



Polarization Extension Unit for LSM Upgrade Kits

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Introduction

Since several years, time-resolved fluorescence measurements can be performed with commercially available Laser Scanning Microscopes (LSM) that have been upgraded with a suited LSM upgrade kit. Anisotropy measurements are, however, not possible with such systems, because the fluorescence emission is usually guided to the detectors using a single multimode optical fiber which does not preserve the polarization information.

Anisotropy is generally used as a measure for the polarization behavior of a sample. The polarization of the emission light depends on the fluorophore orientation, the angle between absorption and emission transition dipole moment as well as depolarizing processes such as rotation or energy transfer. Anisotropy can be used to study, e.g. viscosity and permeability of membranes, molecular aggregation, protein/protein and protein/DNA interaction, molecular orientation, protein folding or molecular crystallization processes. The measurement of fluorescence anisotropy can thus be used for many research areas in chemistry, biology and medicine and it is therefore desirable to have the possibility to perform anisotropy measurements also with upgraded LSMs.

This technical note describes an extension to the LSM upgrade kit that accesses the polarization information. The extension is based on a polarization sensitive beam splitter that is mounted directly at the

LSM exit port. In that way, the fluorescence emission is separated by its polarization before it is guided to the two detectors via two multimode fibers.

Theory of fluorescence anisotropy

The use of linearly polarized light for fluorescence excitation will excite mostly molecules whose transition dipole moment is parallel to the polarized light. During the time between fluorophore excitation and light emission, which takes place in a range of picoseconds to nanoseconds, depolarization of the emission light can occur due to rotation, torsional

Main uses of fluorescence anisotropy

molecular orientations

- membrane structure
- material structure (crystallization, polymers)

molecular rotations

- size determination
- binding processes
- protein folding

energy transfer

- oligomerization
- aggregation

vibrations, Brownian motion, and radiative and non-radiative transfer of excitation energy to an other fluorophore with a different orientation. To analyze the polarization change, the emitted light needs to be separated and directed onto two detectors that measure independently the intensity of the two orthogonal polarization components of the emitted fluorescence (parallel and perpendicular to the plane of excitation polarization). With these two measurements, the fluorescence anisotropy, r , can be calculated from

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2 \cdot I_{\perp}} \quad (1)$$

(I_{\parallel} = Intensity (parallel to the polarization plane)); I_{\perp} = Intensity (perpendicular to the polarization plane) For immobilized single molecules and single photon excitation, the anisotropy value can range from -0.5 to 1, depending on the orientation of the molecule and the angle between the excitation and the emission dipole moments [1]. An ensemble of molecules which are randomly oriented show an anisotropy between -0.2 and 0.4, depending on the angle between the absorption transition dipole moment and the emission transition dipole moment, being 0.4 if both transition dipole moments are parallel.

Fluorescence anisotropy measurements contain plenty of information about the intrinsic properties of a molecule as well as of the environment in which it resides. It can be used to analyze the size and form of molecules, the stiffness of internal structure, and also the viscosity of its environment. The combination of these effects are described by the Perrin equation:

$$\frac{r_0}{r} = 1 + \frac{\tau}{\theta} = 1 + 6 D \tau \quad (2)$$

Where r_0 is the fundamental fluorescence anisotropy, r is the measured steady state anisotropy, τ is the fluorescence lifetime, θ is the rotational correlation time, and D the rotational diffusion coefficient.

θ is a parameter arising from rotational Brownian motion and describes how fast a molecule can rotate in solution. The correlation time is shorter for molecules that can freely rotate and longer for those that are larger or have restricted motion. The anisotropy can be measured on a pixel-by-pixel basis in confocal images. Therefore, one can determine the rotational correlation time, and hence, obtain information on the molecular parameters and environment. The rotational correlation time can be calculated by

$$\theta = \frac{\eta V}{RT} = \frac{1}{6D} \quad (3)$$

and depends on the viscosity (η) of the environment, the volume (V) of the molecule (incl. hydration shell) and the temperature (T). R is the gas constant.

For example, the rotational correlation time for proteins can be estimated taking into account the molecular weight of the protein (M), the protein volume (v_{spec}), which is typically 0.73ml/g, and the hydration volume (h).

$$\theta = \frac{\eta V}{RT} = \frac{\eta M}{RT} (v_{spec} + h) \quad (4)$$

Note that the rotational diffusion time scales with the volume, while translational diffusion times scale with the radius. As a consequence, rotation is much more sensitive to size changes.

Even molecules with moderate weight show a rotational diffusion time which is significantly longer than the usual fluorescence lifetime of common dyes. For measuring these long rotation times, it is important that the label is rigidly attached via a rigid linker as otherwise mainly the dynamics of the label on its linker will be observed.

Slider design

The polarization extension unit is comprised of two special parts: a polarization beam splitter fixed on a slider and directly mounted on the exit port of the LSM and a dual channel detector unit with SPAD detectors, specially modified for the integration of two fiber inputs (see Fig. 1). The slider with polarization beam splitter has two positions that permit to manually insert

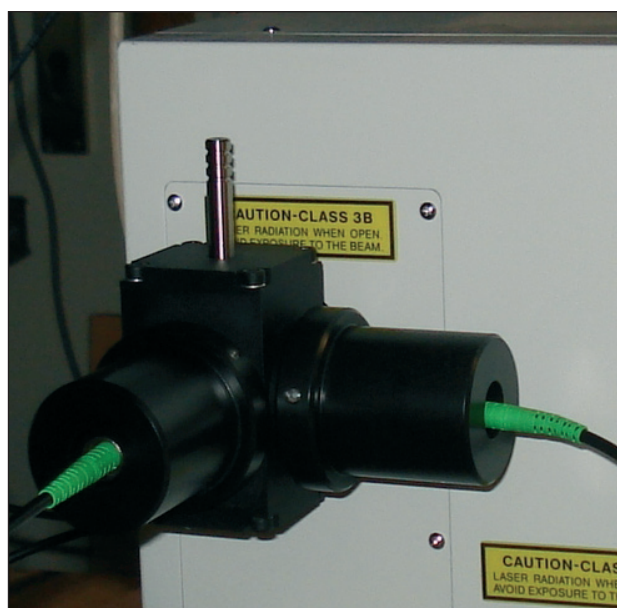


Fig. 1: Polarization extension mounted at the exit port of a Olympus Fluoview FV1000 scan head.

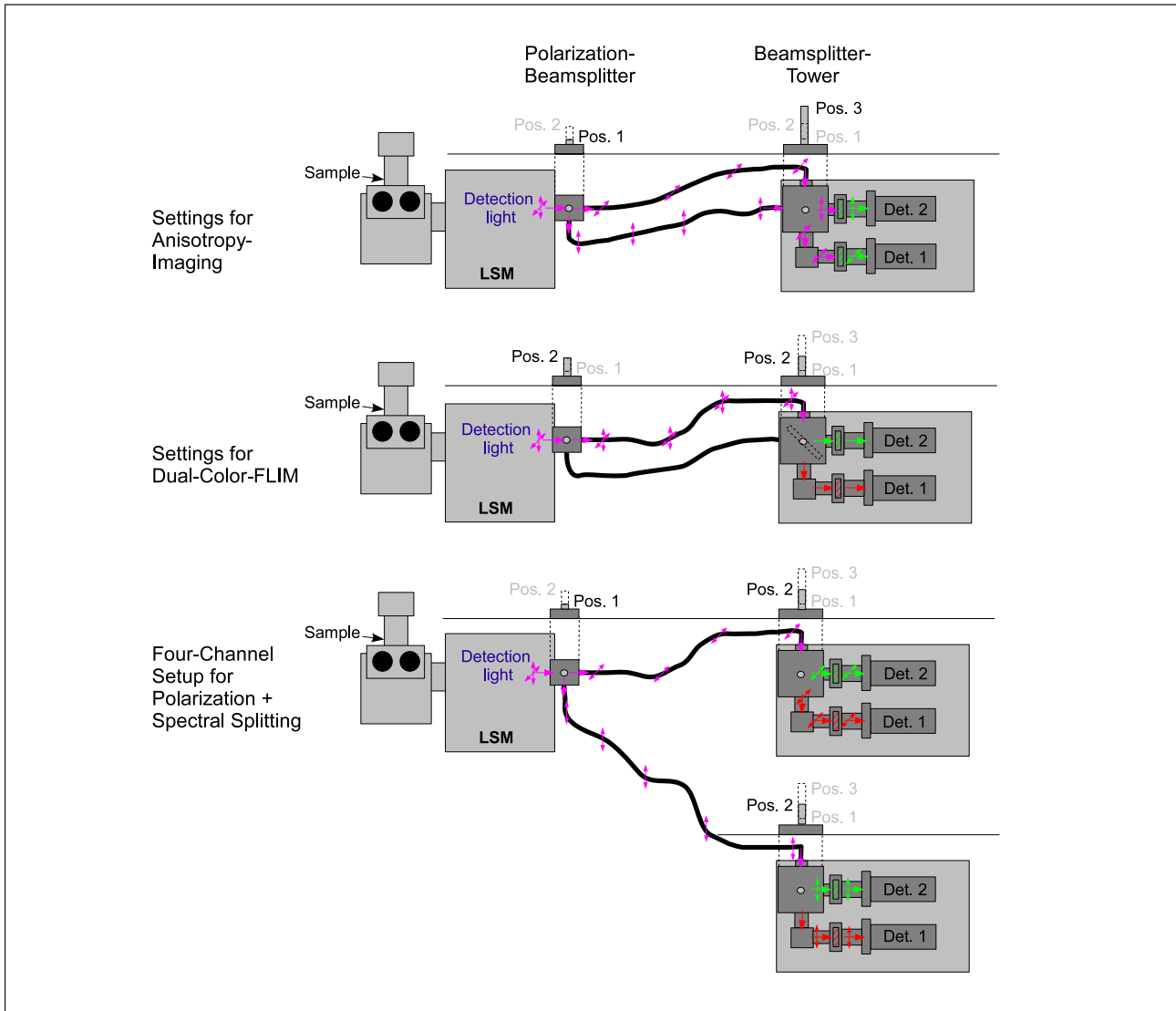


Fig. 2: Switching between anisotropy measurements and multicolor FLIM-measurements.

or remove the beam splitter from the beam pathway (see Fig. 2). If the polarization beam splitter is inserted, the light is separated based on its polarization, and guided via two multimode fibers onto detector 1 (horizontally polarized light) and detector 2 (vertically polarized light). For these measurements it is important to remove the DIC-prism from the beam path. If the slider is in its second position, i.e., if the polarization beam splitter is removed from the beam path, all the emission light is coupled into one fiber. In this configuration the polarization information is lost and the fluorescence can be separated based on its spectral properties. Alternatively, four detectors can be used to simultaneously split the fluorescence into its polarization followed by splitting into spectral components using two dual channel detector units. The polarization beam splitter is suited for the wavelength range between 450 nm and 700 nm. It can be adapted for a wide range of commercial LSMs such as the Nikon A1, the Olympus FluoView FV300 and FluoView FV1000 as well as the Zeiss LSM 510, LSM 710 and LSM 780. For Leica SP5 microscopes,

polarization measurements are realized with a similar solution directly available from Leica. Analyses of anisotropy measurement are possible by a dedicated SymPhoTime software script that is freely available from PicoQuant.

Determination of the G-Factor

In general, the detection efficiency of parallel and perpendicular polarized light is different due to beam splitting elements and variations of the photon detection efficiency of individual detector units. The measure of this difference is the so-called G-factor, that can be defined as:

$$G = \frac{S_V}{S_H} \quad (5)$$

where S_V and S_H are detection sensitivities for parallel and perpendicular polarized emission components, respectively. The G-factor is usually wavelength de-

pendent and must be taken into account when the anisotropy is calculated. Equation (1) is modified to:

$$r = \frac{I_{\parallel} - G \cdot I_{\perp}}{I_{\parallel} + 2 \cdot G \cdot I_{\perp}} \quad (6)$$

In an L-geometry spectrometer setup, such as the FluoTime 300 from PicoQuant [2], it is possible to define two principally different polarization planes: vertical and horizontal (V- and H- hereafter) relative to the plane defined by excitation and detection. In this case the G-factor can be easily determined using H-polarized excitation of a fluorescent solution. H-polarized excitation is isotropic when observed from the side, that is in L-geometry. The G-factor then equals to the ratio of the detected intensities of V- and H-polarized emission components, upon H-polarized excitation.

This procedure is not applicable in a microscope setup due to the completely different excitation/detection geometry. Using linearly polarized excitation light, isotropic excitation cannot be achieved in co-linear geometry (epifluorescence configuration). However, it is feasible to get completely depolarized emission instead. Consider a sample with steady state anisotropy close to zero. Equation (6) transforms into

$$0 = \frac{I_{\parallel} - G \cdot I_{\perp}}{I_{\parallel} + 2 \cdot G \cdot I_{\perp}} \Rightarrow 0 = I_{\parallel} - G \cdot I_{\perp} \Rightarrow G = \frac{I_{\parallel}}{I_{\perp}} \quad (7)$$

In this case G equals the ratio I_{\parallel}/I_{\perp} (equation (7)). A fluorescent solution has a steady state anisotropy close to zero, if the rotation time θ is much faster than the fluorescence decay time τ . This follows from the Perrin-equation (equation (2)), which can be rearranged to the following form:

$$\frac{r_0}{r} = 1 + \frac{\tau}{\theta} \Rightarrow r = \frac{r_0}{1 + \frac{\tau}{\theta}} \quad (8)$$

Small organic fluorophores in water with a lifetime of several nanoseconds are well suited. The steady-state anisotropy can be estimated as follows: For common one-photon excitation and randomly oriented molecules in solution, the fundamental (initial) anisotropy r_0 is maximally ~ 0.4 . In water at room temperature the rotational diffusion time of a small organic fluorophores is maximally 0.3 ns. According to equation (8), already a fluorescence lifetime of 4 ns results in a steady state anisotropy value as low as 0.03. A fluorescence lifetime of 9 ns yields a steady state anisotropy of 0.013. In this context, the best dyes are metal complexes like e.g. ruthenium-tris (2,2'-bipyridine) with a luminescence lifetime of

hundreds of nanoseconds and very low initial (fundamental) anisotropy.

Correction for high numerical aperture objectives

Working with high numerical aperture objectives requires further correction factors to be included in equation (6). (See references 3 and 4 for details.) In this case, the anisotropy is calculated according to:

$$r = \frac{I_{\parallel} - G \cdot I_{\perp}}{(1 - 3l_2) \cdot I_{\parallel} + (2 - 3l_1) \cdot G \cdot I_{\perp}} \quad (9)$$

where l_1 and l_2 are correction factors that compensate for the influence of the high NA-lens used. As the correction factors only affect the denominator, the G-factor can still be determined as described before.

These new correction factors can be determined using common fluorescence decay measurements as described in reference 4. An alternative procedure involves only intensity measurements. At least two different samples with known, significantly different and non-zero steady state anisotropies are necessary. The experimentally determined G-factor, measured polarized intensities (I_{\parallel} and I_{\perp}) and the known steady state anisotropies (r) have to be inserted into equation (9), resulting in a set of two equations for two unknowns: l_1 and l_2 .

Measurement example

Anisotropy measurements on ordered and disordered phases of Giant Unilamellar Vesicles (GUVs)

Cells or compartments in cells are formed and surrounded by membranes, which create and maintain a specific internal environment and provide separation e.g. of different biochemical processes from each other. To maintain a specific internal environment the membrane has to be impermeable for most molecules, but allow for the regulated exchange of specific sets of molecules, as well as signal transduction. Membranes are formed by a bilayer of phospholipids, into which different molecules such as cholesterol, proteins with lipid anchors and proteins with transmembrane domains are incorporated.

A common model system to study the basics of membrane organization and dynamics are Giant Unilamellar Vesicles (GUVs), which have a diameter on the order of 1 μm to 100 μm . GUVs can incorporate fluorophores like nitrobenzoxadiazole (NBD)-conjugated lipid probes into the membrane [5,6] and

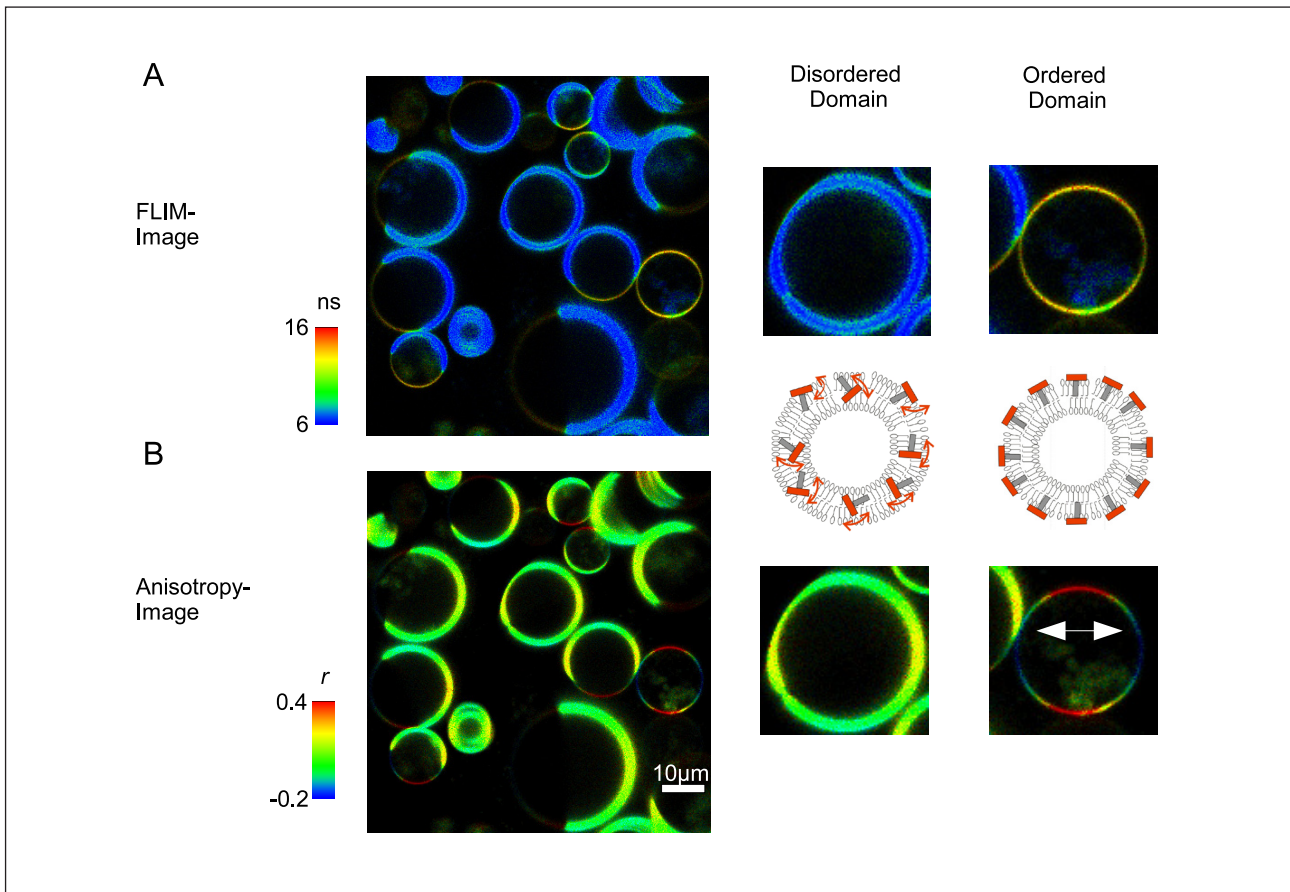


Fig. 3: Fluorescence anisotropy for measuring rotational mobility and orientation in GUVs using NBD fluorescence. The results clearly show that anisotropy allows to distinguish between liquid ordered and disordered domains of the GUV.

differences in membrane composition or viscosity can then be monitored due to differences in the fluorescent intensity, the fluorescence lifetime, or changes in anisotropy.

Depending on the composition, typical biological relevant lipids in a GUV can have spatially different supramolecular organizations (domains), which can be classified as “liquid ordered domains” and “liquid disordered domains” (see Fig. 2). For the highly fluid disordered domain, an equal anisotropy is expected at all positions within the GUV. On the other hand, NBD molecules incorporated into the ordered domain should show different anisotropy values along the phospholipid bilayer, depending on their relative orientation to the polarization plane of the excitation.

Figure 3 shows a measurement result of NBD labelled GUVs. It can clearly be seen that liquid disordered and liquid ordered domains can be clearly distinguished by their anisotropy. In the image of the GUV with liquid ordered domain it can be seen that NBD molecules at the top and the bottom of a xy-section through the equator of the GUV have an orientation which is 90° flipped compared to the NBD molecules situated in the left and right edge of the GUV. This is caused by the fact that the NBDs on top and bottom of the ordered GUV have a transi-

tion dipole moment which is parallel to the excitation light, while the NBDs on the side are perpendicular to the exciting laser polarization. In a liquid disordered environment, the orientation of dipoles is less stringent, rotational motion is much faster and therefore a much more random orientation is possible. This complements the observed difference in the fluorescence lifetime: vesicles with a liquid ordered domain have a longer lifetime than vesicles with in a liquid disordered domain. These results clearly prove that fluorescence anisotropy can very well be used to study the difference in different membrane domains.

Protein oligomerization on a plasma membrane

After infection with a virus, the cell produces viral proteins allowing assembly of new viruses. So-called enveloped viruses surrounded by a membrane, bud from cellular bilayers like for example the plasma membrane of the host cell. In order to study the behavior of a viral envelope protein in different cell compartments, a suited viral protein was fused to mYFP and CHO cells were transfected with the construct.

The viral membrane protein studied locates in the ER, Golgi and the plasma membrane. Anisotropy measurements of such cells (see Fig. 4) show a decreased anisotropy in the plasma membrane. In

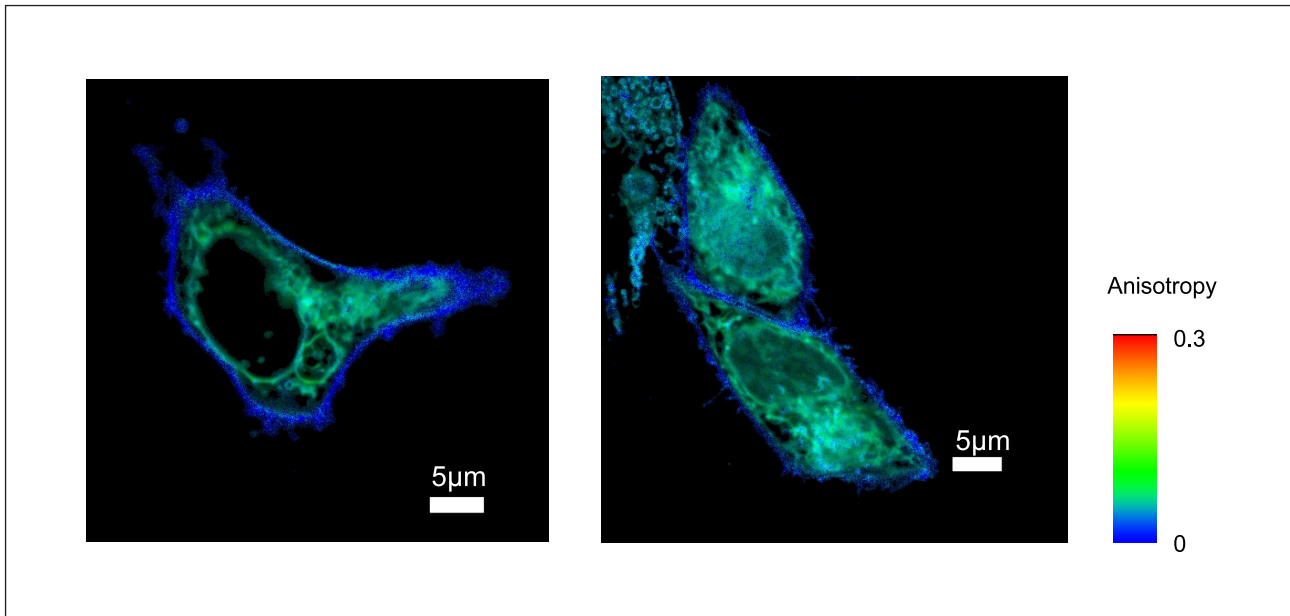


Fig. 4: Left: Live CHO (Chinese hamster overy) cells transfected with a viral membrane protein-mYFP. Right: the same cells after staining of the outer YFPs using a mouse anti GFP antibody and a secondary anti-mouse antibody stained with Alexa568. In both cases, the anisotropy in the plasma membrane is similar, but decreased compared to the protein located in the Golgi and in the ER.

order to determine whether this change is caused by a different rotational mobility of the mYFP in that membrane or by a energy transfer between various mYFP-molecules, e.g. upon dimerization or oligomerization, the outer mYFP was targeted with an antiGFP monoclonal mouse antibody and a secondary antibody labeled with Alexa568. If the anisotropy decrease is caused by a different rotational mobility, it is expected that the antibody binding causes a decrease in the rotational mobility due to the higher molecular weight of the complex. While the binding could be confirmed by imaging the fluorescence of Alexa568 (not shown), the anisotropy remained unchanged. This indicates that the decreased anisotropy is indeed caused by a dimerization or oligomerization of the viral membrane protein-mYFP in the outer cell membrane.

detection system even allows simultaneous measurement of polarization and spectral information, e.g. for multiparameter fluorescence detection on a single molecule scale or multi color fluorescence correlation spectroscopy of molecular rotations.

Conclusion

The polarization extension unit for anisotropy measurements can be mounted to most of the commercially available LSMs. A dual channel detection unit is used in combination with the polarization extension unit to switch between polarization and spectral splitting of the emission light. Such a system permits the measurement of anisotropy, fluorescence lifetime, Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Cross-Correlation Spectroscopy (FCCS). It can also be used to measure molecular rotations which are too slow to be efficiently determined with anisotropy via FCS in the nanosecond range, using the T2-mode of the PicoHarp 300 and HydraHarp 400 photon counting units. A four channel

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