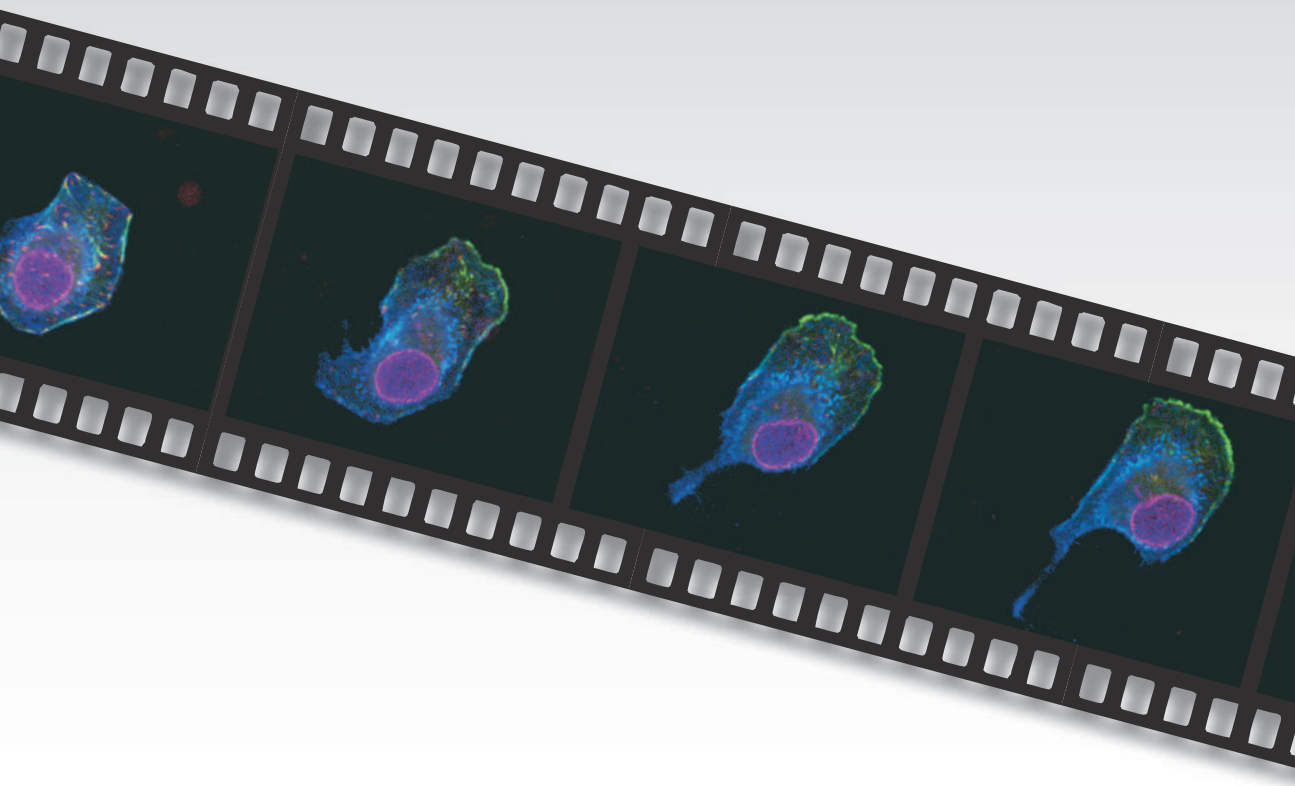


D e c .
2 0 0 7
N o . 2 7

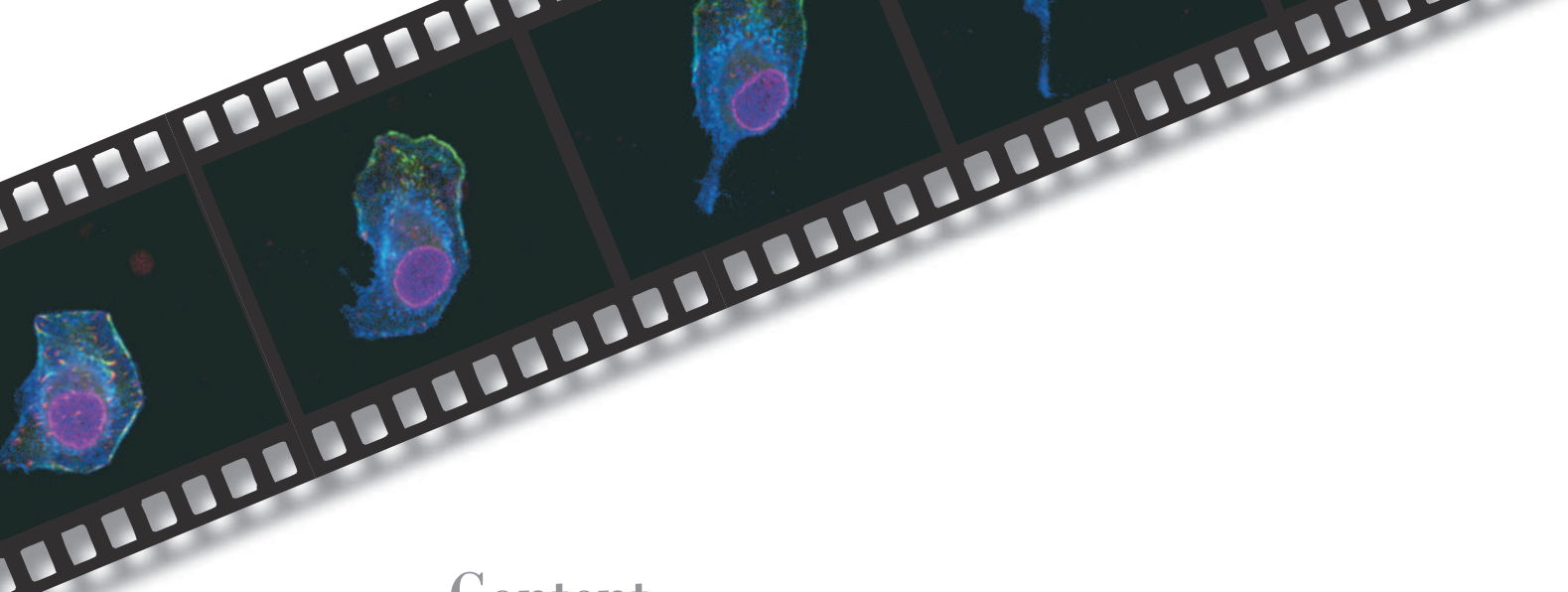
CONFOCAL APPLICATION LETTER

reSOLUTION

Live Data Mode with Leica TCS SP5



Leica
MICROSYSTEMS



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Titel Page:

Cell Type MDA MB-231. Time lapse: time points of acquisition each 20 min. Blue CD44- CFP (plasmatic membrane)
Red Vimentin-mRFP (Focal Adhesions). Green Actin -YFP (Cytoskeleton). Magenta Lamin A-GFP (Nuclear envelope)
Courtesy Dr. Maria Montoya, Confocal Microscopy and Cytometry Unit, CNIO, Madrid, Spain

Live Data Mode in LAS AF with Leica TCS SP5

Introduction

Live cell imaging applications comprise many different kinds of specimen and scientific interests including dynamic studies, drug and cancer research, e.g. interaction between immune cells in cell cultures and following changes of gene expression in developmental biology in live embryos. All these applications require a flexible instrument and software that allows for online interaction with hardware control settings in order to improve experimental conditions and prolong cell viability during an experiment. For more advanced applications it is important to pre-define settings and configure time lapse sequences.

The Live Data Mode tool in LAS AF is designed for live cell experiments and dynamic investigations: the scientist can define an experiment before data acquisition to carry out manipulations on the living specimen, e.g. application of a drug, switching of external devices etc., or pausing the experiment which is a prerequisite for good experimental control.

Direct interaction with the instrument is also highly important: this means the possibility of changing hardware parameters like laser power, scanning speed, acquisition channels or other imaging settings during the running experiment.

1. How to get started

The Live Data Mode tool can be activated from the drop-down list in the menu-line.

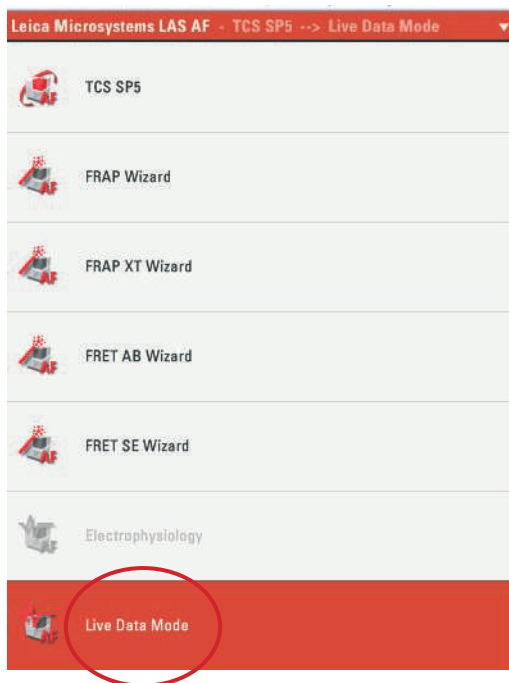


Figure 1: Drop-down list in the menu-line to select Application Wizards, Live Data Mode and TCS SP5 mode

After entering the Live Data Mode the user interface shows two tabs: **Default** (Fig. 2) and **Monitor** (Fig.3). The **Default** tab displays the beam path of a default setting that can be defined and the additional function buttons **Prepare/Apply** (1) for interactive data acquisition on the bottom. Furthermore, one can switch between the **Acquisition** and **Experiment** tab.

Figure 2: Live Data Mode tool Default tab

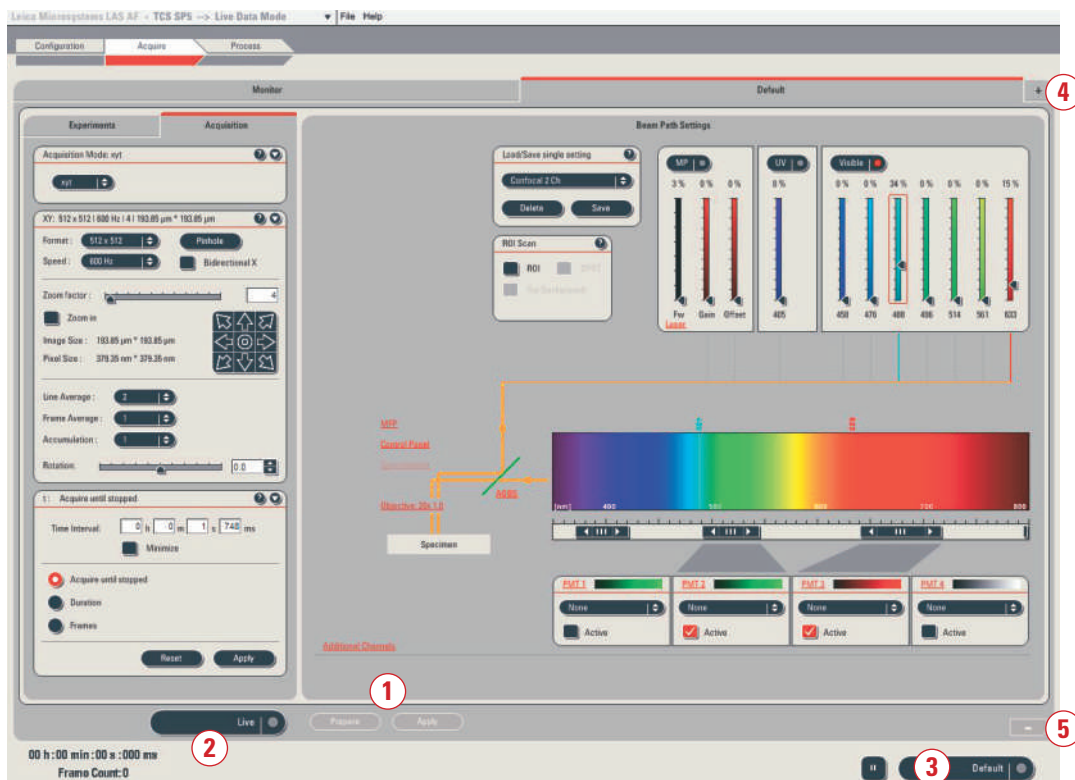
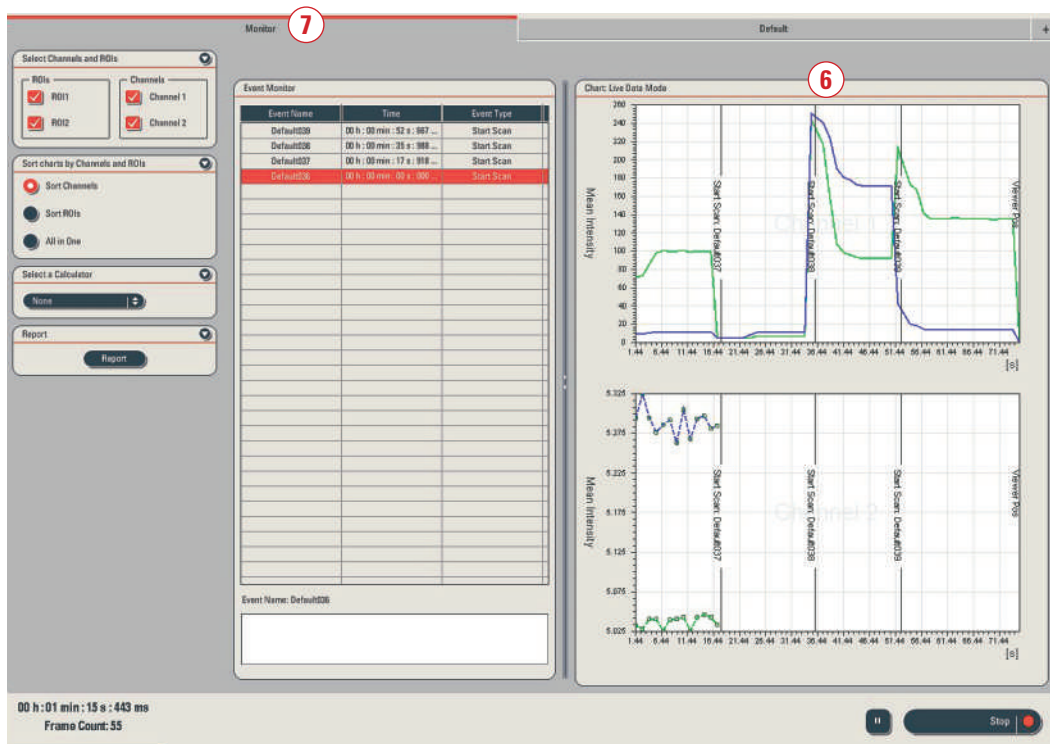


Figure 3: Live Data Mode tool Monitor tab



Live Scan

Before starting an experiment the adjustment of imaging conditions can be carried out during Live-Scan (2). No experiment file is generated in this case.

Default

This function defines the hardware control settings for an experiment or a part of an experiment. Available scan modes are: xt, xyt and xytz. The execution of the **Default** setting (3) automatically generates an experiment folder named "LDM" which contains a file named "Default 001" (see also section *Data Handling*, page 19). Experiment sequences (jobs) can be added by using the **plus symbol** (4) and be deleted by using the **minus-symbol** (5).

Note: The default setting can be freely changed but not be deleted.

Online Quantification

Online quantification of several regions of interest (ROIs) is crucial for live cell imaging application. Especially for the monitoring of changes in fluorescence intensity during the running experiment it is beneficial to see images in the viewer and quantification charts simultaneously.

The **Monitor** tab (Fig. 3) contains the quantification tool with the graph display (**Chart**) (6) for online measurements as well as the **Event Monitor** list (7).

Subsequently, the mean intensity of each ROI is displayed online within the **Chart** (right). The ROIs can be moved during image acquisition. The **Event Monitor** list (middle) shows the time point and type of events, e.g. switching of jobs and triggers.

2. Interactive Data Acquisition

For optimal adaptation of the imaging conditions to the specimen and the experiment, the Live Data Mode tool allows for directly interaction with the hardware control settings so that imaging parameters can be easily optimized during the running experiment.

Interactive changes of hardware settings

During the running experiment (default setting or any added job) the current hardware settings can be changed interactively by clicking on **Prepare** (1) (Fig. 4a). The user interface is now situated in a virtual mode and gives access to all imaging parameters (2) that can be changed and optimized (see also box page 6).

By clicking on **Apply** (3) the new settings are applied to the further imaging. Now the system is ready again for new hardware changes.

After each change of the hardware settings by using the **Prepare/Apply** function the system starts scanning a new series that appears under **Experiments** as a data file in the LDM folder. These changes are indicated in the event list and graph within the **Monitor** tab.

In order to apply certain hardware settings (job) on demand during a running default-setting a new setting (job) needs to be added and defined (see section *Pre-Definition of Experiment Settings*, page 7).

Note: When the job is completed the system switches automatically to scan using the Default setting.

Parameters for imaging that can be changed interactively by using the Prepare/Apply function:

- scan mode
- scan format and speed
- line and frame average
- accumulation
- changes in the t-dialog (minimize = minimized time interval between each frame, definition of time duration or number of frames)
- changes in the z-dialog
- adding/reducing number of PMTs
- switching of additional channels (PMT Trans or NDDs)
- changing of control panel configuration

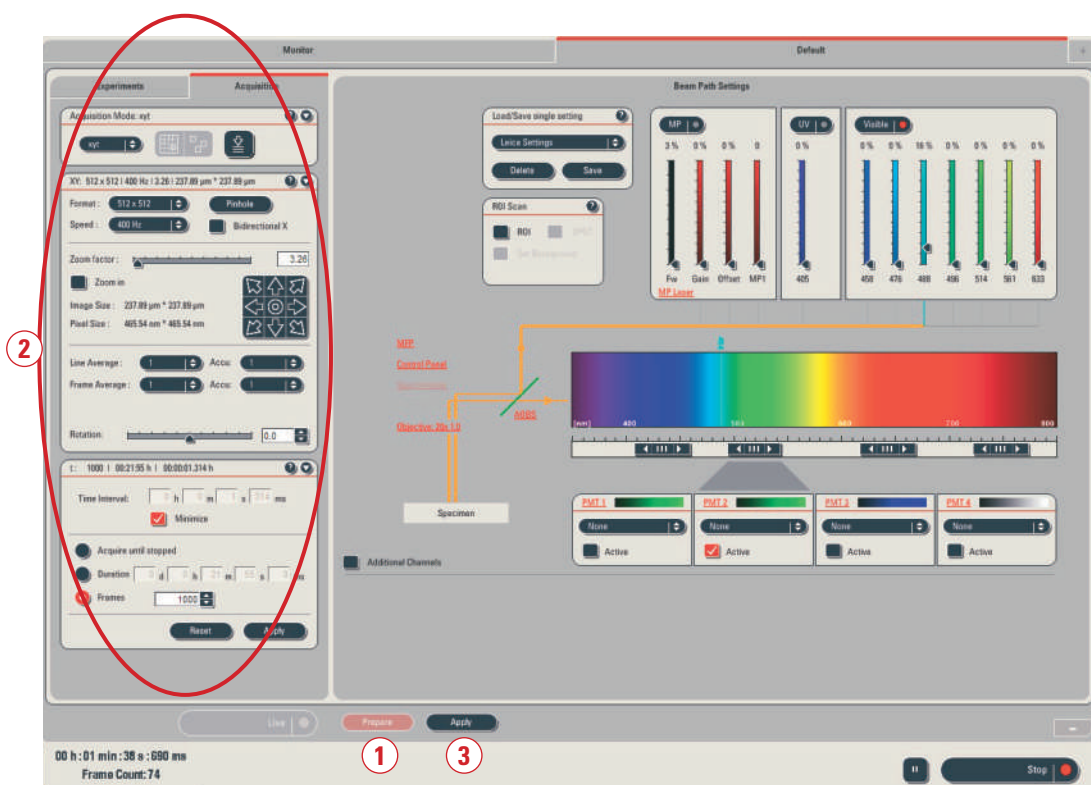
Parameters that can be changed online without use of Prepare/Apply function:

- PMT gain and offset
- laser power by AOTF
- adding/removing of laser lines
- zoom factor
- scan field rotation
- pinhole size
- A OBS settings

Pausing data acquisition interactively

The manipulation of living specimen sometimes requires pausing of the data acquisition during a running experiment. For example cells are monitored without any treatment; the data acquisition is paused while a drug is applied. Subsequently, the data acquisition is continued for the monitoring of cell reactions. Pausing of scanning can be done interactively at any time just by a click on the **Pause** button (4) located bottom right (Fig. 4b). To continue data acquisition the **Pause** button needs to be clicked again.

Figure 4a: Activated Prepare function: hardware settings can be accessed in the Acquisition tab



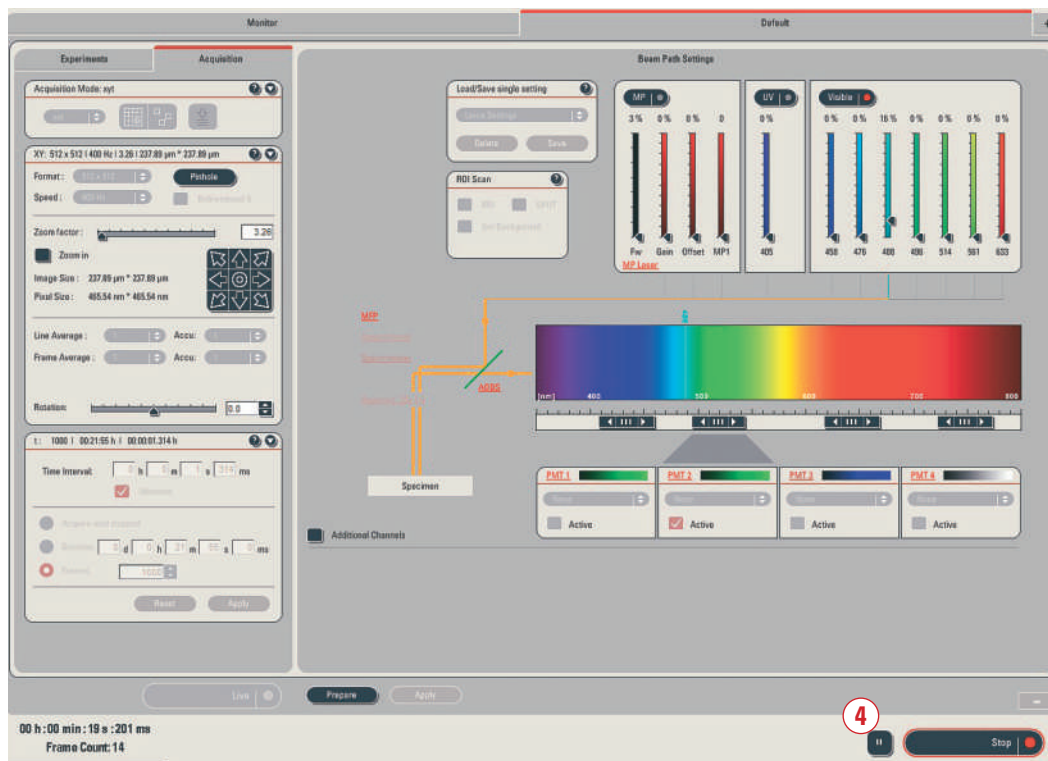


Figure 4b: System is ready for the next interactive hardware change: **Prepare** needs to be pressed again to access hardware settings

3. Pre-Definition of Experiment Settings

Beside interactive data acquisition the pre-definition of individual experimental job settings within time lapses is relevant. With the pre-definition any experimental setting can be used for a certain time. Also the time course of advanced applications can be pre-defined, saved and re-used again for reproducible experiments. The Live Data Mode tool enables the scientist to easily configure these kinds of experiments by defining jobs and job macros.

Adding Jobs

A Job can be added by clicking on the **plus** symbol (1) top right in the beam path window (Fig. 5a). Three options are available from a list (2) (Fig. 5b):

- 1. **Job:** a standard time lapse experiment without sequential scan mode.
- 2. **Job (Sequential Scan):** a time lapse experiment using sequential scan mode. When this

option is selected the sequential scan dialog appears automatically in the **Acquisition** tab on the left side.

3. **Macro:** a tool for configuring Jobs in a certain order, definition of pauses and loops between Jobs, assignment of triggers to jobs, access to trigger settings dialog.



Figure 5a: Plus symbol for adding a job

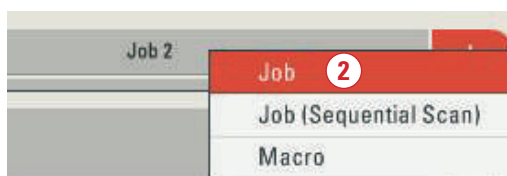
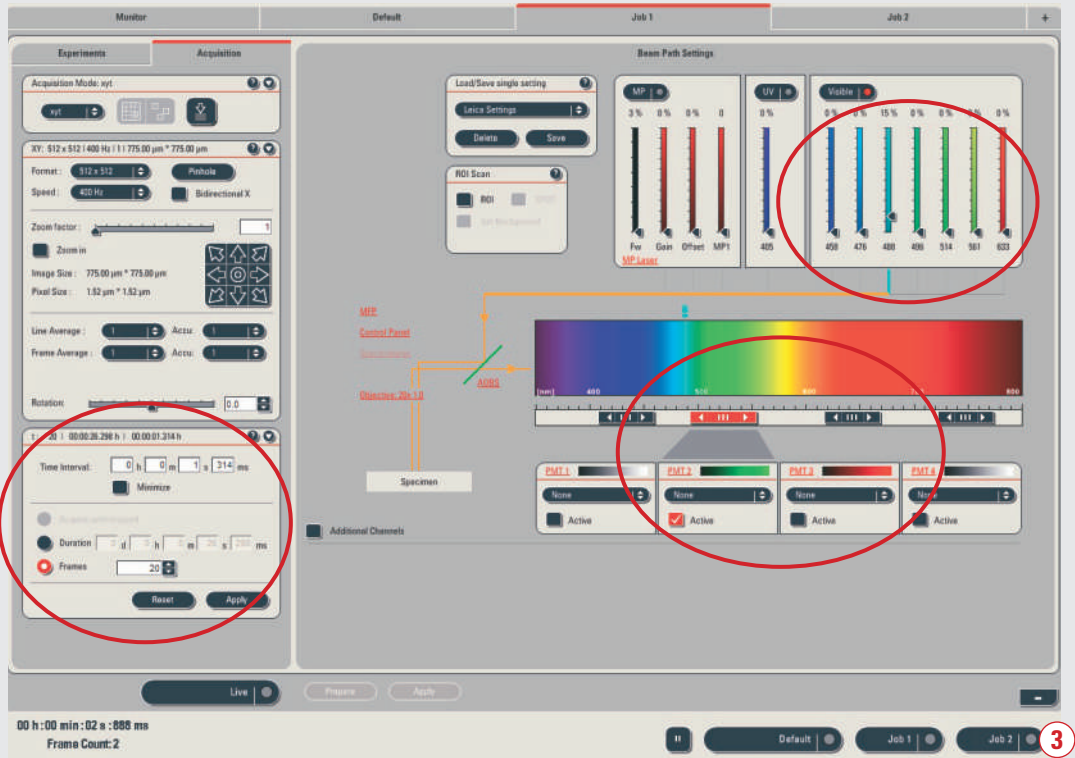


Figure 5b: Options for adding a job

6a Specific settings of Job1:
5 frames, 488 nm ex, detection
window 505-568 nm, PMT 2



6b Specific settings of Job2:
time interval minimized, 20
frames, 633 nm ex, detection
window 635-700 nm, PMT 3

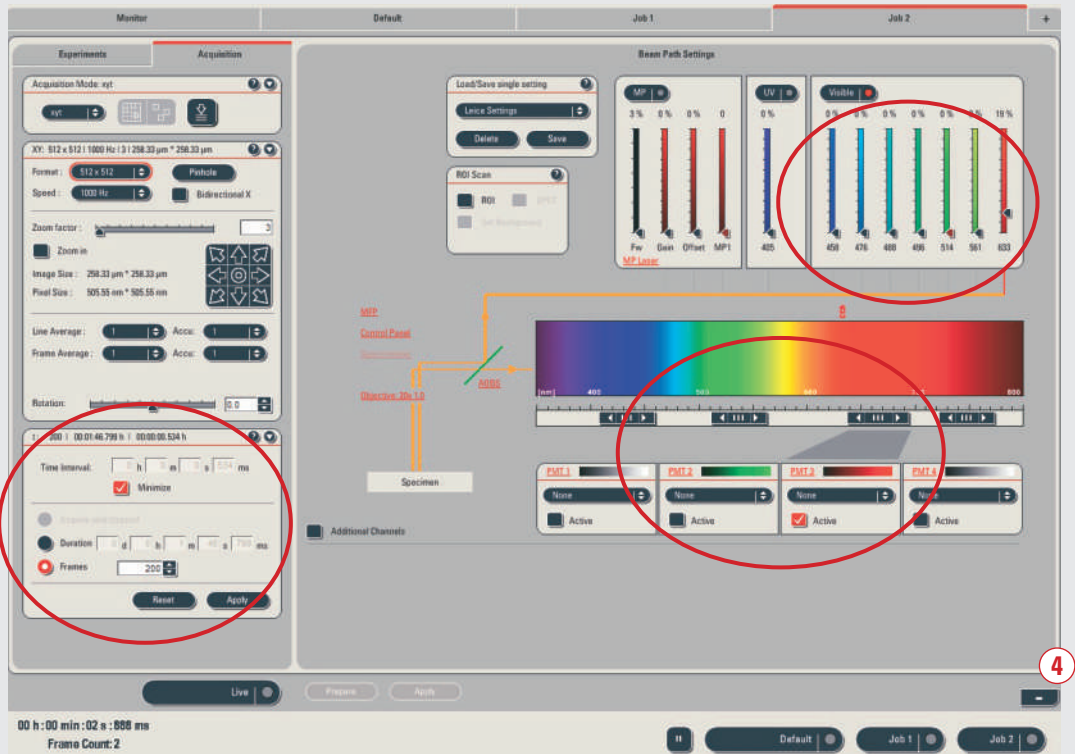


Figure 6: Specific settings of Job1 (6a) and Job2 (6b)

Defining Job Settings

After adding the first job, the hardware settings can be defined independently from the default settings. For each job added the beam path and the settings appear in an individual tab. Thus, by selecting the **Job** tab the settings for each job can be viewed, controlled and modified if necessary (see Fig. 6a and 6b).

The system automatically counts the added jobs (Job 1, Job 2, Job 3 etc.).

If several jobs are to be defined a newly added job will contain the same settings as the job that was selected during the addition, e.g. if the tab of Job 1 is selected the settings of Job 1 are automatically applied to the new job (Job 2).

Jobs can be started with a click on the appropriate **Start** button (3) (Fig. 6a) which appears bottom right below the beam path window. During a running job or default setting interactive chang-

es of imaging parameters can be performed as described earlier in section *Interactive Data Acquisition*, page 5.

Deleting, Renaming and Saving Jobs

A job can be deleted by clicking on the **Job** tab and a subsequent click on the **minus** symbol (4) (Fig. 6b). 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.

Deleting, renaming and saving jobs can also be done with a right mouse click on the **Job** tab. (Fig. 7).

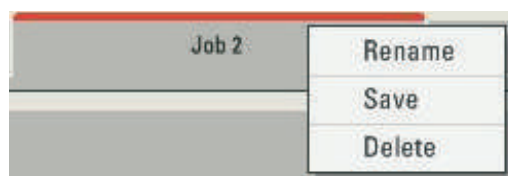


Figure 7: Options for job handling

4. Setting Up an Experiment (Job Macro)

Many applications require a good experimental definition within time lapses. For example, when the effect of a drug is studied on living cells the experiment may be defined as follows:

Job 1 Cells are observed without any treatment for a certain time.

Job 2 After the application of the drug the cells react and the organelles move much faster. Now, imaging parameters can be adapted accordingly by pre-defining short time intervals for data acquisition for a certain time.

Job 3 After 5 min the cells react slowly and data acquisition can be performed with longer time intervals.

In developmental biology the acquisition speed can be adapted to the investigated processes, e.g. the movements of cells within the embryo or cell division. This is done by defining jobs with different settings for the time interval.

The Live Data Mode provides many possibilities to define advanced time lapses by free configuration of jobs and their combination in a job macro. The key features of the job macro are listed on page 10.

Job Macro Key Features

1. Combining different jobs to design a job macro: pre-definition of experiments consisting of several jobs
2. Pause of data acquisition at a certain time point for a defined duration of time
3. Loops: repeated data acquisition using certain imaging parameters that includes looping of one job or a part of an experiment
4. Triggering: synchronizing the scanning process with external devices

4.1 Combining Jobs in a Macro

To define an experiment macro the option **Macro** must be selected after clicking on the plus symbol (Fig. 8).

Then a new tab named **Job Macro 1** is generated (Fig. 9a and b). All previous defined Jobs are listed on the top left (1). For Job and Macro handling the functions **Add, Insert, Remove, Load, Save Macro** are available (2). In a job macro **Trigger Settings, Loop and Pause** (3) can be selected. By a click on a job and subsequently on **Add**, the job is transferred into the grey time line below (4) (Fig 9b).

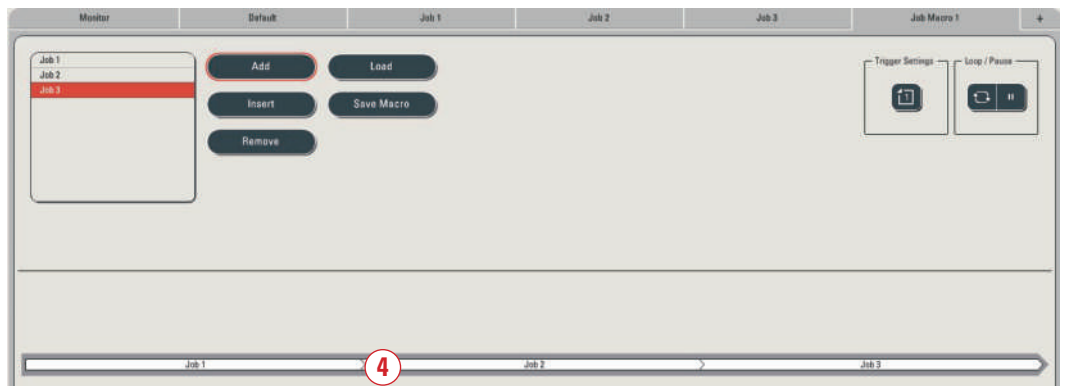
Figure 8: Options for adding



Figure 9a: User interface of Job Macro 1 with listed Jobs and time line



Figure 9b: Jobs have been added into the time line (Job Macro 1)



In order to insert a job at a certain position within the time line, e.g. Job 3 should be inserted between Job 1 and 2, proceed as follows:

1. Select the required job (Job 3) (5) in the list (Fig. 9c).
2. Define the position in the time line where the job should be inserted. This is always on the left side of the selected job (6).
3. Click on **Insert** (7).

A job can be removed from the time line by a click on the particular job in the time line and a subsequent click on **Remove** (8). Jobs that have been removed from the time line are still available as a **Job** tab.

Save and reload of a job macro

In order to get reproducible experiments a job macro can be saved and reloaded by the two function buttons **Save Macro** (10) and **Load** (9) (Fig. 9c). The macro will be saved under a default name given by the software, e.g. Job Macro 2. It can be renamed afterwards.



Figure 9c: Job 3 has been inserted between Job 1 and Job 2 (**Job Macro 1**)

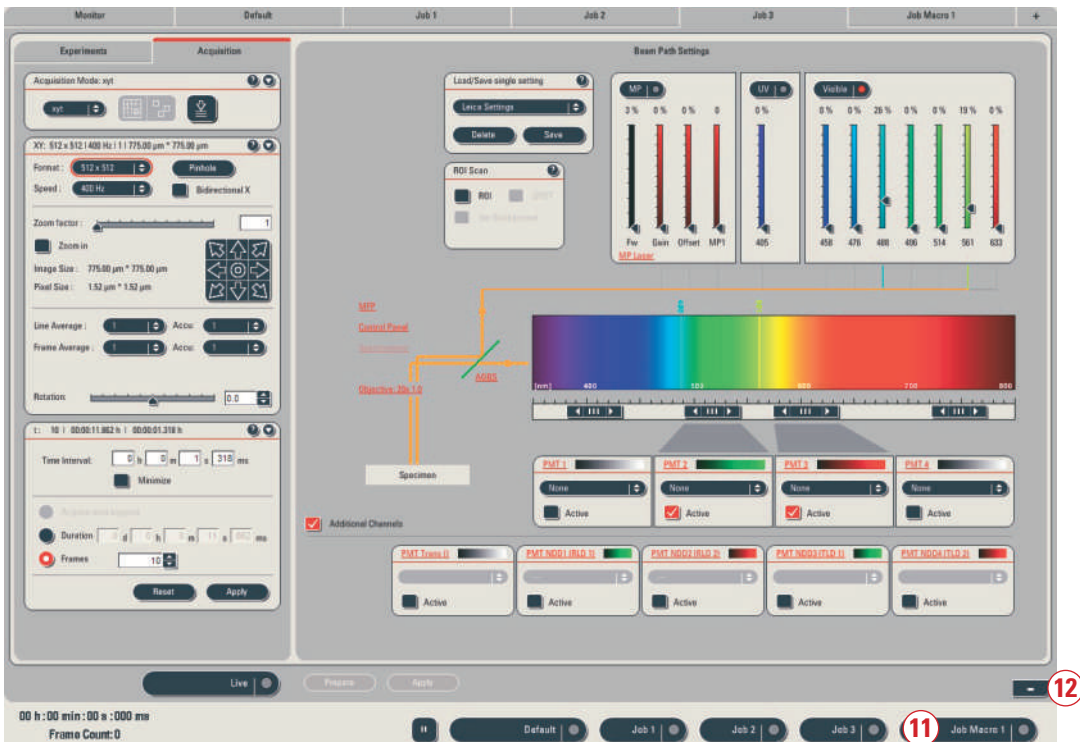
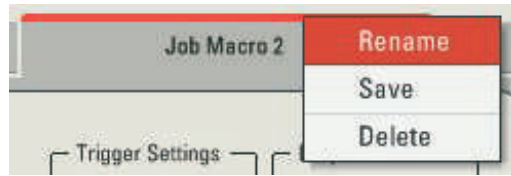


Figure 10: Imaging settings of Job 3. Other jobs and **Job Macro 1** are visible. Start buttons for each job and **Job Macro 1** are available bottom right

Figure 11: Rename, Save and Delete function for a job macro



The job macro is started by clicking the **Start** button (11) (Fig.10). To delete a job macro its tab must be selected followed by a click on the minus symbol (12) (Fig. 10). A further defined job macro will automatically be counted and named **Job Macro 2**. A right click on **Job Macro** displays the functions **Rename**, **Save** and **Delete** (Fig.11).

4.2 Pause

Some experiments require pausing of data acquisition for a defined time, e.g. drug delivery to cells, time of inactivity of cells or developing embryo. During a pause no data are recorded that will save memory space in addition.

A pause can be placed between two jobs. The pause will be inserted left of a selected job in the time line: e.g. a click on **Job 2** (1) followed by a click on **Pause** (2) (Fig 12 a). Now the pause appears within the time line (3) (Fig 12b) left of Job 2. Upon a double click on the pause in the time line a window for defining the duration of the pause appears (4).

Figure 12a: After clicking on the pause symbol in the timeline, the window for defining the duration of a pause appears

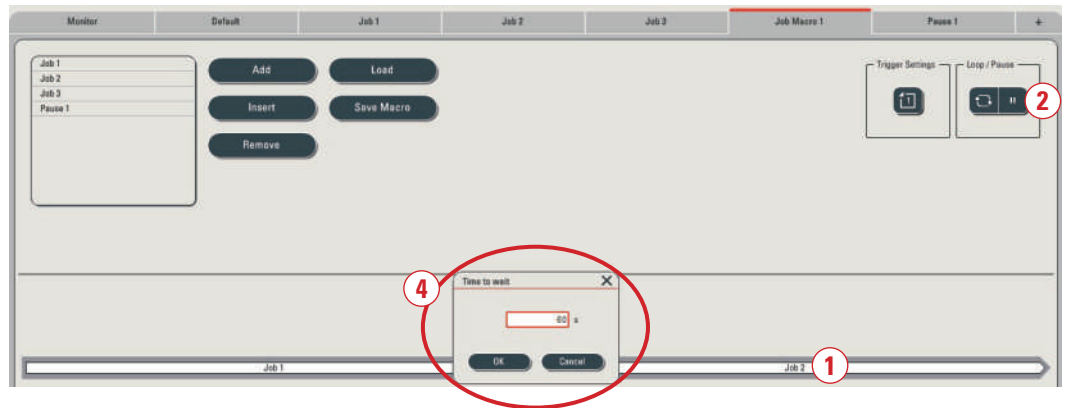
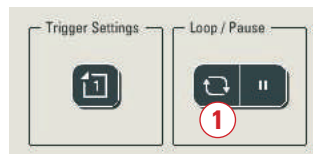


Figure 12b: The duration of a pause has been defined



Figure 13a: Selection of a loop by clicking the **Loop** symbol



4.3 Loops

For repeating the whole experiment or parts of an experiment, loops can be defined.

By a click on the **Loop** symbol (1) (Fig.13a) the **Loop Settings** dialog opens. Start and end of a loop as well as the number of loops can be entered (Fig. 13b). A loop can be defined for one Job, between jobs as well as between a job and a pause.

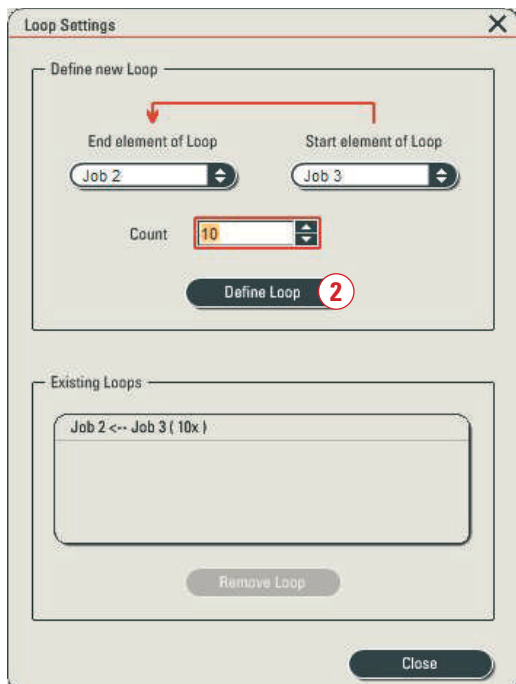


Figure 13b: The **Loop Settings** dialog has opened and a loop can be defined

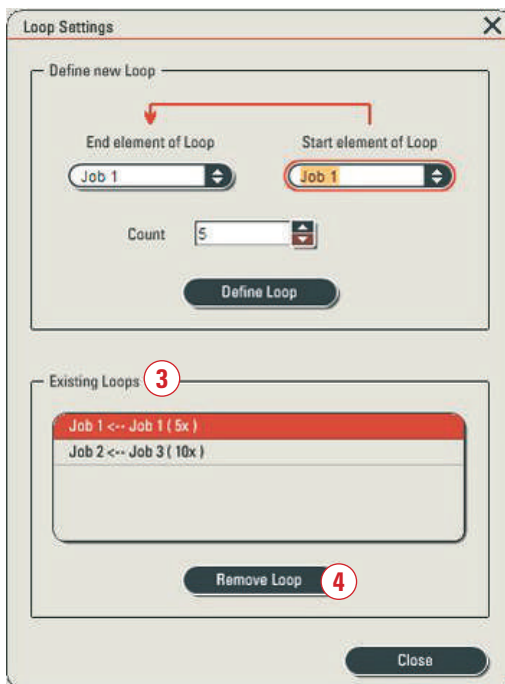


Figure 13c: Selection of a loop for deletion

A click on **Define Loop (2)** applies the Loop to the experiment Macro in the time line (Fig. 14). For removing a loop it has to be selected in the list

of existing loops **(3)** followed by a click on the button **Remove Loop (4)** (Fig. 13c).



Figure 14: A loop is indicated in the job macro

4.4 Trigger Settings

To synchronize the scanning process with external devices (patch pipettes, electrodes etc.) trigger functions can be used. For these applications it is a prerequisite that the system is equipped with the Leica trigger unit.

To assign a trigger to an individual job this job must first be selected in the time line. A subsequent click on the **Trigger** symbol **(1)** (Fig. 15) opens the **Trigger Settings** dialog that allows for setting input (IN) and output triggers (OUT) (Fig. 16).

All defined triggers are recorded automatically in the event monitor list and are indicated by a marker within the graph. Therefore, in the trigger settings dialog checking is not necessary in **Record in Event List** and **Show in Graph**.

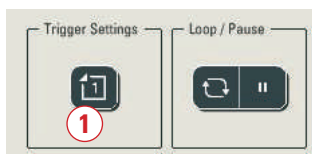


Figure 15: A click on **Trigger Settings** opens the Trigger Settings dialog

Trigger IN

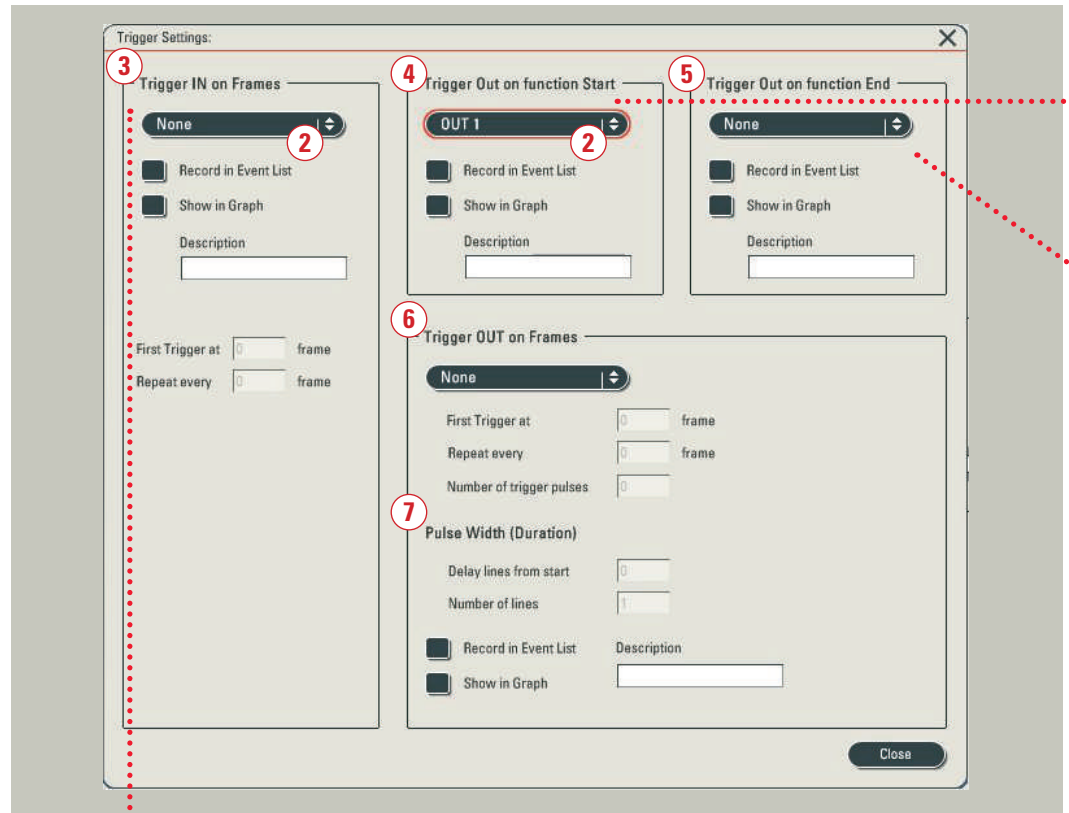
Using a signal from an external device (patch clamp system, micromanipulator) for triggering the start of scanning, two free configurable input triggers are available. The scan starts when the trigger signal arrives in the scan head. When the system is equipped with an extension for FCS only one input trigger is available for the Live Data Mode tool as one input trigger is configured for the FCS-application.

the scan start (**Delta T**) depends on the scan speed and format (see Fig. 19). In addition to the Delta T, a little time is required to position the x-galvo. The maximum time needed is equal to the time needed to scan one line (for 1000 Hz = 1ms). However, input triggers react reproducibly. Without considering the time needed for positioning the x-galvo reproducibility is within 10 μ sec.

Note: A certain time is required to position the y-galvo for scanning a frame: this time duration needed from the beginning of a trigger-pulse to

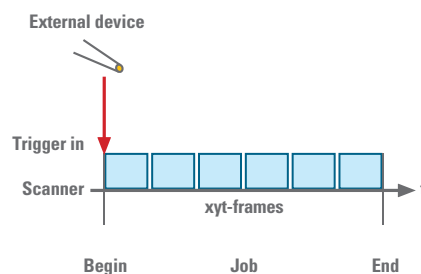
To assign an input trigger to a job the appropriate trigger channel has to be selected from the drop down list (2) (Fig.16) .

Figure 16: Trigger settings



Application:

1. Upon the action of a patch-pipette its signal is used to trigger the start of a job.
2. If a hand-held pipette delivers the drug to the specimen the **Trigger In** signal can be used to start the data acquisition (hand- or footswitch).



(3) Trigger IN on Frames

Input triggers can be defined at begin of a job in the xyt-scan mode.

Note: The functions **First Trigger** at and **Repeat every** are currently not working for input triggers.

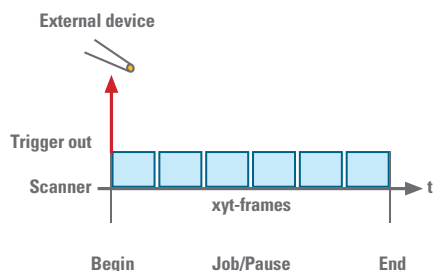
Trigger OUT

To start the action of an external device on a defined time point four freely configurable output triggers are available (Fig. 16). In this case the trigger signal is sent from the scan head to the external device (e.g. a patch clamp system) to start its operation.

To assign an output trigger to a job the appropriate trigger channel from the drop down list (2) has to be selected (Fig.16). The defined trigger is indicated in the time line (Fig. 17).

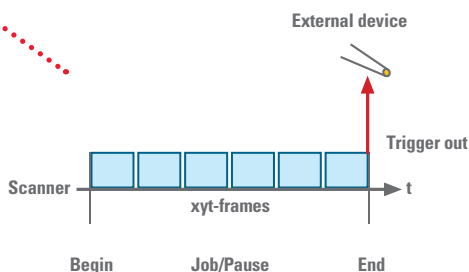
Output triggers can be defined at the begin and the end of a job or pause as indicated below.

Note: The reaction time for **output triggers on function start (4)** and **output triggers on function end (5)** can not be predicted like the input triggers because they are realized by software.



(4) Trigger OUT on function Start:

An output trigger can be set at the beginning of the first frame of a job or at the beginning of a pause.



(5) Trigger OUT on function End:

An output trigger can be defined at the end of the last frame of a job or at the end of a pause.

(Red arrows indicate trigger pulses)

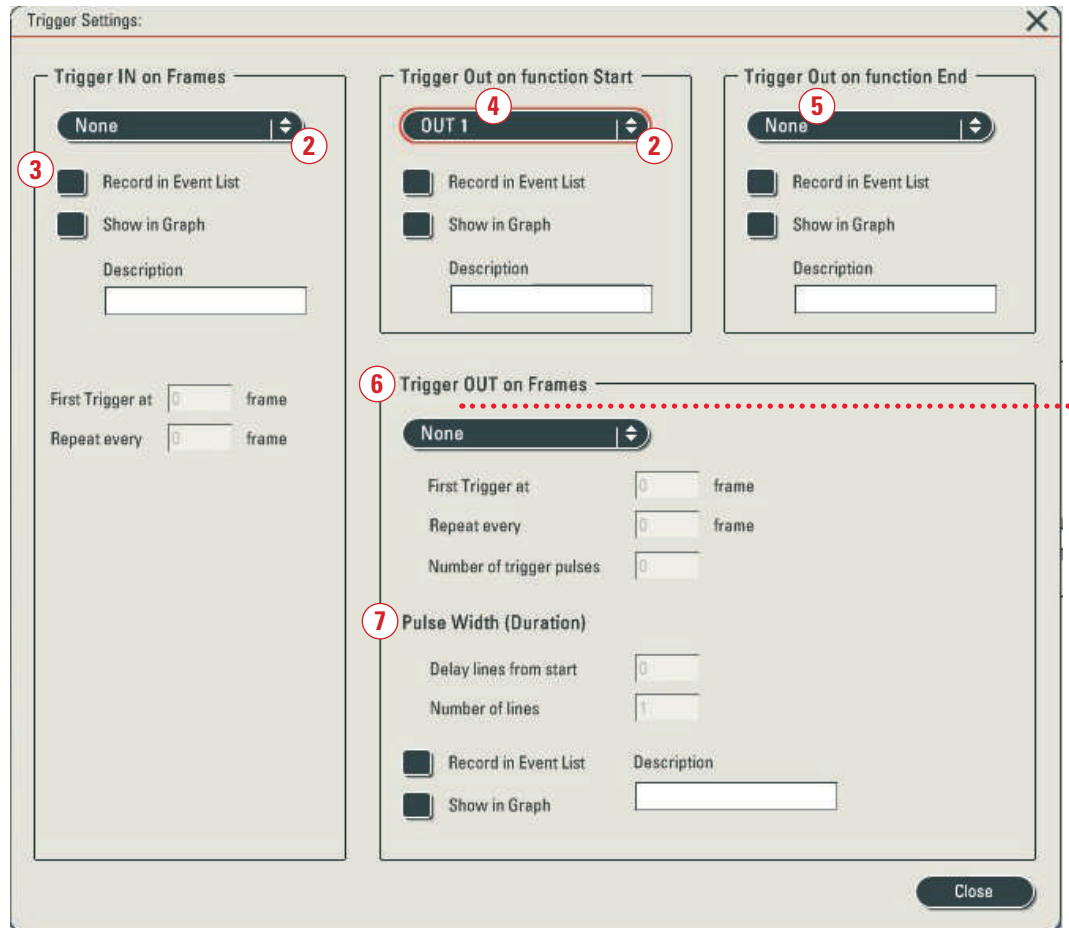


Figure 17: Job macro with activated out-trigger (OUT1) at the begin of Job 2

Applications:

1. Microinjection to manipulate the specimen at a defined time point: a microinjection pipette is attached to the incubation medium. Scanning is performed to acquire control data in Job 1. The **Trigger Out** signal from the scanner can be used to start the injection at a defined time point within the experiment. Thus, at the beginning of Job 2 an output trigger starts the action of a micropipette that delivers a drug to a cell preparation.

2. Adding a certain buffer to the medium at the begin or the end of a job.



Applications:

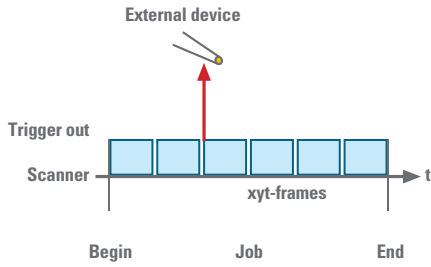
Fast Recordings of

- calcium transients in neurons and neuronal spines in xt-scan mode after electrical stimulation
- calcium waves and sparks in heart muscle cells in xt-scan mode or xyt-strip scan format
- studies on ion channels using a patch clamp system. A trigger out signal from the scanner is used to start the patch clamping

(6) Trigger OUT on Frames

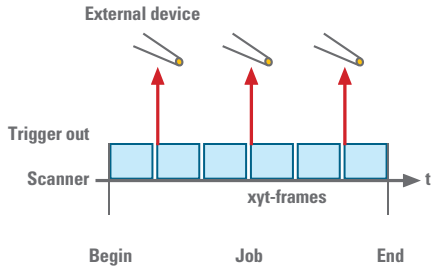
This option can be used for starting an external device with a certain time delay after the beginning of a job. It is also possible to do repeated output triggering within a job. Specifically, it is possible to send out trigger pulses starting at an arbitrary line within a chosen frame.

Note: In **Trigger OUT on frames** triggers are generated very fast and are sent without any delay time at the beginning of a line.

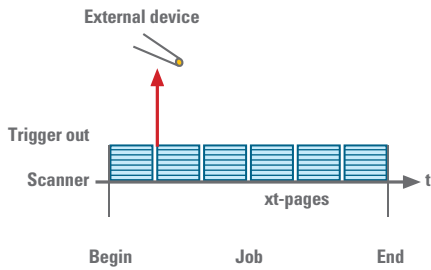


xyt-scan mode

For setting a delay a number needs to be entered in **First Trigger at ... frame**.

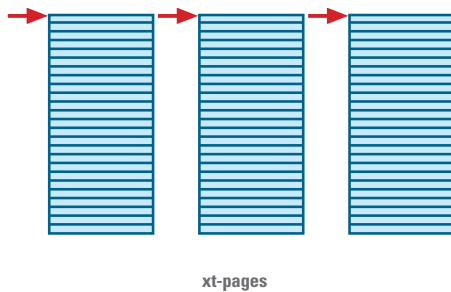


The sending of an output trigger in xyt-scan mode can be repeated within a Job. Repeating frequency has to be set in **Repeat every ... frame**. If a certain number of triggers should be send starting on a certain frame a number needs to be entered in **number of trigger pulses**.



xt-scan mode

A delayed trigger pulse is set at the beginning of a certain xt-page (see left). Here the term "page" corresponds to frame.



Repeated output triggering in xt-scan mode requires the definition of several pages within the time-dialog in the **Acquisition** tab. For fast repeats it is recommended to define a small number of lines per page, e.g. below 1000. The maximum number of lines per page is 8192.

(Red arrows indicate trigger pulses)

(7) Pulse-Position and Width for XT-Scan Mode

A delayed output trigger from the start of a page can be defined by counting lines from start. The

delay needs to be defined in **Delay lines from start** (Fig.16). The duration of a trigger pulse can be set in **Number of lines** (see also Fig. 18).

Figure 18: Setting trigger pulse duration and position (delay) in xt-scan mode by defining lines: This example shows a position of the trigger pulse four lines after the page start. The trigger pulse lasts for another six lines.

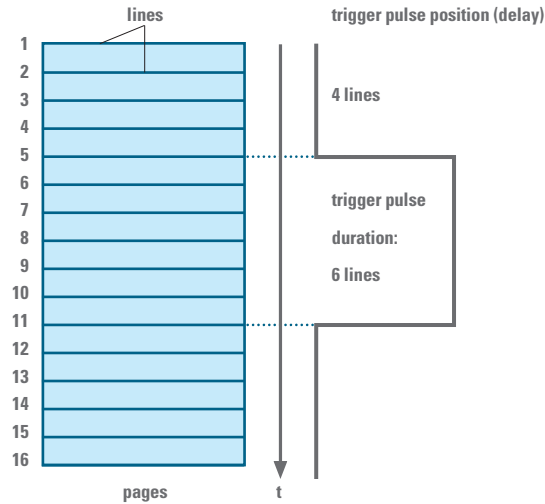
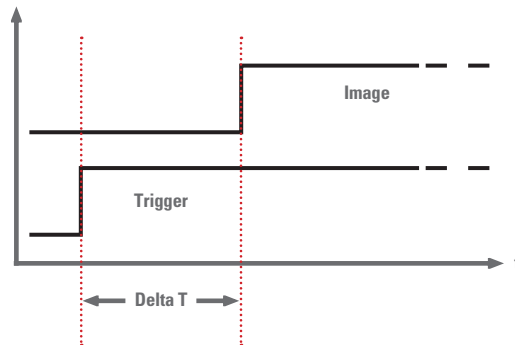


Figure 19a: Scheme on Delta T



Trigger Timing (Delta T) for Input Triggers

The time from begin of a trigger pulse to the scan of the 1st image pixel (Delta T) depends on the scan speed. See scheme and data regarding timing (Fig 19a and 19b).

Delta T for input triggers SP5-Systems (ms)

Scan Speed (Hz) unidirectional (bidir)	Pixelformat									
	16	32	64	128	256	512	1024	2048	4096	8192
100 (200)	320 (160)	620 (310)	100 (50)	100 (50)	100 (50)	100 (50)	100 (50)			
200 (400)	160 (80)	310 (155)	50 (25)	50 (25)	50 (25)	50 (25)	50 (25)			
400 (800)	80 (40)	155 (77.5)	25 (15)	25 (17.5)	25 (15)	25 (15)	25 (15)	25 (15)	25 (15)	40 (15)
700 (1400)	45 (22.8)	88.5 (44.2)	17.1 (15.7)	17.1 (15.7)	17.1 (15.7)	17.1 (15.7)	17.1 (15.7)			
1000 (2000)	32 (16)	62 (31)	16 (16)	16 (15)	16 (15)	16 (15)	16 (15)	16 (15)		
1400 (2800)	22.8 (11.4)	44.3 (22.1)	15.7 (15)	15.7 (15)	11.4 (15)	17 (15.7)	17 (15.7)	17 (15.7)		
Resonant Scanner 8000 (16000)	4 (2)	7.7 (3.87)	2 (1.65)	2.5 (1.87)	3.75 (2.37)	5.75 (3.5)	10.25 (5.62)			

Figure 19b: Table on Delta T in the Leica TCS SP5

5. Data Handling

Each experiment series is saved as a file named "Default000", "Default001" or "Job 1". "Job 2" etc., inside an LDM experiment folder (Fig. 20).

To view all images inside an LDM experiment folder click first on the LDM experiment folder and second play the movie in the viewer. To create an AVI movie this LDM folder can be exported.

There is no size limit for experiment files anymore. Thus, experiment series larger than 3.5 GB can be acquired. The limitation relies solely on the main memory, size of the hard disc and CPU speed.

Note: If the **Acquire until stopped** function is activated in the t-dialog the maximum file size is restricted to 2.1 GB.

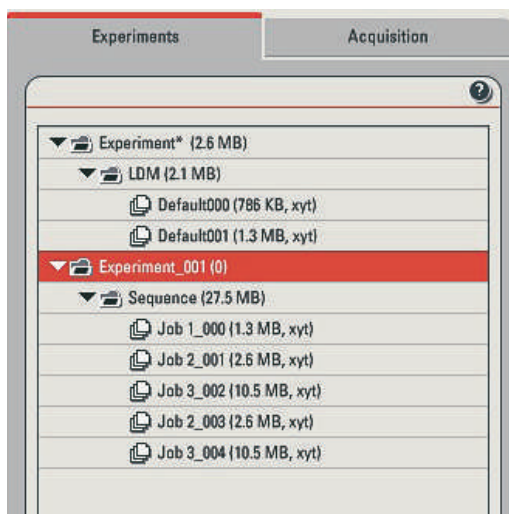


Figure 20: Experiment folder of Live Data Mode

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Leica Microsystems' Life Science Research Division supports the imaging needs of the scientific community with advanced innovation and technical expertise for the visualization, measurement and analysis of microstructures. Our strong focus on understanding scientific applications puts Leica Microsystems' customers at the leading edge of science.

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● Biosystems Division

The Biosystems Division of Leica Microsystems brings histopathology labs and researchers the highest-quality, most comprehensive product range. From patient to pathologist, the range includes the ideal product for each histology step and high-productivity workflow solutions for the entire lab. With complete histology systems featuring innovative automation and Novocastra™ reagents, the Biosystems Division creates better patient care through rapid turnaround, diagnostic confidence and close customer collaboration.

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