Biooptics Newsletter 06/2022

Biooptics Core Facility-in general

The Biooptics Core Facility of the MUI exists since early 2009 and is located in the CCB, Innrain 80-82, first floor, rooms M01.370 and M01.381. We are currently hosting five microscopes in-house, a neuron-tracing instrument in cooperation with the Institute of Pharmacology at Peter-Mayr-Strasse 1a and a 2-photon microscope in cooperation with the Institute of Physiology, Schöpfstrasse 41. You can find more information on the official website.

SP8: Strategies to remove autofluorescence

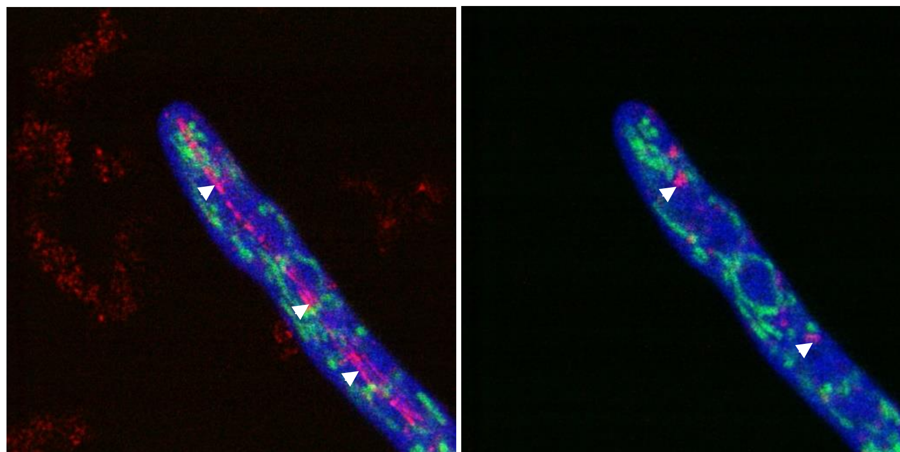
Autofluorescence in the true sense of the word is the - often unwanted - fluorescence of any endogenous biological compound present in a given sample. This will result in fluorescence emission upon excitation with one or several wavelength due to the absorption and emission properties of this specific biological compound. In typical systems there will be more than one fluorescent biological compound. Autofluorescence is not (!) the same as unspecific antibody binding or cross-labelling by insufficient species specificity of secondary antibodies. Although this will also result in non-specific fluorescence and actively contribute to the background, this background fluorescence originates directly from fluorophores used for labelling and not from the sample itself.

Second, also the kinetics of the fluorescence decay i.e. the fluorescence lifetime (τ) will be different between fluorophores used for labelling and autofluorescence. On our SP8, both spectral and fluorescence decay kinetics can be used as strategies to identify and remove autofluorescence.

The pulsed and tunable WLL Laser (WLL, 470 nm – 670 nm), enables very specific excitation of any fluorophore exactly at its excitation maximum (and not only close to it). It is very noteworthy that fluorophore names do not necessarily indicate the optimal excitation wavelength; e.g. Alexa Fluor 488 has its best excitation at 498 nm not at 488 nm. The same is true for many more fluorophores. Autofluorescence will have different spectral properties than the one of specific fluorophores used for labelling. By choosing the optimal wavelength we are able to generate a much better specific signal over the autofluorescent background.

In addition, a "gating" strategy on the HyDs can be used against autofluorescence. Generally, all fluorophores do have a fluorescence lifetime (τ) and thus a specific fluorescence decay kinetics. Mathematically, fluorescence decay kinetics is very similar to the radioactive decay, except that fluorescence decay is reversible. The fluorescence lifetime (τ) is the exact analogon of the radio-active half-life. Typically values of τ are in the range of 2 - 5 ns for many well-known fluorophores (eg. Alexa 488, 4.1 ns). Autofluorescence will also have a certain fluorescence lifetime, which is typically lower than fluorescence lifetime of our typically used fluorophores. "Gating" on the HyDs i.e. the selection of only long-lifetime fluorescence thus enables to largely remove any unwanted background from autofluorescence.

Example: filamentous fungi expressing 3 different FPs in organelles (blue, cytosol, green, mitochondria, red, peroxisomes). **Left (Gate OFF)**: **filamentous artefact** (triangles, very strong autofluorescence) in the red channel (LEFT), peroxisomes not visible. **Right (gate ON):** filamentous artefact completely removed, **specific signal** (triangles) in peroxisomes.



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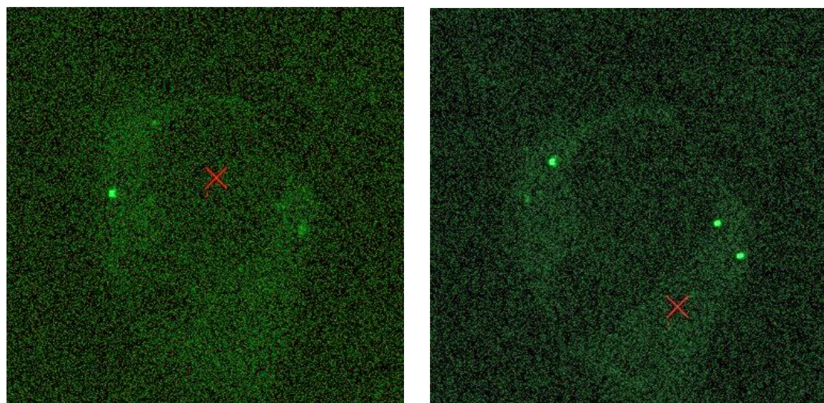
LSM980: Fluorescence Correlation Spectroscopy (FCS)

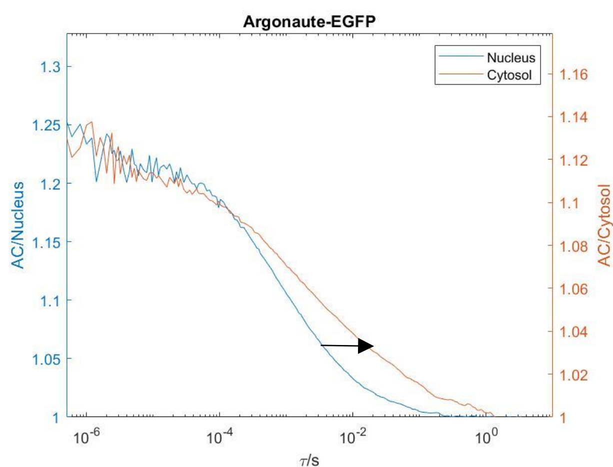
On the LSM980 we have access to is an interesting, quantitative imaging technique – **fluorescence correlation spectroscopy (FCS)** - allowing the measurement at very low FP- expression levels (nM range) in order to study absolute concentrations of proteins and/or binding and/or protein mobility within living cells. The actual FCS measurement (point measurement) is typically preceded by a confocal image to define the point of FCS measurement. The measurement itself is a simple recording of the fluorescent signal over time ("FCS-trace", count rate) typically 5 to 30 sec per trace. Due to molecular movement of fluorophores through the confocal volume, the observed count rate will vary around an average over time. This variation will be the faster, the faster the fluorophores cross the confocal volume. A statistical analysis of these traces involves an incremental time shift over an increment τ of the trace and comparing the shifted to the original trace ("Autocorrelation"). Depending on the analysis performed, we can deduce the absolute concentration of the fluorophore (mol/l), determine the speed of movement (diffusion time τ_D), get information on association states and more.

Example: Argonaute2-EGFP (part of the RISC complex) nucleus and cytosol, X indicates FCS measurement position. Very low expressing cell.

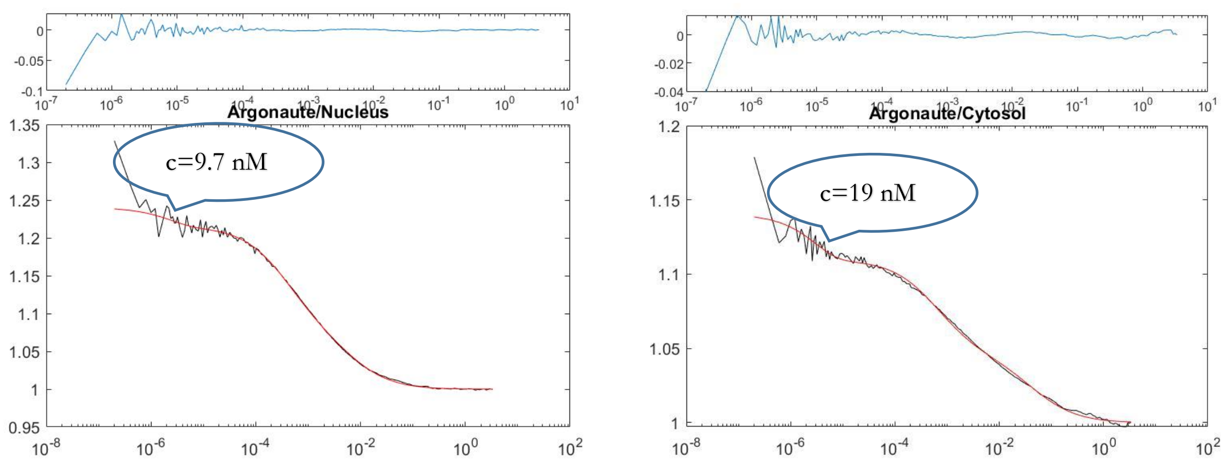


1) **Qualitative** comparison of autocorrelation (AC) in the nucleus and the cytosol.

- Argonaute is slower in the cytosol than in the nucleus (larger complexes in cytosol?); curve in cytosol is shifted to longer times (arrow).



2) **Quantitative** analysis, calibration, curve fitting:



Nucleus:

- 9.7 nM Argonaute in nucleus
- 65% (=6.3nM) pass through the confocal volume within 400 µs (fast population)
- 35% (=3.4 nM): 6.42 ms (slow population)

Cytosol:

- 19 nM Argonaute in cytosol
- 58% (=11.02 nM) diffuse within 654 µs through the confocal volume (fast)
- 42% (=7.98 nM): 44.2 ms (slow)

Small differences in molecular weight such as dimerization of two similarly sized proteins is not directly accessible via FCS i.e. for FCS the difference needs to be huge (receptor-ligand, large complexes). It is possible to address these questions using a second color, resulting in Fluorescence Cross Correlation Spectroscopy (FCCS) - we also have access to FCCS for a limited evaluation period from Zeiss.

FCS is generally available for all interested people- please request a **specific FCS introduction on the LSM980.**

Outlook:

We will receive a **major upgrade on the SP8** this summer, which will substantially increase the sensitivity. The upgrade will be available for all imaging methods (including STED) using the standard scanner (not the resonance scanner) and will result in a 2.5 fold improvement of the sensitivity. It is expected to arrive at end of July 2022.

Contact and further information

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