

Photo-stimulation and photo-bleaching with the A1

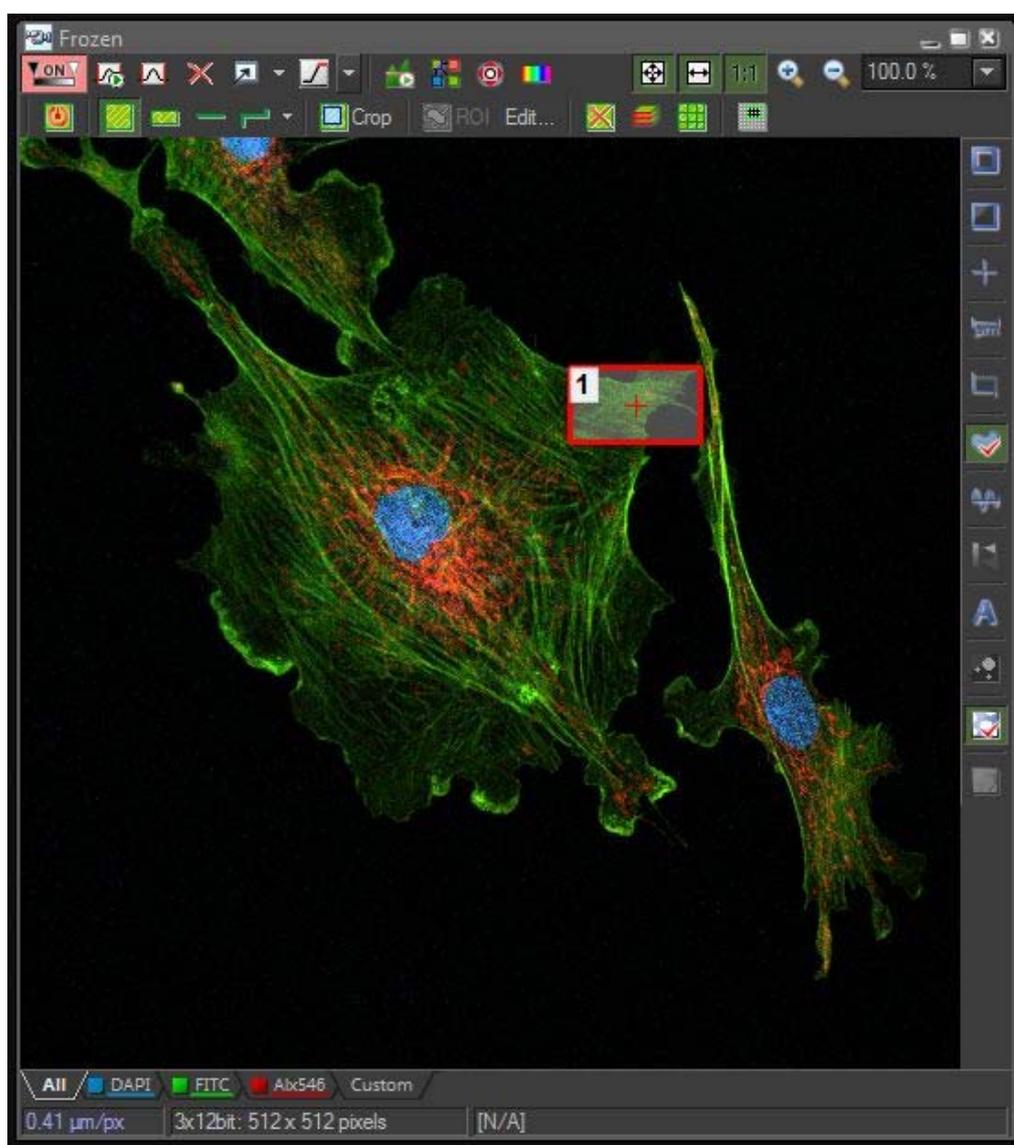
This guide shows how to conduct bleaching and photostimulation experiments using the A1 and A1R.

Sequential Stimulation

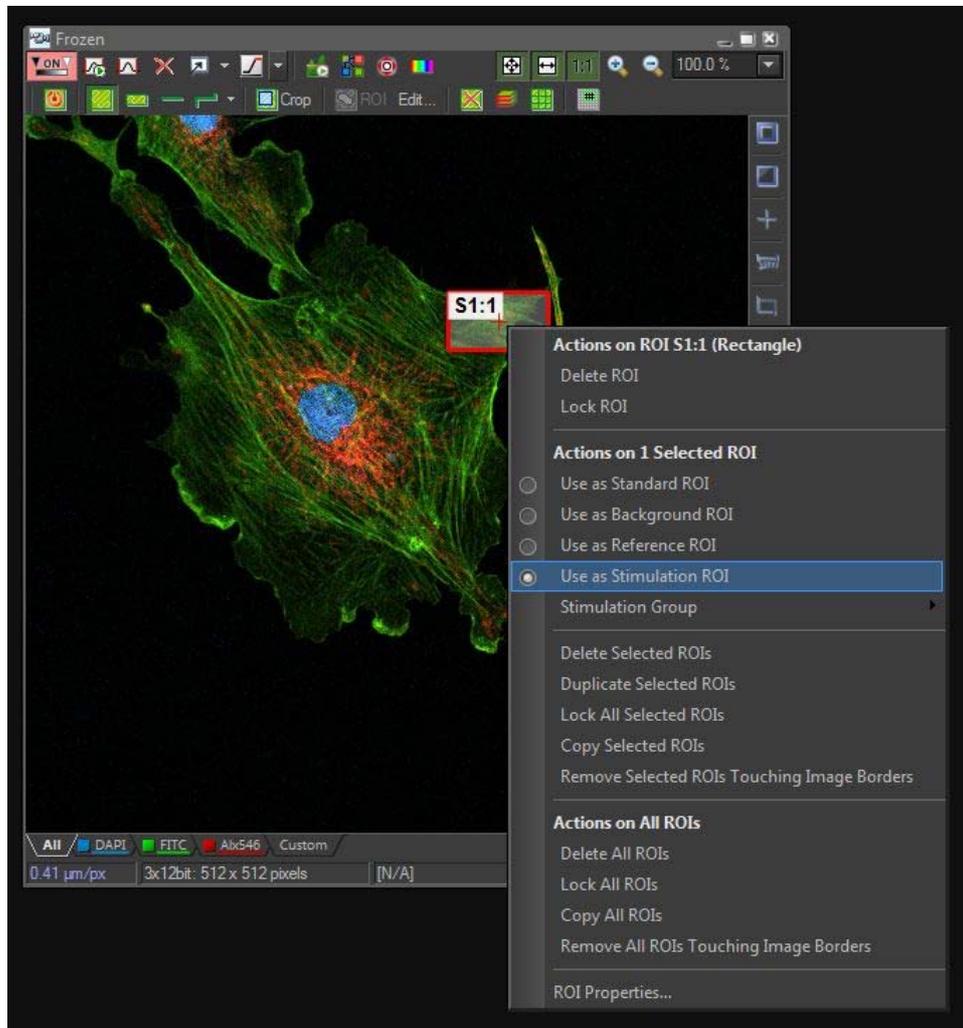
Sequential Stimulation uses the galvano scanners to acquire a series of pre-stimulation images, stimulate (also with the galvano scanners), and then acquire a series of post-stimulation images. No images are acquired during the stimulation phase. This experiment mode is compatible with both the A1 and A1R confocal microscope models.

Setup

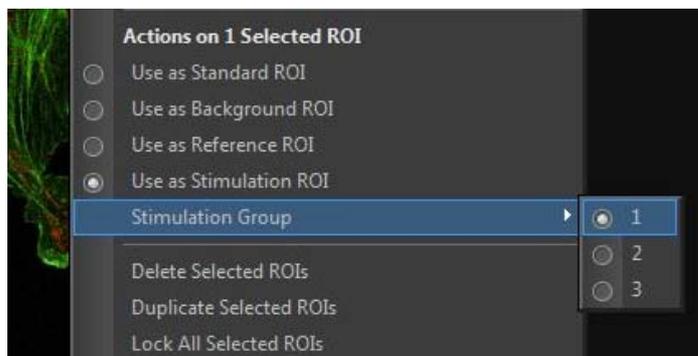
On the frozen image draw an ROI on the area to be stimulated either using the ROI button on the side of the document window or by going into Simple ROI Editor.



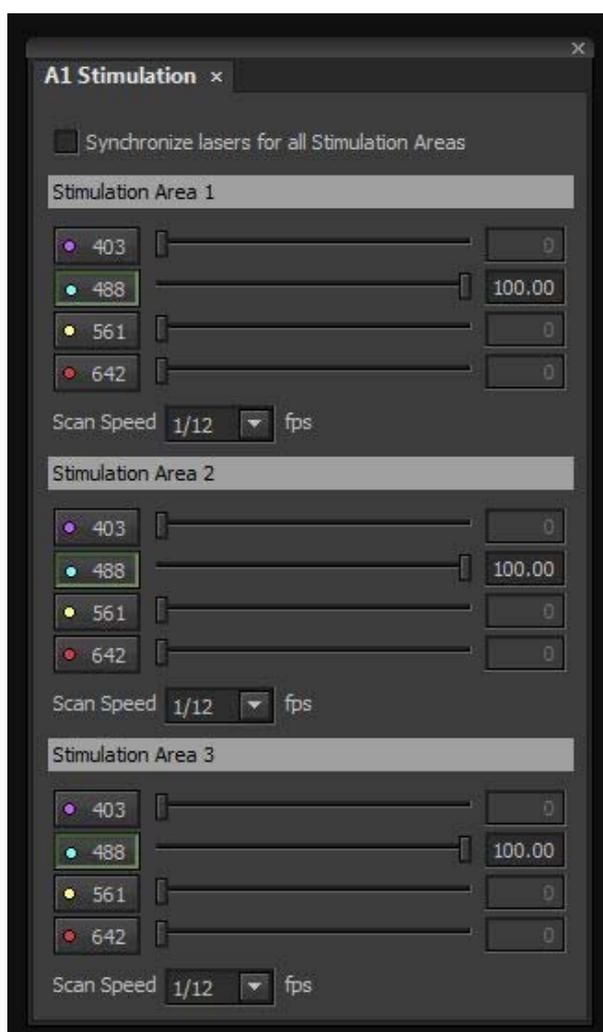
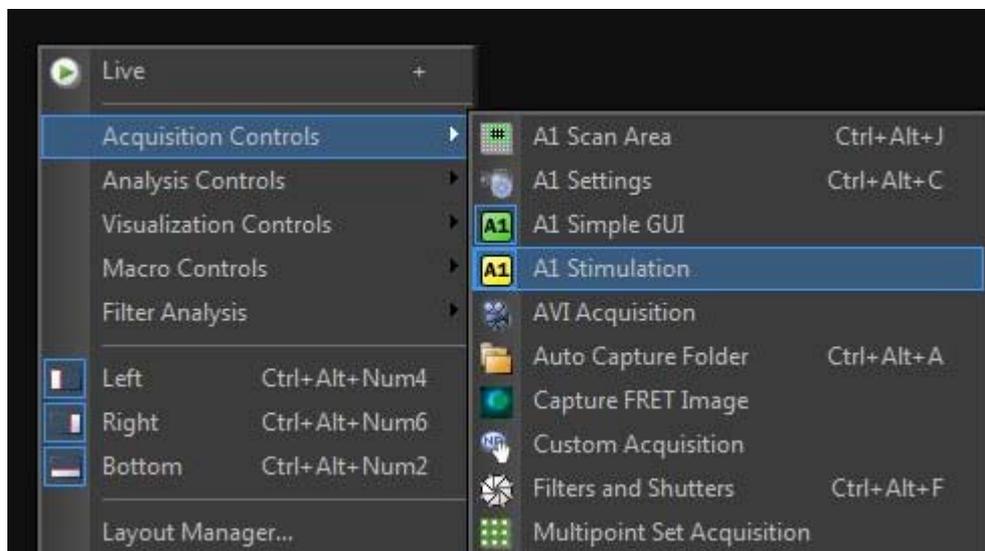
Any shape of ROI can be selected for use as a stimulation or bleaching ROI. Once the ROI is drawn it should be marked as a stimulation ROI by right-clicking the ROI and selecting <Use as Stimulation ROI>. This option should be selected for both stimulation and bleaching experiments.



ROIs can also be grouped into several groups, which share stimulation settings and phases. To assign ROIs to a group, select <Stimulation Group> and select 1, 2 or 3.



In order to set up a stimulation experiment, the stimulation GUI needs to be accessed. The photostimulation GUIs are different depending on the A1 GUI used. For users of the A1 Simple GUI, the <A1 Stimulation> panel can be found by right-clicking the background, under <Acquisition Controls>.

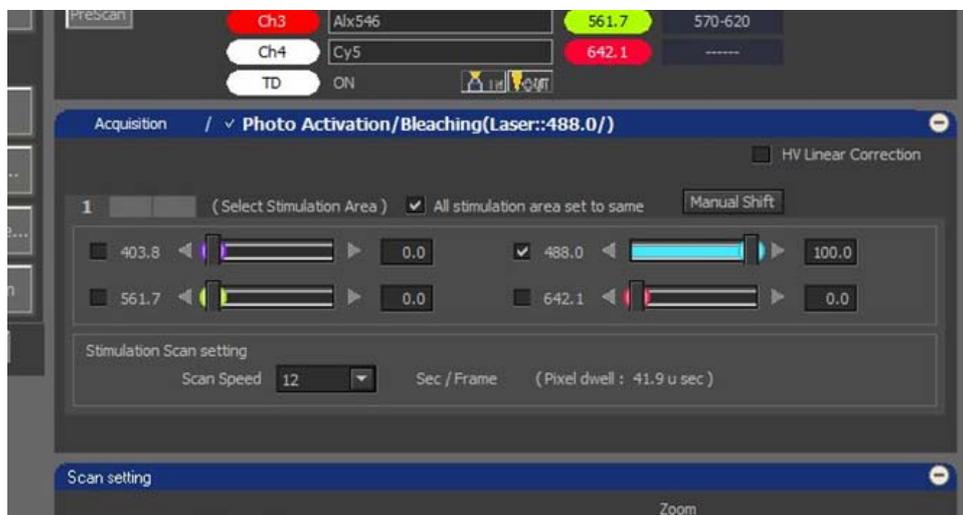


The stimulation settings open in a new panel. The screenshot to the left shows the panel in the fully expanded mode. This mode is used when there are multiple stimulation groups and each group has different laser lines, laser powers or stimulation scanning speeds. If only one group is used, or the settings for all the groups is the same, then the <Synchronize lasers for all Stimulation Areas> option can be ticked, and the panel will shrink to a single section which controls the settings for all the Stimulation ROIs.

The settings show the available laser lines, the powers for each laser and the scan speed to be used for stimulation. Please note that the scan speeds are shown in fps (**frames per second**) e.g. a scan speed of 1/12 fps will mean that a single pass of the stimulation laser will take 12 seconds, whereas a speed of 16fps means a single pass will take 1/16 of a second.



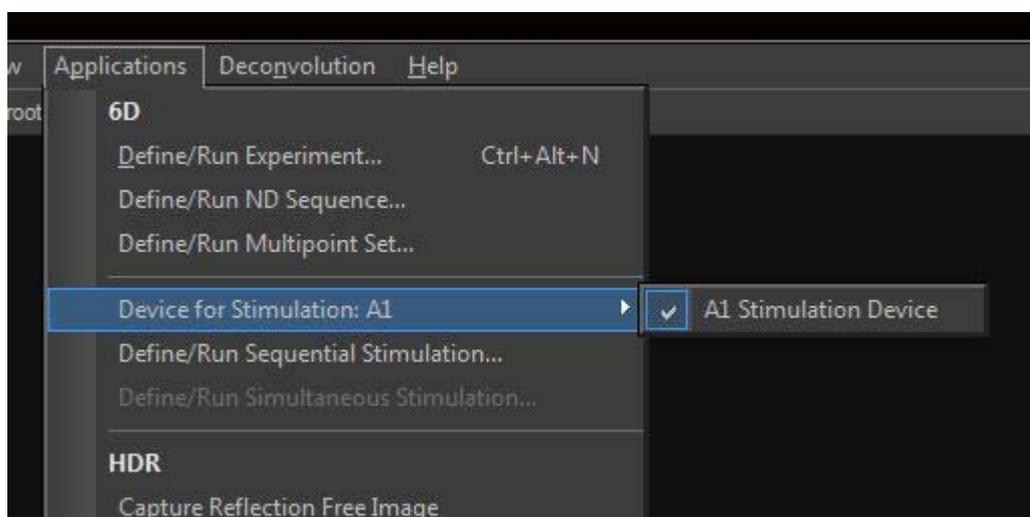
For users of the original A1 Settings GUI, the stimulation controls are within the main window. In the Acquisition part of the panel, where the laser power and gain are set for normal imaging, click <Photo Activation / Bleaching> in the title area to open the stimulation settings.



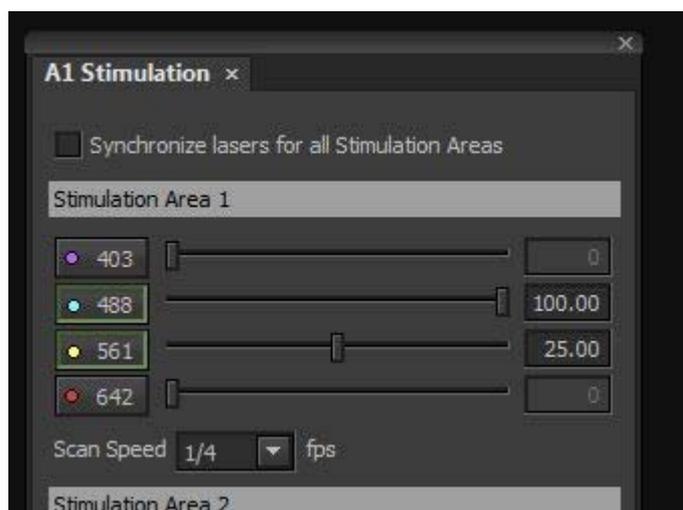
Here the different stimulation groups are displayed as tabs rather than expanded panels. The <All stimulation area set to same> option is also available. This panel contains laser lines, powers and scan speeds. In this GUI, however, the scan speed is shown in **seconds per frame** e.g. a scan speed of 12 sec/frame will mean that a single pass of the stimulation laser will take 12 seconds, whereas a speed of 1/16 sec/frame means a single pass will take 1/16 of a second.

Stimulation Settings

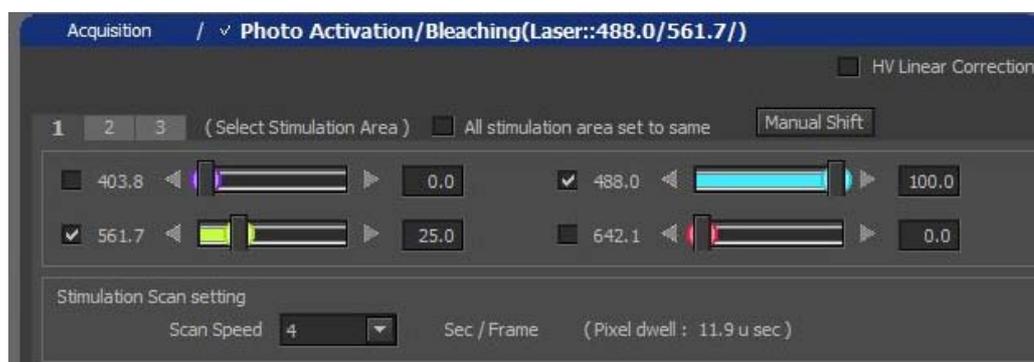
Once the GUI has been correctly set up for bleaching, the A1 should be set as the stimulation device. In most cases this will be set as default. To check this go to <Applications> on the main menu and under <Device for Stimulation> make sure that <A1 Stimulation Device> is selected.



Using your GUI of choice, set up the laser lines, laser powers and scan speed.



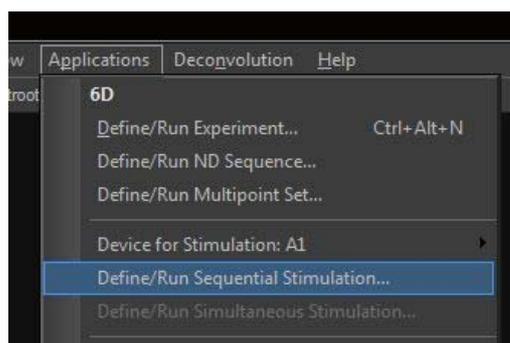
Any combination of available lasers can be used for sequential stimulation. In this example the 488nm and 561nm lasers have been selected. The 488nm laser has been set to 100% power, and the 561nm to 25% power. As there is only one ROI, only Stimulation Area 1 has been set up. The scan speed has been set to 1/4fps, meaning each pass of the laser will take 4 seconds.

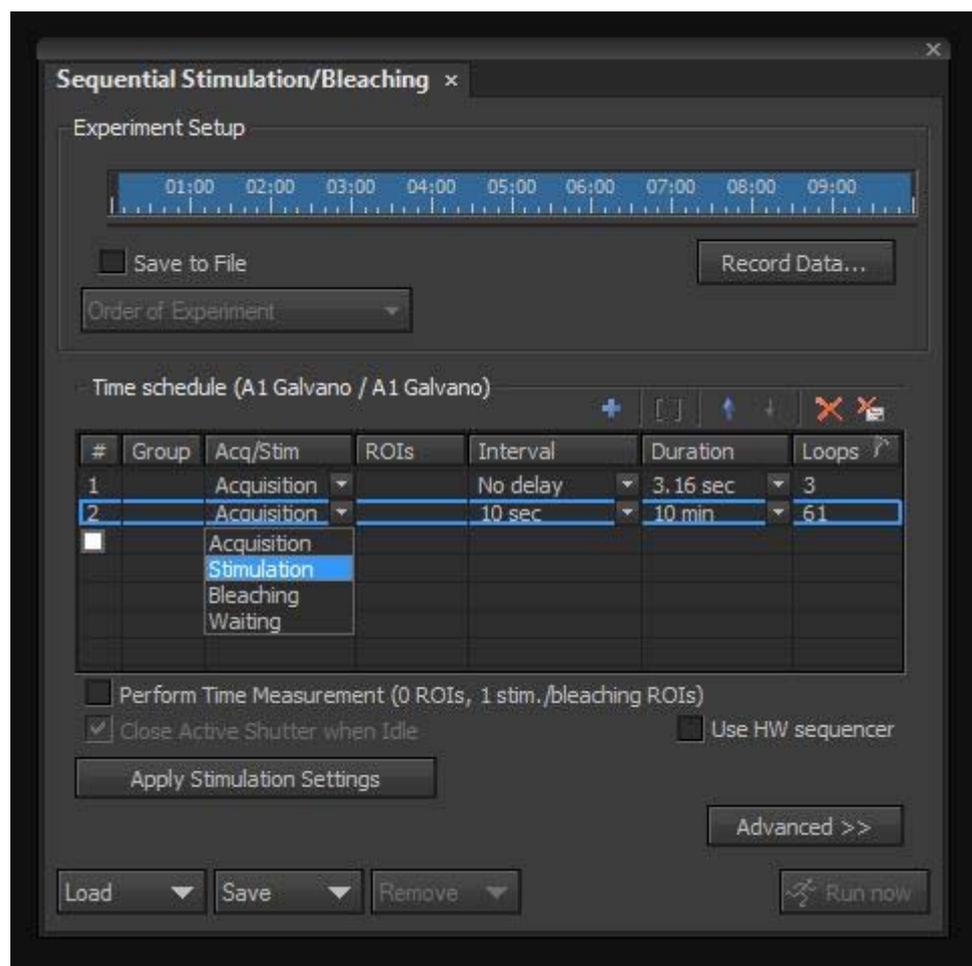


Settings will carry over between the two GUIs, so only one window needs to be set.

Experiment Settings

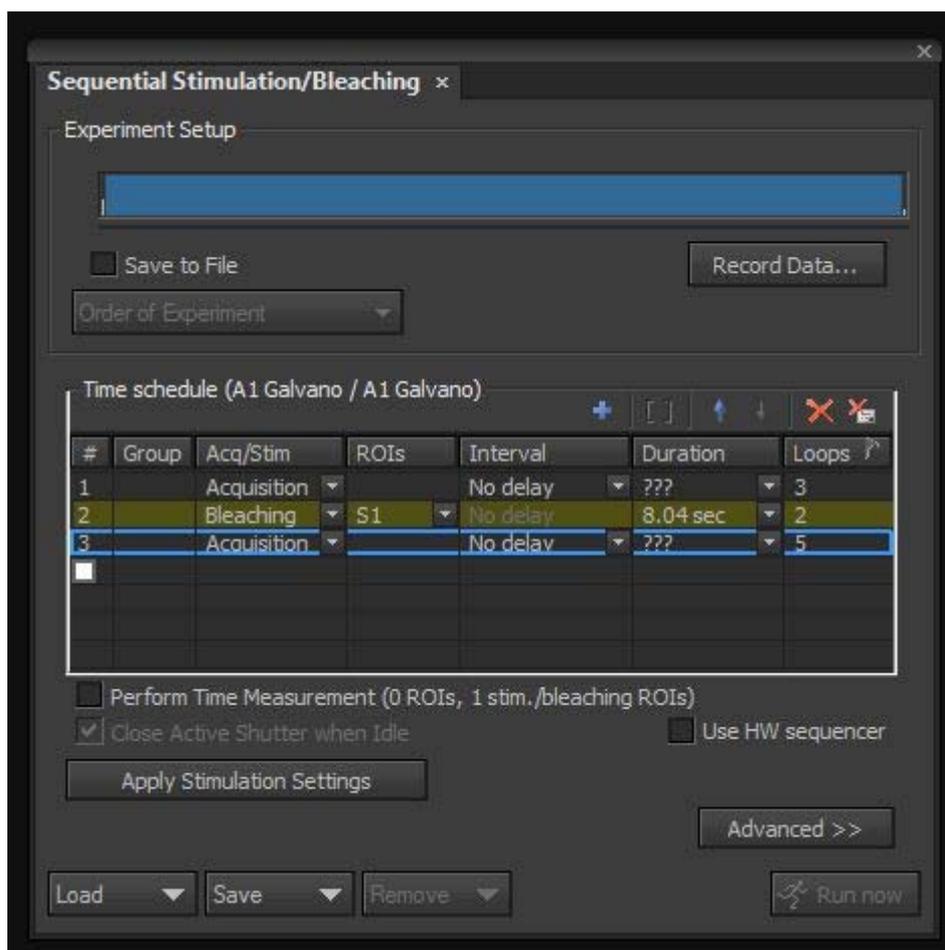
To open the stimulation experiment window select <Applications> from the main menu, then choose <Define/Run Sequential Stimulation>.



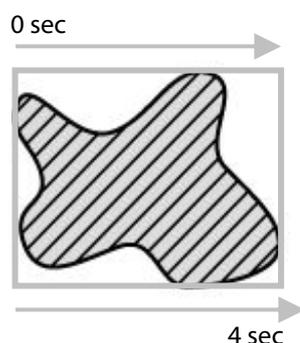


The Sequential Stimulation window looks much like a normal ND Timelapse window. The timeline at the top shows a summary of the experiment. The experiment can be saved directly to disk by selecting the <Save to File> option and specifying a filename and directory. The experiment is divided into phases. These phases can be Acquisition, Stimulation, Bleaching or Waiting.

In <Acquisition> phases images are acquired with the galvano scanner at the specified interval for a set duration or number of loops. In <Waiting> phases no acquisition or stimulation occurs for the duration specified. <Stimulation> and <Bleaching> phases function in the same way and are interchangeable – the ROI is stimulated by the galvano scanner and no acquisition occurs. It is best to specify Stimulation phases in terms of loops. Each loop is a single pass of the stimulation laser. The speed of this pass is determined by the scan speed in the stimulation settings. For example, if the scan speed is set to 1/4fps (or 4sec/frame) one pass will take 4 seconds. If two loops are chosen the total stimulation time will be $4\text{sec} \times 2 = 8\text{ seconds}$.



The scan speed is the time taken for the laser to pass from the top left pixel of the bounding box of the ROI to the bottom right pixel. In a rectangular ROI the laser will be on for the full scan speed time. For other shapes of ROI, for example irregular Bezier shapes, the scanner will zoom to the smallest possible box surrounding the shape and then use the AOTF to turn the laser on and off, so stimulation light only hits the desired regions.



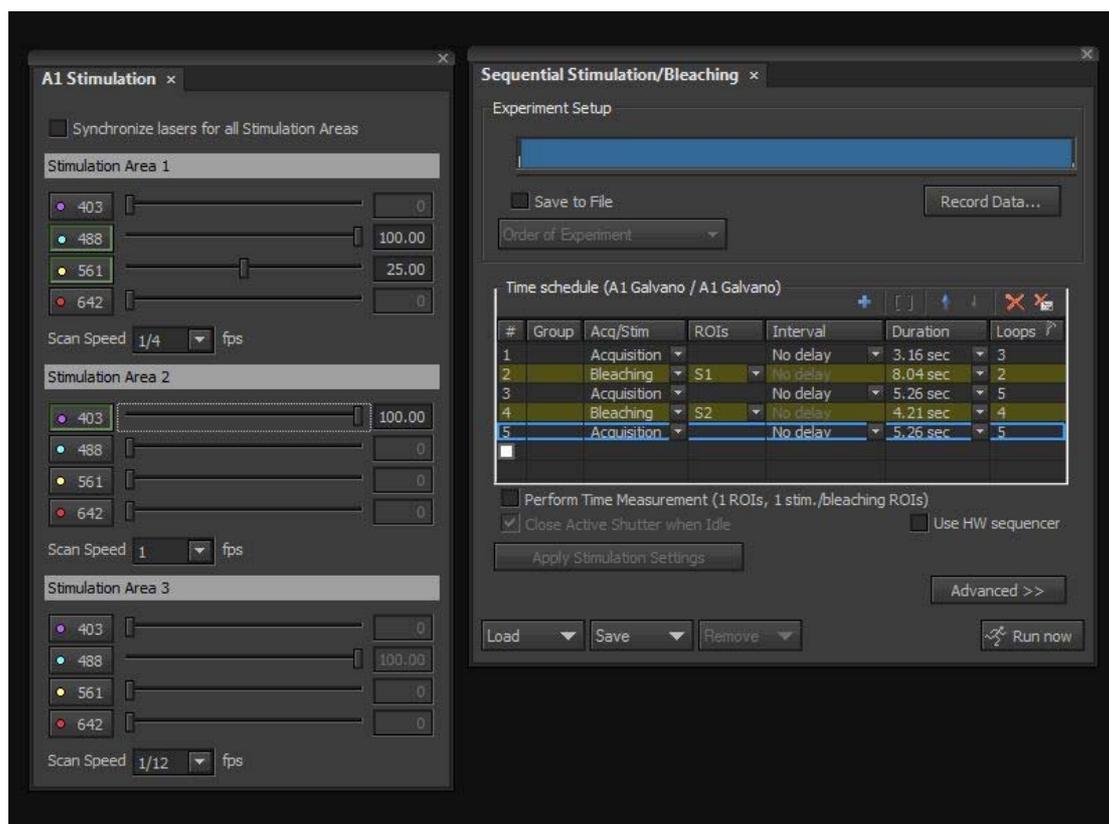
Once the ROI, laser settings and experiment phases have been set up, click <Apply Stimulation Settings> to prepare the experiment. If you move the ROI you will need to click <Apply Stimulation Settings> again to prime the experiment.

To start the experiment, click <Run now>.

Multiple Stimulation Groups

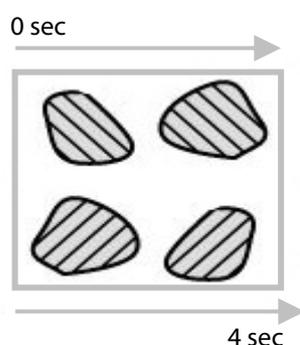
To stimulate/bleach multiple groups of ROIs with settings, draw multiple ROIs and assign them to the three stimulation groups. Using the expanded A1 Stimulation GUI, or A1 Settings, set different stimulation settings for each group. In the example below Group 1 is stimulated with the 488nm laser at 100% and the 561nm laser at 25% simultaneously, with each pass of the lasers taking 4 seconds. Group 2 is stimulated with the 405 laser at 100%, with each pass taking one second.

In the experiment setup select multiple stimulation phases to stimulate each ROI group in turn, or select multiple groups of ROIs for each stimulation phase.



The image shows two software windows. The left window, 'A1 Stimulation', has three sections for 'Stimulation Area 1', 'Stimulation Area 2', and 'Stimulation Area 3'. Each section contains sliders for laser wavelengths (403, 488, 561, 642 nm) and their intensities, and a 'Scan Speed' dropdown menu. The right window, 'Sequential Stimulation/Bleaching', shows an 'Experiment Setup' section with a 'Record Data...' button and a 'Time schedule' table. The table lists five rows with columns for '#', 'Group', 'Acq/Stim', 'ROIs', 'Interval', 'Duration', and 'Loops'. Below the table are checkboxes for 'Perform Time Measurement', 'Close Active Shutter when Idle', and 'Use HW sequencer', along with 'Apply Stimulation Settings', 'Advanced >>', 'Load', 'Save', 'Remove', and 'Run now' buttons.

#	Group	Acq/Stim	ROIs	Interval	Duration	Loops
1		Acquisition		No delay	3.16 sec	3
2		Bleaching	S1	No delay	8.04 sec	2
3		Acquisition		No delay	5.26 sec	5
4		Bleaching	S2	No delay	4.21 sec	4
5		Acquisition		No delay	5.26 sec	5



When using stimulation groups, the scan speed is the time taken for the laser to pass from the top left pixel of the bounding box of the **stimulation group** to the bottom right pixel. In a group with a **single rectangular ROI** the laser will be on for the full scan speed time. For groups with multiple ROIs, regardless of shape, the scanner will zoom to the smallest possible box surrounding the **group** and then use the AOTF to turn the laser on and off, so stimulation light only hits the desired regions. As such, grouping

ROIs that are widely spatially separated into the same stimulation group should be avoided, as this can result in lower bleaching/stimulation efficiency.

Simultaneous Stimulation

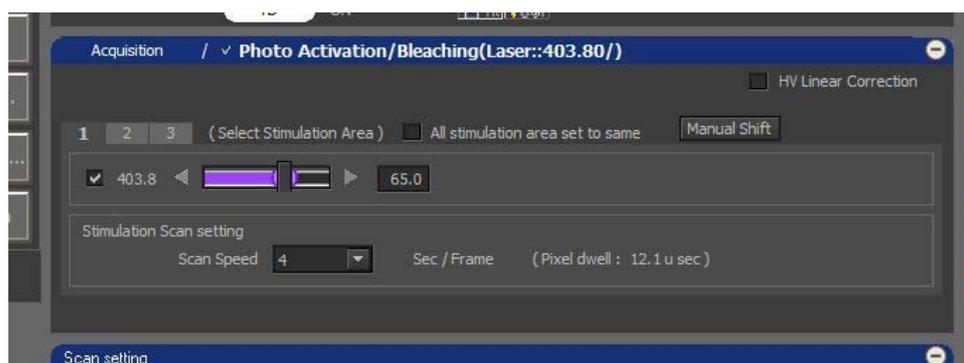
Simultaneous Stimulation uses the resonant scanners to acquire a series of images, while the galvano scanners are used for stimulation. Acquisition continues during the stimulation phase. As this function requires the hybrid scanner technology this experiment mode is compatible with the A1R confocal only.

Setup

Prepare the GUI and create stimulation ROIs as per the instructions for sequential stimulation.

Stimulation Settings

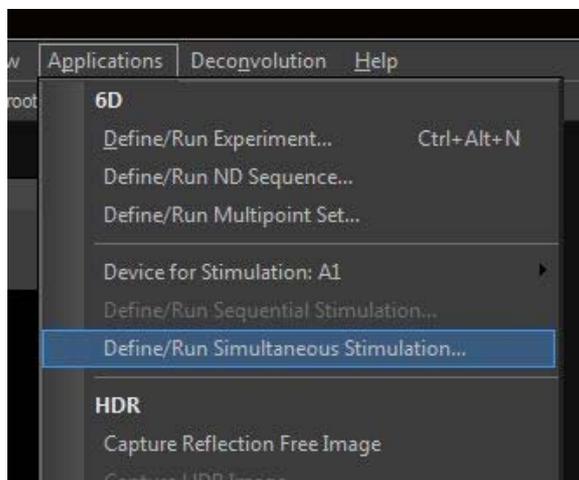
Using your GUI of choice, set up the laser lines, laser powers and scan speed.



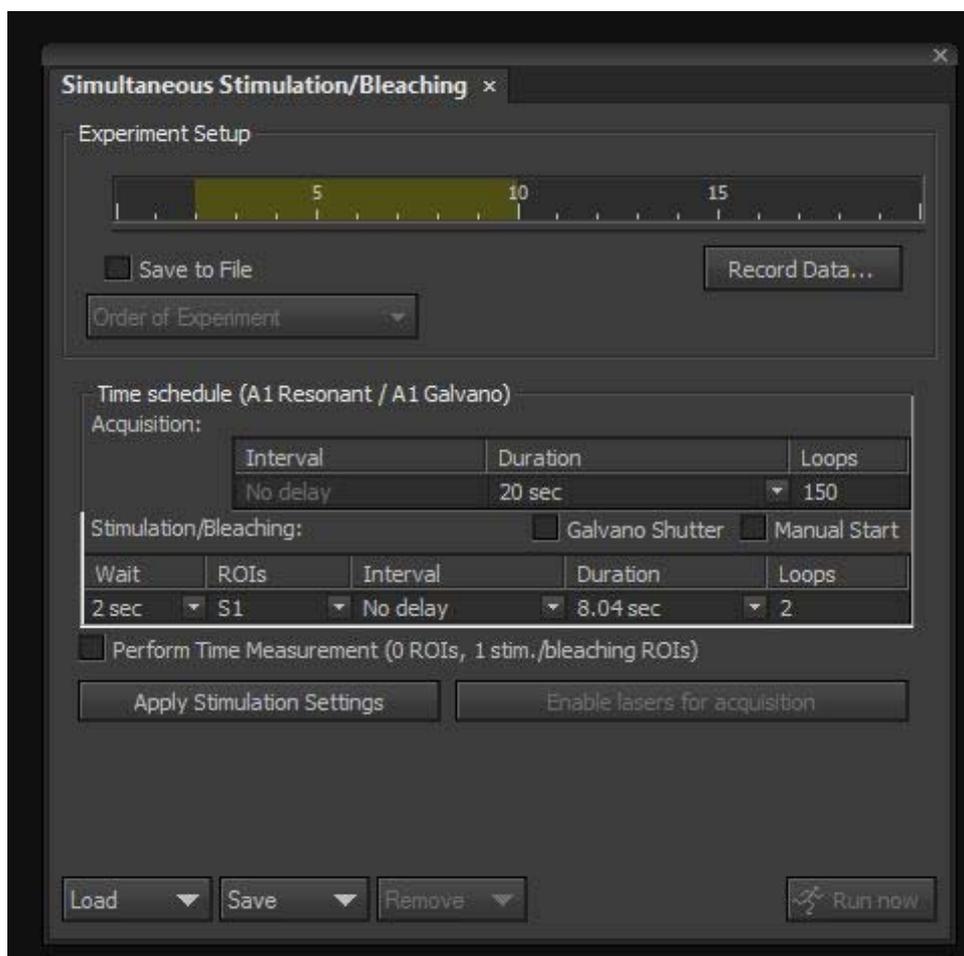
You will note that the choice of lasers is restricted in simultaneous stimulation. This is because the hybrid scanning system uses a dichroic to send the chosen laser line to the galvano scanners, allowing the rest of the lasers to continue to the resonant scanner for imaging. In most systems only the 405nm laser line will be available for stimulation. In this case the 405nm laser will be disabled for imaging. In systems fitted with a blue/blue hyperselector, the 488nm laser can be selected as the stimulation laser (please see brochure for more details).

Experiment Settings

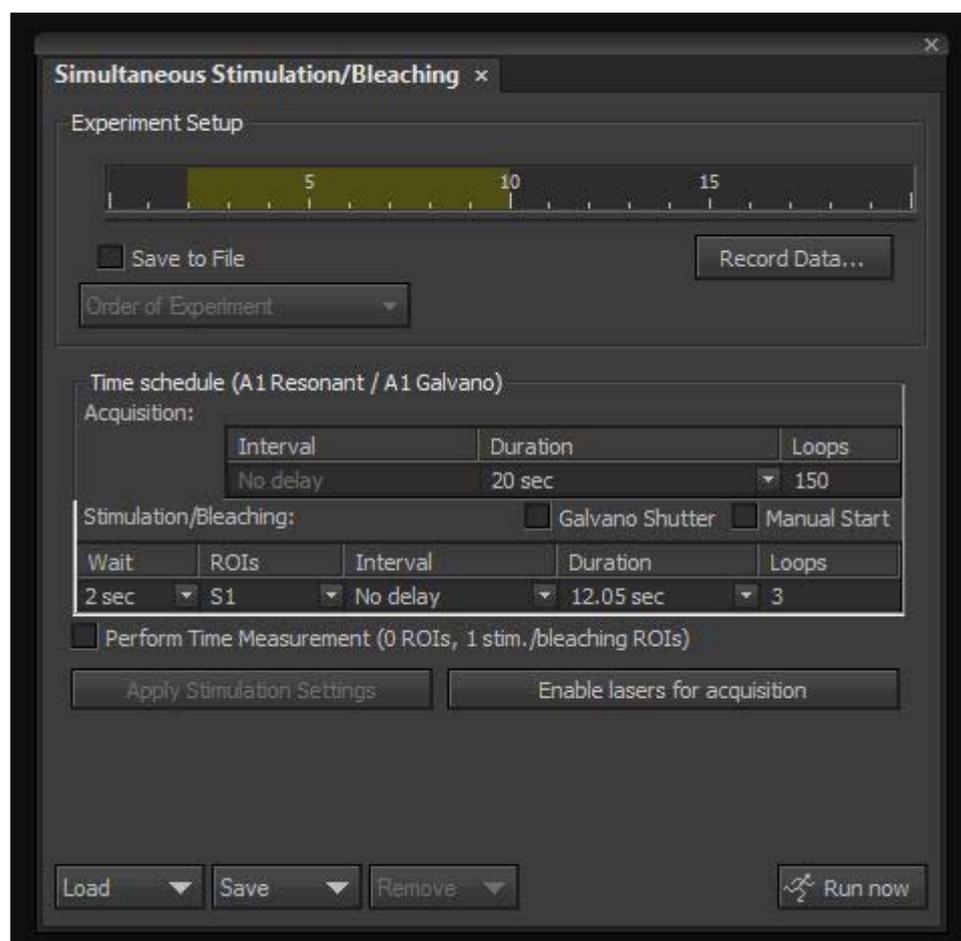
To open the stimulation experiment window select <Applications> from the main menu, then choose <Define/Run Simultaneous Stimulation>.



The Simultaneous Stimulation window looks slightly different from the Sequential Stimulation window. The experiment is set up as a continuous no delay acquisition, and then the stimulation/bleaching phase is “nested” within this acquisition.



First, set the length of the whole experiment in the <Duration box under <Acquisition>. Next, specify how long to wait before starting the stimulation. Select the groups to stimulate, the interval between stimulation passes and the number of loops of stimulation. As in sequential stimulation, each loop is a single pass of the stimulation laser. The speed of this pass is determined by the scan speed in the stimulation settings.



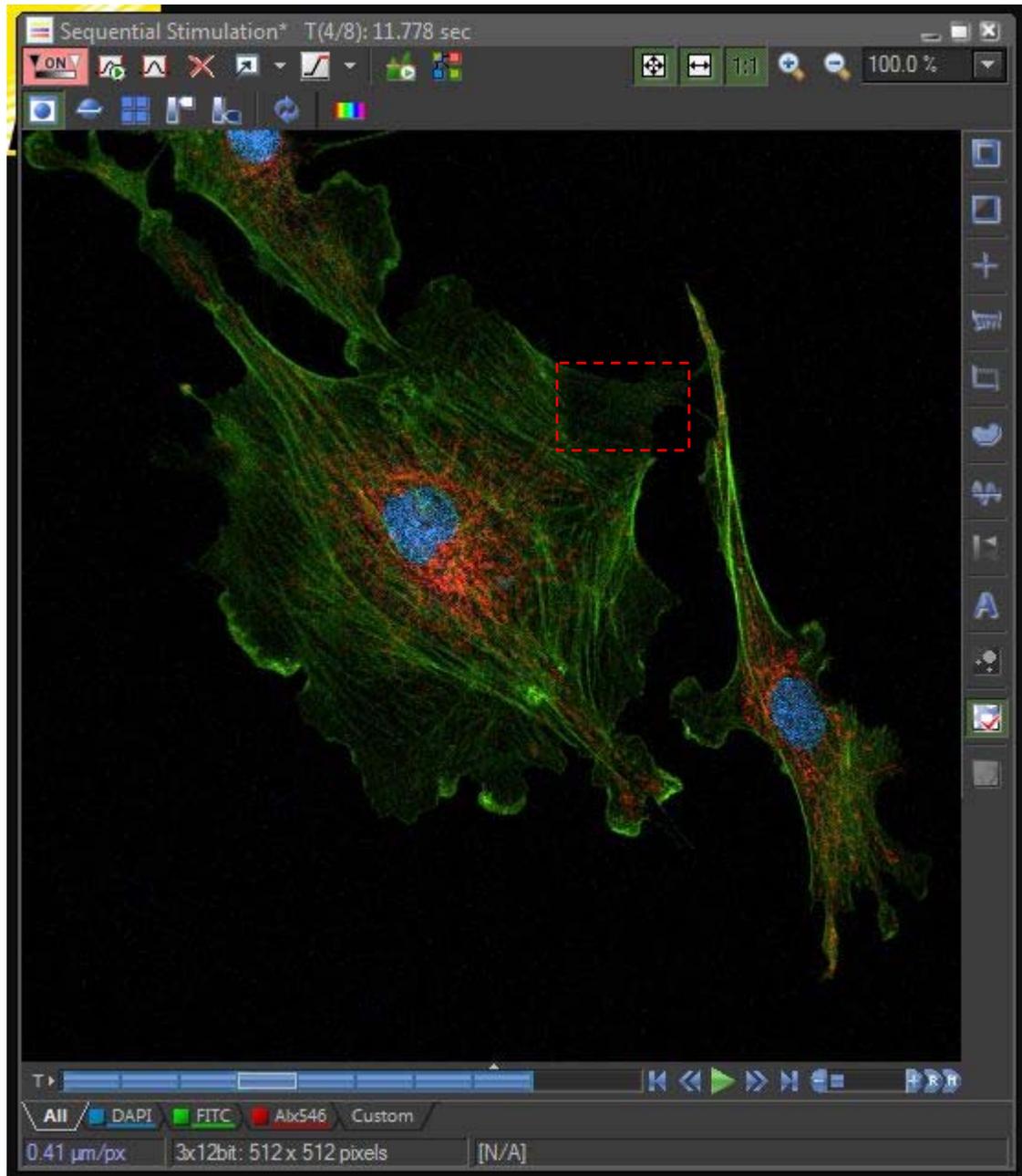
Once the ROI, laser settings and experiment phases have been set up, click <Apply Stimulation Settings> to prepare the experiment. If you move the ROI you will need to click <Apply Stimulation Settings> again to prime the experiment.

If you wish to exit without running the experiment, press the <Enable lasers for acquisition> button to remove the hyperselector from the light path and allow acquisition with all laser lines, then close the window.

Otherwise when you are ready to start the experiment, click <Run now>.

Measurement

The finished document for any stimulation experiment will look like a timelapse, and can be played back as a movie.

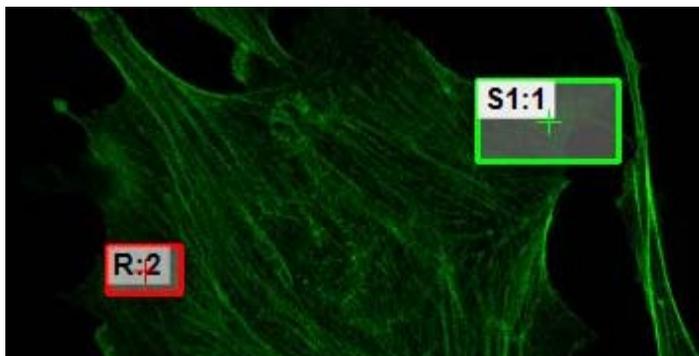


The stimulation ROI(s) can also be measured over the course of the experiment either during or after acquisition.



It is also possible to add a reference ROI. This will measure the overall bleaching of the specimen from imaging, and correct the measurement of the stimulation ROI for the loss in intensity.

To add a reference ROI draw an ROI in a non-bleached area of the specimen and right-click to set it as a reference ROI. It will then be marked with <R:>



In the Time Measurement window, select the <Reference correction> button. When this button is pressed, all measurements will be corrected to the reference ROI.

