CONFOCAL APPLICATION LETTER

reSOLUTION

SEQUENTIAL IMAGE RECORDING

with Leica TCS SP2

between lines/between frames/between stacks
This application letter will help you to find the best way to enhance image quality (e.g. avoiding crosstalk) by sequential image recording. Guided steps of work show you how to create and optimize your own instrument parameter settings (IPSs) and how to combine them for sequential image recording. With the sequential image recording option you can define a combination of n IPSs for n fluorescent dyes. This set you can use to record automatically multiple labelled specimen within one scanning series.

Advantages at a glance

Avoiding crosstalk
Fluorochromes with different excitation wavelengths frequently have emission spectra which show a wide overlap (e.g. FITC and TRITC, see Figure 1). When two or several such fluorochromes are simultaneously excited in a specimen, it cannot be avoided that several fluorescence signals are recorded in a detection channel and can no longer be separated in the images. This so-called crosstalk or bleedthrough can be avoided by using the sequential image recording method.

Optional sequential modes

Depending on your system configuration you can choose from three different modes of sequential image recording:
• between lines
• between frames
• between stacks

«between lines» (Fig. 2)
With this sequential mode, the detection channel and excitation wavelength are changed already after the scanning of a single line. This pattern is repeated until a full image is completed. This mode can only be used if your microscope system is equipped with an acousto-optical tunable filter (AOTF). It is particularly useful to minimize movement artifacts occurring in in vivo investigations.

«between frames» (Fig. 3)
With this sequential mode, first a complete image is recorded with the instrument parameter settings (IPS) adapted to one fluorochrome, before the system switches to a different instrument parameter setting to record the same optical section. This pattern is repeated in the subsequent optical planes.

Scanning multiple stained specimens with a limited number of detection channels
Specimens labelled with multiple fluorochromes can be recorded within one scanning series, even if the microscope system is equipped with less detection channels than needed. Thus it is possible, for example, to image a triple stained specimen with a microscope system that has only two detection channels.
Confocal Application Letter

Sequential Image Recording

Instructions how to perform a sequential image recording using the «between lines» method for example with FITC/TRITC

When you use this recording mode be aware of the fact that there is only a limited number of hardware parameters, namely AOTF, AOBS, PMT, Gain and Offset, which can be changed between the recording of two lines. Other hardware parameters, which you might wish to use for sequential image recording, have to be set to the same value for all IPS. Due to technical reasons, the time required for switching such hardware elements is not as short as the time between the recording of two lines. These hardware parameters are e.g. the detection bandwidth, the excitation beam splitter, the shutter of the laser, the diameter of the detection pinhole or the beam expander lens.

Figure 2: Scanning pattern of the «between lines» mode. Rectangles: Optical sections Black arrow: Recording mode

Figure 3: Scanning pattern of the «between frames» mode. Rectangles: Optical sections Black arrow: Recording mode

Figure 4: Scanning pattern of the «between stacks» mode. Rectangles: Optical sections Black arrow: Recording mode

stained specimen with a two-channel microscope system, since there is sufficient time between the recording of two images to change certain time-critical hardware parameters, which is not the case in the «between lines» mode.

«between stacks» (Fig. 4)
With this recording mode, all optical sections that make up an image series are recorded before the system switches to the next sequential instrument parameter setting and continues to record another image series. This method is only suitable for the imaging of fixed, immobile specimens. In addition, fluorescent dyes prone to bleaching can be recorded in a less damaging way in this recording mode. For example, if the excitation line of a determined fluorochrome simultaneously excites a second, more responsive one in a double stained specimen, it is recommendable to record the complete image series using the IPS which is adapted to the second, more responsive fluorescent dye.

This sequential recording method is particularly time-saving, if you use a TCS NT system.
Sequential Image Recording

1. First, the instrument parameters to be used for the recording of the first fluorochrome (FITC) have to be adjusted and saved as an instrument parameter setting. For this purpose, click on the «Acquire» arrow symbol and then the «Beam» button to open the «Beam Path Setting» dialog window.

2. Select the FITC-TRITC instrument parameter setting in the folder labelled with «L» in the list box in the upper right corner, in order to load hardware settings preset by Leica (see Fig. 5):

3. Deactivate the «PMT» detection channel for TRITC, clicking the «Active» check box.

4. Set the excitation line for TRITC (e.g. 543 nm) to 0%.

5. Select the desired scan format, scan speed and the zoom factor clicking the «Format», «Speed» and «Zoom» button respectively. While running the system in the «Continuous» scanning mode, optimize the PMT Gain and Offset values as well as the intensity of the excitation wavelength for the first fluorochrome in your specimen (488 nm, FITC).

6. Save these optimized settings as user-defined instrument parameter setting, (IPS in the «U» folder) and name it e.g. FITC_seq. To do this click the «Save» button in the «Beam Path Setting» dialog window (see Fig. 6).

7. Then activate the PMT assigned to TRITC and deactivate the PMT assigned to the detection of FITC.

8. Set the excitation wavelength for FITC (488 nm) to 0%.

9. Optimize the PMT Gain and Offset values as well as the intensity of the excitation wavelength for the second fluorochrome (543 nm, TRITC).

10. Save the changed settings as user-defined instrument parameter setting, (IPS in the «U» folder) and name it e.g. TRITC_seq (see fig. 7).

This procedure ensures that neither the detection bandwidth nor the excitation beam splitter, the diameter of the detection pinhole or the beam expander lens are changed when switching from one IPS to another. Depending on the number of available PMT’s in your system, you can use this method to program the recording of triple or quadruple stained specimen, as well.

11. Now click the «Sequential Scan» check box in the lower left corner of the «Beam Path Setting» dialog window (see Fig. 8).
12. Copy the instrument parameter settings you created into the «Sequential scan settings» list box which you find in the expanded «Beam Path Setting» dialog window. To do this use the «Add» button or click on the IPS, keep the left mouse button pressed and move the IPS to the «Sequential scan settings» list box (drag and drop).

13. Select the «between lines» sequential recording method in the list box named «Mode».

14. Select an appropriate scan format (xyz, xzy, xyt, xzt, xt) using the «Mode» button. Start running the system in the «Continuous» scanning mode and then define the begin point and end point using the «Begin» and «End» buttons as well as the step size using the «Sections» button when you want to record a spatial image series. Click the «Time» button to set all necessary parameters if you intend to perform a time series. If you find that the current settings of the PMT Gain and Offset values are not satisfying, you can still alter them now, since always the current settings are used for this parameter.

15. Click the «Save» button to save the complete sequence of sequential parameter settings in a file, the extension of which is «*.seq».

16. Click the «Series» button to start the sequential image recording.

Instructions how to perform a sequential image recording using the «between frames» and «between stacks» methods, for example with FITC/TRITC

Follow the steps 1 through 10 of the above description «instructions how to perform a sequential image recording using the «between lines» method for example with FITC/TRITC». Then continue with step 11 of the following description.

Alternatively, you can adjust the hardware parameters by yourself. **Please note:** The less the difference between the hardware settings of the single IPS, the faster the system is able to switch between the IPS and therefore the faster is the complete image recording. This is an important point to bear in mind, when it comes to time-critical experiments.

Note:
If you change hardware parameters such as the detection bandwidth, the excitation beam splitter, the shutter of the laser, the diameter of the detection pinhole or the beam expander lens between the single IPS, these changed parameters CANNOT be used in the «between lines» mode:
1. Select the excitation wavelength for the first fluorochrome, activate the laser line and set its intensity.
2. Select an appropriate excitation beam splitter (preferably an excitation beam splitter that is suitable for all fluorochromes you use for the sequential recording). If you have a system that is equipped with an AOBS, you can skip this step.

3. Click one of the «PMT» check boxes to select a detection channel, select the fluorochrome you use and define the color look-up table as well as the detection bandwidth.

4. To optimize image quality during image recording (AOTF setting, PMT Gain and Offset values), start the continuous scanning mode with the «Continuous» button after you have defined the scan format, the zoom factor and the scan speed using the «Format», «Zoom» and «Speed» buttons respectively.

5. Click on «Save» in the «Beam Path Setting» dialog window to save the settings of the first recording method as an instrument parameter setting.

6. In the following steps you will set the instrument parameters for the second sequential recording method and save them as instrument parameter setting. Select the next excitation wavelength, activate the laser line and set its intensity. Remember to deactivate the excitation wavelength and PMT that you do not need any more.

7. Select an appropriate excitation beam splitter (preferably an excitation beam splitter that is suitable for all fluorochromes you use for the sequential recording). If you have a system that is equipped with an AOBS, you can skip this step.

8. Click one of the «PMT» check boxes to select a detection channel, select the fluorochrome you use and define the color look-up table as well as the detection bandwidth.

9. To optimize image quality during image recording (AOTF setting, PMT Gain and Offset values), start the continuous scanning mode with the «Continuous» button.

10. Click on «Save» in the «Beam Path Setting» dialog window to save the settings of the second recording method as an instrument parameter setting.

11. Select an appropriate scan format (xyz, xzy, xyt, xzt, xt) using the «Mode» button. Start running the system in the «Continuous» scanning mode and then define the begin point and end point using the «Begin» and «End» buttons as well as the step size using the «Sections» button when you want to record a spatial image series. Click the «Time» button to set all necessary parameters if you intend to perform a time series. After you have started the «between frames» or «between stacks» sequential recording method you can no longer change settings in the «Continuous» scanning mode nor acquire single images using the «Single Scan» button.

12. Click the «Sequential Scan» check box in the lower left corner of the «Beam Path Setting» dialog window.

13. Copy the instrument parameter settings you created into the «Sequential scan settings» list box which you find in the expanded «Beam Path Setting» dialog window. To do this use the Add button or click on the IPS, keep the left mouse button pressed and move the IPS to the «Sequential scan settings» list box (drag and drop).

14. Select either the «between frames» or the «between stacks» sequential recording method in the list box named «Mode», in order to determine when the system switches to the second IPS.

15. In the list box named «Parameter» check the list of parameters which are applied for all sequential recording methods. If the check box of a parameter is ticked, the setting saved with the IPS is used for the sequential recording. If the check box of a parameter is not ticked, the currently selected hardware setting of the parameter is applied. **Please note:** The less saved parameter you select, the faster the system is able to switch from one IPS to another and consequently the faster is the data acquisition!
16. Click the «Save» button to save the complete sequence of sequential parameter settings in a file, the extension of which is «*.seq».

17. Click the «Series» button to start the sequential image recording. Please note: If you want to work in the «Continuous» scanning mode again, (see above), remember to deactivate the «Sequential Scan» mode by clicking the corresponding check box in the «Beam Path Setting» dialog window. For further questions concerning the operation of the Leica Confocal Software, please consult the online tutorials. In the menu click «Help» and «Tutorials».

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

Figure 10: Hoechst/Cy2/Cy3 triple stained specimen

Simultaneous image acquisition (mixture of red & green)
Sequential image acquisition (separation of red & green)