Supporting information

A biological nanomachine at work: watching the cellulosome degrade crystalline cellulose

Manuel Eibinger\textsuperscript{1†}, Thomas Ganner\textsuperscript{2†}, Harald Plank\textsuperscript{2,3*} & Bernd Nidetzky\textsuperscript{1,4*}

\textsuperscript{1}Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Petersgasse 10-12/1, 8010 Graz, Austria.
\textsuperscript{2}Institute for Electron Microscopy and Nanoanalysis, Graz University of Technology, Steyrergasse 17, 8010 Graz, Austria.
\textsuperscript{3}Graz Centre for Electron Microscopy, Steyrergasse 17, A-8010 Graz, Austria.
\textsuperscript{4}Austrian Centre of Industrial Biotechnology, Petersgasse 14, 8010 Graz, Austria.

*Correspondence to: bernd.nidetzky@tugraz.at (B.N.); harald.plank@felmi-zfe.at (H.P.)
†These authors contributed equally to this work.
Supporting Methods

Materials

Unless stated, all chemicals were of the highest purity available from Carl Roth + Co KG (Karlsruhe, Germany).

Preparation of cellulose nanocrystals

Cellulose nanocrystals were prepared from Whatman® qualitative filter paper (Grade 1; Sigma-Aldrich) according to literature (1). Four grams of filter paper was cut into squared pieces of approximately 2 × 2 mm size and hydrolyzed in 70 mL of 64 % (w/w) of sulfuric acid for 45 minutes at 45°C. The hydrolysis was stopped by adding 10 volumes of deionized water. The pH of the suspension was adjusted to 1.9 by repeated centrifugation and washing with deionized water. Finally, a colloidal cellulose preparation was obtained by repeated 1 min-long sonification (Sonoplus; Bandelin electronic GmbH & Co. KG, Berlin, Germany) of the suspension cooled on ice. The final concentration of the cellulose nanocrystal suspension determined by weighing of the dry mass was 15 g L⁻¹. The preparation was stored at 4 °C until further use.

Characterization of cellulose nanocrystals

Cellulose nanocrystals were identified by Raman spectroscopy as allomorph Iβ (2). Wide angle X-ray scattering (WAXS) was used to determine the crystallinity index (Cᵢ) of the cellulose nanocrystal as 90% or greater (2). The morphology of the cellulose nanocrystals was determined using Transmission Electron Microscopy (TEM). The image data were fitted as a normal distribution. The average length was 127 ± 45 nm, the average width was 17 ± 6 nm. The cellulose nanocrystals are approximately rod shaped with tapering ends. The detailed experimental setup and the staining procedure used in TEM investigations were reported elsewhere (2).
The amount of sulfate half-ester groups located on the crystal surface, expressed as degree of surface substitution, was calculated to be below 0.2 (1). We monitored the change in pH upon the addition of cellulose nanocrystals in all reactions, including the set-ups for AFM measurement. The pH change was less than 0.1 pH units so that a significant effect on the enzyme activity can be excluded. The pH was measured before and after the reaction. No change within error limit (0.05 pH units) was observed.

Data fitting and peak deconvolution were performed using Origin 9 (OriginLab cooperation, Northampton, USA). Raman data were analyzed using LabSpec 6 (Horiba, Tulln an der Donau, Austria).

**General procedure of sample preparation for AFM experiments**

Wafers prepared from highly oriented pyrolytic graphite (HOPG) (grade I; SPI Supplies, West Chester, PA, USA) were used as substrate for AFM experiments. The HOPG wafer surface (12 × 12 mm) was prepared by removing the top graphite layer with adhesive tape (“adhesive tape cleaving”). This was followed by immediate incubation of the wafer with 500 µL of a cellulose nanocrystal suspension diluted to a concentration of 0.5 g L\(^{-1}\) with deionized water. Note that incubation conditions were chosen to ensure the presence of mostly individual, non-aggregated cellulose nanocrystals on the HOPG wafer. After 10 min of incubation, the HOPG wafer was rinsed with 10 mL of deionized water, dried by CO\(_2\) spraying and mounted on a vacuum chuck for the following AFM experiments.

**Sample preparation for AFM experiments on a pre-structured silicon wafer**

Pre-cut silicon wafers (10 × 10 mm) with a 5 nm SiO\(_2\) layer were kindly provided by AMS AG (Unterpremstätten, Austria). The silicon wafers were processed by focused ion beam (FIB) milling to obtain a micron-sized squared grid (2 × 2 µm) composed of four isolated segments. FIB milling was done using a FIB Nova 200 (FEI Company, Eindhoven, The Netherlands) with primary ion beam energies and beam currents of 30 kV and 30 pA,
respectively. An appropriately adapted bitmap image was used for milling the pattern with 1024 × 1024 pixels and a pixel dwell time of 100 μs (single pass).

Thus pre-structured silicon wafers were used as substrate for AFM experiments (Fig. S6) in which the degradation of cellulose nanocrystals was monitored over extended times (20 h or longer). Note that the surface pre-structuring was essential to trace individual cellulose nanocrystals before and after the incubation.

To attach cellulose nanocrystals to the surface of the silicon substrate, the FIB-processed silicon wafer were rinsed with isopropanol and deionized water. This was followed by immediate incubation with 300 µL of a cellulose nanocrystal suspension with a concentration of 7.5 g L⁻¹. After 3 min of incubation, the silicon wafer was again rinsed with deionized water, dried by CO₂ spraying and stored in a nitrogen atmosphere until further use.

**Enzyme preparations**

*Clostridium thermocellum* ATC 27405 (3,4) was grown anaerobically in GS-2 media (5) containing resazurin as redox indicator. Avicel PH-101 (5 g L⁻¹) was the sole carbon source and a total volume of 50 mL was used. The cultivation was done at 60 °C with shaking (100 rpm) in 100 mL sealed bottles. Prior to autoclaving and inoculation, the media were sparged with N₂ until the resazurin turned colorless, thus showing the absence of oxygen.

The culture was harvested once the Avicel PH-101 substrate had been largely dissolved (approximately 60 h). Cells and other solids were removed by centrifugation (4500 g, 30 min). The cleared supernatant was filtered through 0.2 μm disposable syringe filter (Sartorius, Germany). The buffer was exchanged to 30 mM MOPS pH 7.0, 100 mM NaCl and 10 mM CaCl₂ using a disposable Vivaspin® (Sartorius, Germany) ultrafiltration device with a 300 kDa molecular mass cut-off. The original volume was concentrated about 10-fold by the ultrafiltration. Size exclusion chromatography was used for isolation of the cellulosomes. A GE Healthcare (Little Chalfont, United Kingdom) FPLC system equipped with a HiLoad® 16/60 Superdex 200 prep grade column was used. The column was equilibrated with 30 mM
MOPS pH 7.0, 100 mM NaCl and 10 mM CaCl₂. It was operated at room temperature and a flow rate of 1 mL min⁻¹. Fractions containing the cellulosomes were pooled, concentrated and stored at 4°C. Analysis of the cellulosome preparation by SDS PAGE revealed a pattern of protein bands (Fig. S7) fully consistent with the literature (6,7).

Complete (noncomplexed) cellulase was obtained from the supernatant of the fungus Trichoderma reesei (strain SVG17) grown on wheat straw as reported previously (8). Clarified cellulase preparation was stored at 4°C supplemented with 0.05% w/v sodium azide. TrCel7A was isolated from the cellulase preparation using reported methods (1) and the apparent homogeneity of the enzyme was demonstrated as described in earlier work (1). β-Glucosidase from Aspergillus niger was from Megazyme (Dublin, Ireland) and stored at 4°C. Protein concentration was measured using a commercial BSA kit (Thermo scientific, Waltham, MA, USA).

**Soluble sugar release on enzymatic conversion of cellulose nanocrystals**

Experiments were performed as biological duplicates or triplicates. A freshly prepared 30 mM sodium acetate buffer, pH 5.5, containing 100 mM NaCl, 10 mM CaCl₂, 10 mM cysteine and 2 mM EDTA was used for cellulosome reactions. A 50 mM sodium acetate buffer, pH 5.0, was used for cellulase reactions. Enzyme comparisons were made in the buffer for the cellulosome reaction. Reactions were carried out in a total volume of 750 µL or 1.8 mL at 50 °C and 500 rpm using an Eppendorf Thermomixer comfort (Eppendorf AG, Hamburg, Germany). The concentration of cellulose nanocrystals was 1.0 or 2.0 g L⁻¹. β-Glucosidase was added to a concentration of 1 µg mL⁻¹ to all reactions. Reactions were started by adding enzyme to a final substrate loading of 5 mg or 10 mg g⁻¹ substrate⁻¹.

Samples were taken at suitable times. For sampling, 50 to 100 µL of the well-mixed suspension was withdrawn. This was mixed with 50 µL of 100 mM sodium hydroxide to stop the reaction. Subsequently, the sample was centrifuged (9,400 g × 5 min) at 8 °C. The cleared
supernatant was analyzed for glucose using a commercial assay (Fluitest GLU HK, Analyticon Biotechnologies AG, Lichtenfels, Germany).

**AFM characterization of enzymatic degradation of cellulose nanocrystals**

Experiments were conducted as duplicates at 50 °C. Incubations were done in a water bath under mild agitation. The total reaction volume was 2 mL and sealed glass vials were used for incubation. A freshly prepared 30 mM sodium acetate buffer (pH 5.5) containing 100 mM NaCl, 10 mM CaCl₂, 10 mM cysteine and 2 mM EDTA was used. The pre-structured silicon wafer having cellulose nanocrystals adsorbed to its surface was used as substrate. The silicon wafer was equilibrated in buffer for 30 min prior to adding the enzyme. The enzyme concentration was 7.5 μg mL⁻¹. Cellulosome or *T. reesei* cellulase was used. β-Glucosidase was added to a concentration of 1 μg mL⁻¹. The reaction was stopped after 30 h by removing the supernatant, rinsing the silicon wafer with deionized water and drying it for one day at 70 °C. Negative controls did not contain enzymes except for β-glucosidase.

**Modification of AFM tips**

This was required for single molecule analysis of the cellulosome at elevated temperature. AFM tip-sample interaction plays a crucial role in imaging (9). Image quality can be significantly lowered by sticking of the AFM tip to the scanned surface or sample molecules attaching to the AFM tip, respectively (10).

Preliminary AFM experiments using cellulosomes showed a severe decrease in image quality over time rendering continuous measurements impossible. In particular, we observed pronounced peak broadening (apparent structure width was enhanced ≥ 5-fold) and a frequently occurring loss of surface contact with the probe. Altering buffer conditions (e.g., adapting salt concentrations) to positively affect electrostatic interactions or using PeakForce Tapping®, where the applied force is tightly controlled in the nano- to pico-Newton regime, showed no improvement. SEM analysis (not shown) of cantilevers (made of Si₃N₄) which
were in contact with cellulosome preparations, hinted at the possibility that cellulosomes or aggregates thereof are attracted to the AFM tip.

In view of this, we performed a surface modification of the FastScan D cantilevers (Bruker AXS, Santa Barbara, CA, U.S.A.). The strategy was to introduce a self-assembling hydrophobic monolayer of octadecyltrichlorosilane (OTS) on the tip surface to reduce the apparent cellulosome-AFM tip interaction.

Modification of FastScan D cantilevers was performed according to Wei and coworkers (10) with some changes described in more detail below. Prior to the modification the tips were cleaned using a series of hydrochloric acid/sodium hydroxide washings that were followed by heating and repeated washings with deionized water and absolute ethanol (10).

Immediately after cleaning, the tips were immersed in toluene containing 5 mM OTS for 20 min at room temperature. After incubation, the tips were carefully rinsed with toluene and transferred into a fresh toluene washing solution. After 5 min, OTS coated tips were transferred again and washed in absolute ethanol and deionized water, respectively, to remove unbound material. The duration of each washing step was 5 min and the tips were carefully moved in the respective washing solutions. Processed tips were dried for 20 min at 120 °C and stored in a nitrogen atmosphere. According to earlier published results (10) such modified tips are stable for an extended period of time.

**Time-lapse AFM experiments**

All AFM experiments were carried out using a FastScan Bio Atomic Force Microscope (Bruker AXS, Santa Barbara, CA, U.S.A.) operated by a Nanoscope V controller. Time-lapse experiments were conducted in a 60-µL flow cell (Bruker AXS, Santa Barbara, CA, U.S.A.). Modified FastScan D cantilevers were used in tapping mode with a nominal spring constant of 0.25 N m⁻¹, a tip opening angle of about 15° and a nominal tip radius of 5 nm.

Prior to continuous image acquisition, the flow cell covering the HOPG wafer was carefully rinsed with reaction buffer (30 mM sodium acetate buffer (pH 5.5) containing, 100 mM NaCl,
10 mM CaCl$_2$, 10 mM cysteine and 2 mM EDTA) using an in-house built syringe-driven injection system until the system was devoid of macroscopic air bubbles. The system was equilibrated for at least 1 h at 50 °C and multiple reference images of varying size were recorded. Continuous image acquisition was started by carefully rinsing the cell with 200-250 µL buffer solution pre-heated to 50 °C containing cellulosomes (25 µg mL$^{-1}$).

The scan rate was varied between 0.5 and 2 frames/min and the pixel count per line was adjusted to obtain a final resolution of 2.5 nm/pix or better. Suitable ranges for parameters, in particular parameters controlling the force load on the sample (e.g. set point amplitude), were selected after preliminary experiments to ensure stable observations and prevent the cellulosomes from being damaged by the tip (Fig. S2). Note: although AFM operation settings cannot be readily converted into the applied force load, a similar range of settings using a related cantilever was previously used successfully for the visualization of individual noncomplexed cellulases on cellulose nanocrystals (1). Briefly, we used small oscillation amplitudes of less than 50 nm (drive amplitude control) and amplitude setpoints of 70 - 90%. Thus, the phase shift was typically kept below 20°, which is sufficient for the visualization of fragile proteins (11). We also excluded influence of the scan rotation, apart from occasionally occurring tracking problems, as typical for low force AFM operation in liquids. Also note that, cellulose nanocrystals are more robust than enzymes and are not damaged by using AFM settings suitable for protein analysis.

Additionally, scanning parameters were adapted during time-lapse AFM observations as required.

AFM image processing and analysis was performed using Gwyddion 2.5 and Nanoscope Analysis 1.50 (Build R2.103555, Bruker AXS, CA, USA). Images were plane fitted at 1st order if applicable (reference images covering areas ≥ 1 µm$^2$) and a median or Gaussian filter was applied unless otherwise stated.
Identification of cellulosomes during time-lapse AFM experiments

Identification of cellulosomes on the surface was done mainly by using the height channel. Typically, cellulosomes and their subunits had an apparent height of $\geq 2$ nm and thus are clearly distinguishable from the surrounding material using a reference image without a cellulosome. Previously, we analyzed TrCel7A on HOPG and calculated average heights of 1.8 nm and 2.8 nm for the cellulose binding module and the catalytic domain, respectively (1). Although this data was obtained at room temperature and from non-moving objects, it provides a reasonable estimation for the apparent heights of a typical cellulase (TrCel7A: $\sim 65$ kDa) using our setup. By comparison, the average molecular mass of the cellulosome-incorporated enzymes is about 80 kDa, calculated from data in the literature (12). Factoring in the challenging environment (thermal sample drift), mobility and flexibility of the cellulosome, we concluded that a height cut-off $\geq 2$ nm would be sufficient to allow for a reasonable distinction between cellulosomes and the pre-characterized cellulose/HOPG background.

Additionally, the location of cellulosomes was confirmed by using the property-sensitive phase information, which shows clear difference between rigid cellulosic material and rather soft biological matter (see Fig. 2A and ref. (13)). If required, amplitude images were used additionally to determine the area covered by the cellulosomes. Detailed examples of this procedure based on the images used in Movie S1 are shown in Figure S7 and S8.

AFM data failing in any of the steps of rigorous analysis were excluded from consideration for single-molecule identification. Despite our best efforts, some of the observed cellulosomes ($\leq 20\%$), however, appear distorted without exactly defined borders. The most likely origins of these imaging problems are (i) the flexibility of the cellulosome itself (14), a feature inherent in many other biomolecules (15,16), and (ii) the challenging AFM measurements at elevated temperature (thermal sample drift) (16).
AFM observations for the cellulosome were validated by a series of rigorous controls. The control lacking enzyme addition did not show particles in any way similar to the cellulosome. Other controls used various enzymes that bind (T. reesei cellulases; purified TrCel7A), or do not bind (β-glucosidase), to cellulose. The enzymes were applied in a protein concentration comparable to the cellulosome experiment. None of these controls showed protein structures (e.g. aggregates) comparable to the observed cellulosomes. Cellulases are occasionally observed as clusters on the cellulose surface (see reference 1), but these clusters differ in size and shape from, and show much higher dynamics on the cellulose surface than, the cellulosome. In summary, identification of the cellulosomes in AFM data was highly reliable.

**Assembly of time-lapse movies**

Movies were assembled from single frames using Fiji (ImageJ 1.51 g), as reported earlier (1). Frames showing insufficient tracking stability, mostly due to error lines, were omitted for convenient viewing. Although these frames are not shown in the movies, they were included in the analysis of the surface-bound residence time of the cellulosome, as long as they were technically reliable. Note that we were able to clearly identify cellulosomes that had undergone damage due to the force applied (see Fig. S2). The final videos were carefully checked for complete and reliable information presented.

**AFM measurements on the pre-structured silicon substrates**

Imaging of pre-structured silicon wafers coated with cellulose nanocrystals was conducted at ambient conditions in air using a non-modified FastScan C cantilever in tapping mode. Reference images of the entire micron sized grid (Fig. S6) and high-resolution images (~1nm/pix) of individual segments were obtained before and after incubation, as described above (see **AFM characterization of enzymatic degradation of cellulose nanocrystals**).

Prior to image acquisition after the enzymatic reaction it was necessary to remove adsorbed enzymes/enzyme fragments and salt crystals from the wafer. This was essential to allow for high-resolution imaging. The washing was performed by immersing the silicon wafer in 1 M
sodium hydroxide in 50% (v/v) isopropanol/deionized water. After 10 min of incubation, the silicon wafer was rinsed with deionized water, dried via CO₂ spraying and immediately subjected to image acquisition. Experiments at varied incubation time for the washing step showed that under the conditions used the amount of cellulose nanocrystals detaching from the wafer was minimal. We also showed that modification of the nanocrystals’ shape and size due to the surface cleaning procedure could be excluded. This is in line with literature (17).

Image processing and analysis of crystal length was done using Gwyddion 2.5.

**Safety Statement**

No unexpected or unusually high safety hazards were encountered.
Fig. S1. AFM images of cellulosomes adsorbed on a wafer. (A) Individual cellulosomes and their ultrastructural organization were recognized in the height channel (bottom panel) and the number of subunits plus the covered surface area was quantified (top panel). (B) Additional examples of individual cellulosomes or cellulosome clusters used for evaluation. (C) Distribution of individual subunits per recognized cellulosome excluding cellulosome clusters. Images were recorded at ambient conditions in air after 12 hours of drying at 70 °C on a silicon wafer. The resolution is ~ 1 nm/pix.
Fig. S2. AFM observation at 50 °C with varying AFM operation settings to study single cellulosomes adsorbed on silicon support. Image acquisition rate was 0.2 frames/min. (A) A single cellulosome (outlined in green) with a rotund shape is visualized using modified tips and typical setting regarding the applied force load. (B) Increasing the amount of force exerted via the cantilever for a single frame by increasing the cantilever drive amplitude (~excitation of the cantilever) 4-fold results in destruction/unfolding of the cellulosome. (C) Restoring the initial operation settings neither promotes the refolding nor stops further unfolding of a damaged cellulosome (outlined in green). However, it allows for visualization of new cellulosomes (outlined in magenta) entering the scanning area. Insets show the data without applying a Gaussian filter. The applied color scale corresponds to heights between 2 and 12 nm for all images. Scale bars are 100 nm.
Fig. S3. Shape analysis of cellulosomes on HOPG. Individual cellulosomes (N = 26) were tracked during time-lapse observations and the ratios of the inscribed and circumscribed circles were calculated to give the “degree of deflection” (DoD). Values of DoD approaching unity indicate a circular shape while larger DoD values indicate a more elongated appearance. The inset shows a replica of Fig. 2D (cellulosome shapes on cellulose nanocrystals) to allow for an easier comparison.
Fig. S4. The mode of action of noncomplexed cellulases in deconstructing cellulose nanocrystals. (A) An exemplary cellulose nanocrystal after 30 h of incubation in the presence of enzyme at 50 °C. Cross section profiles were collected along the dotted lines before (blue, pink) and after the incubation (green, cyan). An identical color scale was used for both images. (B) The crystal length (upper panel) remains unchanged, the height along the longitudinal crystal axis is slightly decreased (~2 nm) and no formation of cavities or of beveled ends (see Fig. 4D and 4E for comparison) is observed. The preferred attacking site for noncomplexed cellulases is located along the lateral axis (lower panel, indicated by a yellow arrow) leading to thinning of the entire crystal. (C) Length distribution of ~300 cellulose nanocrystals before (blue) and after (green) 30 h of incubation with the cellulases. Contrary to earlier observations made with the cellulosomes (Fig. 4C), the distribution becomes broader.
after incubation with the free cellulases and does not involve accumulation of intermediately sized nanocrystals. Scale bars are 20 nm.
Fig. S5. Activity of noncomplexed cellulases, cellulosomes and TrCel7A on cellulose nanocrystals. (A) Hydrolysis of cellulose nanocrystals by either TrCel7A (black circles) or a complete set of noncomplexed cellulases (open circles). The substrate concentration was 2 g L⁻¹. Reactions were conducted in 50 mM sodium acetate buffer, pH 5.0, in a total reaction volume of 1.8 mL. The enzyme loading was 10 mg g⁻¹ of substrate. Standard deviation was calculated from triplicates. (B) Hydrolysis of cellulose nanocrystals by either noncomplexed cellulases (open circles) or cellulosomes (black circles). Reactions were conducted in 30 mM sodium acetate buffer, pH 5.5, containing 100 mM NaCl, 10 mM CaCl₂, 10 mM cysteine and 2 mM EDTA in a total reaction volume of 750 µL. The substrate concentration was 1 g L⁻¹ and the enzyme loading was 5 mg g⁻¹ of substrate. Standard deviation was estimated from biological duplicates. All reactions were conducted at 50 °C with orbital shaking (500 rpm) and β-glucosidase was added to a final concentration of 1 µg mL⁻¹. The specific activity was determined as 0.18 U mg⁻¹, 0.1 U mg⁻¹ and 0.05 U mg⁻¹ for the cellulase, TrCel7A and the cellulosome, respectively. The activities are determined from the amount of released glucose over the first 2.5 hrs (cellulosome and cellulase) or 3.5 hrs (TrCel7A. For comparison, the specific activities of the cellulase, TrCel7A and the cellulosome on a standard
microcrystalline cellulose substrate (Avicel PH-101) recorded under similar conditions were 0.65 U mg\(^{-1}\) (18), 0.07 U mg\(^{-1}\) (19) and 0.4 U mg\(^{-1}\) (18), respectively.
Fig. S6. Exemplary AFM image of a pre-structured silicon wafer. Topography image of cellulose nanocrystals attached to a pre-structured silicon wafer. Nanocrystals located in the central squares (1 × 1 µm each) were analyzed regarding their length. Scale bar is 1 µm.
Fig. S7. Purified cellulosomal protein obtained from *C. thermocellum* separated by SDS-PAGE (8%) and stained with Coomassie Brilliant Blue (Thermo Fisher Scientific, Waltham, MA, USA). Potential candidate enzymes, which are also highly abundant in cellulosomes obtained from cells grown on microcrystalline cellulose, for prominent gel band were tentatively assigned based on their molecular weight (7,12).
Fig. S8. Exemplary identification of a cellulosome. The image sequence was also used for the first 3.5 min of Movie S1. For each individual frame used throughout the movie, at least one additional channel with completely unedited raw data is provided. Typically, the information
regarding the localization, and the principal shape, were derived from the height channel by comparing the actual frame with a reference image recorded prior to the addition of cellulosome. Additional confirmation was provided by using the material property sensitive phase information (see panels A, B, D, E and F). In, both, panel A and D two particles (indicated by blue crosses) were excluded from this particular analysis, because neither they are present in the next frame nor the are unambiguously attached to the crystal. Note that panel B also includes the amplitude channel, which provides enhanced contrast along the edges. This allowed us to further confirm the location of the cellulosome on the crystal (magenta frame, green arrow). The cellulosome of interest located on the cellulose nanocrystal is typically highlighted in green. Scale bars are 25 nm. False color scale for unedited height images is included for each image sequence.
Fig. S9. Exemplary identification of a cellulosome (continued). The image sequence presented here was also used for the second half (4.5 – 8.0 min) of Movie S1. For each individual frame used throughout the movie, at least one additional channel with completely unedited raw data is provided. Typically, the information regarding the localization, and the principal shape, were derived from the height channel by comparing the actual frame with a reference image (see Fig. S8A) recorded prior to the addition of cellulosome. Material
property sensitive phase information was also used in specific cases (panel B and D) to assign fitting borders for the cellulosomes. In panel A, a cellulosome located close to the cellulose nanocrystal was excluded from further analysis (indicated by a blue cross). This is because, as shown in an expanded height image, the cellulosome is attached in a sickle-like shape (red frame, magenta arrow) to a neighboring crystal. (C) Identification of the leaving cellulosome (encircled in green) and of the approaching cellulosome (encircled in magenta) was done in both cases by using only the height frame. (D) Once the approaching cellulosome is attached to the top site of the crystal, a shift in the phase angle was observed, which was further used to confirm the cellulosome’s location. The cellulosomes of interest located on the cellulose nanocrystal are typically highlighted in green. Scale bars are 25 nm. False color scale for unedited height images is included for each image sequence.
Supporting Movies

Movie S1 (separate file). Time-lapse AFM observation of multiple cellulosomes with partly resolved ultrastructure adsorbing and desorbing to a cellulose nanocrystal at 50 °C. In the first minute an elliptically shaped cellulosome (encircled in green) attaches to a cellulose nanocrystal and alters its shape to a filamentous form. The partly resolved ultrastructure is indicated at the 1.0 min mark with yellow arrows. The flexibility regarding the ultrastructure can be seen until the 5 min mark. Desorption can be observed at the 7.5 min mark for the cellulosome (encircled in green) located around the upper crystal tip. Simultaneously, a new cellulosome is adsorbing to the central part of the crystal (encircled in magenta). At the 8.0 min mark the contact area between the crystal surface and the approaching cellulosome has already increased (indicated by a white arrow). Image acquisition rate and resolution was 2 min\(^{-1}\) and 2 nm/pix, respectively. Scale bar and false color scale are included in the video.

Movie S2 (separate file). Time-lapse AFM observation of a single cellulosome at 50 °C adsorbed to a cellulose nanocrystal with varying shape and ultrastructural organization located near a nanocrystal’s tip for ~ 30 min. Image acquisition rate and resolution was 0.5 min\(^{-1}\) and 2.5 nm/pix, respectively. Scale bar and false color scale are included in the video.

Movie S3 (separate file). Time-lapse AFM observation of a single cellulosome (encircled in green in the first frame) adsorbing and partially degrading a cellulose nanocrystal at 50 °C. The initial crystal tip perimeter is outlined in magenta and projected onto the last image of the sequence. Image acquisition rate and resolution was 2 min\(^{-1}\) and 2 nm/pix, respectively. Scale bar and false color scale are included in the video.
References

1. Eibinger, M.; Sattelkow, J.; Ganner, T.; Plank, H.; Nidetzky, B. Single-molecule study of oxidative enzymatic deconstruction of cellulose. *Nat. Commun.* 2017, 8, 894.

2. Ganner, T. et al. Tunable semicrystalline thin film cellulose substrate for high-resolution, *in-situ* AFM characterization of enzymatic cellulose degradation. *ACS Appl. Mater. Interfaces* 2015, 7, 27900–27909.

3. Resch, M. G. et al. Clean fractionation pretreatment reduces enzyme loadings for biomass saccharification and reveals the mechanism of free and cellulosomal enzyme synergy. *ACS Sustain. Chem. Eng.* 2014, 2, 1377–1387.

4. Lamed, R.; Bayer, E. Cellulosomes from *Clostridium thermocellum*. *Methods Enzymol.* 1988, 160, 472–482.

5. Artzi, L.; Dadosh, T.; Milrot, E.; Moraïs, S.; Levin-Zaidman, S.; Morag, E.; Bayer, E. A. Colocalization and disposition of cellulosomes in *Clostridium clariflavum* as revealed by correlative superresolution imaging. *MBio* 2018, 9, e00012-18.

6. Resch, M. G.; Donohoe, B. S.; Baker, J. O.; Decker, S. R.; Bayer, E. A.; Beckham, G. T.; Himmel, M. E. Fungal cellulases and complexed cellulosomal enzymes exhibit synergistic mechanisms in cellulose deconstruction. *Energy Environ. Sci.* 2013, 6, 1858.

7. Gold, N. D.; Martin, V. J. J. Global view of the *Clostridium thermocellum* cellulosome revealed by quantitative proteomic analysis. *J. Bacteriol.* 2007, 189, 6787–6795.

8. Eibinger, M.; Bubner, P.; Ganner, T.; Plank, H.; Nidetzky, B. Surface structural dynamics of enzymatic cellulose degradation, revealed by combined kinetic and atomic force microscopy studies. *FEBS J.* 2014, 281, 275–290.

9. Hinterdorfer, P.; Dufrène, Y. F. Detection and localization of single molecular recognition events using atomic force microscopy. *Nat. Methods* 2006, 3, 347–355.
10. Wei, Z. Q.; Wang, C.; Bai, C. L. Surface imaging of fragile materials with hydrophobic atomic force microscope tips. *Surf. Sci.* **2000**, *467*, 185–190.

11. Uchihashi, T.; Kodera, N.; Ando, T. Guide to video recording of structure dynamics and dynamic processes of proteins by high-speed atomic force microscopy. *Nat. Protoc.* **2012**, *7*, 1193–1206.

12. Doi, R. H.; Kosugi, A. Cellulosomes: plant-cell-wall-degrading enzyme complexes. *Nat. Rev. Microbiol.* **2004**, *2*, 541–551.

13. Ando, T.; Uchihashi, T.; Fukuma, T. High-speed atomic force microscopy for nanovisualization of dynamic biomolecular processes. *Prog. Surf. Sci.* **2008**, *83*, 337–437.

14. Currie, M. A.; Cameron, K.; Dias, F. M. V.; Spencer, H. L.; Bayer, E. A.; Fontes, C. M. G. A.; Smith, S. P.; Jia, Z. Small angle X-ray scattering analysis of Clostridium thermocellum cellulosome N-terminal complexes reveals a highly dynamic structure. *J. Biol. Chem.* **2013**, *288*, 7978–7985.

15. Hansma, H. G.; Hoh, J. H. Biomolecular imaging with the atomic force microscope. *Annu. Rev. Biophys. Biomol. Struct.* **1994**, *23*, 115–140.

16. Dufrène, Y. F.; Ando, T.; Garcia, R.; Alsteens, D.; Martinez-Martin, D.; Engel, A.; Gerber, C.; Müller, D. J. Imaging modes of atomic force microscopy for application in molecular and cell biology. *Nat. Nanotechnol.* **2017**, *12*, 295–307.

17. Knill, C. J.; Kennedy, J. F. Degradation of cellulose under alkaline conditions. *Carbohydr. Polym.* **2003**, *51*, 281–300.

18. Ng, T. K.; Zeikus, J. G. Comparison of extracellular cellulase activities of *Clostridium thermocellum* LQRI and *Trichoderma reesei* QM9414. *Appl. Environ. Microbiol.* **1981**, *42*, 231–240.

19. Guo, Z.; Duquesne, S.; Bozonnet, S.; Cioci, G.; Nicaud, J.-M.; Marty, A.; O’Donohue, M. J. Conferring cellulose-degrading ability to *Yarrowia lipolytica* to facilitate a consolidated bioprocessing approach. *Biotechnol. Biofuels* **2017**, *10*, 132.
