Confocal Application Letter No. 13

Sequential Scan for
Leica TCS NT/SP systems

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Sequential Scan

The new feature Sequential Scan allows image acquisition of multistained specimens to be performed sequentially for different filter settings using a single scanning procedure. This protocol should help you to create your own macro for the sequential scanning procedure.

The Sequential Scan enables:

Easy examination of multilabelled samples even with systems which have fewer detectors than needed for simultaneous acquisition

(e.g. tripple stained sample with a two-channel-system).

Reduction of cross-talk when emission spectra of the fluorochromes have wide overlap

(e.g. DAPI and FITC, see Fig. 1).

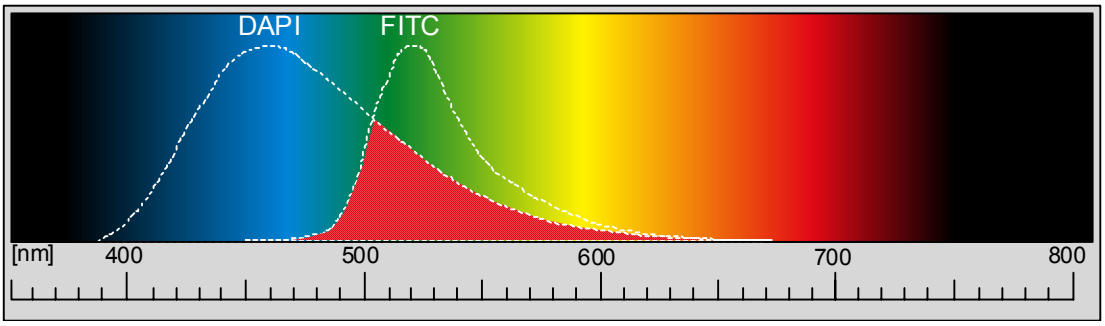


Figure 1: Emission spectra of DAPI/FITC

The emission spectra of DAPI and FITC show a wide range of overlap

In addition, Sequential Scan also allows fast changes of the excitation wavelength by controlling the Acousto Optical Tuning Filter (AOTF). This capability is needed for ratioimaging ion measurements or pH measurements with fluorescent indicators such as Fura 2 or SNARF-1, respectively.

Procedure:

1. Open ***<Filters>***
2. For each label (e.g. DAPI, FITC, TRITC, Cy5), define all filter settings such as filter wheels, mirror devices, selection filters (TCS NT) or mirror positions for the detection band (TCS SP), pinhole, AOTF, PMT (HV), offset, Clut, etc. Deactivate all the PMTs not needed for the actual acquisition. Save the settings for each label in a separate macro in the filter setting dialogue by clicking ***<save>*** and assigning a unique name to each of them (e.g. seq_DAPI, seq_FITC).

   Note: check focal plane for maximal intensity.

   You have now created ”Available methods“ for the various stainings of your specimen needed for step 5.
3. Click on ***<Seq>*** within the filter dialogue box to open the dialogue box for the Sequential scan settings (Fig. 2).

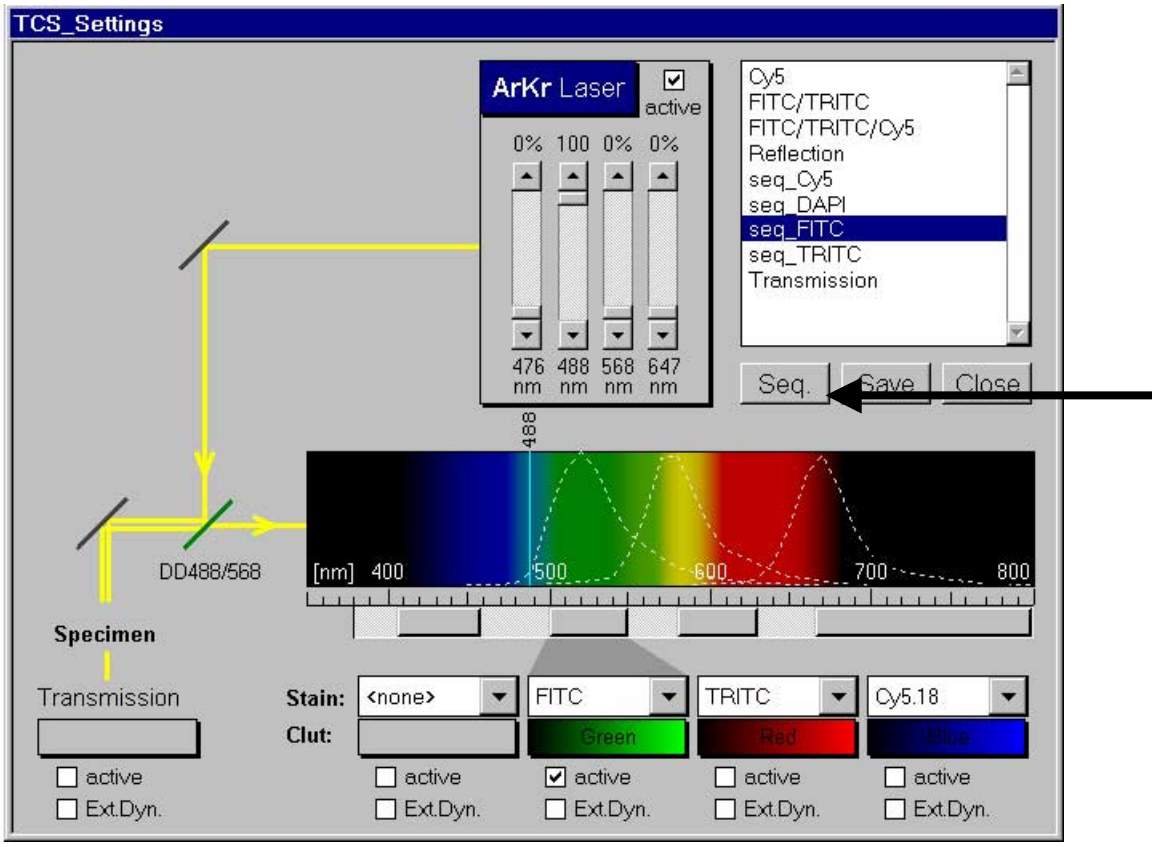


Figure 2: Dialogue for Filter Settings

4. Mark ***<Activate sequential scan>*** on the upper left corner of this dialogue box (Fig. 3).

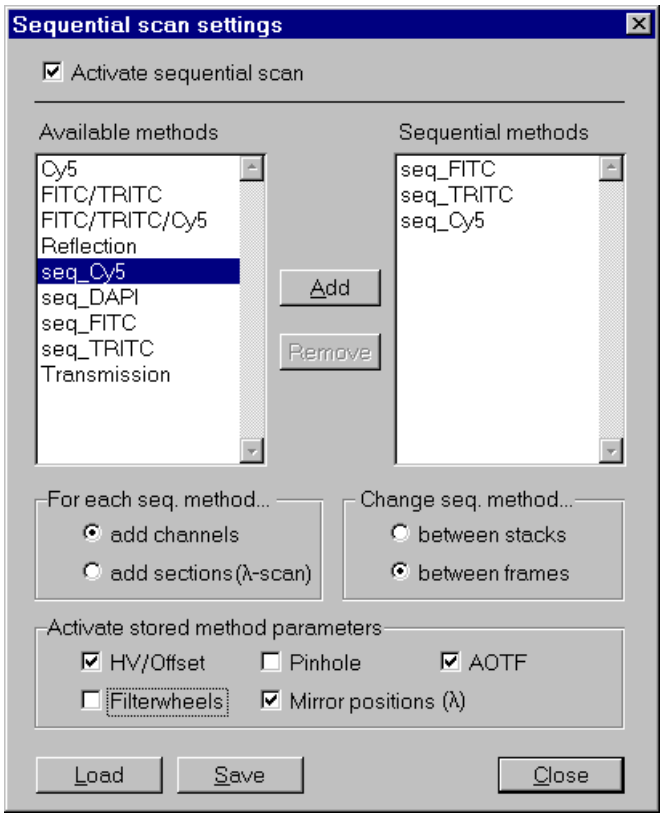


Figure 3: Dialogue for Sequential Scan

5. In ***<Available methods>*** a complete list of all saved macros is shown. Select the newly defined macros (seq_DAPI, seq_FITC,..) and add them to ***<Sequential methods>*** in the desired order.
6. In the field ***<For each seq. method ….>*** select ***<add channels>***
7. If you only have to scan one focal plane, proceed with step 9 below.
   If a stack of several optical sections is to be scanned, you have the option to change acquisition channel ***<between frames>*** or ***<between stacks>.***
   <between frames> acquires the image (frame) for an optical section for one channel, switches to the other channel, acquires the image, then moves to the next optical section and so on. This procedure is preferable in general because of the possibility of dynamics in your sample, such as shifting, thermal processes, etc.

The **<*between stacks*>** option acquires the images for the complete stack of optical sections for one channel, then changes channels acquires the complete stack of optical sections for the second channel.

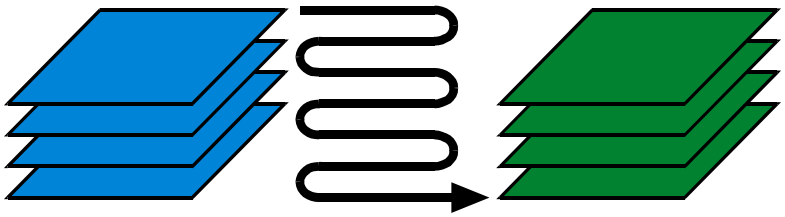


Figure 4: Workflow of option **<*between frames*>**

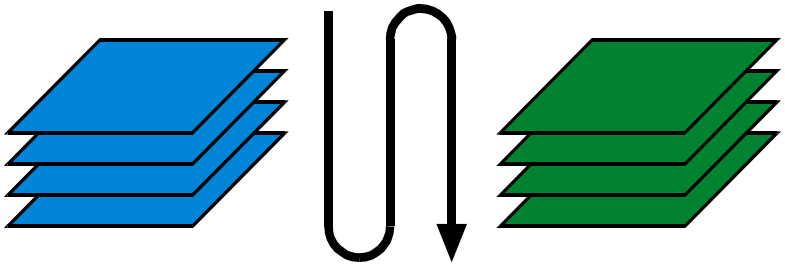


Figure 5: Workflow of option **<*between stacks*>**

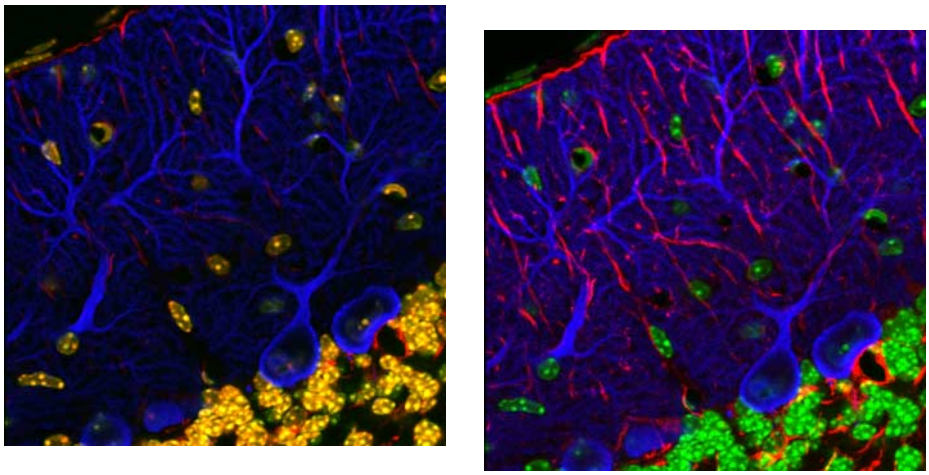
8. In order to activate the stored method parameters in the macros you created in step 2 above, mark **<*HV/Offset*>**, **<*Pinhole*>**, **<*AOTF*>**, **<*Filterwheels*>** and **<*Mirror Positions (λ)*>** in the checkbox **<*Activate stored method parameters*>**. The images will now be acquired exactly as already defined in your macro. By not marking one of the parameters with a check, the system will use the last setting. If, for example, you wish to adjust the PMT during acquisition of the single images, do not mark **<*HV/Offset*>** with a check.
9. The complete set of sequential scan settings may now be saved. It will be saved as type sequential settings (*.seq), and again you must assign to it a unique name.
10. Close the dialogue box.
11. Adjust general parameters such as the focal plane, begin and end of a series, number of sections, accumulation (number of frames to be averaged), zoom factor etc. for one channel. If it is available on your system, adjust these parameters in the simultaneous multi channel scanning mode.
12. Activate the button **<*series*>** in order to start the sequential scan procedure.

Note:

Do not forget to deactivate sequential scan for simultaneous detection.

Remark:

If you want to acquire the images as fast as possible, e.g. for Fura 2 measurements, activate as few predefined parameters as possible in the checkbox ***<Activate stored method parameters>*** (step 8). Only those parameters checked are then controlled by your Sequential Scan macro during acquisition. Unchecked parameters remain unchanged by the macro, i.e. they are fixed to the values present before the macro was activated.



simultaneous acquisition (mixture of red and green = yellow)

sequential acquisition (separation of red and green)

Figure 6: Sample of DAPI/Cy2/Cy3 tripple stained specimen
Parasagittal section of mouse cerebellum immunolabelled with antibodies against glial fiber acid protein for astrocytes (red), with antibodies against calbindin D-28k for Purkinje cells (blue), and counterstained with Hoechst (green).

— Hoechst

— anti-GFAP/Cy2

— calbindin-D28k/Cy3