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CONFOCAL APPLICATION LETTER

No. 47, December 2014

reSOLUTION

Live Data Mode with Leica TCS SP8



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Titel page:

Small images: Heart muscle cell (cardiomyocyte) labeled with Fluo4 for Ca²⁺ imaging, short part of a complex time series.

Courtesy: Leif Hove-Madsen, Catalan Cardiovascular Institute, Barcelona, Spain



Observe user manual

Follow the safety notes and instructions in the user manual.

WARNING



Risk of permanent eye and skin damage from laser radation

Skin and eye damage can occur while using lasers if safety precautions are not taken. Pay particular attention to the laser safety.

1 Introduction

The **Live Data Mode (LDM)** tool is designed for live cell experiments and dynamic investigations such as in cell physiology.

The ability to predefine experimental settings within time-lapses for more advanced applications is crucial. Manipulation of the living specimen by the scientist (e.g. application of a drug, data acquisition start triggered by external devices, or the pause of an experiment) is a prerequisite to good experimental control. To optimize live cell applications with a confocal microscope, it is beneficial to have online signal intensity information from the specimen. Furthermore, the ability to optimize and interact with hardware control settings, such as adjusting laser power, scanning speed or other imaging parameters, can improve experimental conditions and prolong cell viability.

The LDM tool fulfills all these requirements: It allows the scientist to define an experiment before data acquisition. These experiments can be composed of several time-lapses (jobs). A life clock and frame counter record the progress and duration of the experiment. Input and output triggers are adjustable, and ROI-scanning is available for bleaching experiments. For a fast overview, online ROI-based data evaluation is also available.

The software simplifies the general handling and in particular, makes the definition of complex experiments easy to achieve. This document guides you through the functions of Live Data Mode and gives you a fast understanding of how to use the tool. The following terms are used to describe the LDM tool.

An experiment is designed within a “Pattern” that can be composed of one or several parts. A “job” describes one part of the experiment with a specific hardware setting (time lapse or stack etc). A pattern can contain several jobs and pauses (no data acquisition).

Features of Live Data Mode

- Easy design of complex experiments composed of time lapses/image stacks (jobs)
- Focus drift compensation in time lapse experiments by hardware/software based autofocus
- Synchronization of scanning process with external devices (input and output triggers)
- Intuitive user interface control by
 - multiple selections for deleting and removing
 - loop definition
 - repositioning of jobs by drag and drop
 - renaming of jobs, pauses and patterns
- Free time-definition of pauses from milliseconds up to days
- Data acquisitions parameters for each job can be viewed without changing the hardware

2 How to get started

How can the LDM be accessed? Use chosen hardware instrument parameter settings in the LAS AF beampath window, focus on the specimen and begin imaging by pressing the **Live** button. Next, open the **Live Data Mode** tool by clicking “**Live Data Mode**” in the list of wizards (Figure 1).

By entering the **Live Data Mode** tool, the user interface shows an **LDM Pattern** tab. An experiment can be designed using the tool buttons. Inside the **LDM Pattern** menu all subsequently defined jobs, pauses, and patterns are displayed in a time line (Figure 2). All jobs, pauses and patterns can be freely adapted to the experiment conditions.

Note: The LDM Pattern can be freely changed.

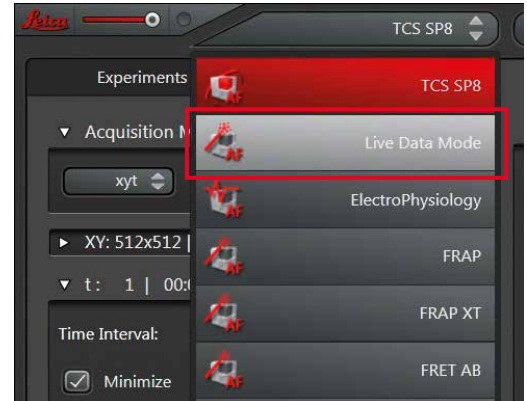


Figure 1 Opening Live Data Mode.

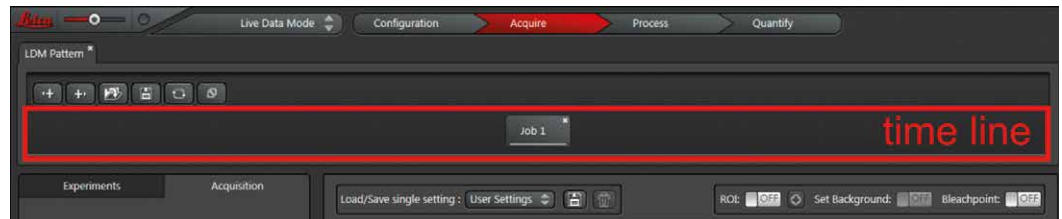


Figure 2 Live Data Mode time line; the job elements are located within the time line.

3 Definition of an experiment

Some experiments can be more complex and require different hardware settings at a certain time within the course of the experiment. For such experiments, Live Data Mode is the tool of choice. First of all, hardware parameters for Job 1 like scan mode and speed, laser line and power, detector etc., should be set.

To add an additional job into the time line, click the **+>** button and choose **New Confocal Job** from the upcoming list (Figure 3). All added jobs are displayed as record elements in consecutive order in the time line. For each selected job, the hardware settings are displayed within the beam path window and **Acquisition** tab (Figure 4).

A job can be defined as a time lapse experiment using all available time scan modes (xyt, xzt, xt, xyzt...). Furthermore, a job can just be one frame or an image stack (xyz, xzy). Consequently, an experiment can be composed by several jobs. Every job can be individually defined to accommodate the different imaging conditions during a complete experiment. All parameters like scanning format, speed, zoom factor, excitation laser, selection of detection channel etc., can be specifically assigned to a job.

A job or a pattern that works well for a certain application can be saved for further experiment sessions. For complex experiment conditions, a pattern can also be integrated into another pattern thereby containing some jobs (Figure 5). Thus, experiment patterns can be easily re-assembled and re-combined. This will save the time for the redefinition of a new pattern.

To add a pattern into the time line, click the **+>** button and choose **New Pattern** from the upcoming list. The added pattern is shown as a record element in the time line (Figure 6).

For every new pattern, a tab appears next to the **LDM Pattern** tab that starts with **Pattern 3**. By clicking on the respective tab, a job can be defined.

Note: As long as a pattern does not contain any job, there is no access to the hardware settings and the beam path window remains empty.

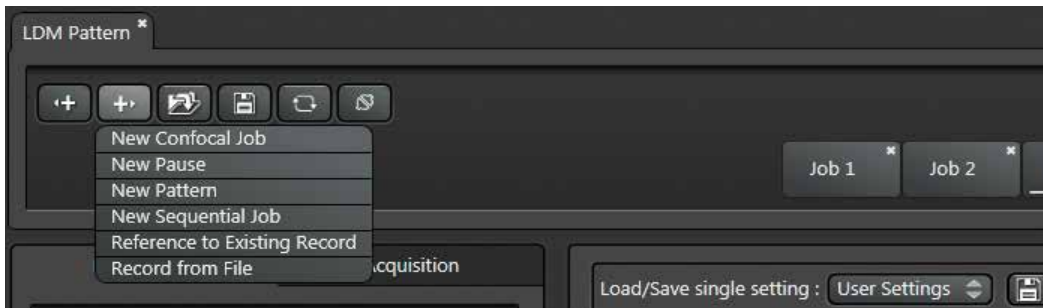


Figure 3 LDM Pattern: Options to add records (job, pause, pattern).



Figure 4 Displayed hardware settings for Job1 (left) and Job2 (right).



Figure 5 A second pattern was defined.



Figure 6 Definition of a new pattern inside the LDM Pattern.

Possible experimental designs – just a few examples:

- Timelapse (xyt, slow speed) – timelapse (xyt, fast speed)
- Timelapse (xyt, slow speed) – pause – timelapse (xyt, fast speed)
- Timelapse (xyt) – stack (xyz) – timelapse (xyt)
- Timelapse (xyt) – timelapse (xyt, triggered by external device for stimulation/drug delivery)
- Multiposition (xy, xyz, xyxt) at different z-position for each timelapse
- Multiposition (xy, xyz, xyxt) with objective change at different positions

3.1 Continuous data acquisition

Continuous data acquisition is a valuable tool to obtain significant information of changes in the sample during a time lapse experiment. This could be the behavior of cells treated with a specific drug with unclear effects, movements of cells within a tissue/tumor/embryos or a group of cells that appear to yield interesting information at a certain time point.

For above described applications, a tool for continuous data acquisition in a time lapse would be helpful. On the other hand, the possibility to assign other scan settings at a certain time point, like higher scanspeed and higher zoom factor to catch all details can be required.

The Live Data Mode can handle such a request by a job that works especially for continuous data acquisition in the **Acquire until stopped** mode. This so-called **Default** job is useful when observing a sample continuously over time. Whenever the experiment makes it necessary, a different hardware setting from a second job with a defined duration can be added (Figure 7). Furthermore, whenever needed for the experiment, it is possible to define and execute several additional jobs with different hardware settings.

To get access to the **Default** job, all displayed patterns need to be deleted. Then the user interface shows the **Default** job (Figure 7).

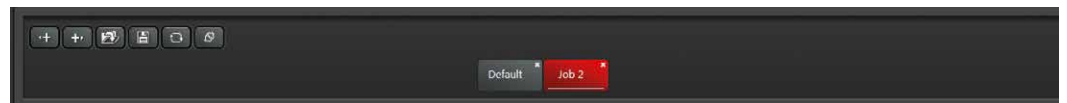


Figure 7 A second job was added to the Default job.

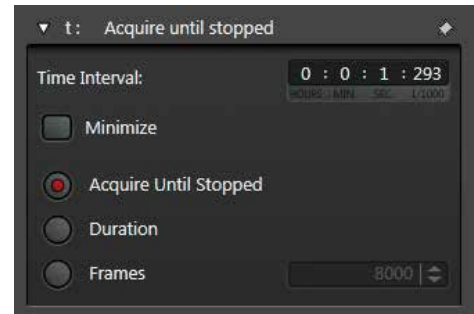


Figure 8 Acquire Until Stopped is active in the default setting.

At any time during the running of a **Default job**, the additional job can be selected and executed by a click on **Start Record**. When this job is accomplished, the system automatically switches back to the **Default job** and continues with the data acquisition. For this purpose the **Default job** is set to the option **Acquire Until Stopped** within the **t** dialog (Figure 8).

Note: The option **Acquire Until Stopped** is only available for the **Default job**. The default setting can be freely changed, but cannot be deleted.

WARNING



Risk of permanent eye and skin damage from laser radiation

From this time on, laser radiation may be present in the specimen area of the laser scanning microscope. Make sure to follow the safety notes for operation of the system.

4 Tools in detail

4.1 The main tool set (Insert, Add, Load, Save, Loop, View jobs without hardware change)

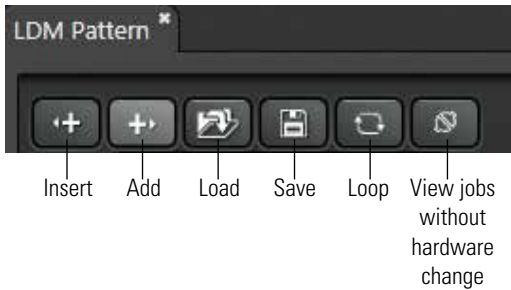


Figure 9 Tools for designing experiment patterns.

The new user interface facilitates the design of complex and long term experiments. The most important tools are located directly above the time line (Figure 9). The following section explains the tools in detail.

Insert and Add

Insert (<+): Integrates a new record (job, pause, and pattern) into the time line on the left side of the selected record element.

Add (+>): Appends the new record at the end of the time line.

Every newly added job will contain the same settings as the job that was selected during the addition. For example, if the element of **Job1** is selected, the settings of **Job1** are automatically applied to the new job.

Upon a click on **Insert (<+)** or **Add (+>)** the following options are available for inserting and adding into the time line (Figure 3):

1. **New Confocal Job:** creates a job for an experiment without sequential scan mode.
2. **New Camera Job:** creates a job for an experiment with camera detection. Only available for systems equipped with a digital camera.
3. **New Pause:** definition of a pause between jobs or patterns
4. **New Pattern:** definition of a new pattern for further definition of jobs
5. **New Sequential Job:** creates a job for an experiment using sequential scan mode. When this option is selected the sequential scan dialog appears automatically in the **Acquisition** tab on the left side.



Figure 10 Example for a reference: Job 1 in the list is the reference of both Job 1 records in the time line.

6. **Reference to Existing Record:** opens a list of record items showing all defined records for the recent LDM session (Figure 10). Each of these records can be inserted into the time line by selecting the respective record and clicking **Insert**. Each inserted record from the list is a reference to the already existing one (e.g. **Job 1**). Thus, changes in one **Job 1** record are conferred automatically to all other **Job 1** records. This provides an easy method of multiplying a record.

Note: Job records with the same name, but not linked as references, get the extension ...01, ...02, etc., in order to differentiate them. This happens when a job is loaded that has already been saved or when a job has been renamed. In this case, changes will not transfer to the other records.

7. **Record from File:** a job, pattern or pause saved on the hard disc can be added or inserted into the time line.



Figure 11 Save and define loop buttons.



Figure 13 View jobs without hardware change.



Figure 12 Setting the execution count for the loop Job1–Job3.



Load

A click on this button opens the load dialog for loading saved jobs or patterns. These records will be integrated into the time line to the left of the highlighted record.

Save

This function opens the save dialog for saving currently selected jobs or patterns. Choose **Selected Record** in the context menu to save a selected record from the time line; choose **Pattern** to save the pattern currently selected in the list of tabs.

Alternatively, right click the respective record element in the time line and choose **Save** in the context menu (Figure 11A).

To save a pattern, right click the respective tab and choose **Save**.

Loop

In live cell imaging, it might be important to repeat the complete experiment or just a part of the experiment one or more times. This can be done in LMD by defining loops.

A loop definition can be freely chosen for a single job, pause or pattern, or for a group of records. To define a loop, a single record or a group of records have to be selected. Multiple selections can be performed by selecting the first record and then clicking the last record of the loop body while the shift key is pressed.

Finally, click the **Define Loop** button above the time line or choose **Define Loop** in the context menu opened by right clicking (Figure 11B).

Now, the loop is defined and indicated by a line. The complete count of executions is indicated in the left corner of the line (Figure 12). To customize the count, double click on the number to open an edit line. To delete the loop, click the cross in the right corner of the loop line.

View jobs without hardware change

Some experiments require viewing and changing hardware settings in different jobs without applying them to the hardware at the same time. The function **View jobs without hardware change** is designed for such requirements.

When the **View jobs without hardware change** button is activated (Figure 13), all data acquisition buttons are inactive. As the changes have to be carried out without active scanning ("offline"), viewing of job settings is done independently from the hardware settings, that means without any hardware changes.

Now, the hardware settings of jobs can also be modified. However, those modifications do not affect the current hardware settings.

This makes it possible to view and modify hardware settings (laser intensity, number of detection channels, duration of time-lapse, etc.) of jobs, containing also individual xyz positions, z positions or even objective lenses without any x, y, and z movement of the motorized xy stage or the z galvo stage. Further, the objective lens will not be changed when the **View jobs without hardware change** function is active.

Click the **View jobs without hardware change** button, go to another job to check the settings, and this job is now highlighted. Finally deactivate the **View jobs without hardware change** button by another click. Now, the frame that marks a job automatically jumps back to the job, from which the **View jobs without hardware change** button was activated.

4.2 Moving and deleting record elements

To move a record element to a different position within the time line, select the element, keep the left mouse button pressed and move the element horizontally along the time line to the desired position. Move at least over the middle of the record you want to jump over. All records can either be removed or deleted. A removed record vanishes from the time line but is still in the record items list.

To remove a record, right click on the respective element and choose **Remove** in the context menu (Figure 14). Clicking the “x” in the upper right corner of the record elements will remove the record from the time line.

Delete takes the record off the record items list, too.

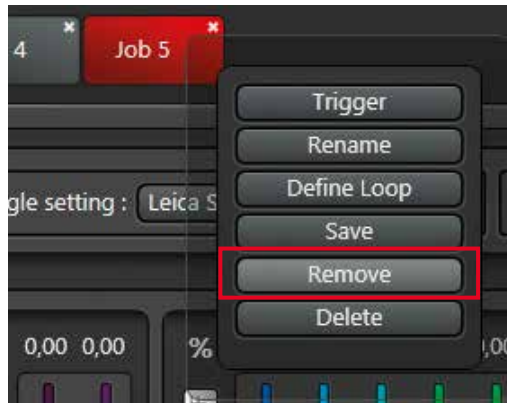


Figure 14 Removing a record.

4.3 Defining Pauses and Triggers

Pauses

Some experiments require pausing of data acquisition for a defined time, e.g. for drug delivery to cells or to pass the time of inactivity in cells or developing embryos.

To pause the data acquisition for a defined time, click on **Insert (<+)** or **Add (+>)** and select **New Pause** (Figure 15).

Right click on the inserted pause. A context menu now appears that shows the pause duration at the bottom (Figure 16). Double click the edit line to change the duration. Pauses can be defined in several milliseconds up to days. During a pause no data is recorded and memory space is saved.

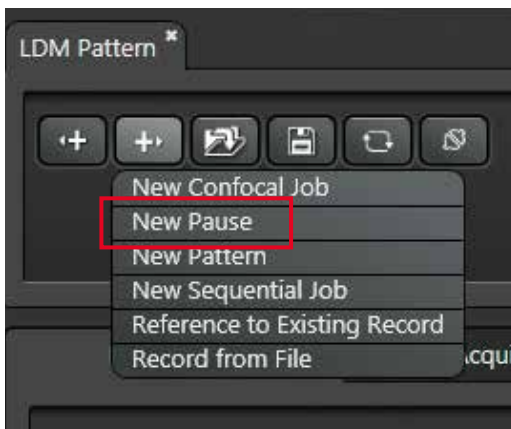


Figure 15 Definition of a pause.



Figure 16 Changing of the duration of a pause.

Triggers

Trigger functions can be used to synchronize the scan process with external devices such as patch pipettes and pumping devices. A prerequisite for these applications is that the system be equipped with the trigger unit by Leica Microsystems. The **Trigger** option can be found with a right click on a job or pause. To assign a trigger to an individual job or pause, select the respective element in the time line. A subsequent right click opens a context menu with several options (Figure 17). By selecting **Trigger**, the **Trigger Settings** window opens (Figure 18).

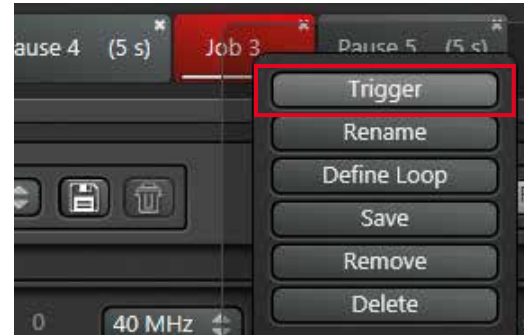


Figure 17 Trigger selection.

In this dialog, input triggers (**Trigger IN**) and output triggers (**Trigger OUT**) are selected. Different options are available, including triggering within xt scans. A defined trigger is automatically assigned to the selected job.

For more information about triggering, refer to Confocal Application Letter 45, *Triggering with Leica TCS SP8 and DMI6000 CFS – An Application Guide*, 1593104032, June 2014.

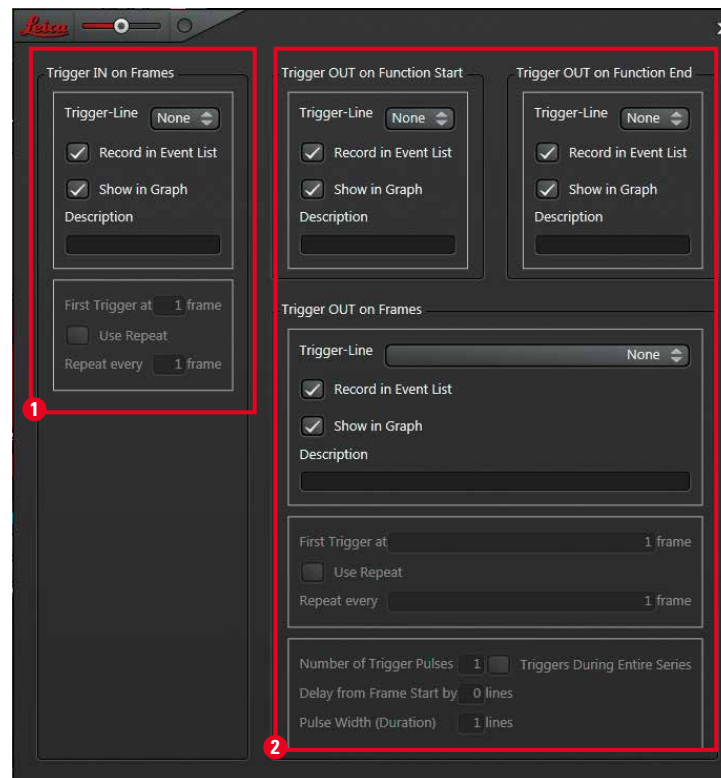


Figure 18 Settings dialog for input (Trigger IN, 1) and output trigger (Trigger OUT, 2).

4.4 Compensation of focus drift

Long term experiments usually need a focus correction over time to compensate for any focus drift. Live Data Mode allows focus corrections to be integrated into the time line, which enables a fast focus correction during a running experiment. There are three ways to correct focus: first using the software based **Best Focus**; second using the hardware based **Adaptive Focus Control (AFC)**; or third using a combination of both.

Upright systems and MP systems do not provide the hardware based AFC. Here, instead, Best Focus is used. Best Focus always finds cells due to intensity or contrast changes – no matter if the cells have been moved in the meantime.

In the following paragraphs, focus drift compensation using Best Focus as well as using AFC are described.

4.4.1 Working Principle of Best Focus

A very fast scanning Best Focus job is defined. Image acquisition jobs can be linked to the **Best Focus** job to assign the corrected focus position to these jobs. Follow the steps below to take full advantage of this functionality:

1. Define a pattern with at least one job in the time line.
2. Insert or define a second job that should be used as a focus job.
3. Click on the **Autofocus** panel (Figure 19, 1). Now the **Autofocus** window (Figure 19, 2) opens and the **Best Focus** function is assigned to the job indicated by a symbol (Figure 19, 3).
4. Define these settings inside the **Best Focus** dialog: **Focusdrive** (z galvo or z wide), **Focus Modes**, **Analyze Type**, **Speed/Accuracy**, and **No. of Steps**. Further options are available for time series upon a click on the **Timelapse** button (Figure 21).
5. Define hardware settings for a fast Best Focus job: Scan mode with z dimension, faster scan speed, smaller pixel format, etc. For a very fast Best Focus job, one can use the **Analyze Type** “Intensity Based Method”, in combination with a small pixel format, high zoom and low laser power (e.g. 64x64, zoom factor 6–12, low % Ar-laser power), set the **Speed/Accuracy**-slider to a middle position.
6. Link a data acquisition job with the Best Focus job. This will assign the focus position of the Best Focus job to the data acquisition job. Therefore, right click on the data acquisition job. Select the option **Use Focus** and choose the job that is defined as the Best Focus job (here Job1, Figure 20A). Link other jobs in the same way.
7. A linked job is indicated with a sign (Figure 20A, red arrow). Undo the link by choosing **None**.

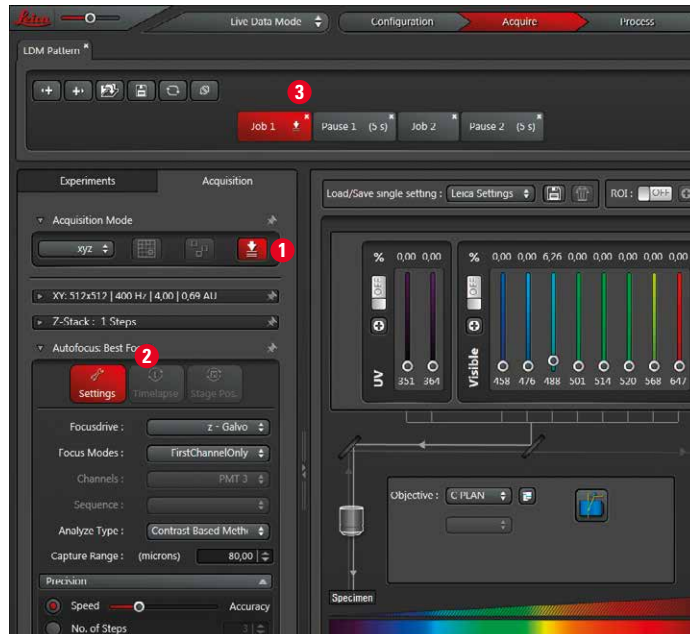


Figure 19 Autofocus/Best Focus window.

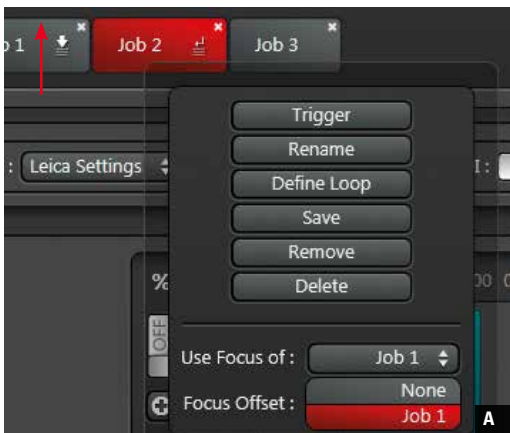


Figure 20 Linking a data acquisition job to a Best Focus Job (A) and defining an offset (B).

WARNING**Risk of permanent eye and skin damage from laser radiation**

From this time on, laser radiation may be present in the specimen area of the laser scanning microscope. Make sure to follow the safety notes for operation of the system.

Offset for Best Focus adjustment

To ensure the stability of the focus with respect to a certain structure within a cell, an offset can be set. The offset is automatically set when focusing on the structure of interest e.g. the cell nucleus. The offset is indicated (Figure 20B) and the z position of the offset is displayed on the control panel.

Best Focus job within a time lapse experiment

The execution frequency of a Best Focus job during a time-lapse experiment (xyt, xyzt) can be defined by clicking the **Timelapse** button (Figure 21). A cycle represents one frame in a xyt experiment or one z stack in a xyzt experiment. The **Timelapse** button is active after a t series has been defined.

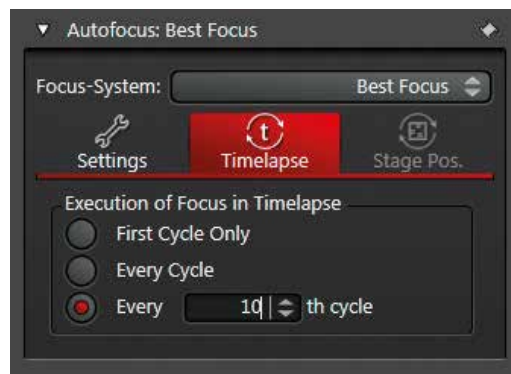


Figure 21 Setting the execution frequency of the Best Focus job.

4.4.2 Adaptive Focus Control (AFC)

Using the hardware based AFC, a focus position can be specified that is maintained while manually searching for the sample and/or throughout the course of an automated imaging experiment, e.g. setup via LMD. The focus position is held in reference to the cover glass of the sample, which is generated by means of an LED-generated reflex at the boundary surface.

Using the AFC, drifts of the whole sample in the z direction, which for example are induced by temperature changes, are corrected by following these movements with the objective focus. Note: Movements of objects within the sample such as migrating cells cannot be corrected. For this purpose Best Focus can be specified. In addition, AFC and Best Focus can be performed in a sequence: The AFC keeps the reference position within the specimen while the Best Focus searches for the z position of cells of interest.

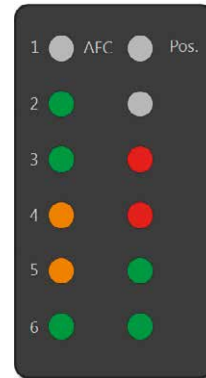


Figure 22 Potential color combinations displaying different states of the AFC in the software.

AFC states

The AFC function can be activated by choosing **Adaptive Focus Control** or **AFC + Best Focus** from the **Focus-Systems** list. Two indicators (AFC and Pos.) show the status of the AFC in the software (Figure 22 and 23) and at the front of the microscope.

The colors of the display (Figure 22) have the following meaning:

AFC gray: Inactive

AFC green: Active

AFC orange: Water immersion objective in use: two boundary surfaces exist (between water immersion and cover slip or between coverslip and medium); it is possible, that a reference plane for defining the focus position cannot be found.

Pos. gray: No focus position set

Pos. red: Focus position defined, but not held

Pos. green: Focus position defined and held

Operation modes: Continuous Mode

The **Continuous Mode** can be used to maintain a specified focus position until the z position is manually changed, e.g. via the focus wheel on the microscope stand, the remote control module (SmartMove) or the SmartTouch Panel (STP6000). Once you have configured the focus position for your experiment, activate **Continuous Mode** with **Hold Current Position**. The specific focus position is then held (and indicated by **Hold** in the graphic display of the operating range).

Note: When using the **Continuous Mode** in an imaging experiment, the AFC performs the focus search prior to the image acquisition.

Operation modes: On Demand Mode

The **On Demand Mode** integrates the AFC into an experiment workflow. During the process of an experiment, the focusing is only carried out at certain points of time and/or at certain positions, which can be defined in the dialogs: **Execution of Focus in Timelapse** and/or **Execution of Focus in Mark & Find**. Once you have configured the focus position, enable **Set Experiment Position** to apply it to the experiment workflow. The focus position is indicated by **Exp.** in the graphic display of the operating range (Figure 23).

Note: If you are working in **On Demand Mode** and wish to acquire z stacks as part of an experiment, follow these steps: Configure the settings for acquiring a z stack in the z stack dialog. At the end, click the **Move To Center** button. This position (center of the z stack) is the focus position for the AFC function; it will be applied to the experiment workflow via **Set Experiment Position**.

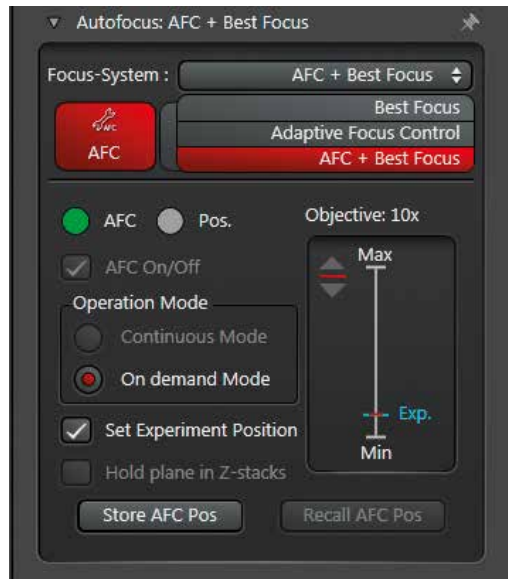


Figure 23 AFC control: AFC is active and the experiment position is set. The experiment position is also indicated within the focus range.

WARNING

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5 Execution of an LDM experiment

The following function buttons for the execution of the LDM experiment are located at the bottom of the acquisition window.



Figure 24 Functions for executing an LMD experiment.

- 1 **Autofocus:** finds the focus of a selected job with the **Focus Mode** selected in the **Autofocus: Best Focus** dialog.
- 2 **Live:** starts a live scan with the settings of the selected job for image optimization.
- 3 **Pause:** interrupts data acquisition and restarts the experiment when the **Pause**-button is clicked again.
- 4 **Leica Infobar:** shows important process notifications.
- 5 **Start Record:** executes a single job by first clicking the respective job element and then choosing **Start Record**. The beam path window displays the hardware settings of the selected job.
- 6 **Start Pattern:** executes the pattern selected from the list of tabs.
- 7 **Skip Job:** skips the currently executed record.
- 8 **Stop:** general stop function that stops the experiment (**Job** and **Pattern**).

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6 Data handling

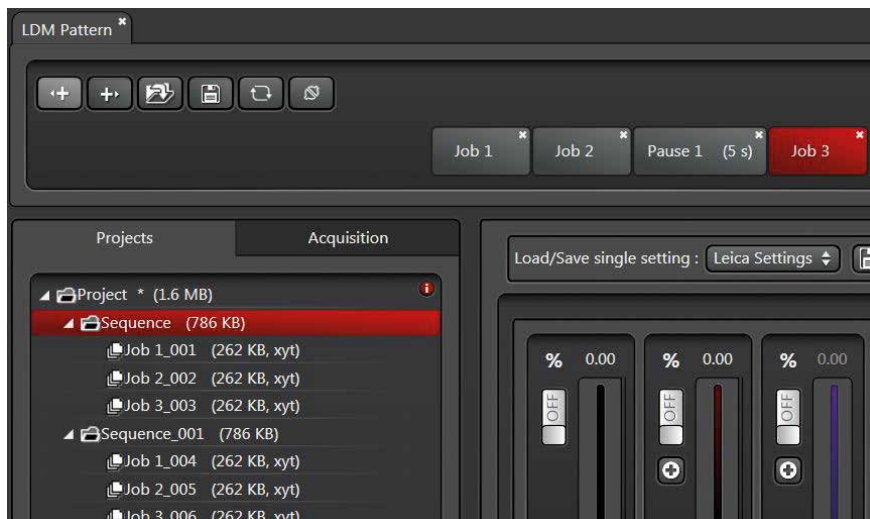


Figure 25 Projects tree.

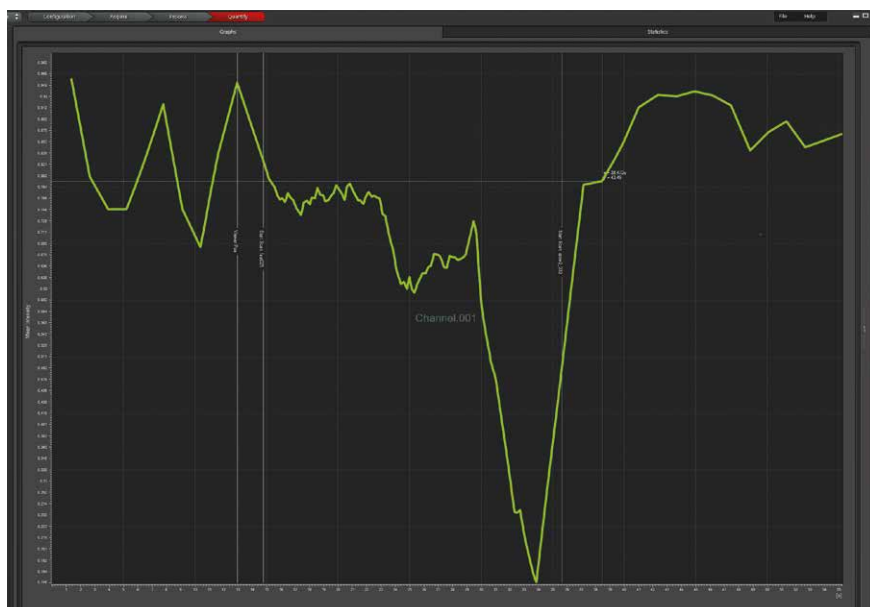


Figure 26 Quantification chart.

Each experiment is saved as a file named “Sequence” containing the executed jobs (Figure 25). Click on the jobs to view the corresponding image series. Data can be exported in the most common file formats (TIFF, JPEG, QuickTime, AVI, etc).

In order to analyze all jobs of a sequence together in a quantification chart (26), click the respective sequence in the projects tree.

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Canada · Concord/Ontario	+1	800 248 0123	847 405 0164
Denmark · Ballerup	+45	4454 0101	4454 0111
France · Nanterre Cedex	+33	811 000 664	1 56 05 23 23
Germany · Wetzlar	+49	64 41 29 40 00	64 41 29 41 55
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Japan · Tokyo	+81	3 5421 2800	3 5421 2896
Korea · Seoul	+82	2 514 65 43	2 514 65 48
Netherlands · Rijswijk	+31	70 4132 100	70 4132 109
People’s Rep. of China · Hong Kong	+852	2564 6699	2564 4163
· Shanghai	+86	21 6039 6000	21 6387 6698
Portugal · Lisbon	+351	21 388 9112	21 385 4668
Singapore	+65	6779 7823	6773 0628
Spain · Barcelona	+34	93 494 95 30	93 494 95 32
Sweden · Kista	+46	8 625 45 45	8 625 45 10
Switzerland · Heerbrugg	+41	71 726 34 34	71 726 34 44
United Kingdom · Milton Keynes	+44	800 298 2344	1908 246312
USA · Buffalo Grove/Illinois	+1	800 248 0123	847 405 0164