

Leica Microsystems' Adaptive Focus Control (AFC)

Be in Focus – Always (Part I)

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.

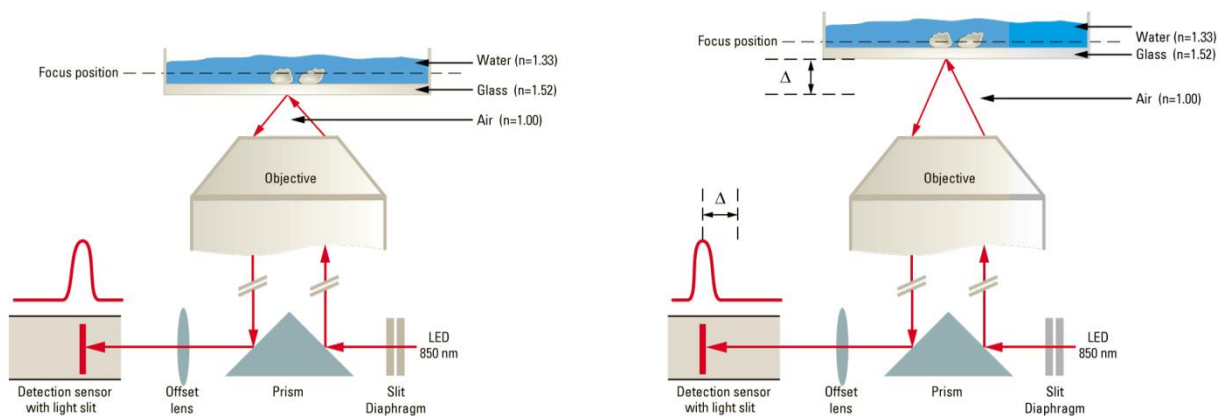


Fig.1: Schematic representation of the working AFC after an induced drift of the sample. After the sample is drifted by Δ the reflection hits the AFC detection sensor at a different position.

Immersion and embedding media – What matters?

The functionality of the AFC mechanism is highly dependent on the reflection measured by the AFC-detection sensor. Reflections occur at every point in the light path where two media with different refractive indices (RI) are in contact. The sensor detects the brightest reflection and regulates the objective focus according to the position of the brightest reflection.

Because of this, it is important to think about the types of media that are in the light path and how they will affect the AFC system.

Air-Immersion:

Dry objectives are the “easiest” case to use with the AFC. With these objectives the AFC detects the first reflection between the air (RI = 1.0) and the glass coverslip (RI = 1.52) itself. This is where the biggest difference between refractive indices occurs (see Fig. 2)

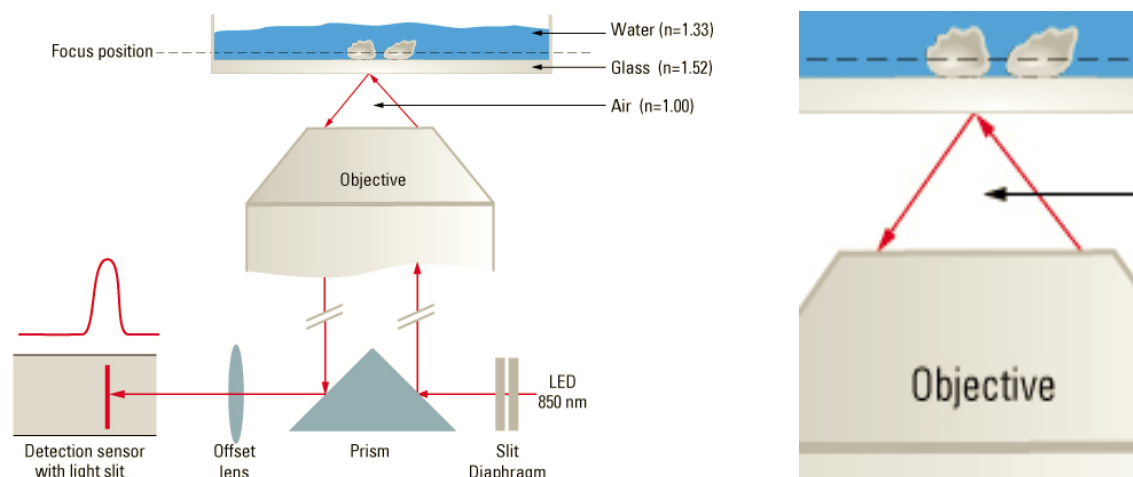


Fig.2: Schematic view of a system used with a dry objective with AFC. With a dry objective the main reflection happens at the air-glass surface.

The AFC works with air immersion objectives independently from the type of sample and embedding medium used. A great advantage when using air immersion objectives is that even plastic dishes can be used with the AFC system. To achieve the best imaging with plastic dishes, it is recommended to use long working distance objectives with a correction collar to correct for the thickness of the plastic bottom. The following objectives have been

From Eye to Insight

tested and work with plastic dishes (and also work with standard 170µm thick glass coverslips / dishes):

11 506 505	HC PL FLUOTAR 10x/0,30
11 506 507	HC PL FLUOTAR 10x/0,30 PH1
11 506 285	HC PL APO 10x/0.40 CS
11 506 284	HCX PL APO 10x/0,40
11 506 286	HCX PL APO 10x/0,40 PH1
11 506 200	N PLAN L 20x/0,40 CORR
11 506 200	N PLAN L 20x/0,40 CORR PH1
11 506 242	HCX PL FLUOTAR L 20x/0,40 CORR
11 506 243	HCX PL FLUOTAR L 20x/0,40 CORR PH1
11 506 297	N PLAN L 40x/0,55 CORR (correct setting of correction collar needed)
11 506 201	HCX PL FLUOTAR L 40x/0,60 CORR (correct setting of correction collar needed)

Oil-Immersion:

Oil objectives need immersion oil between the front lens and the glass bottom dishes. With these objectives, the brightest reflection doesn't occur at the interface between the immersion oil and the glass surface. These materials don't have any difference in refractive indices and therefore don't create a reflection. But nevertheless the AFC can still be used with oil immersion objectives. Here the AFC detects the reflection which occurs at the transition from glass to the aqueous cell culture medium. At this transition, the difference in RI leads to light reflection needed for the AFC (glass: RI = 1.52; water: RI = 1.33) (see Fig.3).

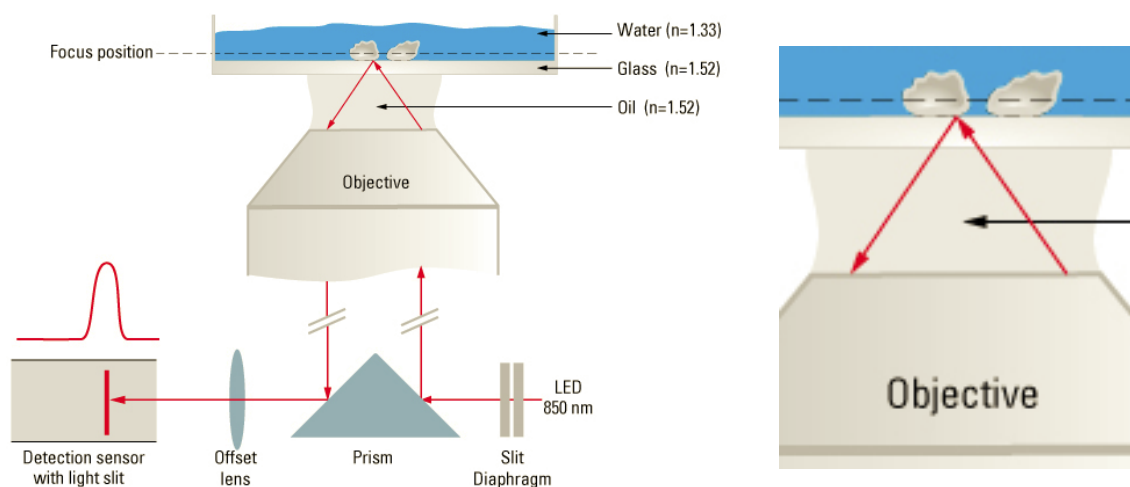


Fig.3: Schematic view on the AFC reflection with an oil immersion objective. With an oil immersion objective the main reflection happens at the glass-water surface.

Water-Immersion:

For live-cell imaging experiments over a long time course, using water immersion objectives delivers the best results in terms of image quality. Using water as an immersion medium most closely matches the RI of the sample, resulting in fewer optical aberrations in the optical system. The AFC can be used with these objectives, but the system operates slightly differently. When using standard living cells in a culture dish with a glass bottom, a special situation occurs in which two main reflections are created instead of one. The first reflection happens at the interface between the immersion water and the glass bottom, and the second one happens at the interface between the glass bottom and the aqueous solution of the culture medium (see Fig.4).

However, this problem actually does not cause an issue with the AFC detection algorithm because it just needs one of the two reflections to keep the focus stable. In 90% of the cases, the first reflection (immersion-water \rightarrow glass) is stronger, caused by the slightly higher difference in RI between distilled water (1.33) and glass (1.52) compared to glass (1.52) and culture medium (1.34- 1.38).

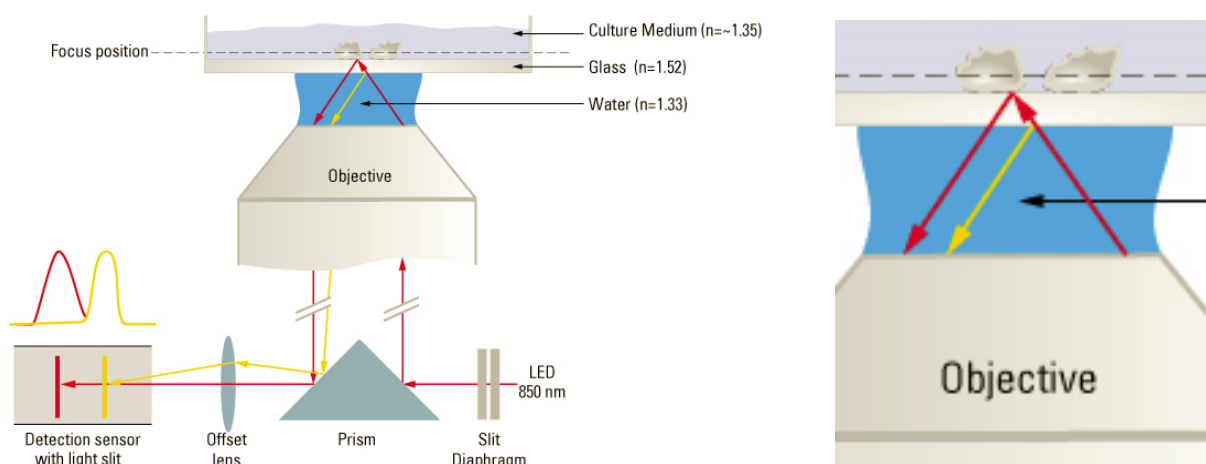


Fig.4: Schematic view on a system used with a water immersion objective and AFC. With a water immersion objective, two reflections hit the AFC detection sensor. Note that the main reflection is the first reflection at the water immersion – glass interface.

Software Control – AFC during experiments

Leica Microsystems' AFC is fully integrated into the Leica Application Suite X (LAS X) software. This easy to use software platform for advanced life science research is available for widefield and confocal imaging systems.

The AFC can be used for a number of different experimental setups, from single image acquisition up to complex multi-dimensional experiments. The user can select between continuous mode and on-demand mode (see Fig.5).

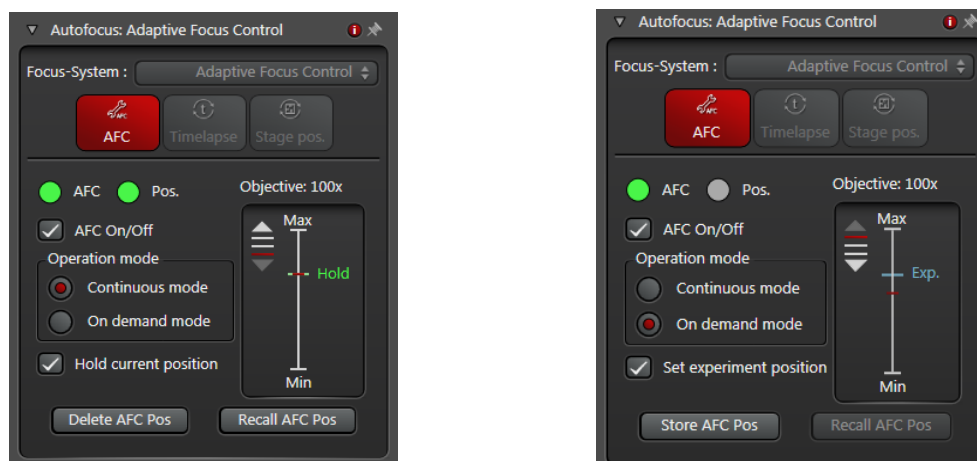
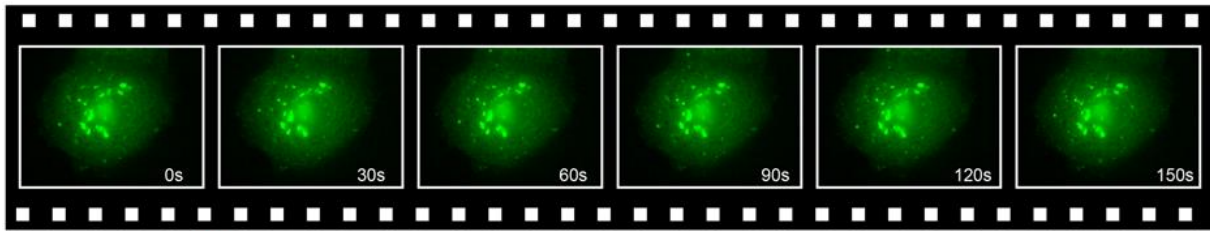


Fig.5: Selection of AFC Continuous Mode (left) or On-Demand Mode (right) in LAS X.

Continuous mode is a mode where the AFC always actively keeps the focus of the sample stable. This mode is helpful for users screening through their samples via the eyepieces using either a manual or motorized stage. Even when the sample holder or the sample itself is tilted, the sample will stay in focus. Another example is experiments where solutions are added to the culture medium during a time-lapse series. The AFC automatically corrects for any induced focus drift caused by the increased weight of the sample after adding the solution (see Fig. 6).

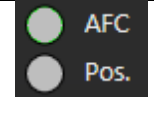
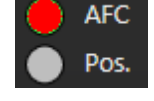
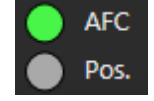












Adding Cold Medium / Incubator open

Fig.6. Single channel fluorescence timelapse experiment with AFC set to continuous mode after adding additional medium with a lower temperature to the cells. After adding the medium no drift was visible.

On-Demand mode is used mainly when the AFC is combined with other image-based autofocus systems. In such an experimental system, the AFC first sets the focal distance based on the last position that was marked. In addition, an image based autofocus is performed to check whether the sample itself has been moved. If a new focus position is found, then the AFC is reset to this position. At the next timepoint, the AFC checks again the actual focus position (according to the newly found one) and adjusts the distance accordingly. The main benefit of using the On-Demand mode is that the images captured can be set to a smaller range in the z direction than that needed for an image based autofocus system alone. In this case, the AFC corrects for any drift of the sample holder or the culture dish, whereas an image based autofocus corrects for any focus differences which occur in the sample (the cells) itself. This minimizes the overall time for refocussing and therefore also the amount of light used to refocus the sample.

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.

	Objective cannot be used with AFC or AFC switched off
	Objective can be used. Red= no reflection on the AFC sensor
	Objective can be used. Green= reflection detected on the AFC sensor AFC holding mechanism on stand-by

 AFC  Pos.	<p>Objective can be used. Green= reflection detected on the AFC sensor</p> <p>AFC holding mechanism busy</p>
 AFC  Pos.	<p>Objective can be used. Green= reflection detected on the AFC sensor</p> <p>AFC found focus (on-demand mode) / continuously holding focus (continuous mode)</p>
 AFC  Pos.	<p>WATER-Objective can be used. Yellow= reflection detected on the AFC sensor</p> <p>AFC holding mechanism on stand-by</p>
 AFC  Pos.	<p>WATER-Objective can be used. Yellow= reflection detected on the AFC sensor</p> <p>AFC holding mechanism busy</p>
 AFC  Pos.	<p>WATER-Objective can be used. Yellow= reflection detected on the AFC sensor</p> <p>AFC found focus (on-demand mode) / continuously holding focus (continuous mode)</p>

Highlights

- AFC is a focus stabilizing device which works with many types of objectives for a wide range of applications.
- Typical applications for the AFC are live-cell-imaging, screening of well-plates, and multi-position experiments.
- The AFC currently works with 87 objectives (dry, oil, water, glycerol) with magnifications from 10x to 100x.
- AFC works with plastic dishes and plastic multi-well plates (long working distance objectives with correction collar recommended).
- AFC is fully integrated into LAS X software and can be combined with image based autofocus systems. The AFC helps to reduce the search range of an image-based autofocus and thereby minimize light stress on the sample during experiments.
- Continuous mode: continuously holds the focus stable during time-lapse experiments and during x-y-movements of the stage when searching for the optimal position of your sample.
- On-Demand mode: designed for combining AFC with z-stacks, multi-position experiments, and image based autofocus systems.