Bacteriorhodopsin is a seven-helix light-driven proton-pump that was structurally and functionally extensively studied. Despite a wealth of data, the single molecule kinetics of the reaction cycle remain unknown. Here, we use high-speed atomic force microscopy methods to characterize the single molecule kinetics of wild-type bR exposed to continuous light and short pulses. Monitoring bR conformational changes with millisecond temporal resolution, we determine that the cytoplasmic gate opens 2.9 ms after photon absorption, and stays open for proton capture for 13.2 ms. Surprisingly, a previously active protomer cannot be reactivated for another 37.6 ms, even under excess continuous light, giving a single molecule reaction cycle of ~20 s$^{-1}$. The reaction cycle slows at low light where the closed state is prolonged, and at basic or acidic pH where the open state is extended.
Bacteriorhodopsin (bR) is a membrane protein and member of the microbial rhodopsin family, a light-driving proton (H\(^+\)) pump formed by seven transmembrane helices (TMHs) and a covalently bound retinal chromophore\(^7\). Photon absorption induces the isomerization of the retinal from all-trans to 13-cis. This event starts a cascade of reactions that lead to H\(^+\)-release on the extracellular side and cytoplasmic H\(^+\)-uptake\(^2,3\), thus H\(^+\)-pumping that establishes an electrochemical H\(^+\)-gradient that powers ATP-production.

The time scales at which the different light-induced intermediates of the reaction cycle occur, span from femtoseconds to millisecond and have been studied by spectroscopy and structural methods\(^8-10\). We prefer to use the terminology "reaction cycle" that represents better the conformational and functional dynamics than the more spectrscopic term "photocycle". In brief, the bR reaction cycle can be subdivided into four main processes. First, upon photon absorption, the retinal isomerizes within the first picoseconds (ground to K state) and then relaxes in the 13-cis conformation (K and L state). Second, a H\(^+\) from the retinal Schiff-base passes to Asp-85 (L to M\(_1\) state) and the proton-releasing group (Glu-194 and Glu-204) releases a H\(^+\) to the extracellular side (M\(_1\) to M\(_2\) state). Third, through a large conformational change of helix-F a funnel to the cytoplasm opens and a H\(^+\) enters via Asp-96 and re-protonates the retinal (N state). Fourth, the cytoplasmic gate closes (N to O state) and the protein resets for the next reaction cycle (O to ground state). The first two steps are accompanied by only small local conformational changes and happen within the first tens of microseconds. In contrast, the third step involves displacements of the cytoplasmic half of helices E and F and the E-F-loop (Fig.1a, b), opening the cytoplasmic gate and rendering Asp-96 and the retinal accessible for re-protonation within the first ten milliseconds. In the fourth step, this conformational change is reversed and the cytoplasmic gate closes. Therefore, structurally, the reaction cycle can be divided into two major parts, the closed state, defined as the state where the cytoplasmic gate is closed (Fig. 1a, pink, PDB 6RNJ) and the open state (Fig. 1a, green, PDB 6RPH) where the E-F loop is displaced and the cytoplasmic gate is open, conceptually matching the alternate access model of membrane transport\(^7\).

The functional aspects of the bR reaction cycle have been studied using spectroscopic methods\(^8-15\). In these experiments a large ensemble of br is exposed to stimulating light pulses and the spectroscopic properties of br and/or pH-sensitive labels is measured. According to these measurements, the K state and retinal isomerization occurs within picoseconds and the L state is reached after ~1.4 µs, a H\(^+\) is released on the extracellular side after ~630 µs, and the M\(_1\) state decays after ~4.7 ms. Finally, the decay of the N-state occurs after ~14 ms concomitant with re-protonation after ~12.1 ms\(^8\). This experiment could however not inform how long a prior active br would need to reset from the O state and allow to be reactivated, and based on these measurements, it was assumed that br cycles at ~100 s\(^{-1}\).

The structural aspects of the bR reaction cycle have been studied by several techniques, some of them are also time-resolved methods: X-ray crystallography\(^4,16-18\), solid-state NMR\(^19-21\), cryo-EM crystallography\(^5,22-25\), high speed atomic force microscopy (HS-AFM)\(^26-28\), and more recently, time-resolved serial femtosecond crystallography (TR-SFX) and X-ray free electron laser crystallography (XFEL)\(^3,7,33\). TR-SFX resolved the conformational changes in the retinal in the first femtoseconds\(^3,34\), XFEL and X-ray crystallography reported about secondary structure conformational changes in the later millisecond range after photoactivation\(^7,35\). Notably, a closed state with 13-cis retinal accumulated in the 0–5 ms-structure, while an open state with large displacement of the E-F loop accumulated in the 10–15 ms-structure with a decay of 23.8 ms. In these experiments the reverse conformational change to the O state could not be observed likely because the crystal contacts did not allow such rearrangements\(^7\). According to this work, the br molecules complete the reaction cycle within 200 ms within the 3D-crystals, thus cycle at ~5 s\(^{-1}\). Yet, the spectroscopic analyses, X-ray and cryo-EM crystallography and NMR based methods provide ensemble averaged data.

Here, we develop and apply HS-AFM line-scanning\(^36,37\) with millisecond temporal resolution coupled to a light activation system\(^26-28\) to monitor single molecule structural dynamics of bR WT and bR-D96N in native purple membranes. We find that large conformational changes to open the cytoplasmic gate occur within <3 ms upon light activation and persist for ~13 ms. Surprisingly, a prior activated molecule cannot be reactivated for another ~37 ms, and thus the maximum single molecules turnover rate is ~20 s\(^{-1}\).

Results
To measure single molecule kinetics of bR upon light activation, we integrated a green laser into our HS-AFM setup to provide 520-nm photons during defined duration and of defined intensity over ~15 µm diameter of the sample around the HS-AFM tip (Supplementary Fig. 1) while recording the protein dynamics (Fig. 1c). Similar to former AFM configurations\(^26-28,30,38\), the infrared (IR) laser (HS-AFM laser) is reflected from the backside of the cantilever to measure its position and operate the HS-AFM feedback loop, while a second laser emitting light at 520 nm (activation laser) is used to stimulate the bR. A beam splitter in the optical path diverts part of the activation laser towards a light meter, which is monitored concomitantly to HS-AFM image acquisition. A signal generator connected to the activation laser driver allows to generate laser pulses of varying duration and intensity.

Sidedness assignment of bR conformational changes. In the TR-SMX structures upon light-activation\(^7\) essentially no conformational changes are detected on the extracellular side of br, while the cytoplasmic side displayed a closed state (Fig. 1a, b, pink, PDB 6RNJ) and an open state (Fig. 1a, green, PDB 6RPH), in which the cytoplasmic half of helix-F and the E-F loop move towards the periphery of the trimer. To assign the br sidedness in our experiments, we first let purple membranes of br-D96N adsorb at high density on the mica HS-AFM sample support, such as to find membranes exposing the extracellular and the cytoplasmic surfaces right next to each other. Second, we acquired HS-AFM movies at the interface of neighboring patches while applying green light (Fig. 1d). We reproducibly observed conformational changes only on one surface type of bR that we assigned to the cytoplasmic surface, in agreement with earlier br sidedness assignments\(^39\), while the extracellular surface remained unchanged (Supplementary Movie 1).

Dynamics of bR-D96N. Focusing on the reaction cycle-dependent conformational changes on the cytoplasmic br side, we acquired high-resolution movies of br-D96N using our laser combined HS-AFM (Fig. 1c). Due to the slow inactivation of bR-D96N, we are able to observe both states at HS-AFM imaging scan rates (Fig. 1e, f). Applying single (Fig. 1e, Supplementary Movie 2) or multiple (Fig. 1f, Supplementary Movie 3) green laser sample illumination periods, the molecular response of br-D96N was monitored and the average topography of the molecules in each frame calculated (Fig. 1e, f, middle). In addition, we measured the light-induced E-F loop displacement, the transition from the M to the N state, of each protomer in the movies by analyzing the peak position shift with respect to the trimer center. The average loop displacement of the protomers in the movies is ~5.0 ± 0.8 Å (Fig. 1e, f, bottom). Recently, we introduced localization AFM (LAFM)\(^40\), a method to
extract high-resolution information from AFM topographies: LAFM maps reveal an E-F loop displacement of 5.5 Å towards the periphery of the bR trimer with a counter-clockwise shift of 9° (Fig. 1e, bottom, insets). These measurements are in good agreement with former HS-AFM, cryo-EM and TR-SXM data (see E-F loop displacement in Fig. 1b). Single protomer dynamics analysis provided additional information: while the average decay time of the open state in bR-D96N after switching off the activation laser, the transition from the N to the O state, is ~10.5 s at pH 7, in good agreement with prior HS-AFM measurements of the D96N mutant. Protomers display individual behavior and may reset swiftly or stay in the open state for ~40 s (Fig. 1e, bottom, gray lines). Another way to visualize the protomer behavior is through plotting the HS-AFM movie as kymographs of profiles across the time.
Fig. 1 Laser combined high-speed atomic force microscopy (HS-AFM) for the study of photo-activated conformational changes in bacteriorhodopsin (bR). a Structure of bR trimer viewed from the cytoplasmic side in the closed (PDB 6RNJ) and open (PDB 6RPH) states. Letters label the 7 helices. Outlines highlight one protomer. b Superposition of the open (green) and closed (red) structural states: The conformational change in helix-F (and to a minor degree in helix-E) are highlighted by arrows. The E-F loop moves by ~5 Å. c HS-AFM setup: Only the main components are shown and labeled: The IR laser (AFM laser) monitors the cantilever position. The green laser (activating laser) is used to excite bR. d Top: Light protocol. Middle: HS-AFM images of bR-D96N patches exposing cytoplasmic and extracellular sides (labeled). Bottom: Averages of the cytoplasmic and extracellular side topographies (labeled cyto and ext in the first pair of images). Only the cytoplasmic side displays measurable conformational changes during and after (bR-D96N has -10.5 s open state dwell-time) light stimulation. Light-induced conformational changes in bR-D96N subjected to (e) a single, and (f) multiple green laser light periods. Top: Light protocol. Middle: High-resolution HS-AFM images and corresponding correlation averages. bR trimer (white dashed circle) and trefoil, de

Openning delay time of bR-WT and bR-D96N following short light pulses. Having measured the open (N) and closed (sum of O, ground, K, L, and M) state single molecule lifetimes in continuous illumination experiments, we next aimed to determine the speed of the opening of bR-WT. The period upon a short light pulse, before the opening of the cytoplasmic gate in the N state,
comprises states K, L, and M, and the period after the N state comprises O to ground state. Thus, this experiment allows us to split the closed state dwell into the two periods before and after the N-state. To measure this, we applied short activating laser-pulses to define the moment of photon capture to a precise time-point. We modulated the laser driver to produce one 0.5 ms (or 0.2 ms) short pulse every second (Fig. 3a, b, top), thus allowing us to measure the time between photon capture by the all-trans retinal (ground to K state) and the conformational change in the E-F loop, i.e., the opening of the cytoplasmic gate that allows re-protonation of D96 (N state). From spectroscopic methods, we know that the all-trans to 13-cis transition is ultra-fast, within ~5 ps\(^3\) - thus measuring the time that passes between photon-capture and the movement of the E-F loop corresponds to the
conformational coupling between the change in the retinal to the end of helix F. A recent TR-SFX study found this conformational change in the 10–15 ms post laser activation structure. In our experiment, we can directly measure two observables on the single molecule level: 1) the time after photon burst to propagate the conformational change from the retinal to the opening of the cytoplasmic gate, which we term the opening delay time; and 2) the time the protein stays in the open state. The same as the open state dwell-time determined in the continuous light experiments (Fig. 2).

To precisely synchronize the activation laser pulses with respect to the HS-AFM-LS recordings, we recorded the photodiode signal in a second data acquisition channel (see Methods). This allows us to precisely calculate the opening delay time from the first pixel when the green laser hits the sample to the pixel where the conformational change of a protomer is identified (Fig. 3a–d, Supplementary Fig. 4). In the presented experiments, scan lines are acquired every 1.667 ms and each pixel is recorded during 2.7 μs (Fig. 3c, d, Supplementary Fig. 4, see Methods). We applied this method to both, bR-WT (Fig. 3a) and bR-D96N (Fig. 3b). Due to the shortness of the activation laser pulses (Fig. 3a, b, top), only few protomers activated upon each pulse (Fig. 3a, b, bottom, dashed outlines). Close inspection of the conformational response of individual protomers showed that the E-F loop moved within the same or in one of the subsequent scan lines upon a green laser pulse, in both, bR-WT (Fig. 3c, left) and bR-D96N (Fig. 3d, left). Plotting the opening event times (with a bin width of 1.667 ms, corresponding to the time of a full scan line cycle), we derived the E-F loop conformational delay upon photo-activation through exponential decay fitting as τdelay = 2.9 ± 0.1 ms (n = 110) for bR-WT (Fig. 3c, right) and τdelay = 3.2 ± 0.1 ms (n = 67) for bR-D96N (Fig. 3d, right), respectively. We also applied a shorter laser pulse of 0.2 ms for bR-WT and obtained the same opening delay time, τdelay-0.2ms-pulse = 2.9 ± 0.1 ms, but from lower protomer activation numbers (n = 100) (Fig. 3e, gray arrowhead). Comparing the opening delay times, bR-D96N was 10% slower than bR-WT, suggesting that not only re-protonation of the retinal is slowed down when aspartate-96 is substituted with an asparagine but also the conformational change to open the cytoplasmic gate was slightly slower; as has been observed for other bR-mutants. The main impact of the D96N mutation is the delay in the re-protonation step. Indeed, the kymographs upon pulse activation revealed that the bR-WT open state dwell-time, corresponding to the duration until the E-F loop regains its initial position, had a time constant τbR-WT = 14.2 ms (Fig. 3c, left, Fig. 3f, g), very similar to τbR-WT = 13.2 ms from the continuous light experiment (Fig. 2g). In contrast, the bR-D96N protomers remained open over extended kymograph acquisition (Fig. 3d, left; Fig. 3b, Supplementary Fig. 5). The bR-D96N open state dwell-time is better measured using HS-AFM imaging, τbRopen-D96N = ~10.5 s (see Fig. 1a, b).26 Our experiment with short light pulses addresses similar questions from a conformational perspective as experiments that analyzed the spectroscopic response of bR and pyrane pH-probes following short laser flashes.8 In these experiments, the rise of the N state occurred ~3.5 ms after flash. The pyrane pH-probe fluorescence decayed ~12 ms and the N state decayed ~14 ms after the laser flash. These results are in good agreement with τdelay = 2.9 ms and τopen = 13.2 ms, found here. In the TR-SMX study the closed state decayed after 4.7 ± 0.3 ms and the cytoplasmic gate open state structure peaked in the 10 ms to 15 ms bin and decayed after 23.8 ± 2.7 ms.7 Thus, our conformational measurements are in good agreement with the spectroscopic data, while the conformational changes in the 3D-crystals appear somewhat slowed.

All the above described measurements were performed at neutral pH 7. However, given that bR is a H+ pump, and that the inactivation step implicates re-protonation of D96, we next examined the opening delay times and open state dwell-times of the bR-WT at different pH (pH 5, pH 6, pH 7, pH 8 and pH 9). Interestingly, the opening delay time remains essentially unchanged over the entire pH-range, with an average value τdelay = 2.7 ± 0.3 ms (Fig. 3e), meaning that the initial steps of the reaction cycle, photo-activation of the retinal, proton release on the extracellular face, and the conformational change along helix-F to the E-F loop, were pH-independent. In contrast, the open state is pH-dependent (Fig. 3f, g): At acidic and neutral pH 5, pH 6 and pH 7, a similar short open dwell with an average time constant τopen(pH5–pH7) = 14.1 ± 1.3 ms is found, while at alkaline pH 8 and pH 9 τopen(pH8–pH9) = 34.5 ± 2.1 ms is measured (Fig. 3h). It is quite intuitive that at acidic and neutral pH, the abundance of H+ for re-protonation of the Schiff base leads to shorter open state dwell-times than at alkaline pH. However, interestingly, the dwell-time distributions show that the fastest and most uniform time constant is found at pH 7, while at lower pH, pH 6 and pH 5 the distributions have long tails (Fig. 3f). Fitting the low pH open state dwell-time distributions with two exponentials, we identify τopen-2(pH5) = 82.2 ± 4.7 ms and τopen-2(pH6) = 51.2 ± 6.1 ms (Fig. 3f, Supplementary Fig. 6). From the fit statistics, we estimate that ~10% of the molecules at low pH are in a slower photocycle with an extended open state dwell. It has been reported that at pH below 6 the proton release to the extracellular medium may directly be accomplished by Asp-85 instead of the proton releasing group (Glu-194 and Glu-204), which may lead to a slower photocycle.66 While the single molecule kinetics show that the individual molecules can inactivate fast (~13 ms, 90%) or slow (~60 ms, 10%), in ensemble
experiments an extended average open state should be found at acidic pH, ~35% slower, and therefore an overall slower H\textsuperscript{+}-pumping in all conditions different from neutral pH. Thus, bR performs best at neutral pH 7, because H\textsuperscript{+}-release and re-protonation are most efficient under such conditions\textsuperscript{46,47}.

Most important, being able to define the rise dwell time, 2.9 ms, between photon capture and opening of the cytoplasmic gate (N state) from the flash activation experiments, allows us to unambiguously assign the dwell time in the closed state minus the rise time, 40.5 ms - 2.9 ms = 37.6 ms, to the transition from the O back to the ground state (see Discussion).

**Discussion**

Since the discovery of the purple membrane and the identification of bacteriorhodopsin (bR) in *Halobacterium salinarum* (formerly *H. halobium*)\textsuperscript{1} various techniques have provided deep insights about its structure and the H\textsuperscript{+}-pumping mechanism. Traditionally, the photocycle has been studied using spectroscopic methods\textsuperscript{13–15} and more recent time-resolved crystallography studies revealed the conformational changes from the femtosecond to the millisecond regime\textsuperscript{3,7,33}.

Here, we applied HS-AFM-LS in a laser-coupled HS-AFM system to characterize the single molecule kinetics of bR. Our study resolves the single molecule rate constants of the conformational changes at millisecond temporal resolution under different light intensities and at different pH: The reaction cycle is optimized to work at high light intensity and neutral pH. In short light pulse activation experiments, we found that the conformational change opening the cytoplasmic gate in bR-WT in the native purple membrane occurred within <3 ms upon photon-capture, and the open state was ~13 ms long. In continuous saturating light experiments, the open state was also ~13 ms long independent of the light intensity, and the closed state shortened with increasing light but could not get shorter than ~40 ms.

Combining the two experiments (Fig. 4), we can calculate the dwell time to reset the molecule after closing the cytoplasmic gate.
back to the ground state, ~37.6 ms (40.5 ms closed state minus 2.9 ms opening delay time, end of N state to ground state). This is much longer than what spectroscopic measurements found, 1 ms to 10 ms, the decay time of those molecules in the ensemble that were in the given state\(^8,15\). In contrast to spectroscopic methods, our single molecule measurements capture precisely how fast an individual protomer in the purple membrane is able to perform, which crucially includes knowing how long it takes until that very molecule can reactivate, and the maximum turnover rate we found is ~20 s\(^{-1}\), mainly limited by the long reset time. Arguably, AFM studies of membrane proteins imply that the sample is deposited on a support, and a buffer layer is trapped between the membrane and the mica\(^{48}\), maintaining the structural and functional properties formed, and a buffer layer is trapped between the membrane and no chemical or biological bond between sample and surface is deposited on a support. However, in the physisorption process, AFM studies of membrane proteins imply that the sample is isomerization to all-trans retinal and stays in the open state structure for 13.2 ms (green), corresponding to state N. The green arrow highlights the movement of the E-F loop to open the cytoplasmic gate. After retinal re-protonation and isomerization to all-trans, bR relaxes to the dark state structure (red). Under continuous light at saturating conditions, the protomer stays in the closed state for 37.6 ms (40.5 ms – 2.9 ms), until it can be activated again. We propose that, during this time, the neighboring protomers cycle (2 x 2.9 ms + 13.2 ms = 32.2 ms). The structure shown are PDB 6RNJ, 6RPH and 6RQP\(^7\).

The physical distance (d) between the Lys216, where the retinal is attached and where the reaction cycle is initiated within picoseconds upon photon absorption, and Arg164 at the very top of the E-F loop is \(d \approx 30\,\text{Å}\). The E-F loop moves \(v_{\text{delay}} = 2.9\,\text{ms}\) later, thus the velocity of the allostERIC coupling through bR is \(v = d/\tau_{\text{activation}} = \approx 30\,\text{Å}/2.9\,\text{ms} \approx 1\,\mu\text{m}\cdot\text{s}^{-1}\). Another system for which we have a direct measure for the beginning and the endpoint of a conformational change are the voltage-gated ion channels: In voltage-gated ion channels, the gating currents report the movement of the voltage sensors and thus mark the starting point, while the ionic currents report the opening of the pore and therefore mark the end point of the conformational transition. The ionic currents occur ~1 ms after the gating currents, and the S4-S5 linker coupling distance is ~25 Å, giving a \(v \approx 2.5\,\mu\text{m}\cdot\text{s}^{-1}\). Thus, the allostERIC coupling speed is of similar order as in bR, and we propose single digit \(\mu\text{m}\cdot\text{s}^{-1}\) velocities as good estimates for conformational coupling velocity in membrane proteins.

At saturating photon availability, the single molecule reaction cycle in the purple membrane is \(\tau_{\text{cycle}} = 53.7\,\text{ms}\), and thus the maximum H\(^+\)-pumping rate is \(k_{\text{H}^+}\text{-pumping} = 1/(\tau_{\text{cycle}}) = 18.6\,\text{s}^{-1}\). The purple membrane can be regarded as a minimal photosynthetic apparatus, where H\(^+\)-pumping through bR fuels ATP-synthase action that generates the cellular energy currency ATP\(^{51,52}\). The ATP-synthase is constituted of two major parts, the F\(_r\) rotor ring and F\(_i\) where ATP formation takes place. F\(_i\) functions in a turbine-like manner and is fueled through the flux of H\(^+\). It consists of about 20 subunits \((\epsilon_{\text{ring}} \approx 10)\) and reaches a maximum rotation speed of \(R_{\text{ATP}\text{-synthase}} \approx 300\text{s}^{-1}\). Thus \(n \approx R_{\text{ATP}\text{-synthase}}\epsilon_{\text{ring}}/k_{\text{H}^+}\text{-pumping} \approx 150\) bR protomers (50 trimers) would be sufficient to fuel one ATP-synthase under saturating light conditions.

In this work, we used HS-AFM-LS to analyze millisecond conformational dynamics in bR. The raw data provides single molecule traces of astonishing clarity at millisecond temporal resolution (Fig. 2a, Supplementary Movie 5). Given that the method is not limited to and influenced by the placement of labels and easily
reports Angstrom-range conformational dynamics at high temporal resolution\textsuperscript{32,33}, we propose HS-AFM-LS as a most powerful method to study membrane protein single molecule kinetics in general.

**Methods**

**Sample preparation.** Purple membranes containing either bR-WT or bR-D96N mutant were isolated from Halobacterium salinarum as described previously\textsuperscript{1}. A 2 μL drop of the purple membrane sample was deposited on a 1 mm\textsuperscript{2} freshly cleaved mica surface. After incubation for 15 min, the sample was rinsed with imaging buffer (10 mM Tris-HCl (pH adjusted to the desirable value for each experiment), 300 mM KCl), and imaged with the HS-AFM.

**HS-AFM data acquisition and analysis.** All images were taken at room temperature with a sample scanning HS-AFM (RIBM, Japan) operated in amplitude modulation mode. Igor Pro version 6.37 was used for HS-AFM data collection. The cantilevers used were 8 μm long cantilevers with nominal spring constant k = 0.15 N/m and resonance frequency f\textsubscript{0} = ~600 kHz in buffer (1200 kHz nominal resonance frequency in air, USC-F1-2-k0.15, NanoWorld, Switzerland). Oxygen plasma etching was used to sharpen the tips. HS-AFM images were taken at 1-frame per second keeping the pixel-sampling ratio constant at 1.667 Å/pix. Movie alignment was carried out in ImageJ with laboratory-build scripts. HS-AFM-LS kymographs were recorded at 1.667 Å/pix and a line scan rate of 1.667 ms. When switching from 2D imaging to HS-AFM line scanning, the central line of the former 2D scan is repeatedly scanned. Typically, a protomer is centered in the 2D scan before switching to HS-AFM-LS. However, owing to the dense packing of bR in the purple membranes, a high success rate for contouring protomers is achieved, wherever the line scan is taken. In brief, we recorded 600 left-right and right-left scan lines per second and each scan line has 300 pixels. Kymographs are composed of left-right scan lines only, each one acquired during 0.833 ms, however, each protomer is only probed once every 1.667 ms. We regard 1.667 ms the deadtime of our measurement: when the conformational change of a protomer is detected for the first time within in a pixel, it could have occurred right then or within the 1.667 ms before. Therefore, we set the activating laser pulse as zero-time mark, and bin events in 1.667 ms bins, e.g., 0–1.667 ms, 1.667–3.333 ms, etc.

For illumination of the sample, a green diode laser (wavelength: 520 nm) was used. The intensity measured after the objective lens was in the range 0.2–1.0 μW. To control laser light emission, a signal generator and a laser diode driver were used (NewPort 505B laser driver).

To analyze the displacement of the protomers in HS-AFM movies we used self-written routines in MATLAB (Matlab, Mathworks, USA) and in ImageJ. First, every monomer position within each frame was detected as the maxima above a given threshold. Second, for every monomer belonging to a trimmer, the trimmer center was determined and used as the origin position to measure the monomer displacement.

To analyze the kinetics of the protomers in HS-AFM-LS height-time traces, we used self-written routines in MATLAB and the Step Transition and State Identification (STaSi) method was used for the identification of the two states\textsuperscript{34,43}. The STaSi algorithm uses hierarchical clustering to assign the optimum number of states.

To analyze the opening delay time, we recorded the photodiode signal along with the topography channel. From these two signals, we correlated exactly in time within in a pixel, it could have occurred right then or within the 1.667 ms before. Thus, the opening delay time as the time since the beginning of the pulse to the observation of the conformational change.

Laser light intensity. To determine the irradiance, we measured the laser power above the fluid cell in the HS-AFM setup, just above the cantilever position. The photon flux was calculated as:

\[
\text{Irradiance or light intensity} = \frac{\text{Power}}{A} \quad (1)
\]

\[
A = \pi r^2 \quad (2)
\]

\[
\text{Photon Flux} = \frac{\text{Irradiance}}{E_{\text{ph}}} = \frac{k(520\text{nm})}{h \cdot c} \cdot \text{Irradiance} \quad (3)
\]

However, in order to determine the activating light intensity, we also need to determine the radius (r) of the laser beam spot at the sample position. This was achieved by covering the laser beam with a 50 μm wide cantilever. In this position, the laser beam is blocked by the wide cantilever (left part of trace). Next, we recorded the laser power while moving the cantilever through the light path in 1.6 μm steps, moving the cantilever completely out of the laser path. We found that the measured laser power increased over a cantilever displacement of ~15 μm (middle part of trace), until it plateaued again (right part of trace) (Supplementary Fig. 1). From this measurement we estimated that the laser beam radius (r) was ~7.5 μm. Together with the measurement of the power meter above the fluid cell, we could estimate the irradiance in our experiments.

**Data availability**

All data supporting the findings of this study are available within the article and its supplementary information files. Data source are provided with this paper. Additional information and raw data are available from the corresponding author upon reasonable request and at the earliest convenience. Source data are provided with this paper.

**Code availability**

Data analysis was performed with custom scripts in Matlab R2018b and ImageJ FIJI v1.52. All codes are either published, ref. [36-37,89] (https://github.com/George-R-Heath/AFM-LineScan-Analyzer) and 43 (STaSi algorithm) or available from the corresponding author upon reasonable request and at the earliest convenience.

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**Author contributions**

A.P.P. and S.S. conceived and designed the experiments. A.P.P. performed the HS-AFM experiments. A.P.P. and A.M. built the HS-AFM light activation system. A.P.P. and S.S. analyzed the data. A.P.P. and S.S. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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**Correspondence**

and requests for materials should be addressed to Simon Scheuring.

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