-Stack** acquisition.
14) Save the image stacks as .oib files and also as .tif files. Names the files as follows:

    <u>IMPORTANT:</u>

    "Lastname_Firstname_Microscope_Platform_MagnificationX_NA_ImmersionMedia_PSF"

    e.g. Brown_Claire_Zeiss710_63X_NA1.4_Oil_PSF)
15) 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).

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