Instrument test protocol

Laser, stage, PMT stability:

Purpose:
Measure laser brightness/ fluctuation and PMT sensitivity/fluctuation over time.

Protocol:
- Warm up lasers for one hour.
- Use the appropriate slide and laser combination. Note: several different laser lines may work with one slide. *The red slide works well for most LASER lines*
- With a 10x or 20x (low NA) lens focus a surface scratch, then focus down ~20um
- Set up acquisition such that: Gain (~½ scale) and offset (> 0) should be set so that no PMT is saturated. The mean value should be ~150 (out of 255 gray levels). These values as well as laser power will vary for each laser used. *If using the same PMT for multiple LASER lines keep the PMT voltage constant and vary the LASER power*
- Collect images every 30 sec for 3 hours. *A couple of line averages per frame can be used.* Use sequential scan to collect as many laser lines as possible, i.e. 1 laser line/ PMT
- Collect images every 0.5 sec for 5 min., one wavelength at a time and scan faster if necessary.
- At the end of the test, shift the slide ~½ field of view and collect another image, measure the intensity across the field to check for photo-bleaching
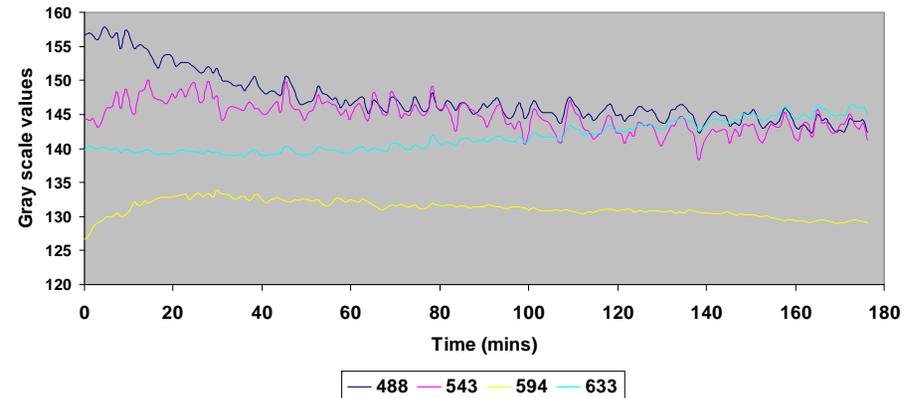
Analysis:

Calculate:
****mean brightness should be ~150****
1. % change from starting value (highest value-lowest value)
    1. Would expect less than 10% change for 3 hr. test
    2. Would expect less than 3% change for 5 min. test
2. Variance of time series
    Please use provided excel sheet when sending data

Laser power Vs. Time

Legend: 488 543 594 633

Notes:

Since this procedure will test several microscope components, care must be taken with the interpretation of the data. For example, if a slow decrease is detected in all laser lines, the problem most likely will be with stage drift. There can be multiple problems superimposed on each other further complicating analysis. If stage drift is suspected a mirror slide would be the preferred test specimen because small changes in Z are more easily detected. Alternatively, the transmitted detector can be used (without a slide) to completely eliminate the stage and thermal drift from the analysis.

If instability is still present after 3 hours, measuring for longer times may be required.

The instability in the above graph is most likely from variability in the optical system and not the LASERs themselves.

Colored slides & bead slides: (will be provided)

Field illumination:

Purpose:
Measure uniformity of illumination across the entire scan field

Protocol:
- Warm up lasers for one hour.
- Use the 488 or 543/561 LASER combination for green/orange slide from chroma slide (cover-slipped area).
- Collect scan such that the intensity is near 150 averaging is OK, zoom @ manufacturers specification, (may not be 1), using as many lens as possible. ***Use sufficient LASER power so that the gain on the PMT is ~ ½ the maximum.***

Analysis:
Using entire image Calculate: perform a scan profile diagonally and horizontally across the image to check for drop off near the edges. The typical 1X zoom variations are 10% in horizontal and 20% in diagonal.

PMT co-registration:

Purpose:
Determine to what extent an object (bead) collected with different PMT register/superimposes to each other

Protocol:
Bead slide: (will be provided) Tetraspeck beads (blue, green orange, dark red 4.0 µm)
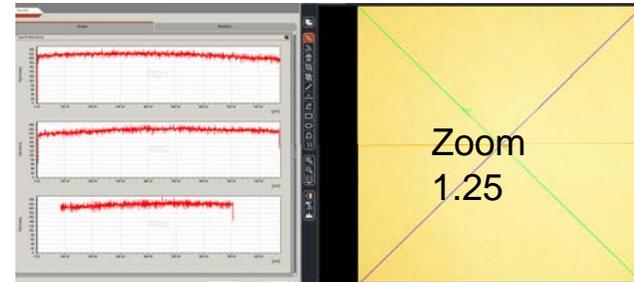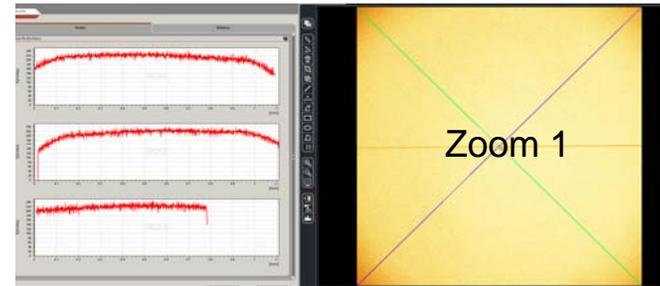Use high NA (>1.2) lens, i.e., 40X or higher. Collect such that the pixel size is half the resolution of the lens. Zoom near 10 will be needed. Use standard three or four color protocol.
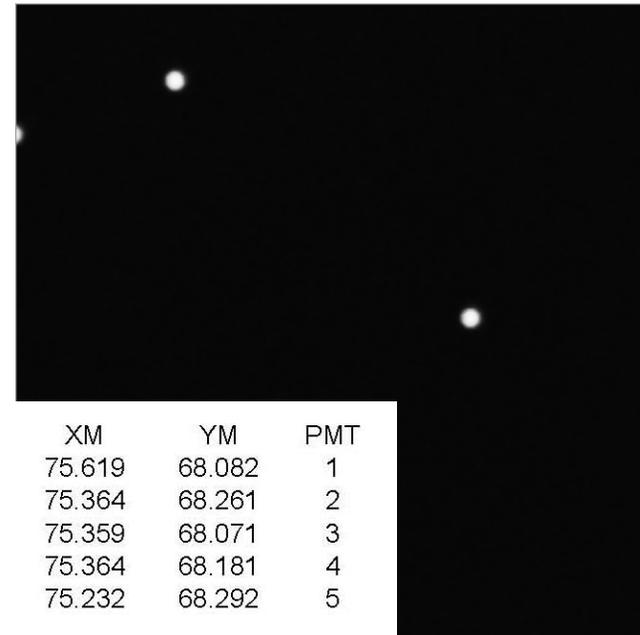Collect Z series using sequential scans of three or more PMTs
Do not forget to use the NDD for MP scopes.

Analysis:
- Using a line scan function, plot the intensities across the bead for each slice in the stack. The brightest slice is the "most in focus" This should be the same Z position for all PMTs.
- Using ImageJ measurement function determine the center of mass for the "most in focus" slice for all the PMTs. Determine the displacement among the PMT's. Performing this on more than on bead will help to separate aberrant beads (i.e. not fully attached). Single beads should be "cropped out" for the measurements.

Zoom 1

Zoom 1.25

Images of fluorescent test slide (20X) & results of line scans

XM	YM	PMT
75.619	68.082	1
75.364	68.261	2
75.359	68.071	3
75.364	68.181	4
75.232	68.292	5

"most in focus" slices from each PMT & center of mass values (µM) for top most bead