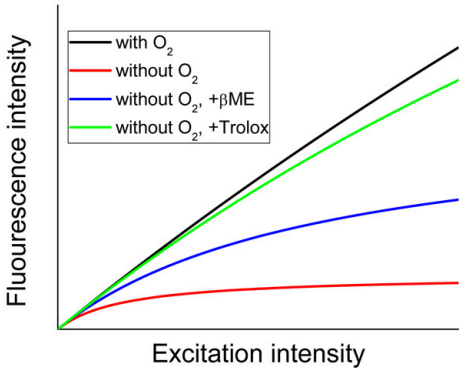
Sonicate TetraSpeck beads and equilibrate them to 37 C. Take note of the lot number of the TetraSpeck beads (the concentration and actual diameter of the beads vary from lot to lot).

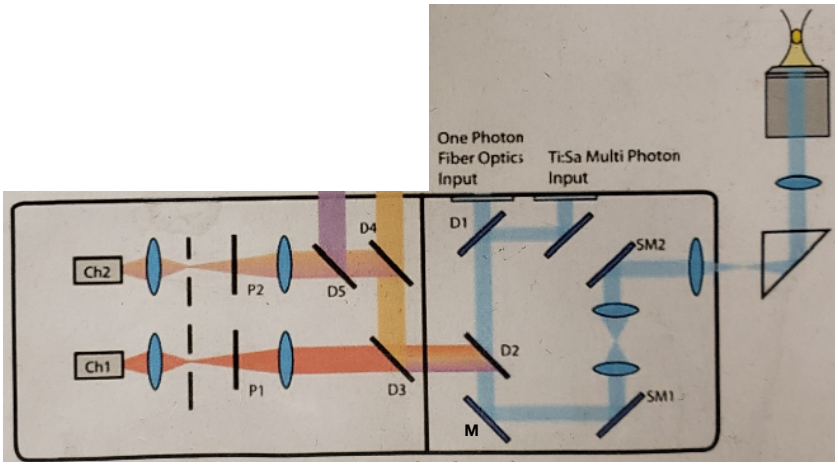
Preprocessing standard fluorescent dye solution using Damon's stocks:
Tween-20: 1:100 (final concentration: 0.05% v/v)
O_2-scavenging: PCA: 1:50 (final concentration: 2.5 mM), PCD: 1:50 (final concentration: 7 ug/mL or 21 uU/mL)
Anti-blinking: Trolox: 1:100 (final concentration: 1 mM)



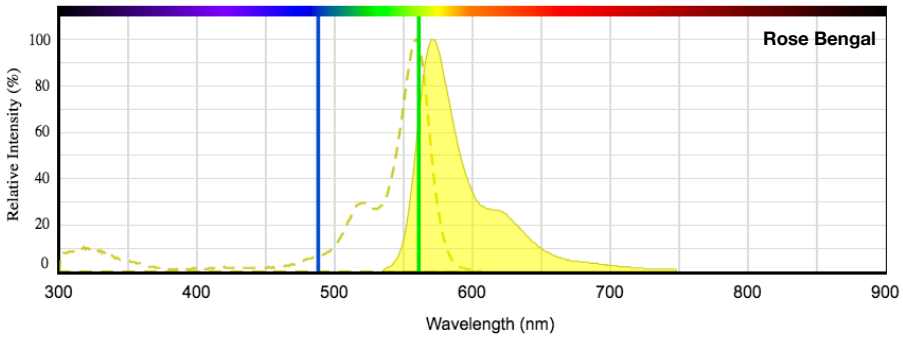
(Ha and Tinnefeld, 2012)

The filter wheel of the Olympus microscope should be set to position #6.

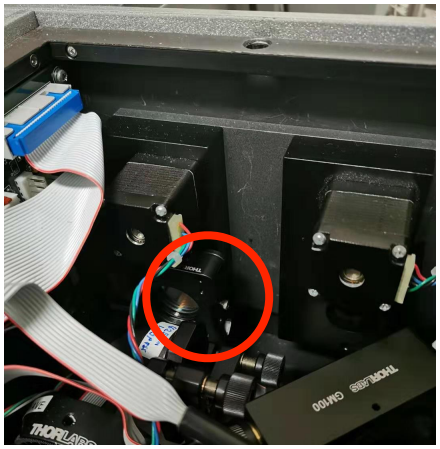
Filter setup:

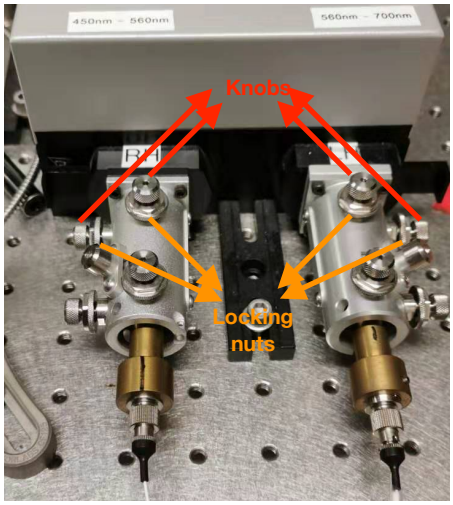


i. Single GFP Channel (Ch 1):
(Ch 1) P1#1 (531/40 bandpass) <- D3#5 (653 shortpass) <-
ii. RFP (Ch 1) + GFP (Ch 2) channels:
(Ch 2) P2#1 (531/40 bandpass) <- D5#3 (empty) <- D4#2 (mirror)
|
(Ch 1) P1#3 (593/40 bandpass) <- D3#4 (562 longpass) <-
iii. IRF measurement [use 1-10 uM Rose Bengal/erythrosine B in 5.6 M KI (aq) instead of LUDOX to avoid the color effect of APD detecters (Szabelski *et al*., 2009); Ch 2 for 488 nm IRF and Ch1 for 561 nm IRF]:
(Ch 2) P2#5 (582/75 bandpass) <- D5#3 (empty) <- D4#2 (mirror)
|
(Ch 1) P1#3 (593/40 bandpass) <- D3#4 (562 longpass) <-
Note: use high power percentage and large pinhole sizes for 488 nm to measure Ch 2 IRF since Rose Bengal has poor excitation at 488 nm:



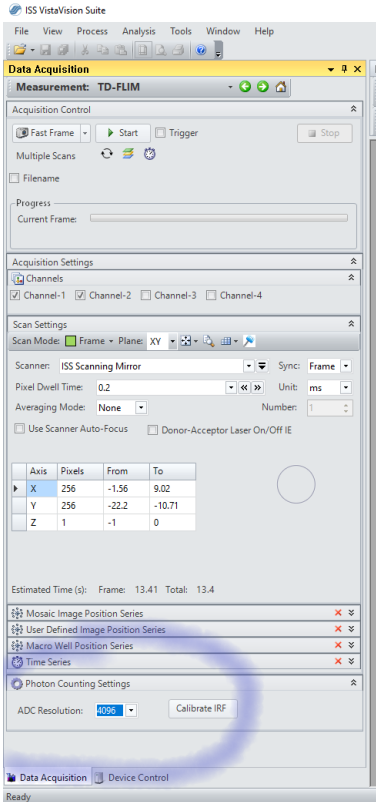
Open the lid of the optical components box and check whether the first filter slider is fully pushed in (leftwards; see the left image below). Set the excitation filter wheel to #3 (405/488/561/635 nm) in VistaVision under Device Control. Make sure that correct single-mode fibers are inserted. The 4 knobs (see the right image below) can be adjusted to align the excitation light. To do the alignment, close APD shutters (or just switch off the channels so that APDs will not accidentally be saturated) and open the lid of the optical components box. Insert a white paper between the filter wheel D2 and the mirror M. Turn on corresponding excitation light through the AOTF panel (set to 100%). Open the excitation shutter in VistaVision under Device Control. Loosen the corresponding locking nuts and adjust the knobs iteratively so that the excitation light spot on the white paper reaches maximum intensity. Then fasten the nuts, remove the paper and close the lid of the optical components box.

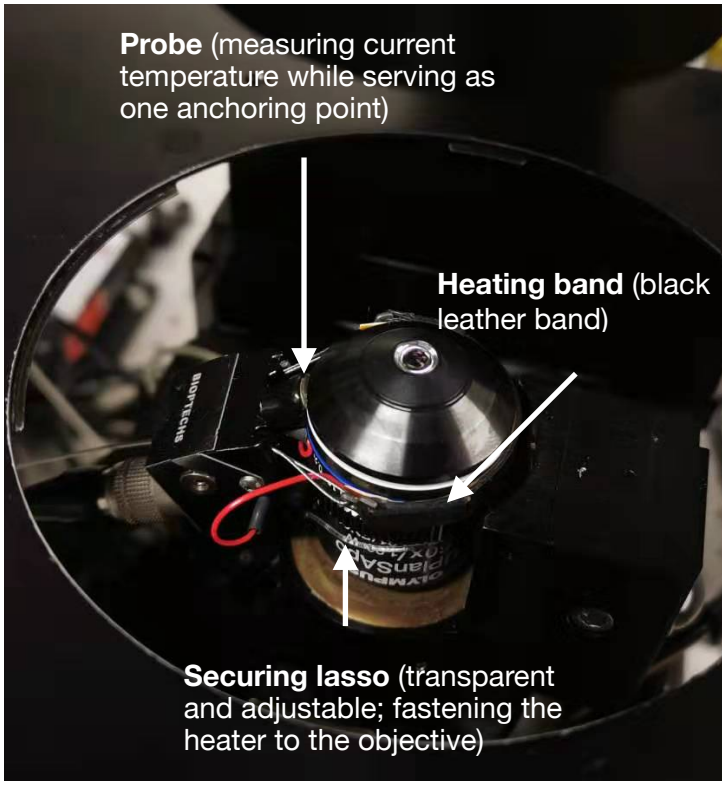




When using different pinhole sizes, re-alignment of pinholes is necessary.

ADC Resolution in TD-FLIM (set to the maximum value 4096):





Setting up the objective heater: 1. Use a hex key of the correct size (5/64") to adjust (from the back) the probe indent so that it is firmly touching against the objective. 2. Adjust the length of the securing lasso.