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COMMUNICATION

Determination of cellulase colocalization on cellulose fiber with quantitative FRET measured by acceptor photobleaching and spectrally unmixing fluorescence microscopy

Liqun Wang,[a] Yiqing Wang[b] and Arthur J. Ragauskas*[a]

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The determination of cellulase distribution on the surface of cellulose fiber is an important parameter to understand when determining the interaction between cellulase and cellulose and/or the cooperation of different types of cellulases during the enzymatic hydrolysis of cellulose. In this communication, a strategy is presented to quantitatively determine the cellulase colocalization using the fluorescence resonance energy transfer (FRET) methodology, which is based on acceptor photobleaching and spectrally unmixing fluorescence microscopy. FRET monitoring of cellulase colocalization was achieved by labeling cellulases with an appropriate pair of FRET dyes and by adopting an appropriate FRET model. We describe here that the adapted acceptor photobleaching FRET method can be successfully used to quantify cellulase colocalization regarding their binding to a cellulose fiber at a resolution <10 nm. This developed quantitative FRET method is promising for further studying the interactions between cellulase and cellulose and between different types of cellulases.

Introduction

Cellulases, naturally produced by a variety of fungi and bacteria, are generally classified into three categories based on their hydrolytic functionalities:[1] endoglucanases that degrade the cellulose polymer chain randomly at internal positions; cellobiohydrolases that release disaccharide cellobioses at the reducing or non-reducing ends of cellulose; and β-glucosidases that cleave cellobiose to glucose monomer. Cellulase is widely used in the textile industry, laundry detergents, pulp and paper industry as well as for food processing and pharmaceutical applications.[2,3] In recent years, its use to deconstruct pretreated cellulosic biomass into simple sugars for subsequent fermentation to biofuels has been intensively investigated.[1] To maximize sugar yields at very low cost, considerable research effort has been made in the past few decades to study the fundamental interactions between cellulase and cellulose and between different types of cellulases,[4] including the adsorption of cellulase to cellulose,[5–7] the competition and synergy among cellulases[8–10] *etc.* Yang *et al.* summarized in their recent review article, due to the complexity of both cellulose substrate and cellulases, that the mechanism of enzymatic hydrolysis of the cellulose substrate and the relationship between the substrate structure and function of cellulases are still not well understood.[4] Further studies and improved analytical tools are needed to better characterize the interactions between cellulose and cellulases and between cellulases.[4]

Non-complex cellulases, such as *Trichoderma reesei* (*T. reesei*) cellulases, have a head–tail shape.[1] They are formed by an ellipsoidal head—catalytic domain (CD) with a diameter ~4.5 nm and a helical tail consisting of a carbohydrate-binding domain (CBM) and a glycosylated peptide linker.[1] Researchers believe that CBM binds to the substrate and CD depolymerizes the carbohydrate polymers.[1,4,11] The fundamental interactions between cellulase and cellulose and between cellulases have seen increasing investigation and notable accomplishments have been reported. Zhang and Lynd have recently reviewed function modes of different types of cellulases and their synergism, cellulase adsorption, and the effect of substrate properties on the cellulase–cellulose interaction, such as degree of polymerization, crystallinity, accessible area, and lignin content.[11] However, analytical methods which can visualize the distribution and interactions of cellulases on the cellulose substrate have not sufficiently developed. Future advances in this field to investigate the interactions between cellulases will continue to benefit from improved, high resolution imaging techniques that can illustrate cellulase–cellulose interactions at a molecular level. Fluorescence microscopy is a technique that has been shown to be capable of tracing single molecular interactions due to its high sensitivity and is frequently applied in the bioanalytical fields. Researchers have recently examined the fluorescence imaging technique to study the cellulase–cellulose interactions. For example, the binding kinetics of fluorophore labeled-cellulase and CBD to the cellulose substrate was directly studied through the fluorescent image of the cellulose substrate by Moran-Mirabal *et al.*[12]

Super-resolution fluorescence microscopy techniques, such as stimulated emission depletion microscopy (STED), saturated structure illumination microscopy (SSIM), photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), have been developed rapidly in the past several

[a]*BioEnergy Science Center, Institute of Paper Science and Technology, School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA, 30332, USA. E-mail: Arthur.Ragauskas@chemistry.gatech.edu; Fax: +1-404-894-4778; Tel: +1-404-894-9701*
[b]*Department of Biomedical Engineering, Emory University, Atlanta, GA, 30322, USA*

years, which have been reported to have a lateral resolution down to 20–30 nanometres.[13–15] These techniques have an obvious advantage for tracing or mapping single molecule components or dissecting the inner architecture of many subcellular structures. FRET is an alternative fluorescence method typically used to investigate the interactions and measure the distance between two molecules.[16] FRET is an optic phenomenon involving an irradiative energy transfer that occurs at distances 10 nm or less between donor and acceptor fluorophores. The steep distance dependence of FRET efficiency makes it useful as a nanoscale ruler.[16–18] It is a technique that has been popularly employed in the biological and biophysical fields to study the spatial interactions between biomolecules,[17] such as mapping protein–protein and protein–substrate interactions.[19] FRET can be quantitatively assessed in several ways, such as sensitized emission—indirect excitation of an acceptor by direct excitation of a donor, and acceptor photobleaching—the donor is de-quenched after irreversible photobleaching of the acceptor.[17,20,21] Synergy and competition of different cellulases have been studied by many researchers,[22] however, how the different cellulases interact with each other, *e.g.* how they distribute or orientate on the fiber surface at a molecular level is not clear yet. Though there are many advanced techniques for nanoscale high resolution fluorescence imaging, in this communication, we present a fluorescence microscopy technique coupled with acceptor photobleach FRET as a powerful tool to quantitatively study cellulase colocalization on the surface of a cellulose fiber.

In the presented work, cellulase was covalently labeled with a fluorescence resonance energy transfer donor (Alexa Fluor 488 succinimidyl ester) or acceptor (Alexa Fluor 568 succinimidyl ester) and subsequently the labeled cellulases were used as the donor and acceptor in the FRET assay. After incubating cellulose fibers in an aqueous solution containing cellulase–donor and cellulase–acceptor conjugates, the resulting fibers were examined with a fluorescence microscope to determine if the FRET phenomenon could be observed between the donor–cellulase and acceptor–cellulase on the surface of the cellulosic fiber (Fig. 1(a)). To determine the FRET efficiency, we used the acceptor photobleaching methodology coupled with a spectral image unmixing technique.[23] This coupling procedure has been reported to have several benefits in the literature. For instance, only a single sample is required, and bleed-through in excitation and cross-talk in emission, which are common FRET problems and need to be carefully corrected in the sensitized method, are overcome in the photobleaching methodology.[23,24] Using this approach the colocalization of cellulases on the cellulose fiber was successfully observed on the scale of several nanometres, and the distribution distances between cellulases were calculated in selected regions of cellulosic fibers.

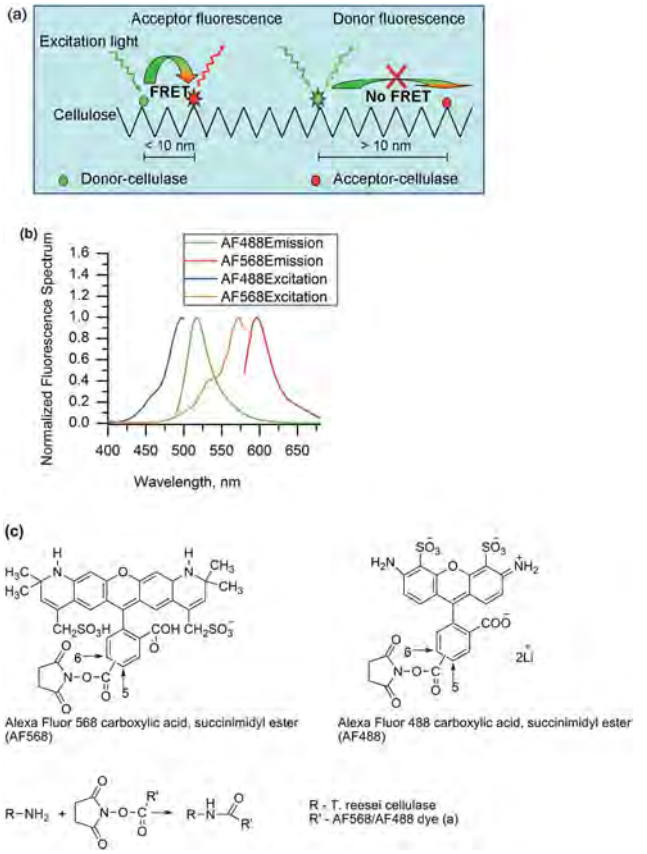

Fig. 1 (a) Demonstration of FRET phenomena when donor-labeled-cellulases are at a close distance (usually <10 nm) to acceptor-labeled-cellulases after they bind to the cellulose fiber; (b) normalized absorption and emission spectra of fluorescence dyes AF488 and AF568 grafted on *T. reesei* cellulase; (c) schematic labeling of *T. reesei* cellulase.

Experimental

Materials and apparatus

Tricoderma reesei (*T. reesei*) cellulase, with an activity of 6 units per mg (6.0 μmol of glucose from cellulose in 1 h at pH 5.0 at 37 °C), was obtained from Sigma-Aldrich (MO, USA). Alexa Fluor 488 succinimidyl ester (AF488) and Alexa Fluor 568 succinimidyl ester (AF568) were purchased from Molecular Probes (Invitrogen, CA, USA). A PD-10 desalting column was acquired from GE Healthcare. Dialysis tubing was obtained from Spectrum Laboratories (MWCO 3500-5000 D). Phosphate buffer (PBS) 10× (pH 7.4) was acquired from G-Biosciences and diluted 10 times (PBS 1×) with D.I. water. Poplar samples were procured from the National Renewable Energy Laboratory (NREL) that had been harvested in 2007 and 2008 from area 0800 at Oak Ridge National Laboratory, TN. The samples were then shipped to NREL in Golden, CO, air dried, and debarked. This substrate was then Wiley milled to pass a 20 mesh screen and kept at slightly below 0 °C.

Optical instrumentation

A wide-field Olympus 1X71 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a mercury light source and coupled with a Nuance multispectral imaging system (Nuance 2.8. Cri, MA, USA) was utilized to collect the spectral fluorescence images. UV absorption measurements were made using a UV/Vis, Shimadazu 2450 spectrometer, Japan. The absorption spectra were acquired from 200 to 800 nm with a scan rate and step size of 1 s per point and 2 nm, respectively.

Preparation of cellulose fiber

Poplar was initially extracted employing a 2 : 1 (v/v) benzene/ethanol solution with an auto-extractor (Soxtex™ 2050, FOSS, USA) for 3.5 h. This material was subsequently holopulped by dispersing 2.00 g poplar in 65.00 ml D.I. water and treated with 0.60 g sodium chlorite

and 0.50 ml glacial acetic acid. After treating the poplar slurry for 1 h at 75.0 °C, another four successive additions of 0.60 g sodium chlorite and 0.50 ml glacial acetic acid were preformed.[25] Upon completion of holopulping the treated pulp was rinsed thoroughly with D.I. water.

The holocellulose pulp was then treated with sodium hydroxide solution following TAPPI method T 203 cm-93 to remove hemicelluloses.[26] In brief, 1.50 g holocellulose poplar was extracted with 17.5% NaOH (100.00 ml), stirred at 25 °C for 30 min and then diluted with D.I. water (100.00 ml) and stirred for another 30 min at 25 °C. The extracted pulp (α-cellulose) was rinsed thoroughly with, suspended in D.I. water, filtered and used for imaging studies.

Preparation of dye–cellulase conjugate

The labeling of cellulase with donor AF488 or acceptor AF568 was accomplished following published methods.[27] In brief, 1.0 mg dye was dissolved in DMSO and added to 1.000 ml of 10.0 mg ml^{-1} cellulase in a 0.10 M pH 8.4 $NaHCO_3/Na_2CO_3$ aqueous buffer solution. After stirring for 2 h in the dark at RT, the reaction solution was dialyzed to remove the free dye. A PD-10 desalting column was then used to further purify the dye–cellulase conjugate. The absorption and emission spectra of AF488 and AF568 grafted cellulase, and the scheme of chemically labeling cellulase with the fluorescence dyes are shown in Fig. 1(b) and (c), respectively. The degree of labeling (DOL) of labeled cellulase was determined by UV-Vis spectroscopic measurements and it was determined to be 4.[27]

The hydrolysis activity of the labeled and starting cellulase with α-cellulose fiber was measured by monitoring the release of reducing end sugars after enzymatic hydrolysis.[28] DNS (DiNitroSalicylic acid) reagent was used to determine the concentration of reducing end sugars in the hydrolysis solutions.[28] Enzymatic hydrolysis experiments were performed at 50.0 °C for 2 and 4 h by incubating 10.0 mg fiber in 1.500 ml of 3.0 mg ml^{-1} cellulase in 0.05 M pH 5.0 acetate buffer. At the end of the incubation, the hydrolysis was stopped by heating the mixture for 10 min in boiling water. Subsequently, the sample mixtures were centrifuged, and the supernatants were filtered. 1.500 ml DNS reagent was added into 1.00 ml of each filtered supernatant solution for the color development following hydrolysis. The absorbance was measured at a wavelength of 540 nm using a UV/Vis spectrophotometer. A standard curve was prepared in the range of 0.4–2.0 mM glucose.[27]

FRET efficiency modeling

The acceptor photobleaching effect was studied in this study for the calculation of FRET efficiency which is a technique that quantitatively measures FRET efficiency by photobleaching an acceptor dye irreversibly. The donor will regain its fluorescence when the acceptor is photobleached at the acceptor excitation wavelength. FRET efficiency (E_{app}) can be determined by the fraction of donor molecules that are involved in FRET before acceptor photobleaching using the following *eqn (1)*:[20]

$$E_{app} = \frac{D_a - D_b}{D_a} \quad (1)$$

where D_a is the donor AF488 fluorescence intensity after acceptor photobleaching and D_b is the donor fluorescence intensity before acceptor photobleaching. The calculation of apparent FRET efficiency includes all donor molecules pairing and non-pairing with acceptor molecules.

Spectral fluorescence imaging

Sample preparation. α-Cellulose fibers were immobilized on the bottom of a glass-bottom Petri dish (P35G-0-10-C, MatTek, USA) by drying a slurry of 0.01 mg fiber per ml water at 70 °C for 1 h.[29] The immobilized fibers were soaked in a pH 7.4 PBS 1× buffer solution for 1 h for rehydration.

Cellulase binding to cellulose. α-Cellulose was incubated in 0.500 ml of 0.2 μM AF488–cellulase PBS 1× for 1 h which was then decanted and the fiber samples were subsequently rinsed three times with PBS 1× and finally immersed in 0.200 ml of this buffer. Time-course fluorescence images of cellulase binding onto the immobilized cellulose fibers were acquired at 3, 5, 15, 30, 45, 60, and 90 min.

Acceptor photobleaching. To obtain the photobleaching rate of the acceptor labeled cellulase, the cellulose fibers were incubated in 0.200 ml of 1.2 μM AF568–cellulase solution for 1 h followed by a rinse with PBS 1× buffer. Spectral fluorescence images were taken on a cellulase bound fiber before and after 10 s photobleaching of the acceptor AF568 by irradiation at 555 nm immersed in 0.200 ml PBS 1× buffer. To investigate possible photobleaching of the donor exposed to 555 nm light, the same process was performed by incubating fibers with 0.200 ml of 0.2 μM AF488–cellulase instead of AF568–cellulase. Since it was determined that monitor bleaching occurred during the fluorescence imaging process, two consecutive images were collected on AF488–cellulase–cellulose or AF568–cellulase–cellulose complexes without photobleaching. The monitor bleach rates were calculated after image analysis, and used to correct the intensities of donor and acceptor after photobleaching for FRET efficiency calculation.[30]

FRET imaging. The specimen of cellulose incubated in 0.200 ml mixture of 0.2 μM AF488–cellulase and 1.2 μM AF568–cellulase for 1 h was also prepared and the corresponding fluorescence images were collected before and after 10 s acceptor photobleach.

All specimens were excited by 488 nm light for collecting spectral images. To minimize the exposure time, specimens were exposed to light only during image acquisition or photobleaching process. Images of specimens were recorded from 520 nm to 720 nm at 10 nm intervals.

Spectral fluorescence image analysis

Linear unmixing was performed on the spectral images using the Nuance program to acquire the donor AF488 and acceptor AF568 fluorescence intensities.[20,23] In unmixing, reference spectral images of each dye–cellulase–cellulose complex were used to determine each dye's contribution to a measured spectrum. Subsequently, unmixing on spectral images of AF488–cellulase and AF568–cellulase or their mixtures binding to cellulose fibers before and after acceptor photobleaching was accomplished.

Results and discussion

Cellulase activity on cellulose fiber

The grafted AF488–cellulase and AF568–cellulase conjugates yielded a DOL of 4 as determined by UV-Vis spectroscopy.[27] The *T. reesei* cellulase used in this work was a mixture of two exo- and five

endo-cellulases, and β-glucosidases. The labeling chemistry was through amide bond formation and primary amine groups from cellulase were involved in the reaction, including the end primary amine and the primary amines from lysine units in the middle of the cellulase chain. Since each cellulase has multiple reactive primary amine groups, for example, exo-cellulase CBHII from *T. reesei* has one end primary amine and 10 lysine units which could react with labeling dye molecules,[31] the exact locations of the multiple grafted dye molecules are not known. The enzyme hydrolysis results are summarized in Table 1 which reveals a slight decrease in activity for *T. reesei* cellulase after labeling. This decrease was tentatively attributed to the steric interactions caused by the dye labels.

Apparent FRET efficiency modeling

The photobleaching of AF488 labeled cellulase under the experimental conditions employed was not observed and therefore only monitor bleaching was considered for the correction of donor intensity after photobleaching. Before substituting D_a and D_b with the values for AF488 after and before acceptor photobleaching into eqn (1), two corrections were performed: the monitor bleach effect of AF488–cellulase and the incomplete photobleach effect of the acceptor.

The corrected donor intensity by donor monitor bleaching is calculated as:[32]

$$D'_a = \frac{D_a}{1 - k_{mb,D}} \quad (2)$$

where $k_{mb,D}$ is the monitor bleaching rate of AF488. To correct for the incomplete photobleach of the acceptor, the difference between AF488 after and before acceptor photobleach ($D_a' - D_b$) was divided by the fraction of AF568 that was photobleached ($A_b - A_a$)/A_b (A_b—acceptor fluorescence intensity before photobleaching; A_a—acceptor fluorescence intensity after photobleaching) and then added to the AF488 intensity before photobleaching (D_b) as previously described in the literature:[20]

$$D''_a = \frac{(D'_a - D_b)A_b}{A_b - A_a} + D_b \quad (3)$$

where the acceptor intensity after photobleaching needs to be corrected by the monitor bleach rate ($k_{mb,A}$):[32]

$$A'_a = \frac{A_a}{1 - k_{mb,A}} \quad (4)$$

Substituting eqn (2)–(4) into eqn (1) yields:

$$E_{app} = 1 - \left[1 + \frac{A_b\,(1 - k_{mb,A})\,D_a - D_b(1 - k_{mb,D})}{D_b\,(1 - k_{mb,D})\,A_b(1 - k_{mb,A}) - A_a}\right]^{-1} \quad (5)$$

Table 1 Hydrolysis activity of AF488-labeled-, AF594-labeled- and non-labeled-cellulases on α-cellulose fiber. Values in the table are the concentrations of reducing sugar in the hydrolysis solutions

Incubation duration/h	*T. reesei* cellulase/mM	AF488–cellulase/mM	AF568–cellulase/mM
2	15.02 ± 0.91	13.86 ± 0.83	14.02 ± 0.77
4	27.28 ± 1.08	25.57 ± 1.14	24.90 ± 0.64

Determination of FRET efficiency and cellulase colocalization

The results of time-course experiments of AF488–cellulase bound on cellulose reveal that the binding equilibrium was reached around 45 min under the conditions employed. Therefore, the spectral images used in this work were collected after 1 h incubation. Non-homogeneous binding distribution of cellulase on the cellulose fiber was observed (Fig. 2). Relatively strong fluorescence emission (or cellulase–dye conjugates) appeared at the end of fibers or other fiber regions showing discontinuity. Previous work by other researchers presented the similar observation. For example, Hildén *et al.* reported that cellulose binding module CBM_{Cel7D} from *Phanerochaete chrysosporium* cellulase showed a non-uniform distribution on spruce pulp fibers with 'hot spots' located at fiber kinks or damaged areas.[33] Blanchette *et al.* observed that both the endoglucanase EG II and cellobiohydrolase CBH I from *T. reesei* bind to the sides of the microfibrillar structure, and CBH I has a specificity for the ends of microfibrils.[34] In our work, both AF488- and AF568-labeled-cellulases showed the same binding preference on the fiber. The labeled cellulase used in our work was a mixture of endoglucanases, cellobiohydrolases and β-glucosidases. This may explain why stronger fluorescence (*i.e.*, more cellulases) was observed at the ends of fibers or other discontinuous regions, where higher reducing or non-reducing ends of cellulose exist and cellobiohydrolases prefer to bind to the fiber ends.

A typical set of fluorescence images of AF488–cellulase and AF568–cellulases binding to a fiber before and after acceptor photobleaching are shown in Fig. 3. Eight regions with different donor and acceptor distributions were selected (red spots in Fig. 3(a)) and the derived eqn (5) was applied to quantify the apparent FRET efficiencies in these regions. The calculated values of E_{app} *vs.* donor intensity before acceptor photobleach are summarized in Fig. 4.

Donor–cellulase and acceptor–cellulase conjugates generated the same distribution tendency on the cellulose fiber. The dependence of measured apparent FRET efficiency on the distribution of donor (acceptor) was observed. An increase of donor and acceptor density resulted in an increase in FRET (see Fig. 4). In order to determine the distance (r) between the donor and acceptor, eqn (6) was used:[35]

$$E = \frac{R_0^6}{R_0^6 + r^6} \quad (6)$$

where R_0 is the Förster distance (62 Å, manufacturer data[36]). Since the FRET efficiency measured in this work was using all donor

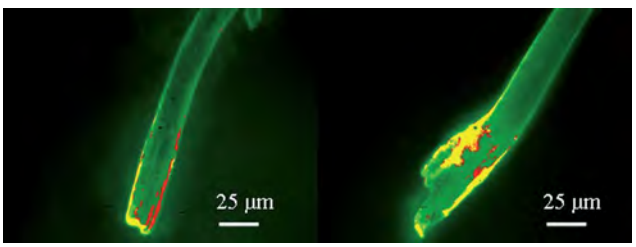

Fig. 2 Colocalization images of adsorption of AF488–cellulase and AF568–cellulases to cellulose fiber. Cellulases showed the preferential adsorption to the end or non-continuous region. Bright green—AF488–cellulase; bright red—AF568–cellulase; bright yellow—colocalization of AF488–cellulase and AF568–cellulase. The intensity threshold was set high such that only the regions with the strongest fluorescence show the colocalization.

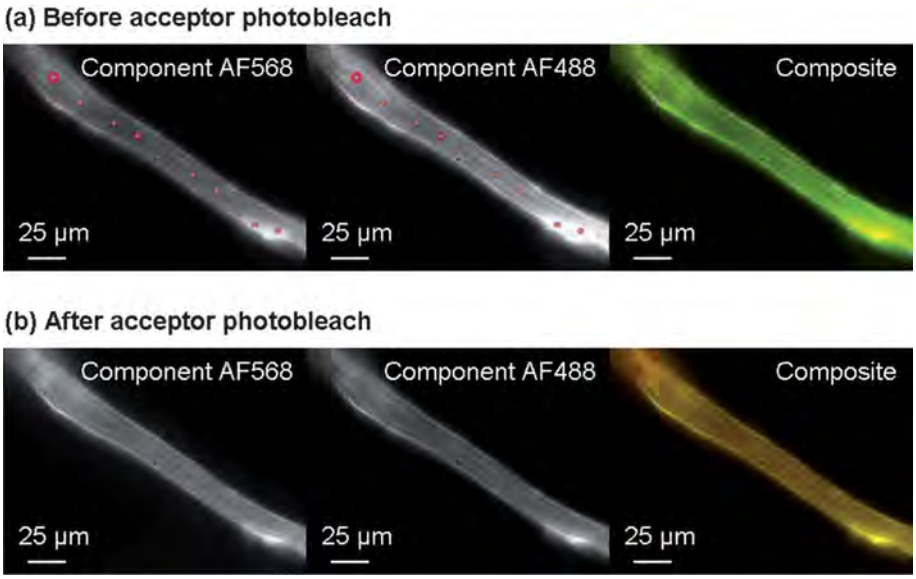

Fig. 3 Fluorescence images of colocalization of AF488–cellulase and AF568–cellulases on cellulose fiber before and after 10 s acceptor photobleaching. Images of component AF568 or AF488 are the spectrally unmixed images showing the distribution of AF568–cellulase or AF488–cellulase conjugate. Intensity indicator is from black (0) to white (255) for the component images. Composite is the pseudo color picture of the overlay of two component images (AF488—green; AF568—red). Red dots are the selected regions for the calculation of FRET efficiency.

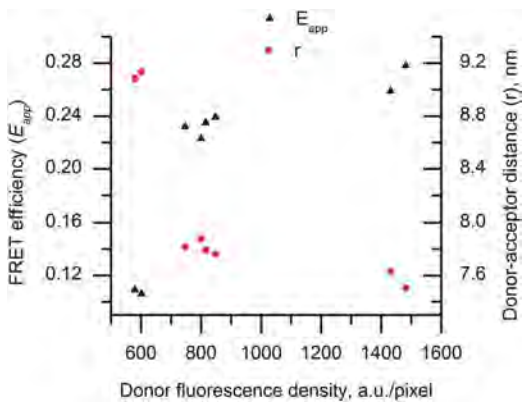

Fig. 4 Calculated apparent FRET efficiency values (E_{app}, see eqn (5) for calculation) and donor–acceptor distances (r, see eqn (6) for calculation) in regions with different donor distributions. $k_{mb,D} = 0.330$; $k_{mb,A} = 0.139$.

molecules, no matter whether they were paired with the acceptor or not, the distance (r) calculated using eqn (6) is the apparent distance, also displayed in Fig. 4. Because FRET decays dramatically with the separation distance at the sixth power, when the donor and acceptor are separated larger than two times of R_0, no FRET occurs.[17] The calculated distances are in the range of 7.6–9.2 nm, which are slightly smaller than the overall length of *T. reesei* cellulases (~10 nm).[37] There is no evidence that the cellulases align themselves on the cellulose fiber in a pattern of head-to-tail. The chance exists for the cellulases to align in a way such that the grafted donor and acceptor dye molecules on different cellulases were close to each other at a distance less than 10 nm.

With an increase in applied cellulase conjugates, the calculated FRET cellulase–cellulase separation was shortened between FRET donor and acceptor cellulases and there is more opportunity for an excited donor to transfer energy to an adjacent acceptor dye grafted onto cellulase. Different methodologies have been applied to study the adsorption/binding of cellulase to cellulose. For example, in the competition and cooperation study of exo-/endo-glucanases, Andersen *et al.* compared the hydrolysis results using single cellulase to those using binary cellulase mixtures, and drew the conclusion based on the cellulase–cellulose interaction in bulk that competition exists for the same binding sites between endo- and exo-glucanases of *Humicola insolens* EG V and CBH II.[38] Moran-Mirabal *et al.* observed using fluorescence microscopy that fluorescence dye labeled endoglucanases Cel5A, Cel6B and Cel9A from *Thermobifida fusca* have the same distribution on the cellulose fiber after incubating the cellulose fiber in an individual cellulase solution.[12] However, limited by the technique used, their work could not provide further adsorption information, such as where the cellulases prefer to bind and how close the cellulases are to each other. Our results show the perspective that, as a spectroscopic nano-ruler, fluorescence microscopy based FRET methodology could provide a visual method to quantitatively resolve the competition or synergy when different cellulases adsorb to the solid cellulose fiber, when an individual cellulase component is available and labeled with an appropriate fluorescence FRET dye pair.

Conclusion

The FRET technique was used for the first time to study cellulase colocalization on cellulosic fibers.[17] *T. reesei* cellulases have an overall length of about 10 nm,[37] which is the proximate distance that FRET could occur between a donor and an acceptor. Therefore, we can conclude from our presented study that some cellulase molecules bind to the cellulose fiber next to each other. As a demonstration of the concept, the dependence of measured apparent FRET efficiency on the distribution of the donor (acceptor) was observed. An increase of donor (acceptor) density resulted in an increase in FRET. Further

application of this developed FRET strategy for the visual investigation of cellulase (or CBM)–cellulase interaction is underway, such as competition or cooperation among two different cellulases (or CBMs) when they adsorb to a fiber at several nanometres distance.

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