

FLIM

Set up SP5 for MP-FLIM imaging

- 1 Turn MP laser to 'on'.
- 2 Activate *Seq* acquisition in LAS AF.
- 3 Load FLIM scan sequence.
- 4 Open on MP laser shutter; make sure Scan 1 pinhole is fully open.

Scan 1=MP-FLIM – set so that signal remains below 'Green' pseudocolor. –Greylevel <80.

Scan 2 = Dx Dm

Scan 3 = Ax Am

Scan 4 = Dx Am

Scan 5 = acceptor bleach/DIC

- To acquire a FLIM image, select scan 1 and go live. Then activate a B&H acquisition (see below).
- To acquire a SE FRET image series and steady state reference image, click "Start".
- To photobleach the acceptor, select scan 5 and go *Live* for your bleach duration; then repeat the post-bleach acquisition for AB-FRET.

BH software setup

Log on to FLIM workstation using your MBF account.

Start SPCM and SPC_Image software on FLIM workstation.

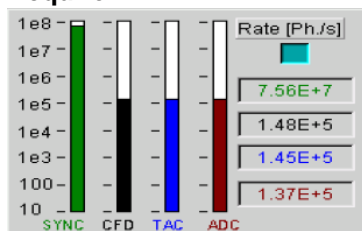
OK each start-up dialog.

In SPCM software load configuration (*Main>Load*); choose setup file and select:
MBF_256_256_sp5.set



In SPCM, set acquisition time
(20 – 60 seconds).

Acquire

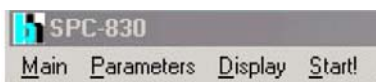


Start continuous scan in Leica SP5 software.

CFD in SPC software should read between $5E+4$ to $5E+6$ to avoid pile up issues.

Lower the laser power in SP5 software if the signal is above this range.

Click '*Start!*'



menu item in SPCM software.

When finished stop scanning (and bleaching) in the Leica SP5 software.

Save

1. **Main>Save** data after acquisition.

Create new file name via the *Browse* button then save the file with the *Save* button

Analyse

1. **Main>Send to SPC_Image** for analysis.

2. In *SPCImage*: set **Bin** setting to '1' (=3×3 pixels (!))

3. Set **Options>Model** parameters

Repetition time = 12.5 (this is a 80 MHz pulsed laser)

Collection Time = time acquired

Dead time = 150 ns

3. Select Menu item **Calculate>Improve Matrix**

Export

1. **File>Export**

Check: tau1; chi2; photons

This will generate text-file images which can be imported with MBF_ImageJ's **Plugins>FLIM>Open B&H export** function.