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Measuring membrane fluidity in *Bacillus subtilis* using TIR-FCS

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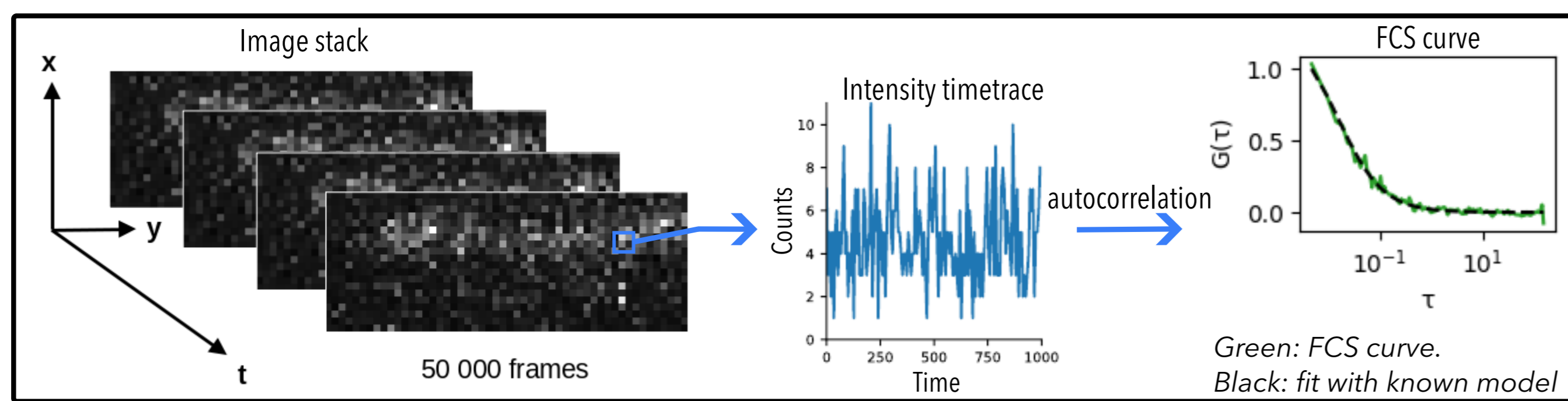
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Bacteria need to adapt their membrane fluidity in response to a wide range of stimuli, whether physical or chemical. For instance, the bacterium *Bacillus subtilis* changes the fatty acid composition of its membrane in response to a change in temperature. Changes in membrane fluidity were either estimated from biochemical methods like fatty acids analysis or indirectly assessed using environment-sensitive probes, but never directly measured. We show here that Fluorescence Correlation Spectroscopy (FCS) used on a TIRF microscope (TIR-FCS) can be used to measure the

diffusion of a well-known membrane marker, Nile Red. Because relative changes in diffusion coefficients are directly linked to a change in membrane fluidity, we can quantify changes in membrane fluidity that could so far only be qualitatively assessed. To facilitate the analysis of the large amount of data generated by TIR-FCS, we developed a simple method to automatically exclude artefactual FCS curves. As a result, we could perform high-throughput FCS measurements revealing the impact of changes in temperature and of sporulation on membrane fluidity.

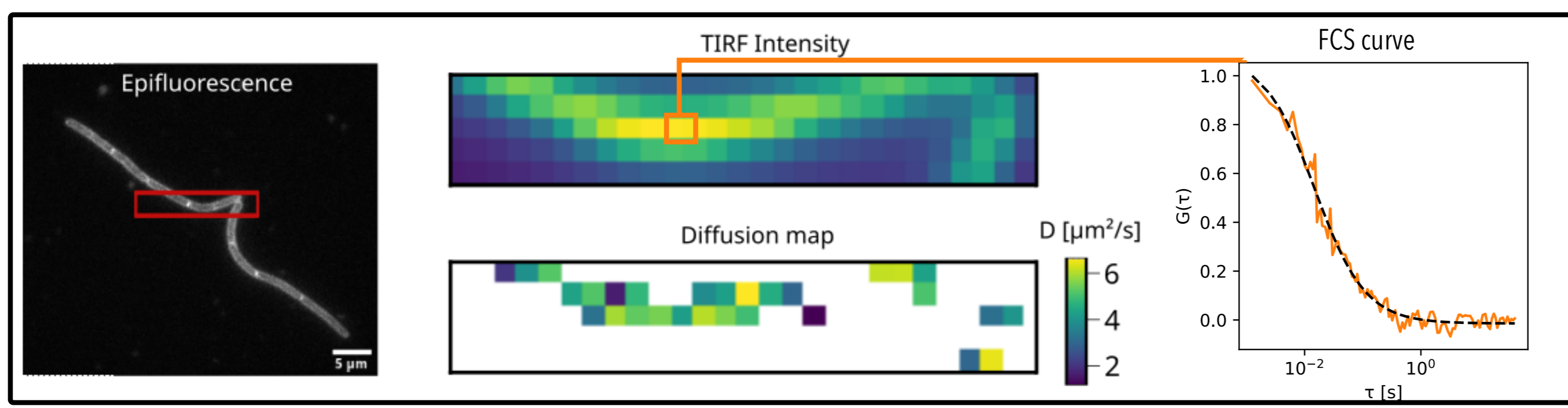
TIR-FCS: FCS on a TIRF microscope

FCS allows, among others, measurements of diffusion speeds. In FCS, fluorescent molecules diffuse in and out of an observation volume, inducing **intensity fluctuations** that can be **autocorrelated** to measure diffusion speed. FCS experiments are usually performed on a confocal microscope. They can also be done on a **TIRF** microscope, to benefit from increased **axial selectivity**, to observe **spatially-varying** diffusion coefficients and to **parallelise** FCS acquisitions.



Acquisition and processing pipeline for imFCS

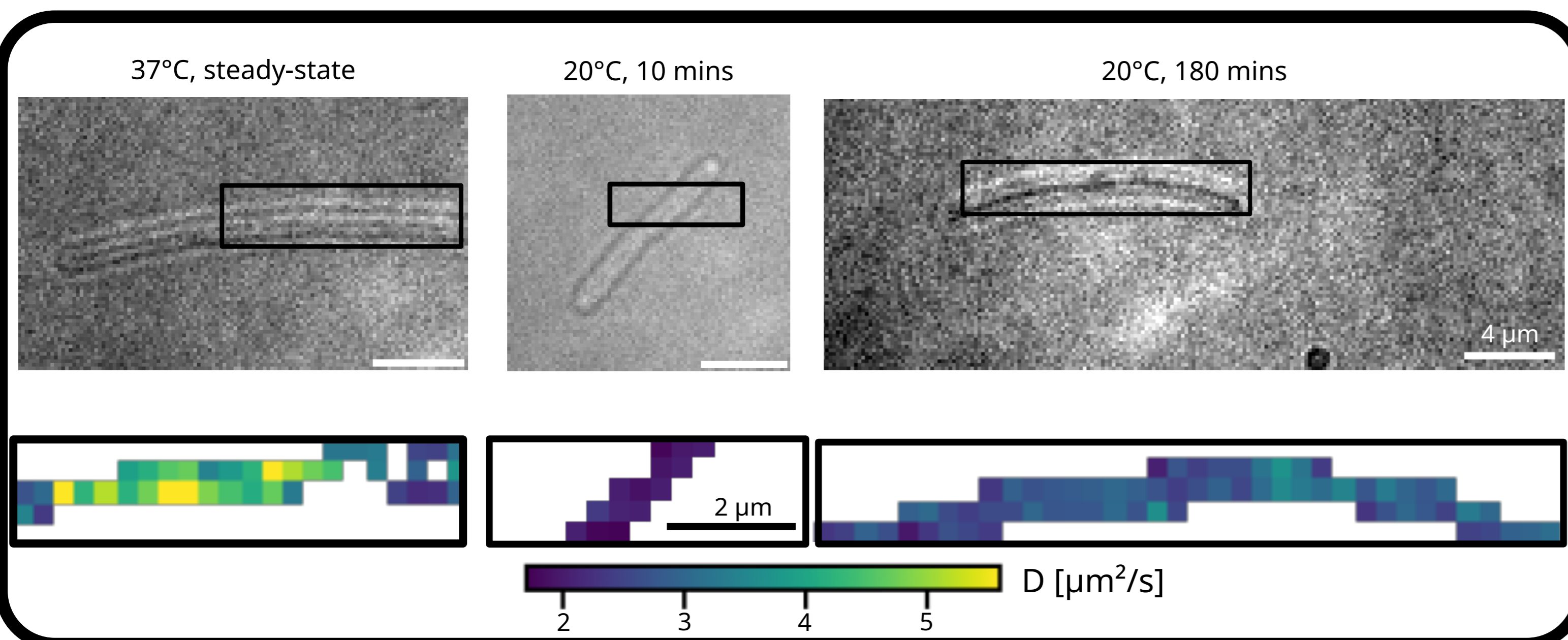
We use FCS to measure the diffusion coefficient of the fluorescent membrane marker **Nile Red** in the membrane of the rod-shaped bacterium *Bacillus subtilis*. Faster or slower diffusion speeds can be linked to higher or lower **membrane fluidity**.



Outcome of a TIR-FCS acquisition on exponentially-growing *B. Subtilis* cells, at 37°C, labeled with Nile Red

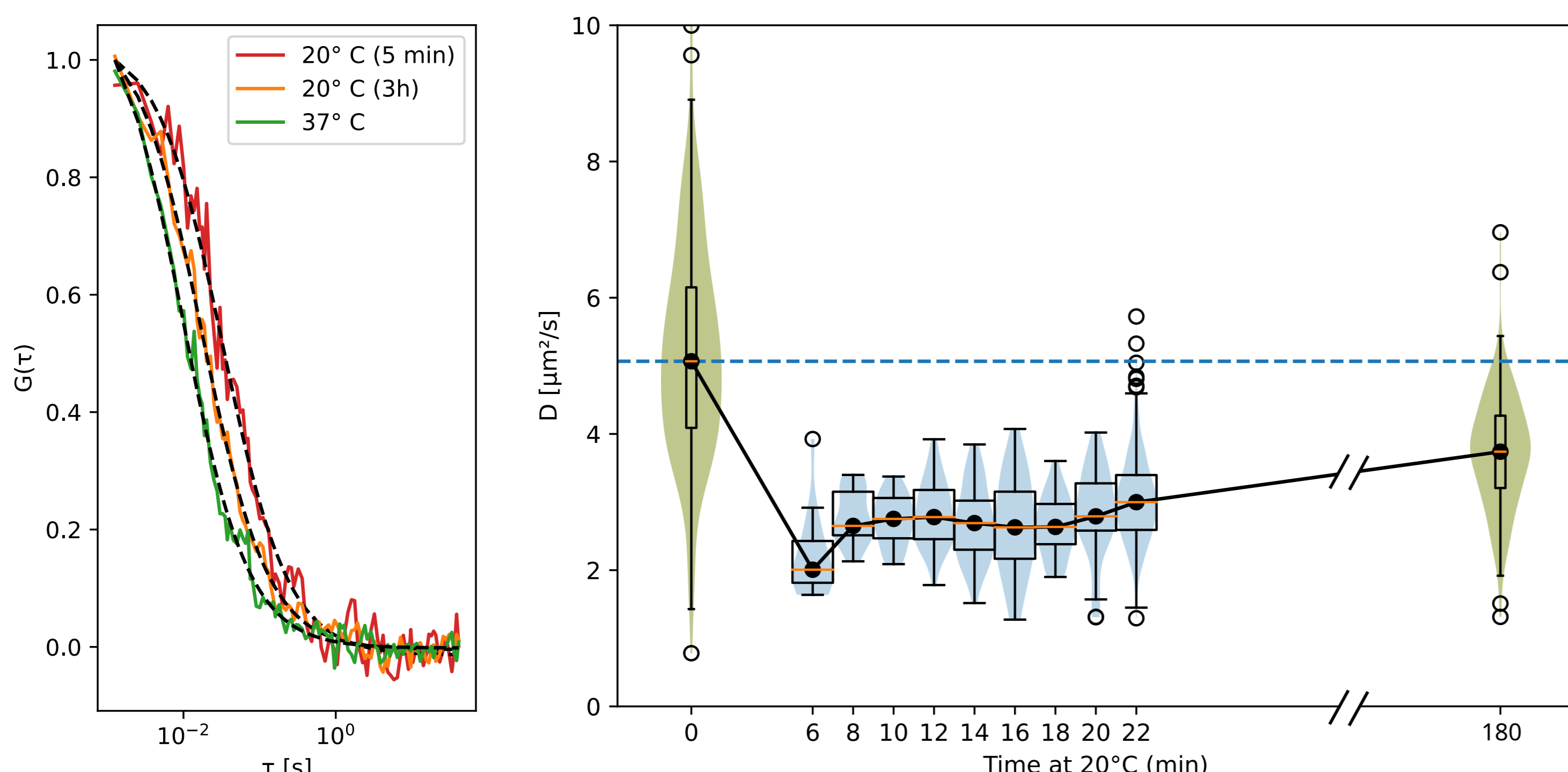
Membrane fluidity and temperature

Cold Shock: A sudden drop in temperature (cold shock) induces a decrease of membrane fluidity. To compensate for this, *B. Subtilis* changes the fatty acid composition of its membrane. We could observe the effect of this remodelling on the diffusivity of Nile Red occurring after cells grown at 37°C were transferred at 20°C. The diffusivity immediately decreases after transfer from 20°C



Bright-field image (top) and diffusion maps (bottom) of Nile Red in *B. subtilis* at 37°C (left), immediately after cold shock (middle) and 3h after cold shock (right)

Incomplete recovery of membrane fluidity: at t=0 and T=37°C, diffusivity of Nile Red is maximum (5 ±1 μm²/s) in the membrane of *B. Subtilis*. Immediately after cold shock, membrane diffusivity is strongly reduced and starts to recover. After 3h at 20°C, diffusivity of Nile Red has increased (3.7 ±0.5 μm²/s) but is still lower than at 37°C.

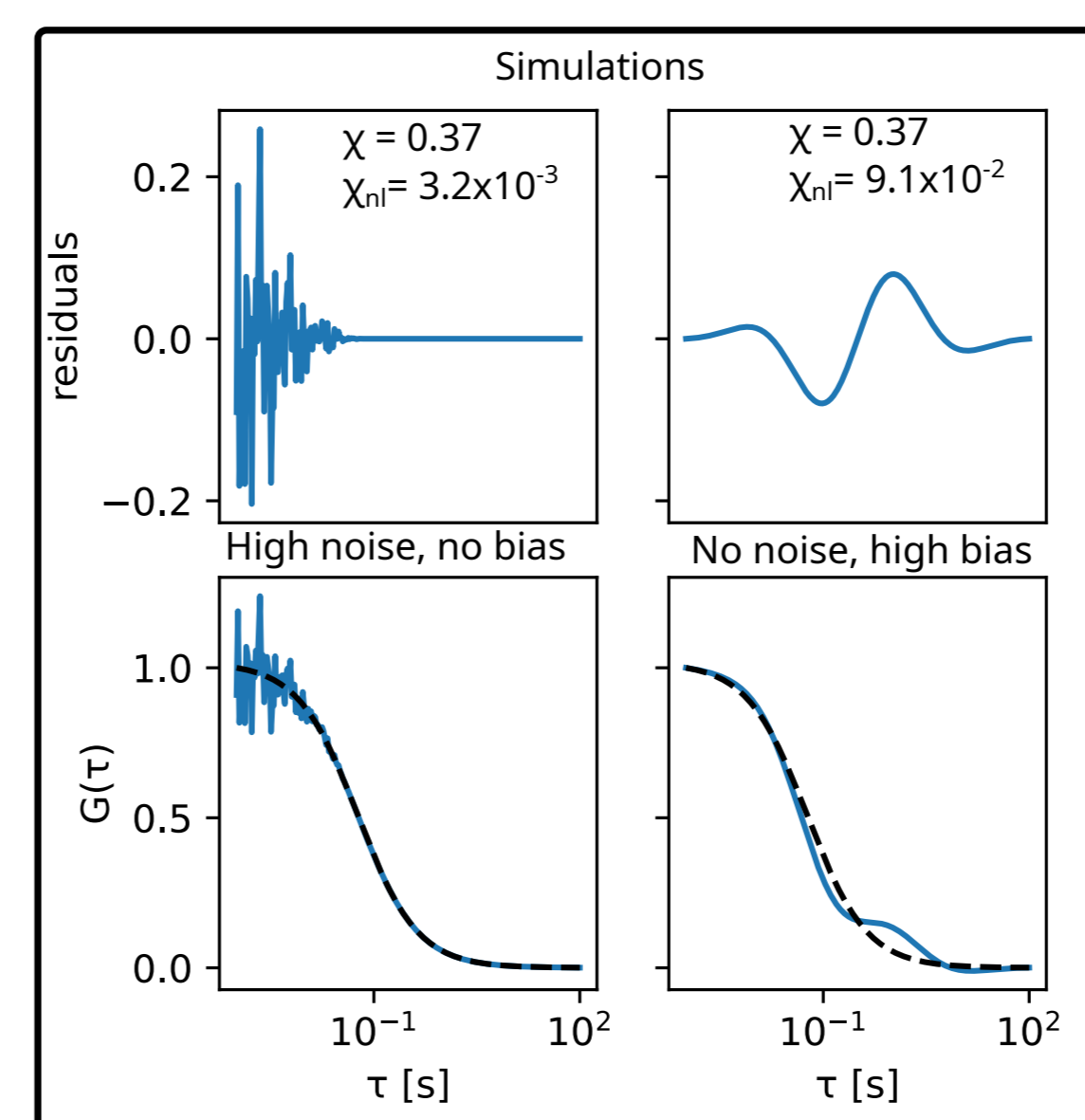


Representative FCS curves acquired in vegetative cells, at 37°C and after 5 mins or 3h at 20°C

Recovery of membrane fluidity after a cold shock in *B. subtilis*. Merging of 3 different experiments: FCS performed at 37°C, 6-30 mins after cold shock or 180 mins after cold shock.

Automated curves sorting

TIR-FCS can generate **hundreds to thousands of FCS curves** per acquisition. As in any FCS experiments, these curves can be subject to **artefacts**, caused for instance by molecular clusters entering the observation area. Several methods exist to detect those, for instance using a **quality metric** based on the deviation of the curve to the fit. Unfortunately these also measure varying signal levels. Here we instead penalise **fitting bias**: a high penalty is given to fitting residuals with several adjacent points of the same sign (either positive or negative).

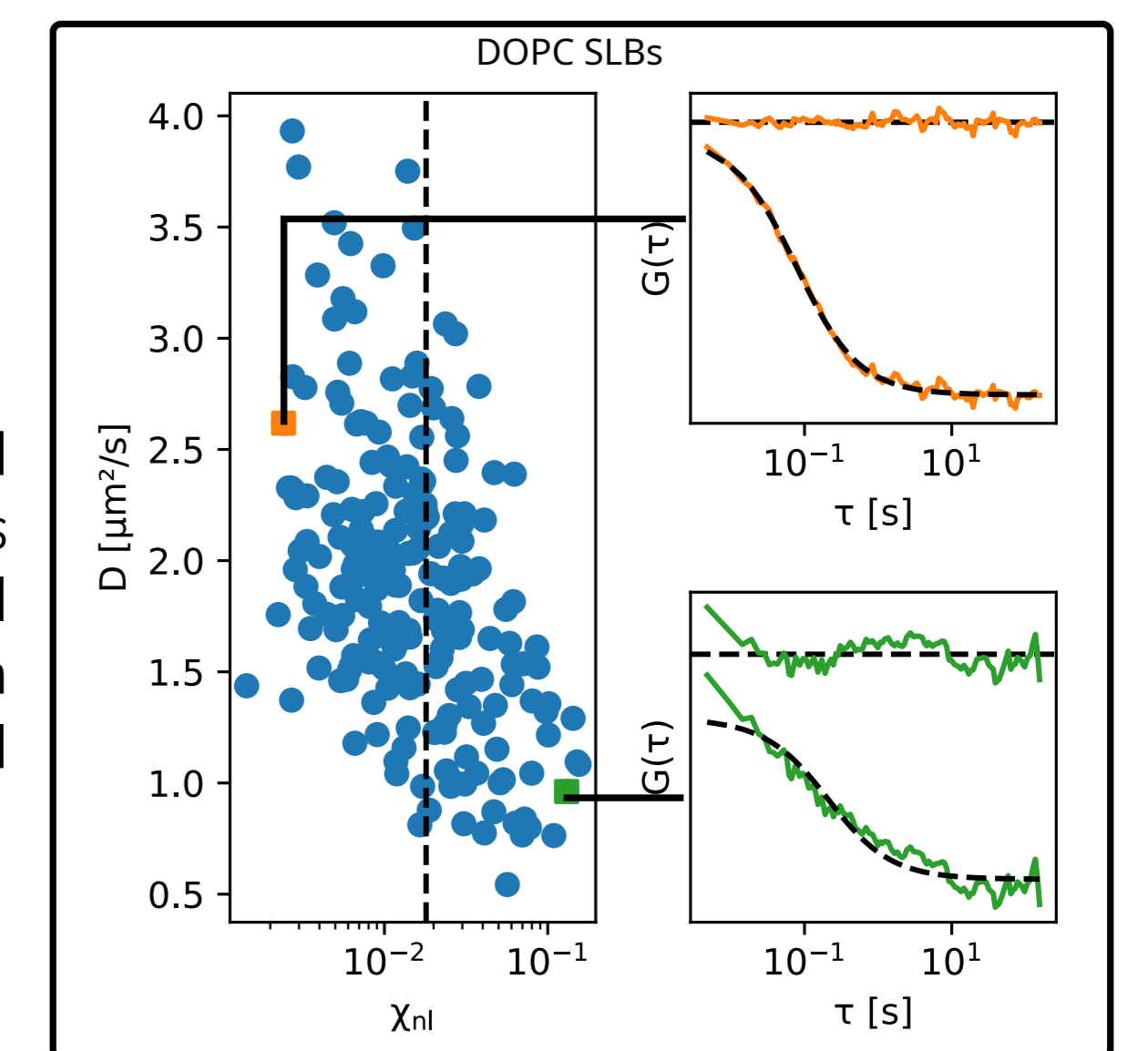


$$\chi = \sum (y_i - \hat{y}_i)^2 \quad \text{Residuals}$$

$$\chi_{nl} = \sum n_{adj,i} (y_i - \hat{y}_i)^2 \quad \text{non-linear Residuals}$$

y_i : value of FCS curve at point i , \hat{y}_i : value of the fit, n_{adj} : number of adjacent points on the FCS curve with residuals of same sign.

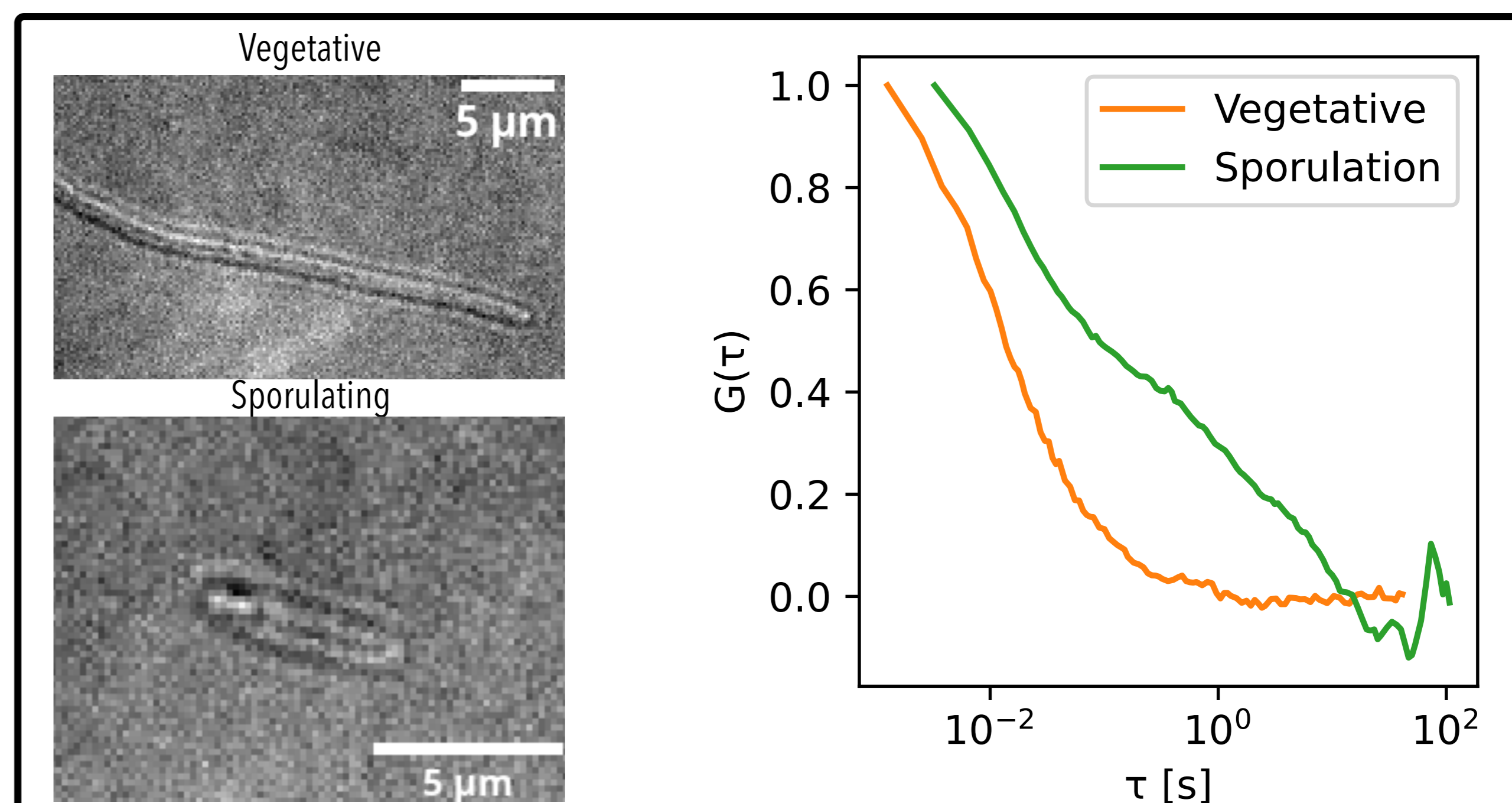
A noisy FCS curve exhibits high residuals (χ) but still represents a valid measurement. Adding a penalisation term for fitting bias (χ_{nl}) allows to discriminate artefactual curves from valid but noisy curves.



We tested this metric experimentally using a supported lipid bilayer (SLB). TIR-FCS in a DOPC SLB generates artefactual curves (bottom right) that can bias overall diffusion measurement. Filtering these curves based on their χ_{nl} (vertical dotted line) allows for unbiased diffusion coefficient measurement.

Membrane remodeling during the sporulation process

Upon starvation, bacteria of the *Bacillus* genus are able to differentiate as resilient **spores**. During its development, the spore is isolated from the environment by a **mother cell**. Here we compared diffusion of Nile Red in the membranes of vegetative cells and in the membranes of the mother cell at a late stage of sporulation. We found that Nile Red slows down considerably in the mother cell. It appears also that there are 2 components of diffusion present during sporulation, perhaps caused by a membrane heterogeneity or interaction with an unknown element in the cytoplasm.



Left: Bright-field images of a vegetative and sporulating cell. Right: average of FCS curves acquired in these cells.

Conclusion

- * TIR-FCS can measure the diffusion speed of membrane markers like Nile Red in the bacterial membrane.
- * We can use this readout as a proxy for membrane fluidity, a crucial element of bacterial phenotype.
- * We used TIR-FCS to monitor membrane remodeling during cold shock and sporulation.
- * This study paves the way for multiple new investigations in different bacteria, on the effects on the membrane of antibiotics, compounds or changes in environment.