CONFOCAL APPLICATION LETTER

Live Data Mode with Leica TCS SP5
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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 xyzt. 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.

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
- AOLS 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

![Image of software interface with highlighted areas for Prepare function access and pause button location]
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 reused 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 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 changes 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).

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.
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 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)

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**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
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.

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**Figure 9c:** Job 3 has been inserted between Job 1 und 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.
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 timeline: 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).

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.
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).

### 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.

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**.

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).
**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.

**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 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 reproducible. Without considering the time needed for positioning the \( x \)-galvo reproducability is within 10 \( \mu \)sec.

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

**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).

![Figure 16: Trigger settings](image)
**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 send 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)

**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.
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.

**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

**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)

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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.
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