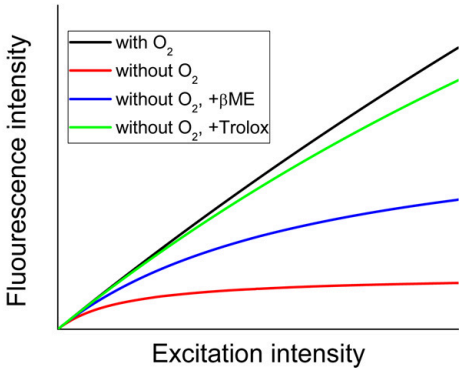
Alba v5 time-resolved confocal microscope (at the SMART center of the University of Michigan) operations manual for GFP/RFP FLIM/FCS/FCCS

Last updated: 9/2/2021 by Chu Chen (Dr. Ajit Joglekar's Lab)

Sonicate TetraSpeck beads and equilibrate them to 37 °C. Take note of the lot number of the TetraSpeck beads (**the concentration and the actual diameter of the beads vary from lot to lot**). The SMART center's 50% SiO_2 collidal (~ 0.02-μm) dispersion in water is from August 2021 (Alfa Aesar 43109, lot# T26H019).

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 μg/mL or 21 μU/mL)
Anti-blinking: Trolox: 1:100 (final concentration: 1 mM)

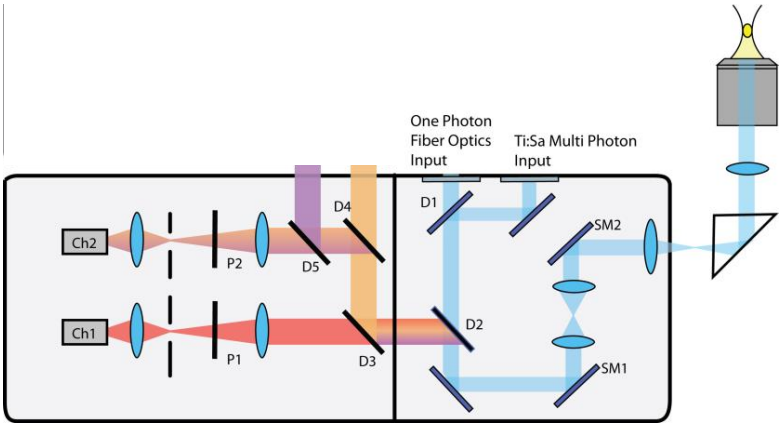


(Ha and Tinnefeld, 2012)

The filter wheel of the Olympus IX-81 microscope should be set to position #6. The "fine" focusing mode moves the sample in the *z*-direction 1 μm per graduation.

The excitation light source is a Fianium WL-SC-400-8. The wavelength is picked by an AOTF (acousto-optic tunable filter). The APD detectors are Perkin Elmer SPCM-AQRH-15. The TCSPC (time-correlated single photon counting) electronics (to register detected photon events in relation to the excitation pulses) is Becker & Hickl SPC-830. When measuring the IRFs for the early/late pulse, shut off the physical AOTF switch for the late/early pulse (instead of just setting the power to 0% in the AOTF controlling software UI due to detectable leakage at 0%).

Filter setup:

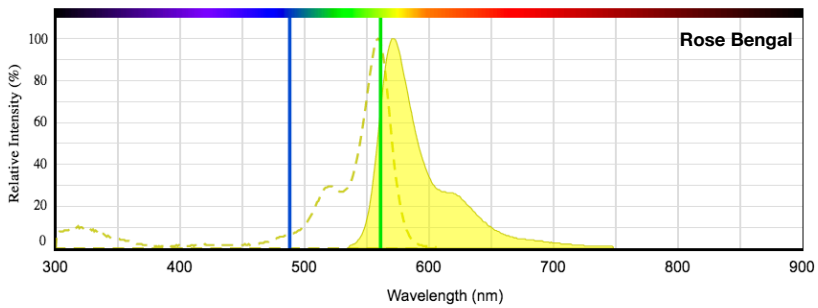


D1 is empty in the setup (there is no Ti:Sa multi-photon input). SM: **s**canning **m**irror. D3#4: 562 longpass **d**ichroic mirror (FF562-Di03, Semrock). D3#5: 653 shortpass dichroic mirror (655DCSPXR, Chroma). P1#1 & P2#1: FITC emission filter (FF01-531/40-25, Semrock). P1#3: TRITC emission filter (FF01-593/40-25, Semrock; can also use #5 630/75 emission filter). P2#5: Cy3 emission filter (FF01-582/75-25, Semrock).

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 (if using SiO_2 collidal/milk/mirror to measure the IRF of Ch2: change P2 to glass/empty)
(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 using 1-10 uM Rose Bengal/erythrosine B in 5.6 M KI (aq) [avoiding the color effect of APD detecters (https://www.sciencedirect.com/science/article/pii/S0009261409001389, 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:



iv. IRF measurement using the mirror (Ch 2 for 488 nm IRF):

(Ch 2) (empty) <- D5#3 (empty) <- D4#2 (mirror)

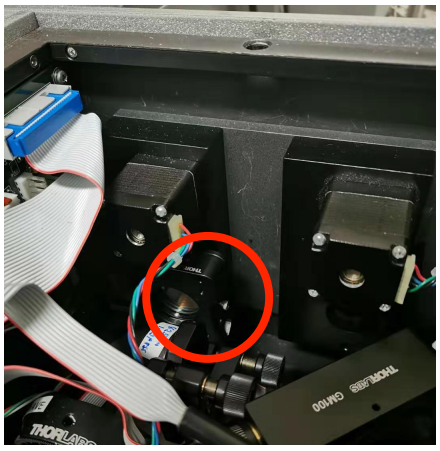
|

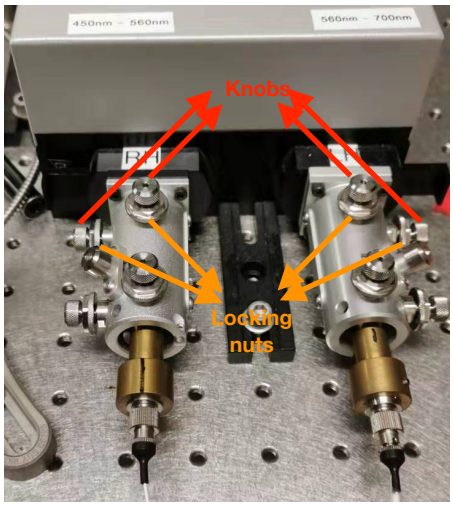
D3#4 (562 longpass) <-

Note: use a low power percentage. The mirror should be loaded on top of a coverslip (objective->water->coverslip->mirror).

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 (D2) to #3 [405/488/561/635-nm quadband dichroic mirror (Z405/488/561/635rpc, Chroma)] in VistaVision under Device Control. Make sure that correct single-mode fibers are inserted.

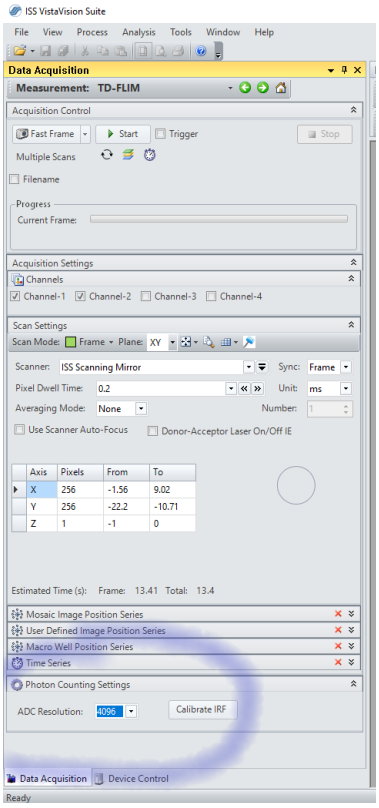
The 4 knobs (see the right image below) can be adjusted to align the excitation light. To do the alignment, close APD shutters (or just switch off the channels so that APDs will not accidentally be saturated) and open the lid of the optical components box. Insert a white paper between the filter wheel 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.

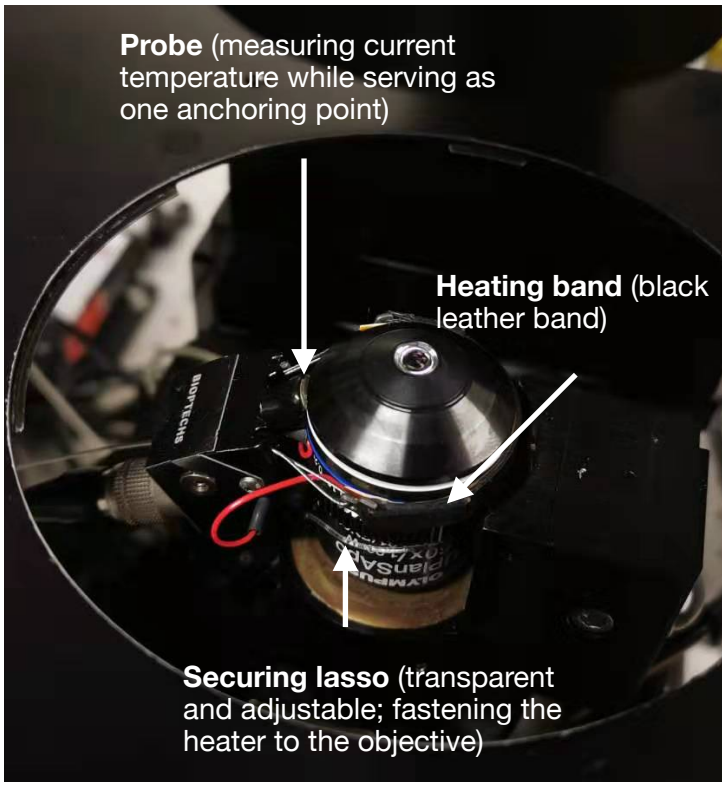




When using different pinhole sizes, re-alignment of pinholes is necessary.

Set “ADC Resolution” in TD-FLIM to the maximum (4096 - see the left figure below, which means that each bin spans ~ 50/4096 ns. Note that the frequency of the excitation pulse is only roughly, but not exactly, 20 MHz). Note that the default unit of “Pixel Dwell Time” is ms.





Setting up the objective (60 ×, 1.2 NA, water immersion, Olympus PlanSApo, with an adjustable objective ring to account for coverslip thickness) 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 (see the right figure above).

Make sure shutters are all closed before closing VistaVision.