Ultraviolet optical horn antennas for label-free detection of single proteins

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Single-molecule fluorescence techniques have revolutionized our ability to study proteins. However, the presence of a fluorescent label can alter the protein structure and/or modify its reaction with other species. To avoid the need for a fluorescent label, the intrinsic autofluorescence of proteins in the ultraviolet offers the benefits of fluorescence techniques without introducing the labelling drawbacks. Unfortunately, the low autofluorescence brightness of proteins has greatly challenged single molecule detection so far. Here we introduce optical horn antennas, a dedicated nanophotonic platform enabling the label-free detection of single proteins in the UV. This design combines fluorescence plasmonic enhancement, efficient collection up to 85° angle and background screening. We detect the UV autofluorescence from immobilized and diffusing single proteins, and monitor protein unfolding and dissociation upon denaturation. Optical horn antennas open up a unique and promising form of fluorescence spectroscopy to investigate single proteins in their native states in real time.
One of the ultimate goals of molecular biology is to watch how single proteins work in their native state. While single-molecule fluorescence techniques have achieved impressive results towards this goal, the requirement for fluorescent markers can potentially lead to severe issues altering the protein structure or modifying its reaction with other species. Therefore, label-free alternatives to detect single molecules are actively investigated. The protein autofluorescence in the ultraviolet (UV) is an appealing route to rule out all the issues related to external fluorescence labeling. More than 90% of all proteins contain some tryptophan or tyrosine aminoacid residues which are naturally fluorescent in the UV. Being able to detect the UV autofluorescence from a single (label-free) protein would be a disruptive method offering many benefits of fluorescence techniques (signal-to-noise ratio, temporal dynamics, sensitivity...) without introducing the labeling drawbacks.

However, proteins are orders of magnitude dimmer as compared to conventional fluorescent dyes, so that single protein UV detection has remained a major challenge so far. Hence, new nanotechnology tools need to be introduced to intensify the emission from single proteins. One of the main limiting issues is that close to a planar dielectric interface, a significant fraction of the light from a single dipole is emitted at large angles above 65°. This fundamental phenomenon is known as supercritical or forbidden light. Microscopes operating in the visible spectral range use objectives of high numerical apertures of 1.4 or above to maximize the fluorescence collected from a single molecule. In the UV, however, the choice of microscope objectives is strongly restricted, UV objectives have a numerical aperture typically below 0.8, which corresponds to a maximum collection angle of 33° into the quartz substrate of 1.48 refractive index. Collecting the forbidden UV light emitted at high angles is crucial to maximize the auto-fluorescence signal and unlock single label-free protein detection.

In analogy to radiofrequency antennas, optical antennas offer a way to control and intensify the emission of single quantum emitters. Intense fluorescence enhancement factors have been achieved with strongly absorbing dyes in the visible, but most optical antennas designs remain unsuitable for UV protein detection due to their narrowband spectral response, challenging nanofabrication, or requirement for solid-state integration. Alternative designs must be developed to offer a highly efficient platform, reaching the needs of high photon count rates, microsecond time resolution, background-free operation, and full compatibility with the UV detection of proteins.

Here, we introduce an optical horn antenna platform for label-free detection of single proteins in the UV with unprecedented resolutions and sensitivity. Our approach combines (i) a conical horn reflector for fluorescence collection at ultrahigh angles with (ii) a metal nanoaperture for fluorescence enhancement and background screening. To experimentally demonstrate the usefulness of our approach and its direct application to biochemical challenges, we detect the UV autofluorescence signal from immobilized and diffusing single proteins, and we monitor the unfolding and dissociation upon denaturation of a widely used protein. Optical horn antennas open up a promising form of fluorescence spectroscopy to investigate single proteins in their native states in real time. As additional advantage of our dedicated design, the improved brightness achieved with our optical horn antenna enables a 100-fold reduction of the experiment integration time as compared to the confocal reference. While the horn antennas are primarily developed here for UV protein detection, the concept is intrinsically broadband, and is straightforward to extend into the visible range to improve molecular sensing, single-photon sources, and non-linear light-emitting devices.

**Results**

**Optical performance and fluorescence enhancement assessment.** Our optical horn antenna platform addresses specifically the challenges of label-free single protein UV detection. It combines a reflective unit with a nanoaperture (Fig. 1a–c) and is the UV analog of a microwave horn antenna (Supplementary Fig. S1). The central nanoaperture of 65 nm diameter concentrates the light in an attoliter detection volume to isolate a single protein and enhance its autofluorescence, while the reflective conical unit covered with a 100 nm thick aluminum layer steers the autofluorescence light toward the microscope objective. Contrarily to the Yagi-Uda or Bull’s eyes resonant designs, the conical horn is intrinsically broadband, covering the full 300–400 nm bandwidth, independently of resonance or interference effects. The detection volume provided by the 65 nm central aperture is three orders of magnitude below that of a diffraction-limited confocal microscope, enabling single-molecule detection at micromolar physiological conditions, and circumventing the need for sub-nanomolar dilutions in conventional confocal microscopy.

The horn antenna performance is assessed using p-terphenyl, a 93% quantum yield UV fluorescent dye (Fig. 1d–g). The conical reflector angle mainly determines the antenna collection efficiency into the 0.8 NA objective (Supplementary Figs. S2, S3). We have fabricated horn antennas with various cone angles from 10 to 55° (Supplementary Fig. S4–S6). The raw fluorescence time trace already shows a 4× larger signal with 32° horn as compared to the bare nanoaperture, indicating an improved collection efficiency by the same ratio (Fig. 1d). Fluorescence correlation spectroscopy (FCS) analysis measures the fluorescence brightness per molecule (Fig. 1e and Table S1) from which we compute the fluorescence enhancement as compared to the confocal reference. The enhancement factor clearly depends on the cone angle with an optimum around 35° (Fig. 1g). While a single nanoaperture improves the p-terphenyl brightness by 4×, the horn reflector brings it to 15×. These values are lower than previous reports using gold antennas in the red part of the spectrum, yet this is explained by the ultraviolet range and the simple non-resonant design of the horn antenna. The major goal here is not to compete with plasmonics in the visible, but rather to enable UV autofluorescence detection of single proteins above the background noise. The collection efficiency gain has been numerically simulated using finite-difference time-domain (FDTD, solid curve in Fig. 1g). The simulation results match well with the experimental data trend, confirming the dependence with the cone angle.

The fluorescence lifetime measurements on Fig. 1f and Table S2 indicate that the p-terphenyl fluorescence lifetime is reduced by 3× in the antennas as compared to the confocal reference. This lifetime reduction is independent of the cone angle and is similar to the lifetime reduction found for the single aperture without the conical reflector. This shows that the emitter’s fluorescence lifetime (and thus the local density of optical states) is mainly set by the aperture diameter. Having a similar local density of optical states between the nanostructured samples, we can conclude that the supplementary gain brought by the optimized horn antenna is directly related to the increase in directivity as compared to the bare nanoaperture. This confirms the idea of the conical reflector as a collection unit to steer the emitted light towards the microscope objective. Based on the gain respective to the bare nanoaperture, the maximum collection angle is estimated to be around 85° for our best system with 32° cone angle. Our
nanophotonic platform collects the fluorescence light emitted at high angles, even beyond the supercritical angle. For a description of the aluminum nanoaperture influence on the fluorescence process, our group has recently published a detailed characterization using label-free proteins in the UV.  As shown by the data in Fig. 1, the presence of the horn reflector improves the collection efficiency, but the fluorescence excitation and emission enhancements occurring in the nanoaperture are not affected. We independently confirm the fluorescence enhancement by quantifying the noise reduction in the correlation data (Supplementary Fig. S7). The high brightness observed with the optimized horn antenna directly improves the FCS signal-to-noise ratio allowing to reduce the experiment integration time as compared to the confocal reference while keeping the same accuracy.

Single immobilized protein detection. We next focus on the label-free detection of single immobilized proteins using our optimized UV horn antennas with 32° cone angle (Fig. 2). The surface of the central nanoaperture is functionalized with silane-polyethylene glycol-biotin to graft individual β-galactosidase-streptavidin proteins. β-galactosidase from Escherichia coli (156 tryptophans) has been modified to bear a streptavidin anchor (24 tryptophans). We first assess the distribution of the number of proteins inside the central nanoaperture using control experiments where the proteins are labeled with an Atto647N-biotin red fluorescent dye (Fig. 2a, b). Specific care was taken to ensure a 1:1 labeling ratio before the surface immobilization so that on average every protein carries a single fluorescent label (see “Methods” for details). The experiments are repeated for two protein concentrations of 5 and 0.1 nM, allowing to report the evolution with the protein concentration. With the Atto647N label, the fluorescence intensity time traces show fast step decays typical of single-molecule fluorescence photobleaching (Fig. 2a, b). These traces are analyzed using the automated software AutoStepfinder to count the number of bleaching steps from which we deduce the number of proteins. The histogram of the
Respectively, 35, 21, and 23 antennas were probed for the different cases from top to bottom. Throughout these experiments, the cone angle is 32° and the white square marker denotes the average value with the bars extending to one standard deviation. The green vertical line indicates the median. The aperture diameter is 200 nm (see Supplementary Fig. S13 for a discussion on the aperture diameter).

Fig. 2 Horn antenna-enhanced label-free detection of immobilized single proteins. The surface of the horn antenna has been functionalized with biotin-PEG-silane to bind β-galactosidase-streptavidin and pure streptavidin proteins. In (a, c, e), the total protein concentration is 5 nM while it is reduced to 0.1 nM in (b, d). a, b Additional Atto647N-biotin red fluorescent label is added as a control to quantify the number of proteins inside individual horn antennas. Typical Atto647N fluorescence time traces are shown on the left panels in (a, b), together with their step-function fit (yellow line) allowing to count single molecules. The traces are vertically shifted for clarity. The right panels in (a, b) display the histogram of the number of detected molecules (bars) together with a Poisson distribution fit (line and dots). For (c–f), the experiments are performed with label-free proteins in the UV using 5 nM β-galactosidase-streptavidin (c), 0.1 nM β-galactosidase-streptavidin (d) and 5 nM streptavidin (e). In (c–e), the left panels show autofluorescence time traces and their exponential decay fit recorded on different individual horn antennas (the traces are vertically shifted for clarity). The right panels represent the histogram of the exponential fit amplitudes. For (c) 35 horn antennas were probed, for (d) 21 and for (e) 23. (f) Scatter plot of the exponential fit amplitudes corresponding to the histograms in (c–e). The points are vertically shifted using a uniform statistical distribution to better view the results. The white square marker denotes the average value with the bars extending to one standard deviation. The green vertical line indicates the median. Respectively, 35, 21, and 23 antennas were probed for the different cases from top to bottom. Throughout these experiments, the cone angle is 32° and the aperture diameter is 200 nm (see Supplementary Fig. S13 for a discussion on the aperture diameter).

number of molecules per horn antenna follow the expected Poisson distribution, with an average number of molecules of 2.3 for the 5 nM concentration and 0.7 for the 0.1 nM case. The evolution of the average number of molecules does not scale exactly with the concentration (although there is an obvious dependence) as the 30 min incubation time and possible steric hindrance between neighboring proteins may limit the number of proteins able to bind on the horn antenna surface. Importantly, for the 0.1 nM protein concentration, there is less than one protein per horn antenna on average, so this case is representative of single-molecule experiments.

UV autofluorescence time traces are shown in Fig. 2c–e and Figs. S8–S10. These label-free experiments have been performed in exactly the same surface immobilization conditions as for Fig. 2a, b, so it is fair to consider that the distributions of the number of proteins are unchanged. Due to the high number of tryptophan residues in each protein, the autofluorescence time traces no longer bear step-like decays but follow an exponential decay due to photobleaching. Control experiments performed in the absence of the protein using only the photostabilizing buffer show that the background noise does not show the exponential signal decay upon UV illumination (Supplementary Fig. S11). In presence of the proteins, each trace stemming from a different horn antenna is fitted with an exponential function to extract the decay amplitude. The histograms of the autofluorescence signal amplitudes are displayed in Fig. 2c, d for 5 and 0.1 nM protein concentration, while the raw data, average, standard deviation, and median values are summarized in Fig. 2f. Dividing the average fluorescence intensity (Fig. 2f) by the average number of proteins (determined from Fig. 2a, b), we estimate the average brightness per β-galactosidase-streptavidin protein in the horn antenna. We find 210 ± 30 counts/s for the 5 nM concentration and 310 ± 50 counts/s for the 0.1 nM concentration, which yield statistically quite comparable values (the difference is twice the standard deviation).

The experiments are reproduced with pure streptavidin, which has 24 tryptophan residues as compared to the 156 residues of β-galactosidase. A separate calibration using diffusing molecules shows that the average brightness per protein is 3× lower for streptavidin as compared to β-galactosidase. Although streptavidin has a 6.5× lower absolute number of tryptophan residues, their average quantum yield is estimated to be around 3.5% while...
Detecting single diffusing proteins. To confirm the single-molecule sensitivity claim, we perform experiments with diffusing β-galactosidase-streptavidin proteins at very low concentrations so that the average number of proteins present in the detection volume is significantly below 1 (Fig. 3). For these experiments, we use a 200 nm diameter nanoaperture to increase the protein residency time inside the horn antenna. For the highest concentration of 20 nM, the calculated average number of proteins inside the 200 nm nanoaperture is 0.12, which corresponds well to the regime required to observe fluorescence bursts from single molecules. Moreover, we add 55% sucrose to the buffer solution to increase the viscosity and ensure the proteins stay in the regime required to observe fluorescence bursts. Without sucrose or any other viscous medium, the diffusion time of proteins across the nanoaperture would be below 1 ms which is not sufficient to record enough photons and clearly resolve the UV autofluorescence bursts stemming from a single protein. The presence of impurities in sucrose leads to a higher background noise level and limits the maximum amount of sucrose that we can use (glycerol mixtures lead to similar observations). We find that a 55% w/w sucrose mixture is a good compromise between increased viscosity and tolerable noise level.

Figure 3a shows typical autofluorescence time traces recorded with increasing protein concentrations from 0 (background only) to 20 nM. Without the β-galactosidase-streptavidin proteins, we detect 14 events per minute above the threshold of 310 counts per 30 ms bin time (corresponding to 2.5× the standard deviation of the background noise, dashed horizontal line in Fig. 3a). In the presence of the protein sample, the number of events exceeding this threshold increases with the protein concentration (Fig. 3a, b inset). Zooming in on some selected bursts (star markers in Fig. 3a), the signal is above the average for several binning times, which indicates that these bursts are not spurious single-time bin events. The photon count histograms confirm an increasing difference with the background level as the protein concentration is increased. Comparing the respective maxima, we estimate that the brightness for diffusing molecules is about 600 counts per second, which stands in agreement with an independent FCS calibration at micromolar concentrations. It is only of 1.6% in β-galactosidase due to a higher quenching by nearby amino acids. The experiments on immobilized streptavidin proteins are performed using a 5 nM concentration to work in the same conditions as for Fig. 2a, c. The average amplitude found for streptavidin is 2.5× lower than for β-galactosidase (Fig. 2e, f), confirming the expected evolution of the signal with the number of tryptophan residues and their average quantum yield. Finally, the experiments with immobilized proteins are reproduced for single nanoapertures without the horn microreflector (Supplementary Fig. 5). The signal is about 3× brighter with the horn antenna as compared to the nanoaperture on a flat substrate, which goes along with the results found for p-terphenyl in Fig. 1g and the improved collection performance of the horn antenna.

Altogether, the data presented in Fig. 2 and S8–S12 demonstrate that the autofluorescence from a single protein can be recorded on a horn antenna. The signal scales with the protein concentration, the number of tryptophan residues, and the collection efficiency. Experiments using a fluorescent marker allow an independent measurement of the number of proteins. This realizes label-free single protein detection in the UV. In addition, fluorescence lifetime histograms can be extracted for traces corresponding to a single protein (Supplementary Fig. 14), a highly challenging task owing to the limited total photon budget that has to be distributed among the histogram time bins.
Fig. 4 Application of UV horn antenna to study denaturation of label-free proteins. a Auto-fluorescence spectra of β-galactosidase in presence of increasing concentrations of urea. b Following the approach in \(^{40}\), the auto-fluorescence intensity at 320 nm is used to monitor the denaturation of β-galactosidase as a function of the urea concentration. Data are presented as mean values, with the error bars corresponding to a 7% deviation accounting for the measurement uncertainty. The measurements have been repeated twice. c FCS correlation functions of β-galactosidase with increasing urea concentrations. The color lines are numerical fits. The arrows indicate the half-width of the correlation. d Evolution of the protein average hydrodynamic radius (left axis) and the mean number of detected molecules (right axis) as a function of the urea concentration. Error bars are standard deviations of the FCS measurements. Each measurement has been reproduced independently at least two times. The yellow line is a guide to the eyes based on a sigmoid function fit \(5 + 15/(1 + e^{t-1})\) where \(c\) is the urea concentration in molar.

Label-free protein denaturation and unfolding. β-galactosidase has gained importance as a model system to study protein folding\(^{39}\). Ensemble-level spectroscopy measurements such as the data in Fig. 4a, b and Supplementary Fig. S16 are commonly used to follow β-galactosidase denaturation in presence of urea\(^{40}\). It is believed that the tetrameric β-galactosidase first unfolds into a globular structure which then dissociates into unfolded monomers as the urea concentration is increased\(^{39,40}\). However, this pathway has never been studied at the single-molecule level and only indirect ensemble-averaged measurements are available. Single-molecule resolution is important here as it allows to clearly distinguish the dissociation into monomers by counting the number of molecules. An approach using fluorescence labeling would be very complicated as it would require all the β-galactosidase monomers to be labeled with a fluorescent dye, which is challenging to achieve and would lead to inter-chromophoric quenching due to the close proximity of dyes\(^{41}\). Another motivation for single-molecule resolution is to be able to simultaneously measure the protein hydrodynamic radius to clearly evidence unfolding.

Here we use label-free FCS enhanced by the optical horn antenna to study β-galactosidase denaturation (Fig. 4c, d). Without the horn antenna, the FCS data is too noisy to reliably measure unfolding and monomer dissociation (Supplementary Fig. S15). Thanks to the horn antenna, the UV auto-fluorescence brightness of β-galactosidase tetramers is increased by 10× which directly reduces the FCS noise by the same factor (Supplementary Fig. S15). In these conditions, the number of detected proteins and their hydrodynamic radius can be assessed as the urea concentration is increased (Fig. 4c, d and Supplementary Fig. S17). The number of molecules informs about the dissociation of the β-galactosidase tetramers into monomers while the hydrodynamic radius indicates expansion or compaction of the protein structure. As additional advantage, the attoliter detection volume of the horn antenna seems beneficial to avoid observing large aggregates which are a perturbation source for ensemble measurements\(^{40}\). While the auto-fluorescence spectrum of β-galactosidase is red-shifted up to 10 nm by the presence of urea (Fig. 4a and Fig. S16a, b), the total integrated intensity in the 310–410 nm detection range remains almost unaffected (Fig. S16c) as well as the auto-fluorescence lifetime (Fig. S16d).

Our experimental data show that below 3.5 M urea, β-galactosidase remains as a tetramer (the number of molecules is constant) while its hydrodynamic radius increases from 6.5 to 9.5 nm indicating protein unfolding (Fig. 4d). Our analysis takes into account the viscosity change as the urea concentration is increased (Supplementary Fig. S17) as well as the influence of the nanoaperture calibrated in ref. 33. In the absence of urea, our 6.5 ± 0.6 nm value for the hydrodynamic radius corresponds well to the 6.85 nm (experimental) and 6.7 nm (calculated) values determined previously in ref. 42. Above 3.5 M urea, the hydrodynamic radius decreases down to 4 nm and the number of molecules increases by more than 2×. These are clear evidences of the tetramer partial dissociation into unfolded monomers, providing a confirmation of the β-galactosidase denaturation pathway established using ensemble methods\(^{39,40}\).

Discussion

While label-free alternatives to fluorescence labeling are actively searched\(^{9–11}\), fluorescence spectroscopy remains by far the most widely used approach for single-molecule detection. The optical horn antennas developed here make a significant step forward by enabling the direct detection of single label-free proteins via their
natural ultraviolet fluorescence. This dedicated design combines simultaneously plasmonic fluorescence enhancement, efficient fluorescence collection, attoliter detection volume, and strong background rejection, allowing to achieve unprecedented protein autofluorescence brightness. Improving the net detected UV photon count rate is key to enable the biophysical applications investigating single proteins in their native state in real-time. Photodamage of aminoacids due to UV illumination is a potential issue that may affect the protein structure. For diffusing proteins, the short illumination time balances the negative impact of the UV photodegradation. However, for immobilized proteins, the risk of photodamage limits the maximum UV power exciting the protein. We have used so far the lowest possible power of 0.3 µW, yet this phenomenon is currently setting the limit for the achievable signal-to-noise ratio. UV detection of single molecules is still at its infancy, and we hope that this work will stimulate more studies in this direction. Moreover, optical horn antennas are also beneficial to improve the collection efficiency in the visible regime, and analyze single proteins in their native state in real-time.

Methods

Optical horn antenna fabrication. The fabrication process involves several steps (Supplementary Fig. S4). Briefly, we first mill the horn antenna by focused ion beam (FIB). Then a 100 nm aluminum layer is deposited to ensure a good UV reflectivity of the horn antenna walls. Last, we carve a 65 nm or a 200 nm diameter nanoaperture by FIB in the center of the top plate of the horn antenna. The substrates are cleaned NG51 quartz microscope coverslips of 150 µm thickness (Neosys). Aluminum layers are deposited by electron-beam evaporation (Röhm Syrus Pro 710) with 10 nm/s rate at a chamber pressure of 10−4 mbar. FIB milling is performed using a gallium-based system (FEI dual beam DB235 Strata) with 30 kV acceleration voltage and 300 pA current for milling the horn antenna and 10 pA current for milling the central nanoperture. All nanoapertures have a 50 nm deep undercut into the quartz substrate to maximize the signal enhancement. Last, a 12-nm-thick SiO2 layer is deposited by plasma-enhanced chemical vapor protection (PECVD, PlasmaPro NPG800 from Oxford Instruments) to protect the aluminum surface against corrosion.

Protein samples and photostabilizing buffer. β-galactosidase from Esherichia coli (156 tryptophan residues, PDB 1DP0), β-galactosidase-streptavidin conjugate (180 tryptophan residues), and streptavadin from Streptomyces avidinii (24 tryptophan residues) are purchased from Sigma-Aldrich. The proteins are dissolved in a Hepes buffer (25 mM Hepes, 300 mM NaCl, 0.1 v/v% Tween-20, 1 mM DTT, and 1 mM EDTA 1 mM at pH 6.1) which was reported to stabilize β-galactosidase conformation and avoid aggregate formation. All the protein stock solutions have been centrifuged for 12 min at 140,000 x g (Airfuge) before the optical measurements, GODCAT oxygen scavenger (100 mM glucose oxidase, 830 mM catalase, 0.4 v/v% Tween20 is added to the buffer to minimize aggregation of residual afterpulsing from the photon counting detector and/or metal quenching.

Surface immobilization of proteins. The horn antennas are thoroughly cleaned with UV-ozone (Novascan PSD-UV 100 W), ethanol rinsing, and oxygen-plasma (Diener Zepto 50 W, 0.6 mbar, 10 min). The silica surface is then coated with silane-modified polyethylene glycol (PEG-silane) by immersion into an ethanol solution containing 0.75 mg PEG-silane 1000 Da and 1.2 mg biotin-PEG-silane 2000 Da. PEG-silane and biotin-PEG-silane are purchased from Nanocs. After overnight incubation, the sample is rinsed with ethanol and dried. The protein solution with either 0.1 or 0.5 mM concentration is placed on the surface for 30 min allowing the streptavidin anchor to bind the surface-grafted biotin. Before the optical measurements, the surface is rinsed three times with water and covered with the GODCAT photostabilizing buffer.

Control experiments with fluorescent labels. A solution of β-galactosidase-streptavidin is mixed with a solution of Atto647N-biotin in a 1:1 ratio and kept at 4 °C overnight. The proteins were centrifuged by using a microcentrifuge (Tecan Spark 10 M) to ensure proper labeling of the proteins with a single Atto647N dye. In this configuration, on average each protein is expected to bind only one fluorescent tag via the streptavidin-biotin bridge. Assuming that each β-galactosidase-streptavidin has 5 binding sites for the biotinylated fluorescent tag (one binding site is taken by the anchor between β-galactosidase and streptavidin), the probability that a protein bears more than 1 fluorescent label is estimated to be below 4%. The surface grafting of the labeled β-galactosidase-streptavidin is performed using exactly the same protocol used for the label-free counterpart. The fluorescence readout is performed on a confocal microscope described in ref. 26 using 1 µW laser power at 635 nm. To promote Atto647N photostability and minimize bleaching, we use the GODCAT oxygen scavenger system together with 1 mM Trolax (c)–6–Hydroxy-2,5,7,8-tetramethylchromanе-2-carboxylic acid). Prior to the experiments, the Trolax stock solution in DMSO is illuminated with a UV lamp for 25 min to ensure a proper ratio of Trolax and its Trolax-quinone derivatives.

Urea denaturation. A 1.7 µM β-galactosidase protein solution is incubated with urea at various concentrations from 0 to 6 M (pH 7) for 90 min at room temperature. 0.4 v/v% Tween20 is added to the buffer to minimize aggregation of denatured proteins. To avoid non-specific adsorption of proteins, the horn antenna surface is passivated with PEG-silane 1000 Da (Nanocs) by immersion into a 1 mg/ml PEG-silane solution ethanol with 1% acetic acid for 3–4 h followed by ethanol rinsing. GODCAT photostabilizing system is added to the protein buffer just before the optical measurements and the urea concentration is adjusted to keep a constant value to avoid protein refolding.

UV microscope. We operate a custom-built confocal microscope with a LOMO 58X, 0.8 NA, water immersion objective. Experiments on p-terphenyl use a 266 nm picosecond laser (Picosquant LDH-P-FA-266, 70 ps pulse duration, 80 MHz repetition rate) with 80 µW average power, while experiments on proteins use a 295 nm picosecond laser (Picosquant Visu-UV-295-590, 70 ps pulse duration, 80 MHz repetition rate). The 295 nm wavelength selectively excites tryptophan residues, as tryptophan and phenylalanine having a higher absorption at 295 nm. For immobilized protein detection, we use the GODCAT UV power for immobilized protein detection is 0.3 µW, while for diffusing molecules we use 8 µW. The urea denaturation experiments on diffusing proteins are performed at 10 µW. Both laser beams are spatially filtered to ensure a Gaussian profile filling the objective back aperture, they pass through a short-pass filter (Semrock FF01-311/SP-25) and are reflected by a dichroic mirror (Semrock FF310-D01-25-D). The optical horn antenna is positioned at the focus with a 3-axis piezoelectric stage (Physik Instrumente P-517.3CD). For the immobilized protein experiment, we use the microscope LED illumination to localize the horn antenna. The laser illumination is turned on immediately at the start of the acquisition to avoid bleaching the proteins which was used in the control experiment.

Fluorescence light is collected back by the microscope objective and separated from the laser light by the dichroic mirror and two emission filters (Semrock FF01-300/LP-25 and Semrock FF01-375/110-25). The spectral range for fluorescence detection goes from 310 to 410 nm. Confocal detection is performed using a 200 nm focal length double lens (Thorlabs ACA254-200-U) and a 80 µm pinhole. Single-photon counting uses a photomultiplier tube (Picoquant PMA 175) connected to a photon counting module (Picoquant Picharp 300 with time-tagged time-resolved mode). The integration time is 2 to 3 min.

Fluorescence time trace analysis. The fluorescence data is analyzed with Symphotime 64 (Picoquant) and Igor Pro 7 (WaveMetrics). For the fluorescence time trace analysis, time intervals were determined by measuring the long-time fluctuations of the fluorescence data. For the control experiments using Atto647N labels, the traces are analyzed using AutoStepfinder, a recently developed software for automatic step detection.

For the experiments on diffracting proteins at very low concentrations (average numbers of proteins in the nanopore <0.12, Fig. 3), we apply a 1 Hz high-pass filter to remove all long-term drifts and fluctuations. These drifts have a small amplitude (typically 5 to 10 counts per 30 ms) and long periods (seconds), yet due to the low signal-to-noise ratio at low protein concentrations, the long-term fluctuations have to be removed before computing the photon count histogram. The average value of the initial trace is then added to retrieve the filtered count histogram.

FCS correlations are fitted using a three-dimensional Brownian diffusion model with a blinking term. The fluorescence correlation function is calculated using a three-dimensional Brownian diffusion model with a blinking term.

\[
(G(t) = \frac{1}{N_{mol}} \left[ 1 - \frac{I_0}{F(t)} \left( 1 + \eta_I \exp \left( \frac{\tau}{\tau_B} \right) \right) \right] \frac{1 + \tau_B}{\tau_B} \left( 1 + \frac{\tau}{\tau_B} \right)^{-0.5} \tag{1}\]

where \(N_{mol}\) is the total number of detected molecules, \(B\) the background intensity, \(F\) the total fluorescence intensity, \(\eta_I\) and \(\tau_B\) are the blinking amplitude and characteristic time, \(\tau_B\) is the mean diffusion time and \(\kappa\) the aspect ratio of the axial to transversal dimensions of the detection volume (\(\kappa = 8\) for the confocal illumination and \(\kappa = 1\) for the horn antenna). Note that the fast kinetics components determined by \(\eta_I\) and \(\tau_B\) may not be only related to the blip blinking, they could also account for residual afterglowing from the laser light. These fluctuations can result from the small time window during which the protein diffuses in nanometer proximity to the aluminum surface. The FCS fit results are summarized in the Supplementary information.
The fluorescence lifetime decays are fitted by an iterative rerconvolution taking into account the measured instrument response function (IRF). As noted in our previous work on single apertures, a three exponential model is needed to correctly interpolate the experimental data. A fixed 10 ps component accounts for the metal photoluminescence and Raman scattering background, while a long component with a lifetime similar to the confocal reference corresponds to a residual fluorescence stemming from molecules away from the optical antenna. The intermediate lifetime component (which has a dominating intensity) corresponds to the lifetime of molecules inside the horn antenna. All the fit results are detailed in the supplementary information.

Numerical simulations. The electric field intensity radiated by a point dipole is calculated with finite-difference time-domain (FDTD) method using RSoft Fullwave software. We set a fixed emission wavelength at 350 nm. Horizontal and vertical orientations of the source dipole are computed separately and averaged for the final output to represent the emission of a molecule with nanosecond rotational time. Each simulation is run with 2 nm mesh size and is checked for convergence after several optical periods. To compute the enhancement displayed in Fig. 1g, 10 horn antennas with different cone angle are simulated. For each of them, we compute the gain into the 0.8 collection NA of the microscope objective as computed with the Finitedifference time-domain (FDTD) method using RSoft Fullwave software. We set a fixed emission wavelength at 350 nm. 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Author contributions
A.B. performed and analyzed measurements, P.R. performed and analyzed measurements and simulations, J.B.C. performed nanofabrication, J.W. designed and supervised research and prepared manuscript with the help of all authors.

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