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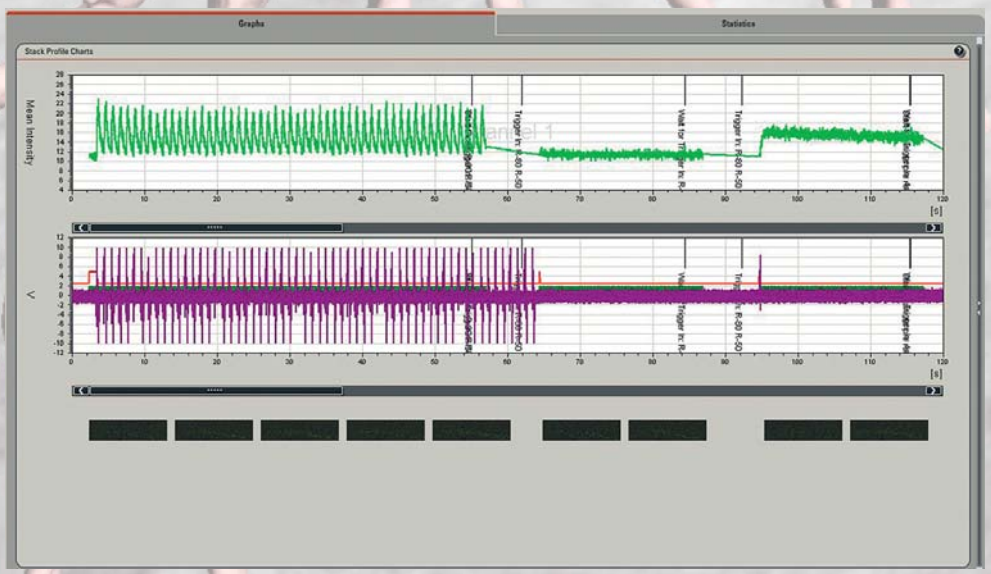
reSOLUTION

Software Tools for Applications in Electrophysiology



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Content

Introduction

Physiological experiments require flexibility in terms of hardware and software tools. The Leica DM6000 CFS is based on a TCS SP5 confocal- or multi-photon system. It can be further equipped with an IR-sensitive CCD-camera to easily visualize the sample and, for example, the micropipettes. The software allows for fast switching between scanner and camera mode. Correlated intensity and electrical data are displayed for fast evaluation of experiment results.

Specialized software, the electrophysiology tool, serves for defining experiment procedures. These macros can consist of different experiment parts with individual hardware settings. Experiment procedures can be looped, saved and reused. The data acquisition can be synchronized with external instruments, e.g. a patch clamping setup, by different input and output triggers that can be freely assigned to specific parts of the experiment.

Cover picture:
3D-reconstruction of a mouse pyramidal neuron layer 5 filled with Alexa 594. The rendering has been performed by Imaris software and later on combined with the scanning gradient contrast image.

Courtesy of Dr. Thomas Nevian, Inst. of Physiology, University of Bern, Switzerland

1. How to Get Started

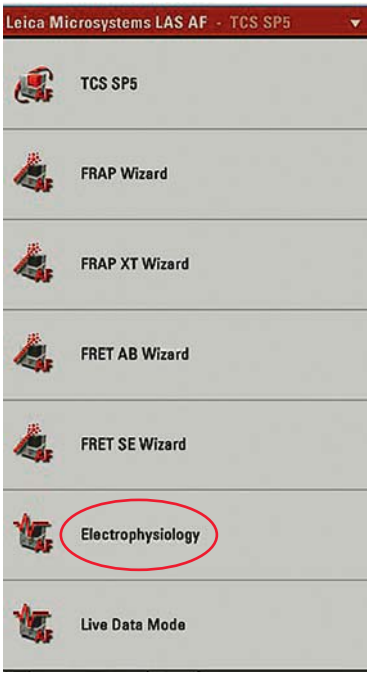


Fig. 1: Drop-down list for selecting **Electrophysiology**

The electrophysiology tool in LAS AF can be accessed from the drop-down list in the menu-line (Fig. 1).

The system opens the user interface for the confocal mode (see Fig. 2). The user interface is similar to that of a standard SP5. In addition, a tool for configuring an experiment containing different jobs is placed on the top – the **Record Pattern**. The current running job is indicated in green. On the bottom right, an individual job or the record pattern can be executed by a click on **Start Job** or **Start Pattern** (Fig. 2).

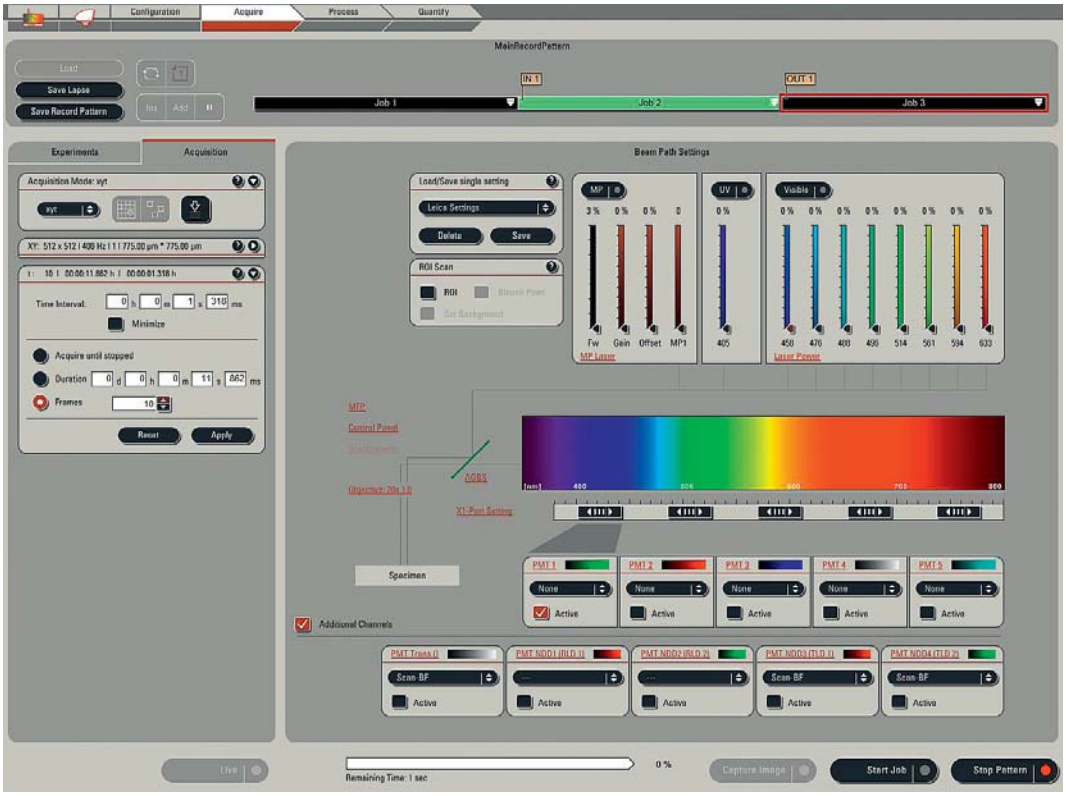


Fig. 2: Scanner mode

2. LAS AF Supports Fast Switching from Camera to Confocal Beam Path

For patch clamping experiments it is most useful to adjust the proper position of specimen and pipettes while imaging with the camera prior to scanning. This allows for overview and detailed imaging. Thereafter, one can switch for imaging with the scanner mode.

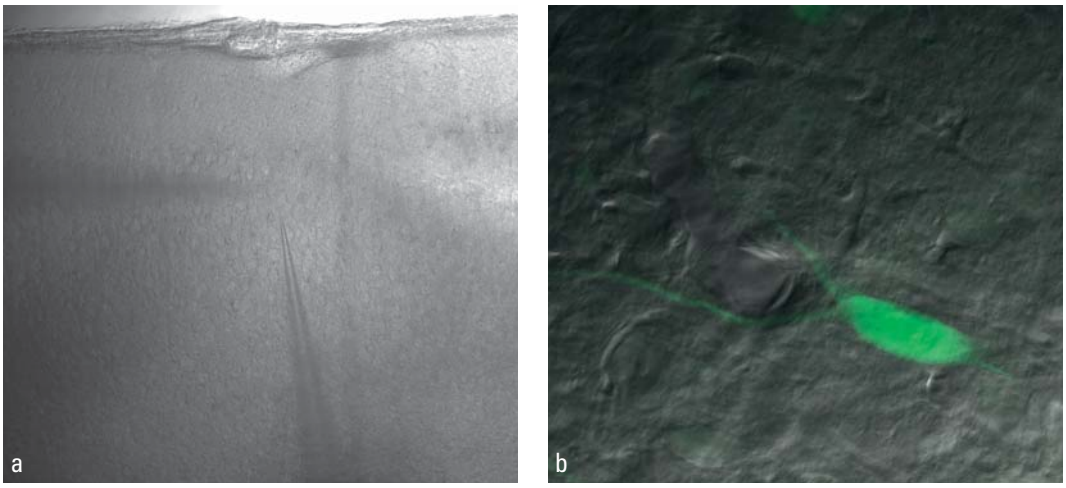


Fig. 3: a) Mouse brain slice: transmitted light image acquired by CCD-camera (Leica DFC360 FX) showing an overview of a part of a mouse brain slice and two micropipettes – one of which is not in focus. **b)** Mouse brain slice: overlay of IR-scanning gradient contrast and fluorescence.

Thus, a fast switch from the camera to the confocal mode will facilitate the work. The Leica software LAS AF has integrated the CCD-camera beam path as well as the confocal beam path. You can toggle between the camera and confocal beam path simply by clicking on the appropriate symbol (Fig. 4).

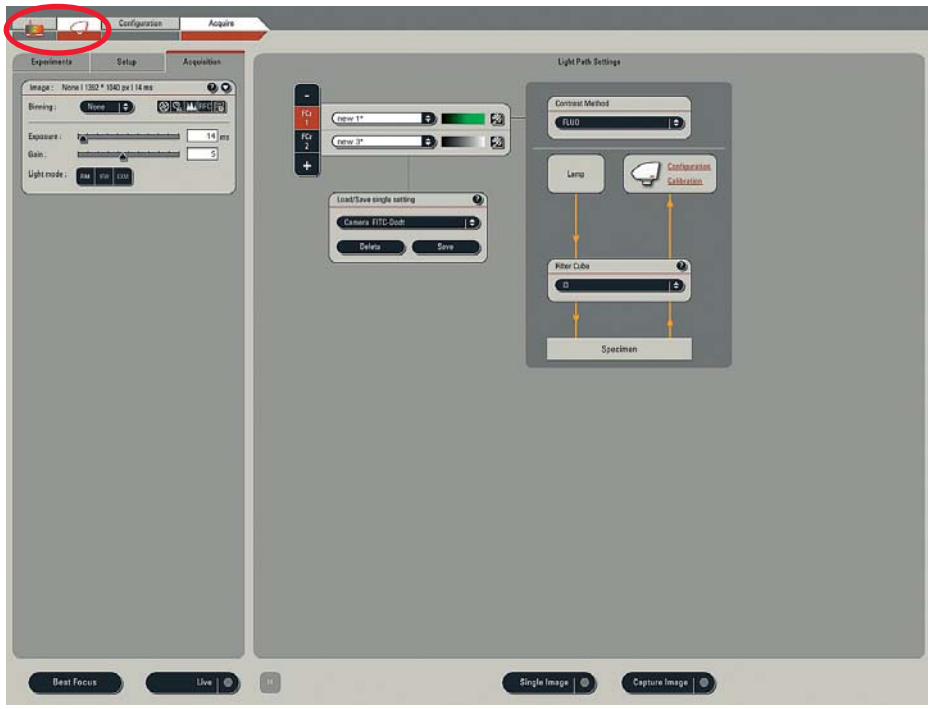


Fig. 4: Camera mode: setting for imaging with the camera can be easily defined within the camera beam path window.

3. Correlation of Intensity and Electrical Data and Images

In many physiological studies the reaction of cells upon different stimuli are investigated. The intensity data usually refer to the intra cellular calcium concentration or pH-value. The reactions of the cells are measured regarding their electrical response as well as their response concerning the intracellular calcium concentration. Both measurements have to be performed simultaneously. For a fast evaluation of the experiment a correlation of intensity and electrical data is indispensable.

Recorded signals like voltage or currents, as from a neuron, are typically amplified. Then the analog signal from the amplifier is sent to a **NI Data Acquisition Box (DAQ box) from National Instruments**. Here the signals are digitalized and sent to the PC via a fast USB connection.

In addition, the DAQ box is connected to the trigger unit to receive signals from the scanner as a command for the data acquisition start. This allows for the perfect synchronization of data received from an external instrument, like a patch clamp electrode, with the data received from the scanning process. Thus, all data, in particular electrical data and intensity data, as well as images, can be displayed in the LAS AF software (see Fig. 5, 6 and 7b). The sampling rate of the DAQ box can be defined within the software.

As a prerequisite for the intensity- and voltage data correlation; the setup must contain the data acquisition box and the Leica trigger unit.

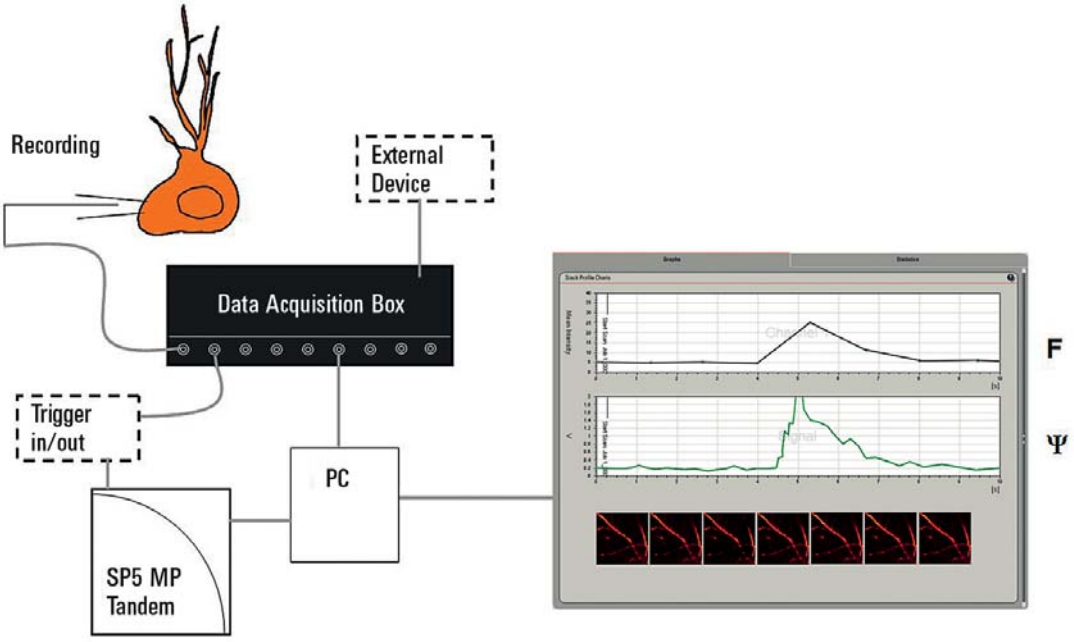
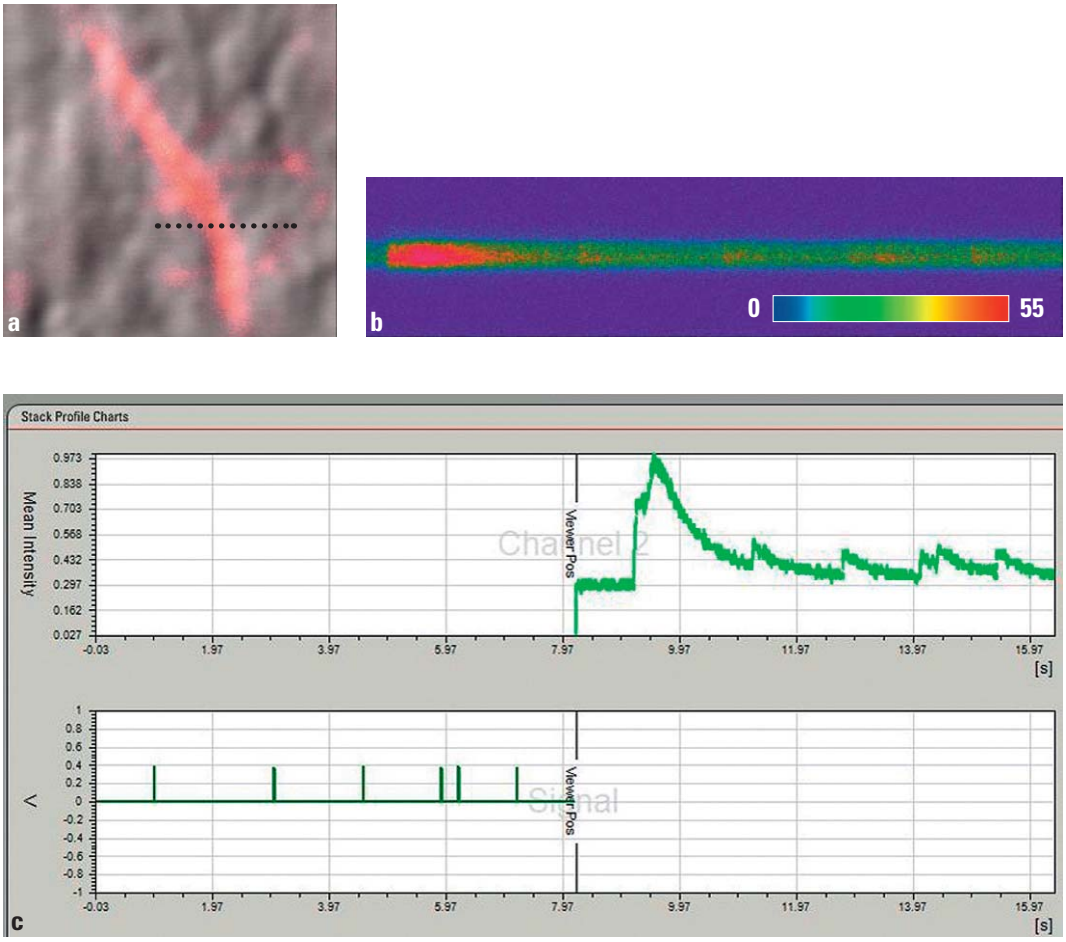


Fig. 5: Scheme of connected components for correlation of optical data, electrical data, and images. An external device can be any instrument like a patch clamp setup, a pulse generator, a pump, etc.

3.1 Combining Electrophysiology and Imaging

The reaction of cells upon electrical stimulation can be studied by combining imaging and electrophysiology. When a neuron is filled with a calcium sensitive dye, e.g. Oregon Green Bapta 1, calcium changes can be investigated in respect to the cell morphology when observing the scanned images. Fluorescence intensity changes measured in a region of interest (ROI) over time can be correlated with the arrival of stimulation pulses. Thus, one can study the time cells need to react to various stimuli (Fig. 6). Furthermore, recorded electrical signals from the cell can be correlated with the fluorescence signal (Fig. 7a and b).



Fluorescence intensity data from scanning

Electrical data (stimulation pulses) from an pulse generator digitalized and synchronized

Fig. 6: Stimulation of a neuron in a living mouse brain slice by trigger pulses using a pulse generator. The neuron has been filled with the Calcium-sensitive dye Oregon Green Bapta 1. Extra-cellular stimulation was performed close to the axon of the neuron by trigger pulses sent from a pulse generator. Time point, strength and duration of the pulses were adjusted manually. The pulse generator has been connected to the DAQ box for data correlation.
(a) Overlay of IR-scanning gradient contrast and fluorescence. The line indicates where xt-scans have been performed.
(b) Xt-line scan: calcium concentration changes are visualized by sensitive color.
(c) Correlated data displayed in the Quantify-tool: stimulation pulses on the bottom (V-graph) and calcium transient (mean intensity-graph).

3.2 Currents in Heart Muscle Cells

Isolated cardiomyocytes from trout are labeled with the calcium-sensitive dye Fluo4. They are stimulated by different trigger pulsing regimes using patch clamping (HEKA EPC-10 double). Their response regarding intracellular calcium concentration and ionic current is measured by means of fluorescence imaging and electrical recordings. The stimulation protocol on the patch clamp setup was synchronized with the confocal time lapse series using a trigger on the patch clamp setup to mark events in individual frames and on the time axis when analyzing specific ROIs (Fig. 7a).

The software allows for a synchronized correlation of electrical and fluorescence intensity data, e.g. voltage recordings are correlated in synchronization with the fluorescence intensity data, and are automatically displayed in graphs (Fig. 7b). This reveals a fast and direct overview on the experiment progress and online data evaluation. Furthermore, images are displayed below the graphs to get fast information on the morphology of the cell.

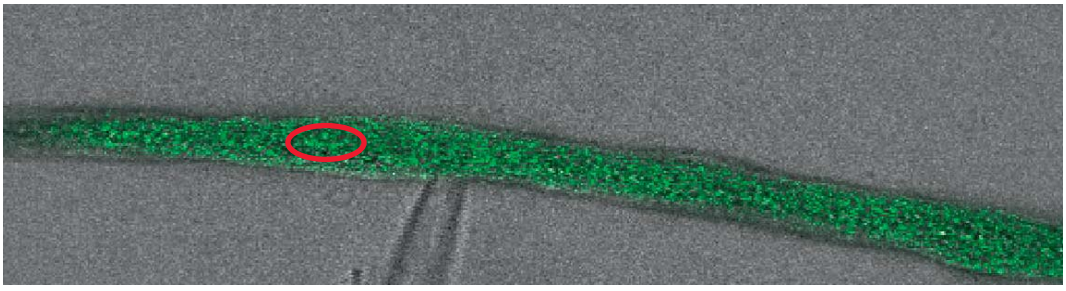


Fig. 7a: Heart muscle cell (cardiomyocyte) with attached patch pipette. Overlay of fluorescence (Fluo4) and transmitted light channel. Fluorescence signal measurements are taken inside the ROI.
Courtesy: Leif Hove-Madson, Catalan Cardiovascular Institute, Hospital Sant Pau, Barcelona, Spain.

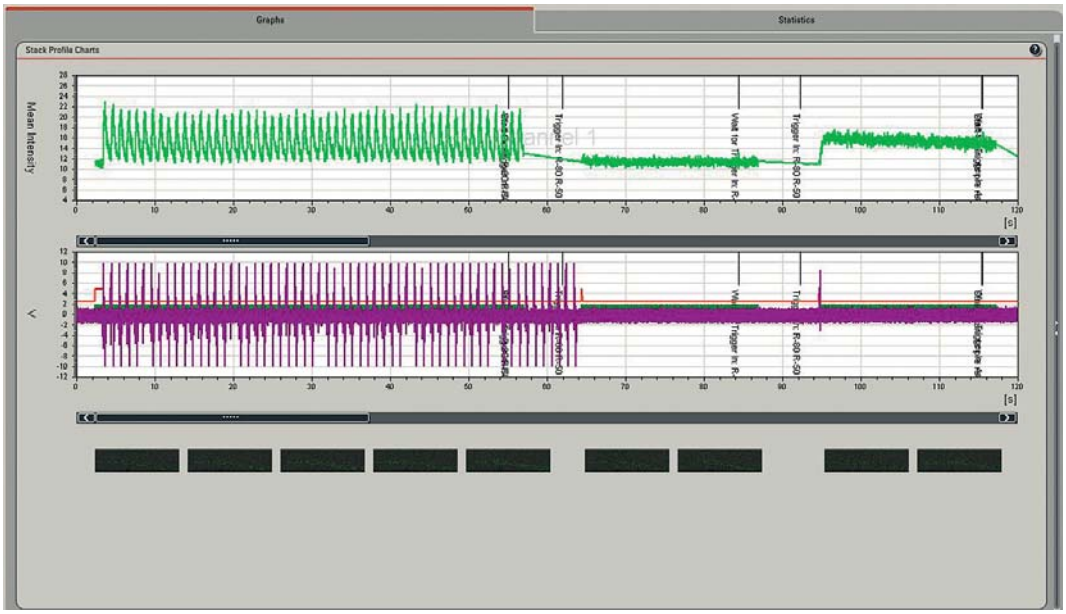


Fig. 7b: Quantification of the experiment described in the text. Intensity graph (top), electrical data (middle) and images (bottom) recorded during a patch clamp experiment in an isolated cardiomyocyte. Electrical data: Current recorded (purple), trigger pulses for stimulation of cells (red) and line trigger from the scanner (green).
Line triggers are recorded in order to have the exact correlation of scanning process, fluorescence signal intensity and electrical response of the cell. These triggers are automatically generated by the scan head. For line triggers it means: whenever a line is scanned, it can be recorded and displayed in the quantification chart when the appropriate connection of the trigger unit channel with the DAQ box was set up.

4. Specific Settings for Electrophysiology

In the following section, the settings that need to be adjusted for a proper use of the DAQ box and the trigger unit are described.

Upon a click on the E-Phys-button in Configuration (Fig. 8,1) specific settings for Electrophysiology appear within the following window:

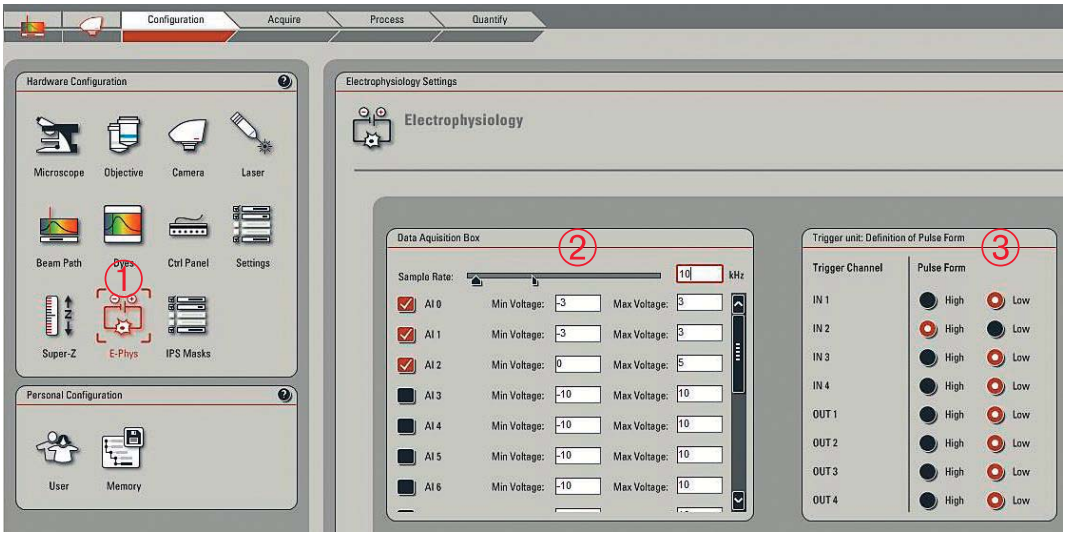


Fig. 8: Selection of analog channels for data acquisition box (2); Selection of Pulse Form for trigger unit (3)

4.1 Data Acquisition Box – Selection of Analog Input Channels (Fig. 8,2)

The analog channels used on the DAQ box have to be selected. Up to 16 analog channels are available and have to be selected (AI 0, AI 1, etc.) according to the external device that is connected to the data acquisition box.

The sampling rate can be freely defined. However, the max sampling rate depends on the number of analog channels selected. The maximum rate of 200 kHz is for one activated channel. It will be halved if 2 channels are activated – and so on. As a default the sampling rate is set to 10 kHz.

For each analog channel a maximum and a minimum Voltage value can be set. The entered values are automatically taken for the scaling of the y-axis in the voltage graph that is displayed below the intensity graphs. The default values are -10 V and 10 V, respectively.

Note: For fast scanning and triggering it could be required to enhance the sampling rate of the DAQ-box in order to get all signals displayed.

4.2 Definition of Pulse Form for Trigger Unit (Fig. 8,3)

Triggering with the SP5 is controlled by the "edges" of the trigger pulses – falling or rising edges. The hardware recognizes the change from high to low or vice versa. To ensure a proper communication between the trigger unit and an external instrument the pulse form can be individually configured accordingly. This can be done for each trigger channel - input and output triggers – in the window "Definition of pulse form". Select "high" for a rising edge; select "low" for a falling edge. By default, the SP5 uses a negative slope for the trigger in signal (Fig.9).

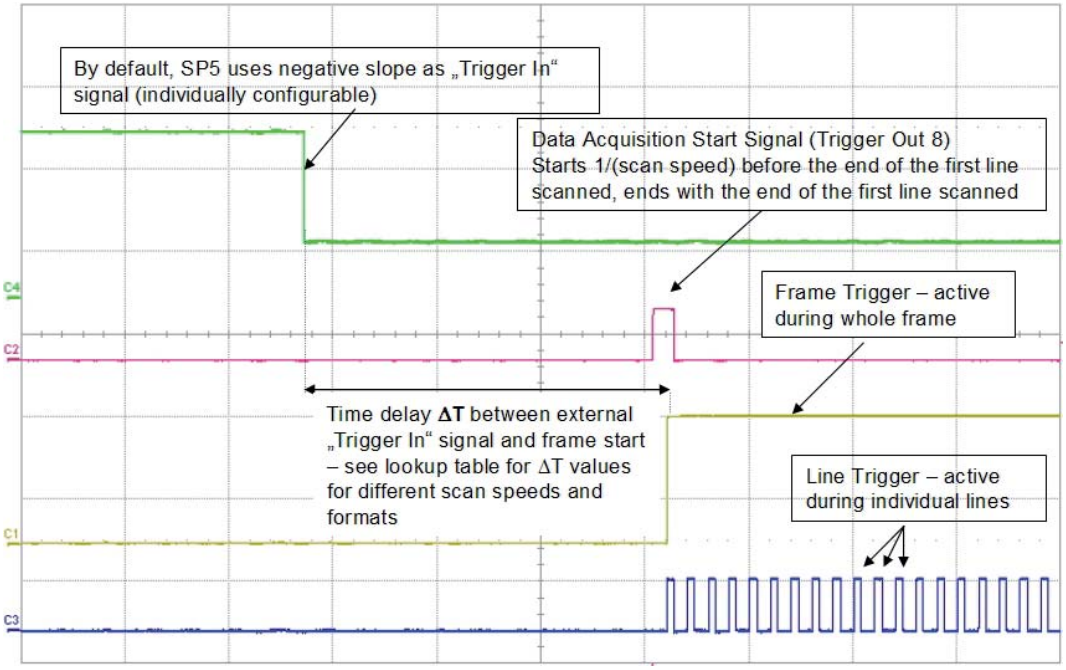


Fig 9: Triggering with the Leica DM6000 CFS showing 4 traces of trigger signals measured and displayed on an oscilloscope.
Green: C4 – Trigger In 1 from function generator
Red: C2 – Trigger Out 8, ("Out5/CTR 0 OUT"), signal for data acquisition start for the DAQ box
Yellow: C1 – Trigger Out 6 from trigger unit, automatic generated Frame Trigger
Blue: C4 – Trigger Out 7 from trigger unit, automatic generated Line Trigger

5. Free Configuration of Experiments

The pre-definition of individual experimental settings within time lapses is crucial for physiological experiments. The Electrophysiology software tool enables the scientist to easily design these kinds of experiments by defining an experiment macro called Record Pattern (Fig. 10). Experiments can consist of different sequences (Jobs) that have individual configurations for data acquisition, e.g. different scanning speeds or different trigger settings etc. A complex experiment can be defined consisting of different jobs, pauses, loops and defined of input- and output-triggers. Each individual experiment sequence as well as complex procedures – the Record Pattern – can be saved and reused again.

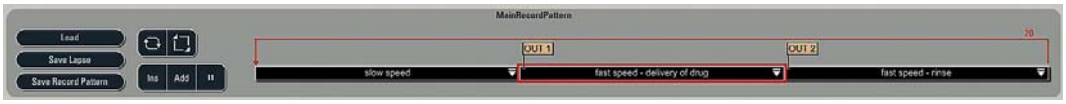


Fig 10: The Record Pattern. A 20x looping has been defined for the whole experiment. In Job2 and Job 3 a trigger out has been set up.

5.1 Adding and Inserting Jobs

Adding jobs

On entering the Electrophysiology software tool a first job is already present in the time line. Further jobs can be easily added or inserted. For adding and inserting a job several options are available, as shown in Fig 11.

Upon a right mouse click on **Add** one can select from the following options:

Normal: for a normal job (not sequential)

Sequential: for a job with sequential scanning; the sequential scan window appears within the **Acquisition** tab.

New Pattern: adds a new record pattern that can also be configured with several jobs.

Existing Records: actual defined jobs, pauses or record patterns can be selected to be added.

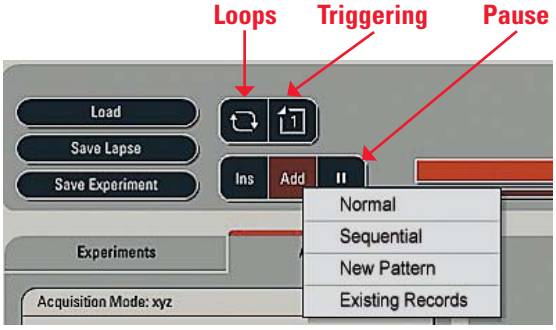


Fig 11: Tools for experiment definition with following functions: Adding and inserting jobs, pauses, loops and triggers, save and load functions.

By selecting a job within the record pattern the scan-settings can be viewed and modified. The system automatically names added jobs (Job1, Job 2, Job 3 etc.). In addition, jobs can be renamed individually later on. If the acquisition settings for the first job are defined a newly added job will automatically contain these settings. Then the settings can be modified for the new job.

Inserting a job to a certain position
To insert a job to a certain position within the record pattern one needs to click first on a job. The new job is always inserted on the left of the job selected. Secondly, by a click on **Ins** the job is now inserted to the desired position. A job can also be selected from the list of **Existing Records**.

5.2 Saving and Loading of Jobs and Record Patterns

In order to get reproducible experiments a job and a complete record pattern can be saved and reloaded by the following functions (Fig.11):
Load: for loading jobs or record patterns that have been saved

Save Lapse: for saving a job
Save Experiment: for saving a whole record pattern

5.3 Deleting, Removing and Renaming of Jobs and Record Patterns

Upon a click on the white arrow inside a job the functions **Delete, Remove** and **Rename** are available (Fig 12).

A job can be deleted by selecting it and a subsequent click on **Delete**. A deleted job is not available in the list of existing records anymore. The system continues its way of counting jobs no matter if a previous job has meanwhile been deleted. So if an old job (Job 3) was deleted and another job is added it will be named Job 4. Renaming can be done at any time.

By a click on **Remove** a job is removed from the record pattern but still available in the list of existing records.

To rename a Job, use the **Rename**-function.

Record Patterns can be renamed, saved and deleted. These options are also available upon a right mouse click inside the top of a **Record Pattern** tab.

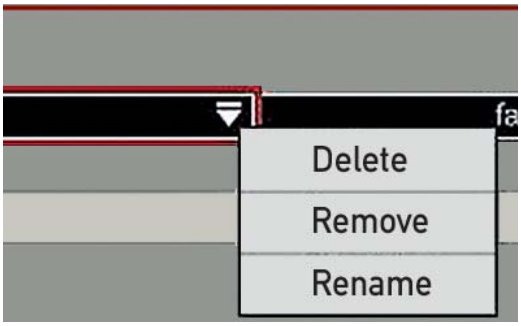


Fig. 12: Options for job handling.

5.4 Loops

For repeating the whole experiment or parts of an experiment loops can be defined. Checking on the **Loop** symbol opens the **Loop Settings** dialog. Start and end of a loop as well as the number of loops can be entered (Fig. 13 a). A loop can be defined for the same job, between different jobs as well as between a job and a pause. A click on **Define Loop (1)** applies the loop to the experiment in the time line. Defined Loops are indicated in the Record Pattern (see Fig. 10).

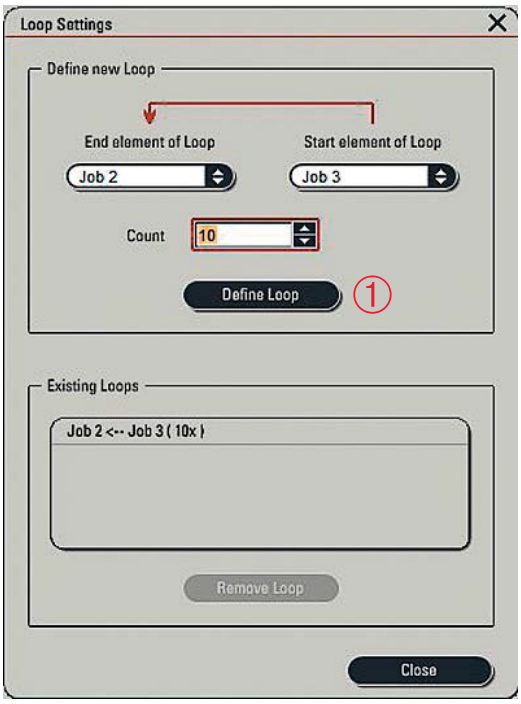


Fig.13a: The Loop Settings dialog has opened and a loop can be defined

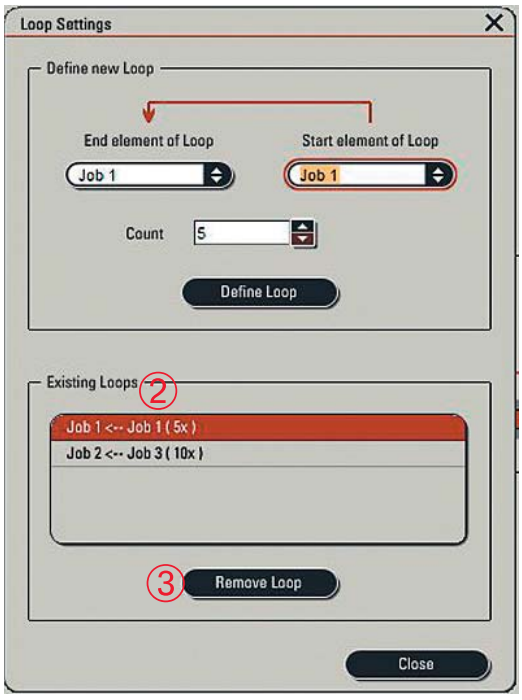


Fig. 13b: Selection of a loop for deletion

For removing a loop it has to be selected in the list of existing loops **(2)** followed by a click on the button **Remove Loop (3)** (Fig. 13b).

5.5 Triggering

To synchronize the scanning process with external devices (patch pipettes, electrodes etc.) trigger functions can be utilized. For these applications it is a prerequisite that the system is equipped with the Leica trigger unit. To assign a trigger or triggers to a certain job, the job first needs to be selected in the record pattern. Secondly, the **Trigger Settings** window (Fig. 14) has to be opened by a click on the trigger button (Fig. 11). Defined triggers are indicated within their assigned job (Fig. 10).

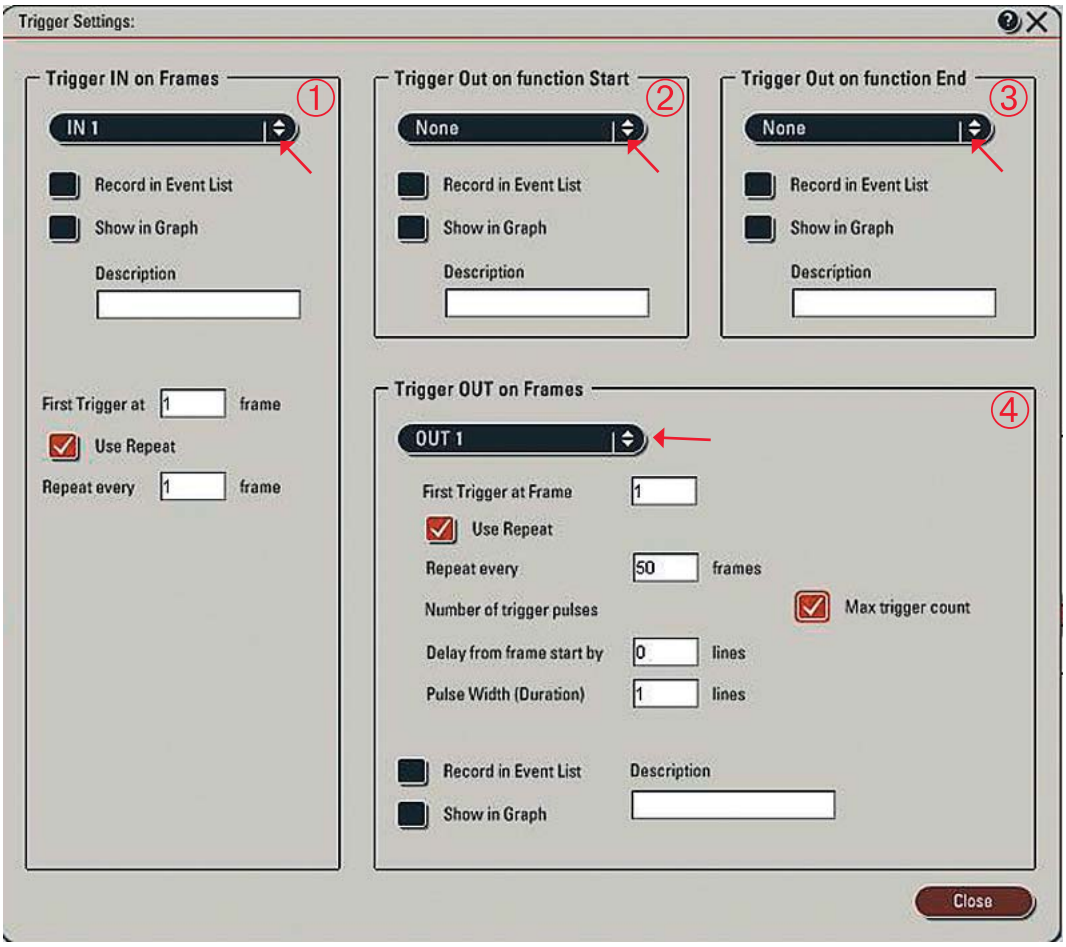


Fig. 14: Trigger Settings window for setting up input- and output triggers.

There are two free configurable input triggers and four free configurable output triggers available. A trigger has to be selected from the pull down list that is located on the top of each of the four parts of the trigger settings window (see arrows). Several options are provided to set up the trigger in an experiment:

(1) Trigger In on frames:
Here input triggers can be defined at the beginning of a job. Incoming trigger signals from an external instrument can be synchronized with the start of the data acquisition. Furthermore, delayed triggering can be performed. The trigger in signal can be defined to arrive at a certain time point within the job. The data acquisition will be continued only after the arrival of the trigger in signal.

The delay has to be set in **First Trigger at … frame**. Repeats of the trigger in signal can be defined in **Repeat every … frame**.

(2) Trigger Out on function start:
To synchronize the start of an external instrument with the data acquisition of the scanner, an output trigger is sent out at the beginning of a job or a pause.

(3) Trigger out on function end:
For the synchronization of the action of an external instrument with the end of the data acquisition of the scanner, an output trigger is sent out at the end of a job or a pause.

(4) Trigger out on frames:
This option can be used for starting the action of an external device with a certain delay after the beginning of a job. The delay has to be defined in **First trigger at … frame**.
Repeated output triggering can be done within a job. The repeating frequency has to be set in **Repeat every … frame**.
The number of trigger pulses that should be send out can be defined in **Number of trigger pulses**.
In addition, it is possible to send out trigger pulses starting at an arbitrary line within a chosen frame. The start of sending out trigger pulses needs to be set in **Delay lines from scan start**.
The duration of the trigger pulses that are send out can be defined in **Number of lines**.

All options described here are working in the xyt- as well as in the xt-scan mode.

Note: For fast scanning and triggering it could be required to enhance the sampling rate of the DAQ box in order to get all signals displayed.

5.6 Pause

Some experiments require a pause of data acquisition for a defined time, e.g. drug delivery to cells. No data are recorded when not necessary to save memory space. A pause can be placed between certain jobs. First, a job needs to be selected. A pause is always inserted on the left of a job that was selected. By a click on the **Pause** symbol (Fig. 11) the Pause is now inserted on the desired position. Upon double clicking inside the Pause, a window for defining the duration of the Pause appears (Fig 15).

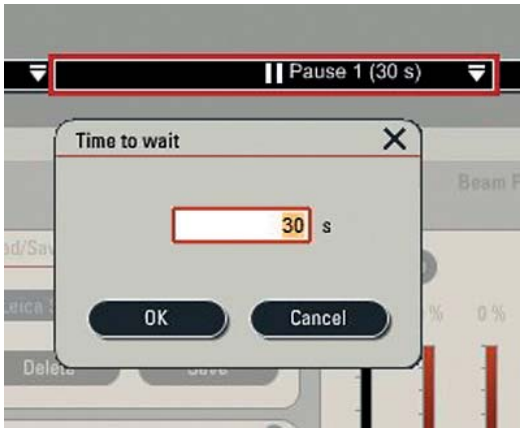


Fig. 15: Pause of data acquisition within an experiment; definition of the duration of a pause.

6. Data Handling

Upon running a record pattern data files are automatically organized in the experiment tree in the following way (Fig. 16):
All data of a record pattern are collected in the **Sequence** folder. This folder contains a Graph-file and the data files of the individual jobs. The data of each job – fluorescence intensity data in the **Quantify** tool and images in the viewer – are displayed when a job is selected.

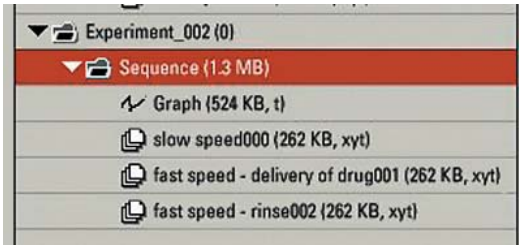


Fig. 16: Experiment tree created when using the electrophysiology software tool.

Upon clicking on **Sequence** all data – fluorescence intensity data, electrical data from an external instrument and images are displayed in the **Quantify** tool; images are shown in the image viewer. In order to just see the electrical data in the **Quantify** tool, a click on **Graph** is required.

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