Adaptive Focus control (AFC) for live cell imaging over time (time course) on Stellaris

INTRODUCTION

The autofocus capacity of the Stellaris system enables to control focus drift over time for live cell imaging. Typical applications for the AFC are live-cell-imaging, screening of well-plates, and multi-position experiments.

There are two autofocus mechanisms available on the Stellaris that users can apply for time course imaging for living samples:

- Adaptive focus control (AFC): hardware-based. AFC corrects for any drift of the sample holder or the culture dish.

- Best focus: software-based. Best focus corrects for any focus differences which occur in the sample (the cells) itself.

This manual is mainly about how to use AFC.

Note: AFC is fully integrated into LAS X software and can be combined with image based autofocus mechanism. The AFC helps to reduce the search range of an image-based autofocus and thereby minimize light stress on the sample during experiments.

1. Theory of AFC

The AFC works like a distance meter in modern cars. It measures the distance between the objective front lens and the surface of the sample carrier. The way it works is quite simple. Light from an LED emitting a wavelength of 850nm is guided through the objective and is reflected at the sample carrier. The reflected light beam then travels back through the objective to a prism which reflects light with a wavelength longer than 850nm onto a line CMOS sensor (AFC-detection sensor). The position of the reflected light on the sensor area is determined by the angle of the light hitting the sample carrier. In turn, the angle of the light reflecting off of the sample carrier depends on the distance between the objective front lens and the sample carrier itself. If the sample carrier is moved in the z direction this means that the reflected light hits the AFC-detection sensor at a different position. The AFC focus drive corrects this difference by moving the objective until it reaches the original position of the reflection on the AFC-detection sensor.
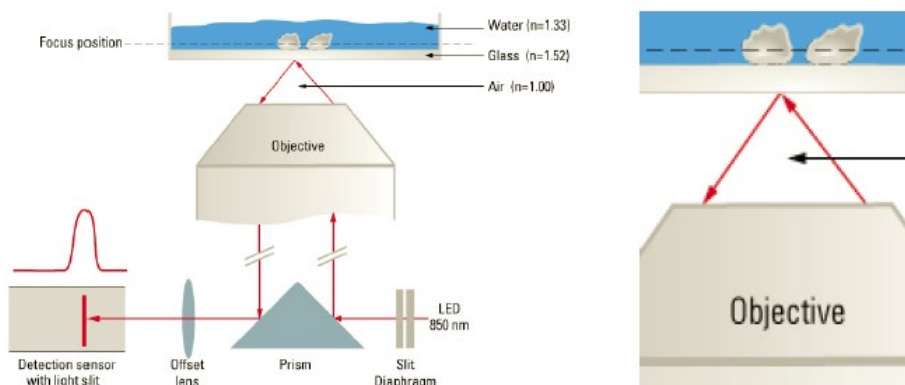
Practically, this means that the objective is focused back to its original focus position relative to the sample carrier.

Schematic view of a system used with a dry objective with AFC. With a dry objective the main reflection happens at the air-glass surface.

2. Objectives and AFC

- Dry objectives: easiest to use with the AFC
- Water objectives: works with AFC
- Oil objectives: may not work if mounting medium RI is too close to that of glass (1.52)

Note: AFC works for the 20x, 40x water and 63x O on the Stellaris but for 2.5x nor 4x (they are used as DLS illumination objectives).

ACTIVATE AFC IN AN XYZT EXPERIMENT
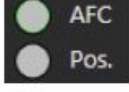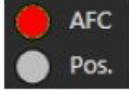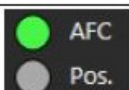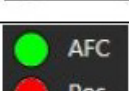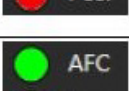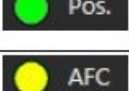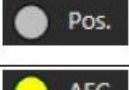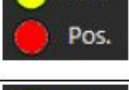
1. Acquisition mode: choose XYZT
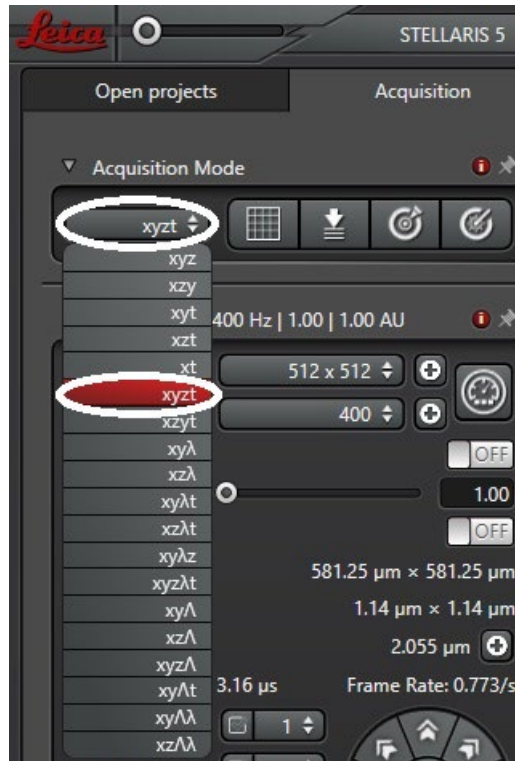
2. Turn on AFC, then the AFC window will be activated as shown below (the screenshot on the right-handed side).
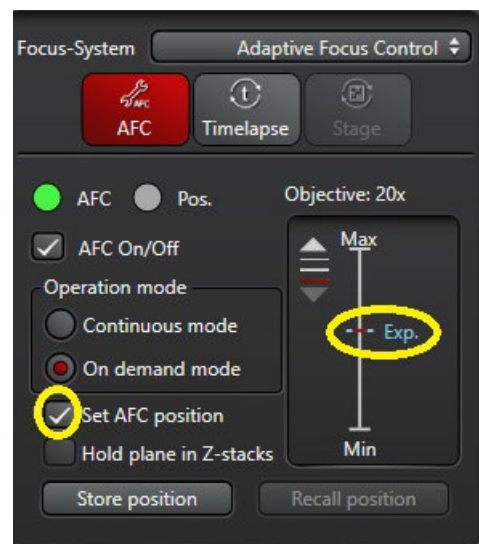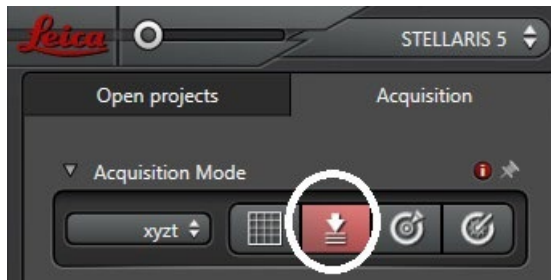
- Continuous mode: AFC always actively keeps the focus of the sample stable. Example application: when continuous medium injection happens during time course.
- On demand mode: used mainly when the AFC is combined with other image-based autofocus systems. It works in most cases and minimizes the overall time for refocussing and therefore also the amount of light used to refocus the sample.
- Set AFC position: once this box is checked, the current AFC position will be used for the experiment ("Exp" in blue color will appear once Set AFC position is ticked).
- Hold plane in Z-stacks: rarely used unless continuous injection of medium to bring changes to every single plane,
- Store position: leave it off (no need to store the position).

In LAS X, the AFC status is displayed using color-coded circles. The upper one ("AFC") shows whether the current objective is compatible with the AFC. The lower one ("Pos.") shows whether the AFC focussing mechanism is in use or on stand-by. In the following table you can find a list of typical status displays.

|	
○ AFC ○ Pos.	Objective cannot be used with AFC or AFC switched off
● AFC (red) ○ Pos.	Objective can be used. Red= no reflection on the AFC sensor
● AFC (green) ○ Pos.	Objective can be used. Green= reflection detected on the AFC sensor AFC holding mechanism on stand-by
● AFC (green) ● Pos. (red)	Objective can be used. Green= reflection detected on the AFC sensor AFC holding mechanism busy
● AFC (green) ● Pos. (green)	Objective can be used. Green= reflection detected on the AFC sensor AFC found focus (on-demand mode) / continuously holding focus (continuous mode)
● AFC (yellow) ○ Pos.	WATER-Objective can be used. Yellow= reflection detected on the AFC sensor AFC holding mechanism on stand-by
● AFC (yellow) ● Pos. (red)	WATER-Objective can be used. Yellow= reflection detected on the AFC sensor AFC holding mechanism busy
● AFC (yellow) ● Pos. (green)	WATER-Objective can be used. Yellow= reflection detected on the AFC sensor AFC found focus (on-demand mode) / continuously holding focus (continuous mode)

3. Choose Adaptive Focus Control (AFC) in the drop-down list to use AFC only as an example.

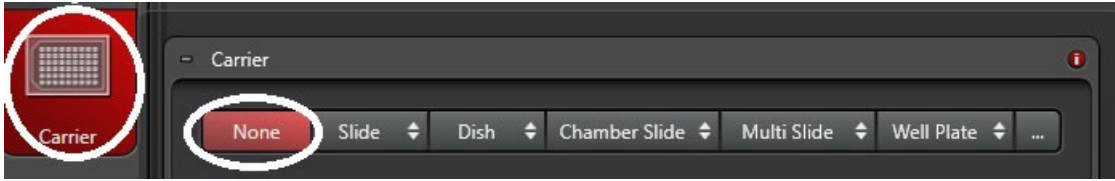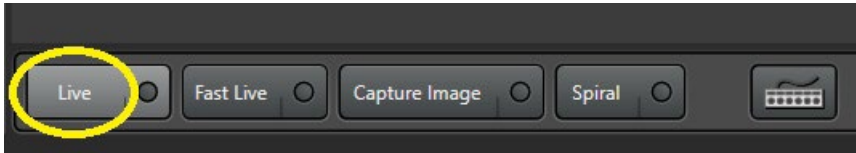
EXAMPLE: USE AFC ONLY TO IMAGE MULTIPLE POSITIONS IN AN XYZT EXPERIMENT

1. Choose "XYZT" acquisition mode. Then go to Navigator—Carrier—None.
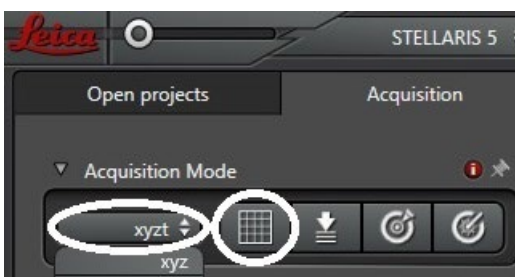
2. Now look for an interesting position through microscope eyepieces and bring to good.
3. In Navigator, check how the current position looks like in live window on the monitor. If needed, adjust Z-garvo to fine tune focusing.

4. Now Mark this position so the current X/Y/Z positions are registered. Once marked, this position will appear in the task list.

Mark current stage position (single image)
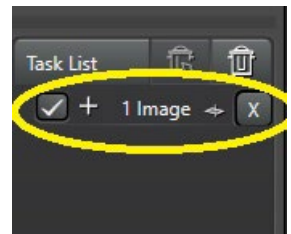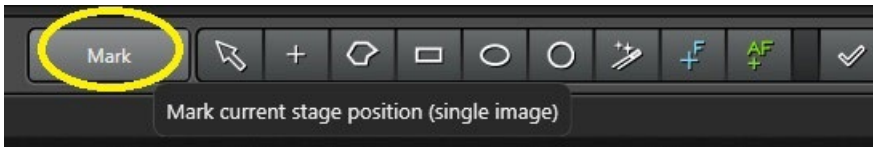
5. Once the position is marked, you can now set the Z stack for this position.

Note: if you want different stack depths for different positions, turn off Same Stack Size for all Regions.

6. In Task List, click Refine Stack to update the Z stacking settings. Now the position icon is changed from XY to XYZ.

Note: once the above position and its stack are done, it is suggested to cancel the stacking settings so next position will be listed in the Task List window as a 2D position (this way is easier for you to know what position is what).
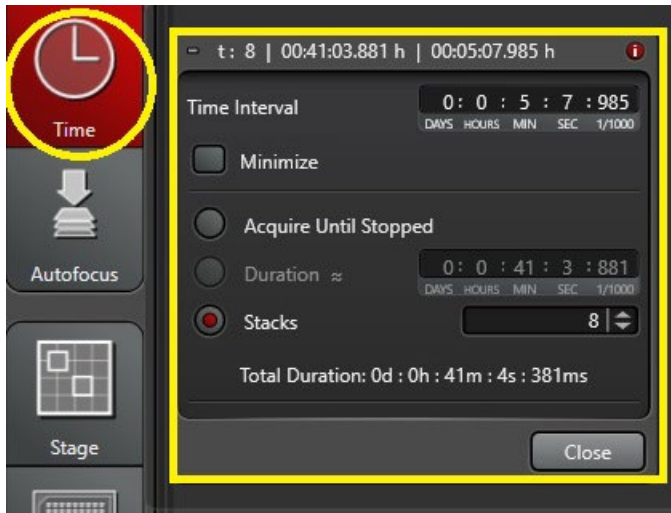

7. Repeat the above steps 2-6 to mark more positions (X/Y/Z). In this case, there are total 2x positions are marked and you can find the details of each position by right click.
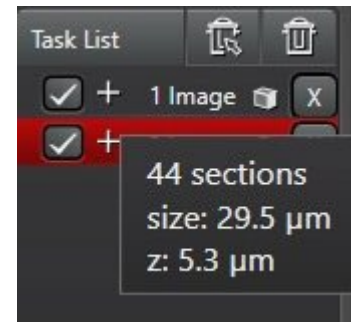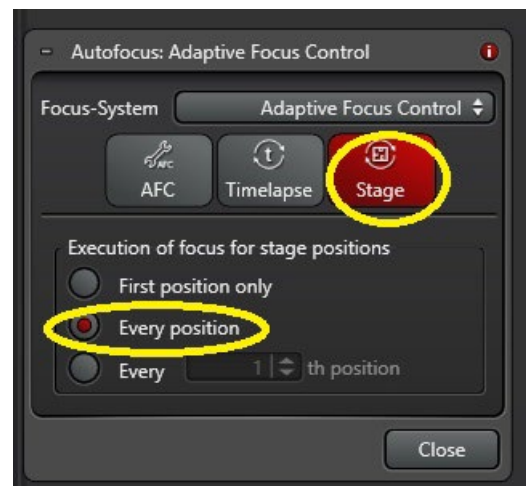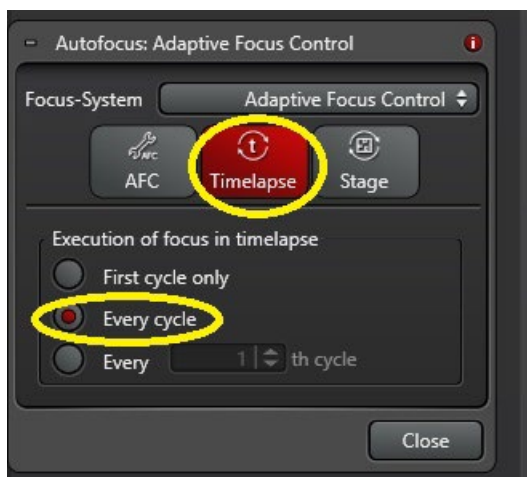

8. Before initialise imaging process, ensure the resolutions of XY and Z are good.
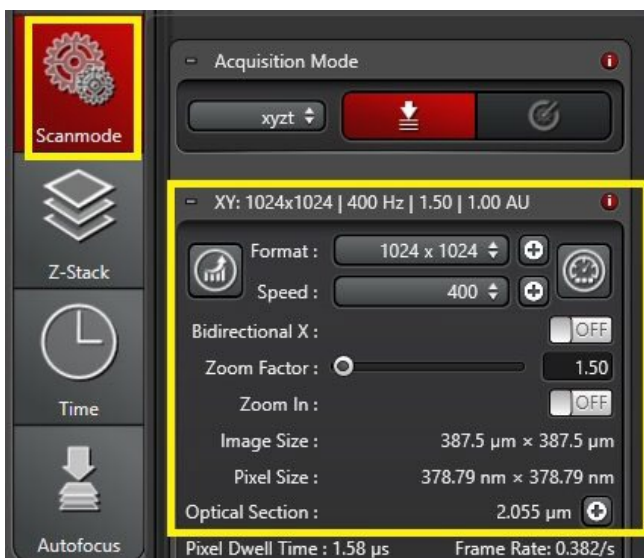
9. Set up the time course.

10. Go to Autofocus, double check AFC settings. In addition, decide how you want AFC to be applied for Time Course and Stage.

11. Finally, click Start to initialise imaging for your XYZT experiment. The imaging progress will be shown at the bottom of the screen.

Note: the top red line shows the progress of the time course. The bottom red line shows the progress of the total XYZ experiment.