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Single-molecule detection of ultrafast biomolecular dynamics with nanophotonics

Mark F. Nüesch^a, Miloš T. Ivanović^a, Jean-Benoît Claude^b, Daniel Nettels^a, Robert B. Best^{c*}, Jérôme Wenger^{b*}, Benjamin Schuler^{a,d*}

^aDepartment of Biochemistry, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

^bAix Marseille Univ, CNRS, Centrale Marseille, Institut Fresnel, 13013 Marseille, France

^cLaboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892-0520, USA

^dDepartment of Physics, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

ABSTRACT: Single-molecule Förster resonance energy transfer (FRET) is a versatile technique for probing the structure and dynamics of biomolecules even in heterogeneous ensembles. However, due to the limited fluorescence brightness per molecule and the relatively long fluorescence lifetimes, probing ultrafast structural dynamics in the nanosecond timescale has thus far been very challenging. Here we demonstrate that nanophotonic fluorescence enhancement in zero-mode waveguides enables measurements of previously inaccessible low-nanosecond dynamics by dramatically improving time resolution and reduces data acquisition times by more than an order of magnitude. As a prototypical example, we use this approach to probe the dynamics of a short intrinsically disordered peptide that were previously inaccessible with single-molecule FRET measurements. We show that we are now able to detect the low-nanosecond correlations in this peptide, and we obtain a detailed interpretation of the underlying distance distributions and dynamics in conjunction with all-atom molecular dynamics simulations, which agree remarkably well with the experiments. We expect this combined approach to be widely applicable to the investigation of very rapid biomolecular dynamics.

Investigating the entire spectrum of biomolecular dynamics is essential for understanding the mechanisms of biological processes at the molecular scale. A powerful class of methods for probing a broad range of timescales is single-molecule fluorescence spectroscopy¹⁻⁸. The application of single-molecule Förster resonance energy transfer (FRET), e.g., is particularly common for obtaining information on molecular distances and dynamics from milliseconds to minutes. However, monitoring more rapid intramolecular distance dynamics often remains a challenge.

Timescales down to the sub-microsecond regime can be reached with fluorescence correlation spectroscopy² (FCS) combined with single-molecule FRET between a donor and acceptor fluorophore attached to specific sites on biomolecules³⁻⁴. One important application of this nanosecond fluorescence correlation spectroscopy (nsFCS) has been the investigation of the rapid chain dynamics of unfolded polypeptides and intrinsically disordered proteins¹⁰⁻¹¹. By globally analyzing the donor-donor, acceptor-acceptor, and donor-acceptor fluorescence intensity correlations, distance dynamics can be quantified and distinguished from other contributions, such as

fluorescence quenching or rotational motion¹¹⁻¹². Moreover, sub-populations can be separated and their dynamics extracted even for heterogeneous mixtures of conformational states^{3-4, 10}. Disordered proteins of 50 to 100 residues typically exhibit chain reconfiguration times of tens to hundreds of nanoseconds.¹¹

However, nsFCS currently suffers from two major limitations. First, data acquisition times of ten hours or more are often required for reliably quantifying dynamics in the sub-microsecond regime, which limits sample throughput. Given typical photon count rates below 10^6 s⁻¹, the probability of detecting photons separated by nanoseconds – the key requirement for retrieving information on this timescale – is intrinsically low. Second, the shortest accessible times are limited by the fluorescence lifetimes of the fluorophores, typically a few nanoseconds. If the distance dynamics of interest are not well separated from this range, distinguishing the two contributions becomes difficult and has limited the use of nsFCS to distance dynamics above ~20 ns.¹¹

Here we use nanophotonics in zero-mode waveguides (ZMWs) to overcome these limitations. ZMWs are subwavelength apertures (Figure 1a, Figure S1) that were first introduced as a means of reducing the observation volume to the attoliter range and thus enabling single-molecule detection at much higher concentrations than previously possible¹³⁻¹⁴. However, another major advantage of ZMWs for single-molecule spectroscopy is the enhancement of fluorescence emission by the local environment in the nanocavity¹⁵⁻¹⁷: The fluorescence brightness is increased, which improves the signal-to-noise ratio and reduces data acquisition times. Simultaneously, fluorescence lifetimes are decreased, which enables access to a broader range of dynamics in the nanosecond regime.

Figure 1 shows the effect of ZMWs produced by focused ion-beam milling in a 100-nm aluminum layer on the confocal single-molecule detection of the commonly used Alexa Fluors 488 and 594 attached as FRET donor and acceptor, respectively, to the termini of the short, disordered peptide G(AQG)₆AGC. As expected from the smaller observation volume in the 120-nm ZMWs, the translational diffusion time of this AGQ peptide is much shorter than in confocal measurements without ZMW (Figure 1d, Figure S2). More importantly, however, the fluorescence lifetimes of the dyes are reduced by about twofold in the ZMWs (Figure 1b), and the average count rate per molecule (CRM) is enhanced by about six- to eightfold (Figure 1c, see Methods). This nanophotonic enhancement of the electric field and the connected effects on the decay and transfer rates of donor and acceptor in the ZMWs are expected to cause a change in the distance dependence of the FRET

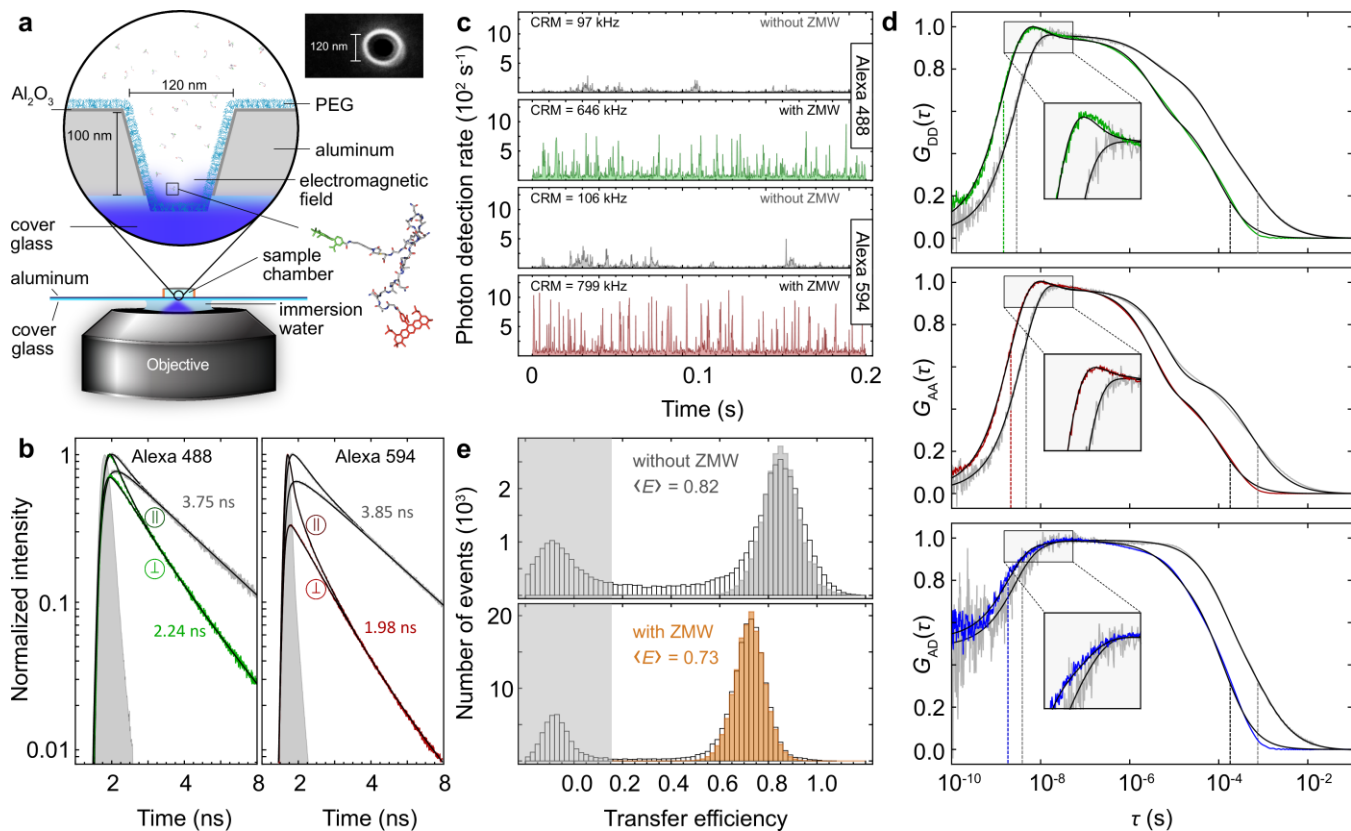


Figure 1. Enhancing nsFCS with zero-mode waveguides (ZMWs). (a) Schematic depiction of confocal measurements of the AGQ peptide labeled with Alexa 488 and 594 in a ZMW. Inset: scanning electron micrograph of a 120-nm aluminum ZMW nanoaperture (Figure S1). (b) Polarization-resolved fluorescence lifetime decays of donor and acceptor measured confocally without (gray) and with ZMW (color; darker: parallel polarization, lighter: perpendicular polarization; instrument response function shaded in gray; average lifetimes from the fits (solid lines) are indicated). Donor decays are from the donor-only population, acceptor decays from the FRET population after direct acceptor excitation (see Methods). (c) Time-binned (0.2 ms) fluorescence traces of donor and acceptor emission without (gray) and with ZMW (green, red), from measurements with similar average numbers of molecules in the observation volume (confocal: ~ 0.2 , ZMW: ~ 0.24); average count rates per molecule (CRM, see Methods) are indicated. (d) Normalized correlation functions from picoseconds to milliseconds of the labeled peptide measured without (gray, 40 h acquisition time) and with ZMW (color, 7 h acquisition time). Global fits with Eq. 5 (see Methods) are shown as black solid lines. Insets show magnified views of the time range where distance dynamics dominate the correlations. The timescales of photon antibunching and translational diffusion with (colored, black) and without ZMW (gray) are indicated as dashed vertical lines. (e) Transfer efficiency histograms (see Methods for corrections) observed without (top) and with ZMW (bottom); black: measured, shaded: shot noise-limited photon distribution analysis⁹. All measurements shown are in 50 mM sodium phosphate, 7.4 M urea, pH 7.4

efficiency¹⁸. The small shift in efficiency we observe (< 0.1 , Figure 1e) indicates only a moderate effect on the energy transfer process¹⁷ that can be accounted for by calibration (see Methods, Figure S3) and shifts sensitivity towards slightly shorter distances. Single-molecule FRET and nsFCS are thus feasible in ZMWs with this commonly used dye pair.

We use the Alexa488/594-labeled disordered AGQ peptide to assess the enhancement of nsFCS by ZMWs (Figure 1d, Figure 2). In 7.4 M urea, where the peptide is more expanded than in the absence of denaturant (Figure S3), its rapid end-to-end distance fluctuations result in a correlation time of only about twice the natural fluorescence lifetimes of the fluorophores (Figure 2a, Figure 1d). This situation is challenging for conventional nsFCS,¹⁹ because the correlation caused by conformational dynamics overlaps with the pronounced drop of the curve at short times caused by photon antibunching (Figure 2a, Figure S2). With the ZMWs, however, the reduced fluorescence lifetimes lead to a narrower photon antibunching component, and the rapid conformational dynamics can be clearly resolved in the correlation functions (Inset Figure 1d, Figure 2b, Figure S2). We extract a characteristic relaxation time corresponding to conformational dynamics of 8.8 ± 0.3 ns (Figure 2b). Even in the absence of urea, where the AGQ peptide is more

compact (Figure S3) and the conformational dynamics are faster still, nsFCS in ZMWs can resolve the contribution from chain dynamics (Figure 2d). The observed correlation time of 4.2 ± 0.5 ns is very close to the natural fluorescence lifetimes of the dyes and thus beyond the reach of measurements without ZMWs (Figure S4). Furthermore, the nanophotonic enhancement of the fluorescence emission rates enables a marked reduction in the data acquisition time required for a quantitative analysis of rapid chain dynamics (Figure 2b), even in mixtures of different proteins (Figure S5). Instead of the typical measurement times of ten hours or more, adequate statistics can be achieved in tens of minutes (Figure 2a,b; Figure S6).

The rapid dynamics accessible with nsFCS measurements in ZMWs enable a direct comparison with molecular dynamics (MD) simulations to gain a detailed picture of the underlying polypeptide chain dynamics. We thus performed all-atom explicit-solvent MD simulations of the AGQ peptide, including explicit donor and acceptor dyes²⁰⁻²¹, using a force field and water model suitable for disordered proteins, Amber99SBws/TIP4P2005s²², and a previously optimized parameterization of the fluorophores²³ and urea²⁴ (Figure 2e and Video S1). Extensive sampling of peptide conformations was ensured with 16 μ s of total simulation time (Figure

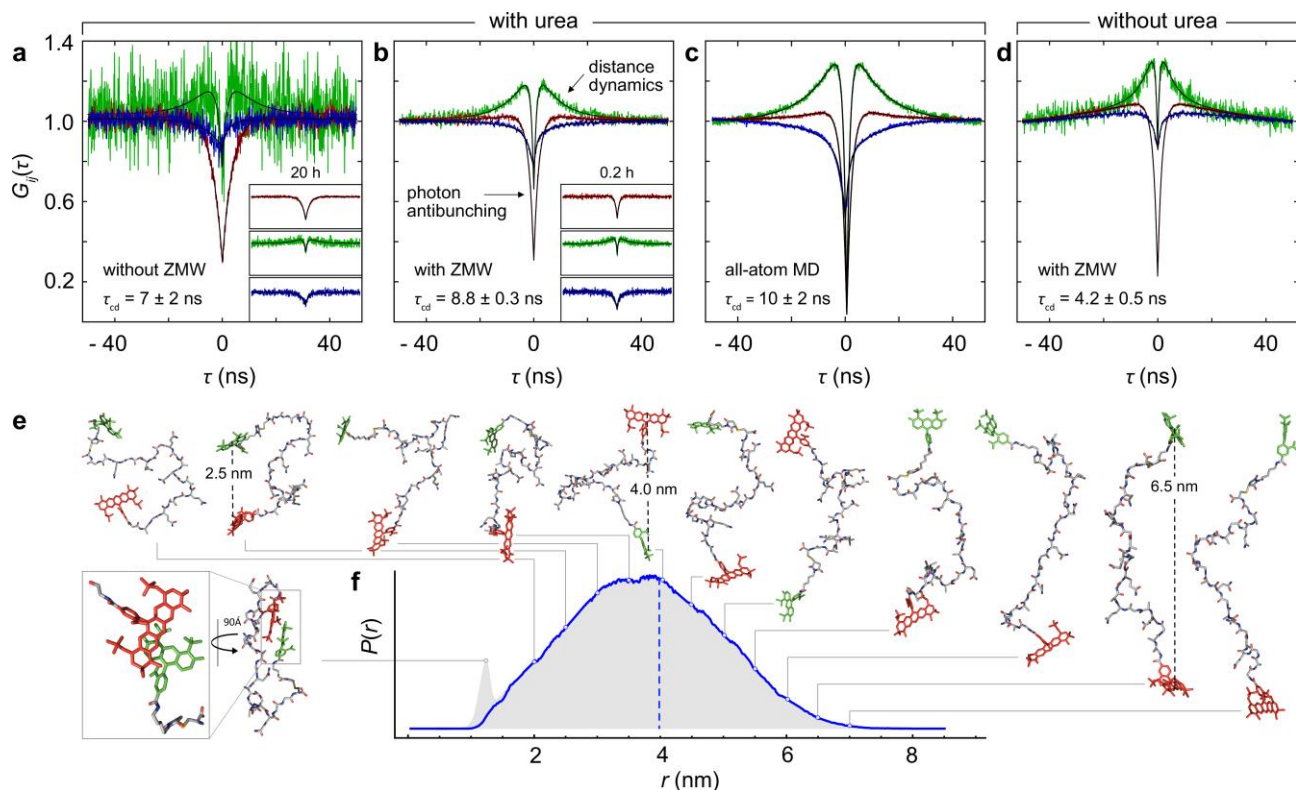


Figure 2. Probing the rapid dynamics of a disordered peptide with enhanced nsFCS. (a,b,c) Overlay of donor (green) and acceptor (red) fluorescence autocorrelations and donor-acceptor crosscorrelations (blue) from measurements of the disordered AGQ peptide in 7.4 M urea without ZMW (a) and with ZMW (b) after 7 h of data acquisition (inset in (a) after 20 h and in (b) after 0.2 h of data acquisition) with 100-ps time binning, and comparison with fluorescence correlation curves simulated based on all-atom molecular dynamics (MD) trajectories (c) using photophysical parameters corresponding to measurements in ZMWs (black solid lines are fits with Eq. 8, see Methods, with resulting fluorescence intensity correlation times, τ_{cd} , as indicated, with uncertainties estimated from three independent measurements; see Figure S7 for uncertainties of τ_{cd} in (c)). (d) nsFCS measurement with ZMW of the AGQ peptide without urea. The slight additional correlation decay visible for longer times is caused by contact quenching between donor and acceptor. (e) Representative snapshots from MD simulations of the disordered G(AGQ)₆AGC peptide with explicit donor and acceptor dyes, Alexa Fluors 488 (green) and 594 (red) and various inter-dye distances. (f) Distance distributions, $P(r)$, from MD simulations in 7.4 M urea (gray histogram: complete distribution; blue histogram: emissive conformations excluding dye-dye contacts) with corresponding root-mean-square inter-dye distance, $\langle r^2 \rangle^{1/2}$ (dashed vertical line)

S7). To enable a direct comparison with the measurements rather than with derived quantities, we simulated photon emission based on the MD trajectories, using the fluorescence lifetimes, excitation rates, and detector crosstalk corresponding to the experiments, including the influence of the ZMWs on the photophysics. In the simulation, the exact dye configuration is known for every simulation frame, so both the distance- and the orientation-dependence of Förster transfer can be taken into account explicitly (see Methods for details). The resulting fluorescence correlation functions (Figure 2c) are in remarkable agreement with the experimental data (Figure 2b), suggesting a faithful representation of the peptide dynamics in the simulations. In the absence of urea, the more rapid chain relaxation is also reproduced accurately, but an additional slower component points towards a slight overestimate of dye interactions (Figure S8). The agreement between the average transfer efficiencies from simulations (0.80 ± 0.01 with urea, 0.92 ± 0.01 without urea) and experiments (0.82 ± 0.03 with urea, 0.94 ± 0.03 without urea) provides an additional indication that MD simulations using current force fields with appropriately balanced protein-water interactions^{22, 25} can provide a realistic picture of the dynamics and conformational ensembles of disordered peptides and proteins²⁶⁻²⁸. Moreover, by comparing simulations of the AGQ peptide in urea with and without fluorophores, we can infer that the end-to-end distance correlation time of the unlabeled peptide is ~30 % lower than that of the labeled one (see SI Methods). This difference

is not unexpected given the size of the probes compared to this relatively small peptide (the relative contribution will be less for larger IDPs), but an accurate value cannot easily be inferred from the experimental data alone.

In summary, nanophotonics-enhanced nsFCS provides new opportunities for probing rapid biomolecular dynamics. The extended range of accessible timescales in the low nanosecond regime together with the pronounced reduction in measurement time eliminate previous limitations of nsFCS. The method will thus enable the investigation of peptides, disordered protein regions, nucleic acids, and other biomolecules in a time range that has so far been difficult to monitor, with much higher throughput than previously possible, and with less stringent demands on long-term sample stability. The method is complementary to the structural information and dynamics available from techniques such as NMR²⁹⁻³⁰, and we expect it to be directly transferable to other single-molecule fluorescence techniques, such as photo-induced electron transfer³¹. Finally, results from nanophotonics-enhanced nsFCS can provide stringent benchmarks for the further optimization of molecular dynamics force fields, and in synergy with the resulting simulations, they will help to obtain an increasingly detailed understanding of biomolecular dynamics over the full range of relevant timescales.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Materials and methods section and supplementary figures (PDF); Video of all-atom molecular dynamics simulation of AGQ peptide in 7.4 M urea (mov)

AUTHOR INFORMATION

Corresponding Authors

*robert.best2@nih.gov, *jerome.wenger@fresnel.fr,
*schuler@bioc.uzh.ch

Notes

The authors declare no competing financial interests.

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