Confocal Application Notes
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The Sequential Scan Tool

In Sequential Scan mode, images will be recorded in a sequential order instead of acquiring them simultaneously in different channels. Each sequence can be recorded using an individual set of user-defined parameters to optimize performance and image quality. For example, simultaneous image acquisition of double or triple-stained samples can result in crosstalk since all dyes will be excited at the same time. Defining parameter sets specific for each dye and executing them in a sequential order can eliminate this crosstalk.

Let's use the following example to explore the Sequential Scan tool.

A scientist would like to use secondary antibodies conjugated with Cy5 (ex633/em660) in his experiments. In addition, the tissue sample has been stained with Propidium Iodide (PI, ex488/em620) to visualize DNA in the nuclei. The broad emission spectrum of Propidium Iodide is overlapping with the Cy5 spectrum and it may be difficult to separate both signals. Simultaneous imaging of both channels may result in some bleed-trough; a part of the PI fluorescence emission will be visible in the Cy5 image. To better separate both signals, each channel can be scanned with optimized settings for this channel using the Sequential Scan tool.

Before we can start a sequential scan we need to specify the instrument parameter settings (IPS) for our experiment. We will create two sets of parameters, one for Cy5 and one for PI.

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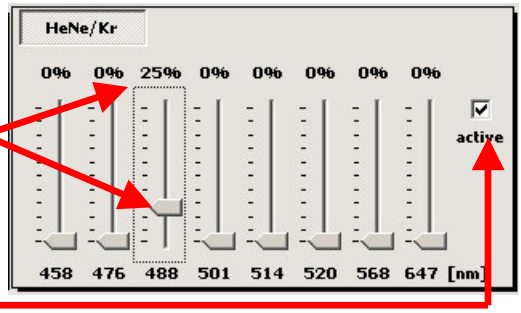
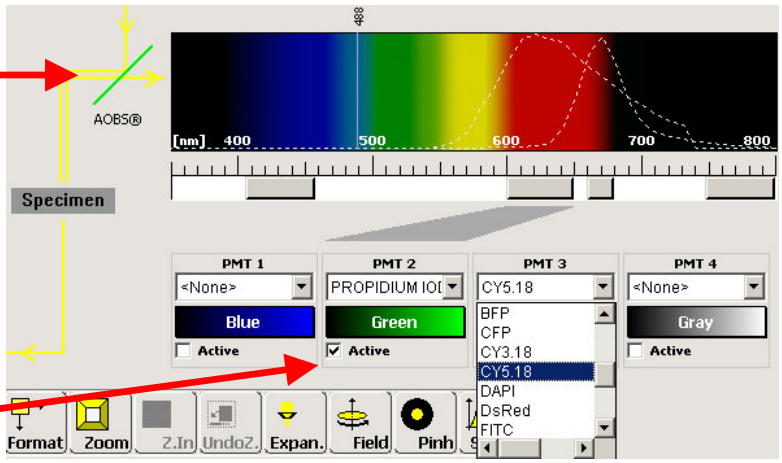
Setting the instrument parameters for each channel/dye

First we will create the appropriate IPS for Propidium Iodide. This can be done in the Beam Path Settings window. Open this window by clicking on 

1. Select the excitation wavelength. The intensity of the laser line can be set by dragging the slider or by double-clicking the number and entering a value. A suitable excitation wavelength for PI is 488 nm. Make sure the “active” box is checked!



2. Next select the beam splitter by clicking on the beam splitter icon. Select TD 488/543/633.



3. Set the active detector and select the fluorescent dye from the drop-down list. Set the detection bandwidth by either moving the slider or double-click the slider of the selected PMT. Enter the wavelength for the beginning (600 nm) and end (650 nm) of the detection band. Double-click the icon of the color look-up table of the active detector to select a display color for that channel.

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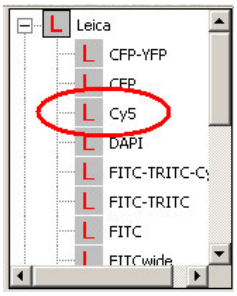
4. Select parameters like Mode, Format or Speed that you would like to use. For our example we will choose the “xyz” mode, an image size of 512 x 512 and a scan speed of 400 Hz.

5. Start *Continuous Scan* 

and set the focal plane (Z position), PMT gain and offset, pinhole, etc. to optimize the image. When you are satisfied with the image, stop the scan and save the instrument parameters as “PI-seq”.

Now repeat these steps for the second dye. Cy5 has already a predefined IPS (**L**) that can be used. Activate this setting by double-clicking on it. Our sample displays only a weak fluorescence signal from the Cy5 label and we need to improve the image using the *Line Average* tool.

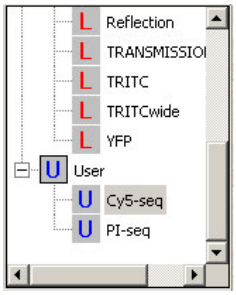


Click on 

and select the number of lines you would like to average.

Start *Continuous Scan* and optimize your settings. Save them as “Cy5-seq”.

Both IPS will appear as user settings (**U**)



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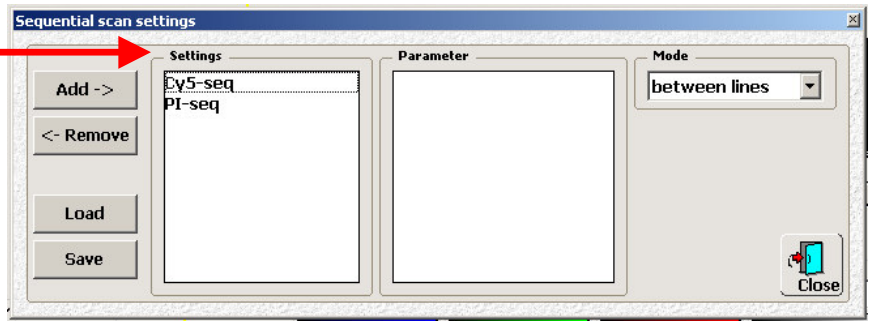
Setting the parameters for the Sequential Scan

Now we have two optimized IPS settings, one for each dye, and we can proceed to define the parameters for the sequential scan. Click on 

in the Beam Path window. This will open the dialog window below.

1. Settings



You can now drag and drop your settings from the IPS window. Selecting an IPS and clicking *Add* will achieve the same result. Sequential Scan settings can be saved and will be available at a later time or can be shared with other users just like other IPS.

2. Mode

Next we need to select the *Mode* for the sequential scan. Here we will choose when to switch between the settings Cy5-seq and PI-seq. For our example we will select *between frames*.

Between lines: after one line has been recorded using Cy5-seq, the parameters are changed to PI-seq and the same line is recorded in the PI-channel. Settings are changed back to Cy5-seq and the next line of the image is scanned in the Cy5-channel. This method is especially suitable for any time-dependent measurements.

Between frames: in this Mode the method is changed after recording a single image. By selecting "*Between frames*" we would first scan one image using the Cy5-seq settings. After that scan is

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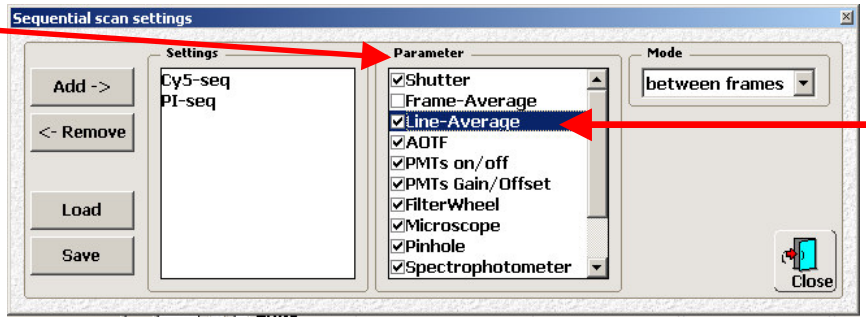
complete, settings will be changed to PI-seq and the corresponding image will be recorded in the PI-channel.

Between stacks: here a series of images, for example a xyz-stack will be recorded with the Cy-5 seq setting and only after completion of this stack the settings will be switched to PI-seq and the corresponding series of images will be recorded.

Please note that the available options; *between lines, frames* or *stacks*; depend on the Mode (xyz, xt, etc.) you choose in the Beam Path window and vice versa. For example, recording in *xyt* will enable only *between lines* and *between frames* whereas Mode *xyz* allows for *between stacks*.

Always keep in mind that sequential scanning will introduce a time difference between the recorded channels. If your experiment is time sensitive, use "*between lines*", the time difference between two correlating pixels in each channel will be minimal (only the time it takes to finish scanning one line).

3. Parameters



This option will only be available if either *between frames* or *between stacks* has been selected. Check the box next to the parameter to use different settings for each sequence as specified in the IPS, uncheck the box to use the same parameter settings for both sequences. Since we would like to use *Line Average* with the Cy5 recording we need to check the appropriate box in the parameter settings.

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With this all the parameters for the Sequential Scan are set and can be saved.

We can now specify the settings for the xyz series (current Z position, begin and end positions, number of sections) and start the recording.

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