DOPI and PALM Imaging of Single Carbohydrate Binding Modules Bound to Cellulose Nanocrystals

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ABSTRACT

We use single molecule imaging methods to study the binding characteristics of carbohydrate-binding modules (CBMs) to cellulose crystals. The CBMs are carbohydrate specific binding proteins, and a functional component of most cellulase enzymes, which in turn hydrolyze cellulose, releasing simple sugars suitable for fermentation to biofuels. The CBM plays the important role of locating the crystalline face of cellulose, a critical step in cellulase action. A biophysical understanding of the CBM action aids in developing a mechanistic picture of the cellulase enzyme, important for selection and potential modification. Towards this end, we have genetically modified cellulose-binding CBM derived from bacterial source with green fluorescent protein (GFP), and photo-activated fluorescence protein PAmCherry tags, respectively. Using the single molecule method known as Defocused Orientation and Position Imaging (DOPI), we observe a preferred orientation of the CBM-GFP complex relative to the *Valonia* cellulose nanocrystals. Subsequent analysis showed the CBMs bind to the opposite hydrophobic <110> faces of the cellulose nanocrystals with a well-defined cross-orientation of about ~ 70°. Photo Activated Localization Microscopy (PALM) is used to localize CBM-PAmCherry with a localization accuracy of ~ 10nm. Analysis of the nearest neighbor distributions along and perpendicular to the cellulose nanocrystal axes are consistent with single-file CBM binding along the fiber axis, and microfibril bundles consisting of close packed ~ 20nm or smaller cellulose microfibrils.

Keywords: Single Molecule, PALM, DOPI, GFP, TIRF, Cellulose, Carbohydrate Binding Module, Bioenergy, Biomass.

1. INTRODUCTION

Processing biomass to biofuels is considered a potentially sustainable route to solving a future global energy crisis. Although lignocellulosic biomass is believed to be the most abundant source that can be converted to simple sugars suitable for fermentation and biofuels production, the bioprocesses to deconstruct the polysaccharides and other polymers in biomass are inefficient, thus fundamental breakthroughs in our understanding of biomass structure and chemical properties are needed [1]. Lignocellulosics consists chiefly of plant cell walls: an intricate mat of cellulose microfibrils embedded in a matrix of complex polysaccaharides (hemicelluloses and pectin) and other polymers (mainly lignin) [2]. While cellulose is the primary polysaccaharide targeted for renewable feedstocks, its spatial-chemical structure and its interaction with degrading enzymes and microbes are not well understood at the nanometer scale. Cellulases are a class of enzymes that hydrolyze the β-1,4-glycoside linkage of cellulose chains to produce glucose[3-5]. Most cellulases contain a catalytic domain and a binding domain, the function of the carbohydrate-binding module (CBM) is in locating the enzyme to the cellulose surface, and bringing the catalytic domain in proximity of its substrate, a critical step in cellulase action. We investigate the interaction of single CBMs binding to cellulose nanocrystals, using single molecule methods. In so doing, a biophysical understanding of the CBMs role in enzymatic biomass conversion may be gained.

Figure 1 shows a schematic of the CBM binding to the cellulose nanocrystal surface by its aromatic amino acid residues interacting with the glucose residues of cellulose chains. As shown in the figure, we have genetically tagged the CBM with a green fluorescent protein (GFP) so as to track the composite molecules position and orientation with single molecule precision. As will be discussed, this labeling scheme is used repeatedly in this work, to implement both defocused orientation and position imaging (DOPI) and photo-activated localization microscopy (PALM), to reveal details of the cellulose-CBM interactions at the molecular level.

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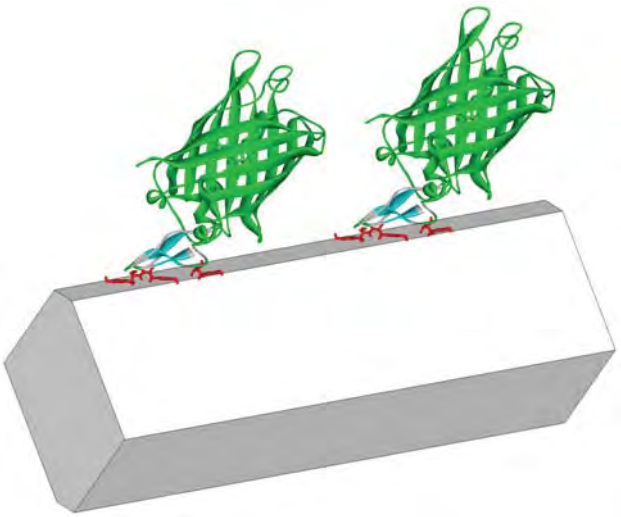


Figure 1: Schematic representation of carbohydrate binding modules tagged with green fluorescent proteins bound to cellulose nanocrystal on its hydrophobic (110) face.

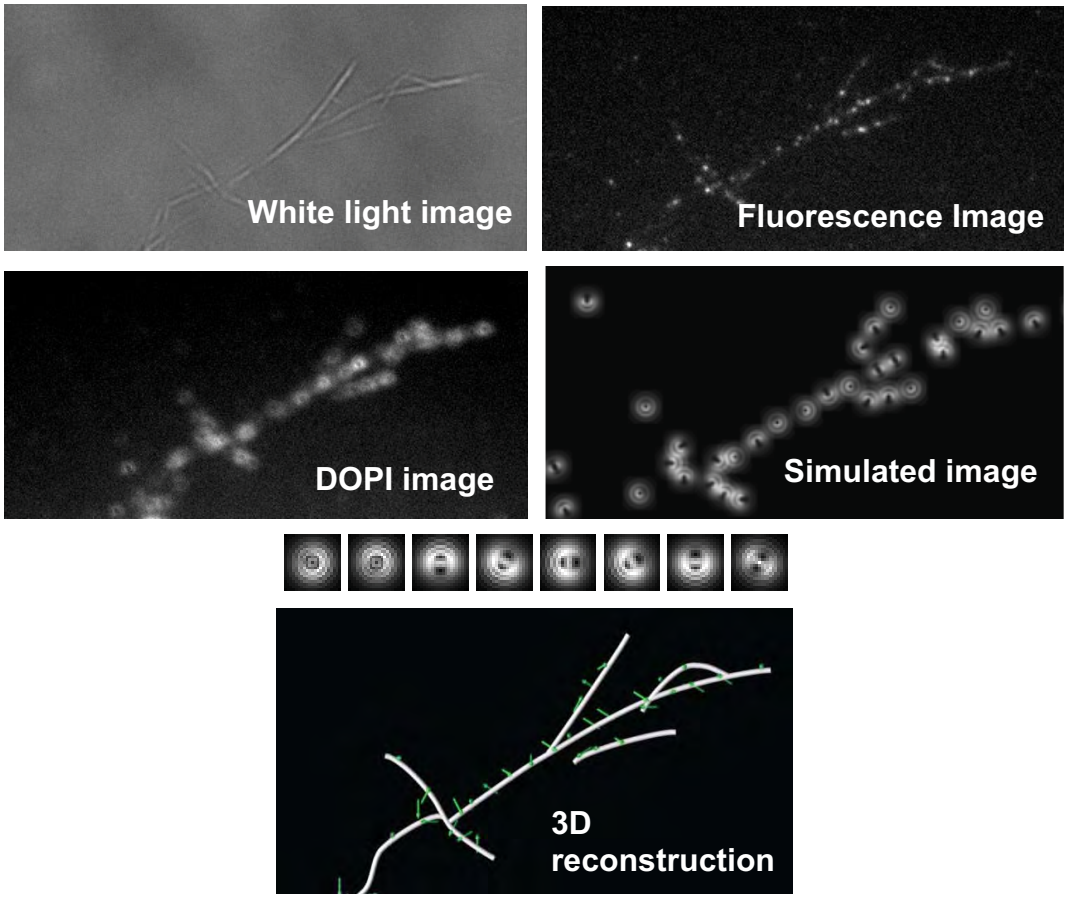


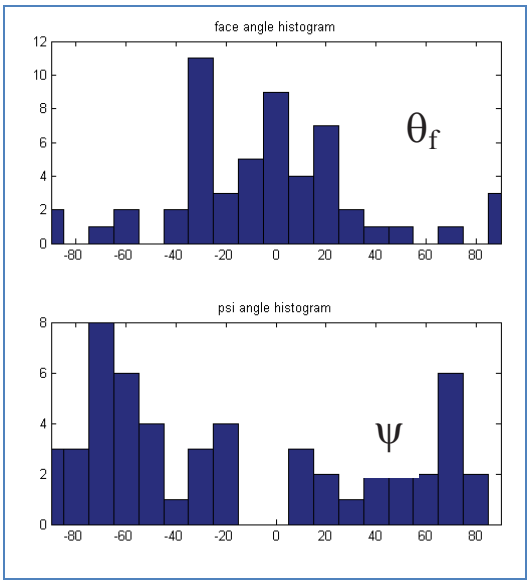
Figure 2: Left to right: White light image of *Valonia* microfibrils, TIRF fluorescence image of GFP-labeled CBM's bound to *Valonia* microfibril, defocused fluorescence (DOPI) image, 2D dipole patterns and simulated images, and (bottom) 3D reconstructed image.

2. DOPI IMAGING OF SINGLE CARBOHYDRATE BINDING MODULES

Single molecule emission is inherently polarized, according to the orientation of the transition dipole of the molecule. In close contact with a planar dielectric substrate, the emission pattern of the dipole is modified by the polarization field within the dielectric slab. This emission is characteristic of the experimental details, including the orientation of the emitter itself, and is highlighted by slight defocus of the optical system. This is the basis for the method known as defocused orientational imaging (DOPI), a single molecule method sensitive to the orientation of single molecules [6-7]. As shown in figure 2, these emission patterns can be predicted by theory, and the 3D orientation of molecules determined relative to the substrate.

Figure 2 shows a summary of DOPI data taken in our lab. In this case, a family 3 cellulose binding CBM, the *Ct*CBM3 was derived from cellulosome scaffoldin CipA of the bacterium *Clostridium thermocellum* (PDB 1NBC).[8] This CBM was genetically engineered to tag with a *C*-terminal GFP to form *Ct*CBM3-GFP with a short linker peptide. The detailed protocol for CBM cloning, expression, and purification was described in previous reports.[8,9] *Valonia* nanocrystals were then exposed to high concentrations of purified *Ct*CBM3-GFP in aqueous buffer, and imaged using a home-built total internal reflection fluorescence (TIRF) microscopy system. The fluorescence images were used to obtain the centroids of each individual molecule, where single-molecule locations were extracted from the in-focus fluorescence images using standard techniques developed for particle tracking (http://www.physics.emory.edu/~weeks/idl/). The white light images were used to obtain the *Ct*CBM3-GFP location relative to the cellulose microfibrils. The defocused images were then compared to theoretical models, computed using code obtained from Enderlein (http://www.joerg-enderlein.de/?292), and the molecular orientations were thus obtained. With these, and the white light image of the microfibrils, the 3D orientation of *Ct*CBM3-GFP bound to *Valonia* nanocrystals were obtained, as illustrated in the lower portion of figure 2. It is clear by inspection that the binding of the CBM along the microfibril is non-random, and registered to the microfibril. Also, a systematic cross-orientation of ~ 70° was observed. To obtain a clearer picture of these angles, a coordinate transformation to a system centered about the microfibril, as defined in the right-hand portion of figure 3, was performed. As shown below in the lefttmost portion of figure 3, a statistical analysis revealed the CBMs bind preferentially to opposite <110> faces of the elementary fibril, with a preferred cross-orientation of ~ 70° relative to the cellulose crystal basis.

We are currently studying the structure of the GFP-CBM bio-conjugate to interpret this preferred orientation. However, the observation of a well-defined angle suggests the CBMs recognize the chemical-spatial structure of the substrate, and therefore bind to the surface in a highly constrained manner. Such experimental observations could be invaluable inputs to molecular models aimed at understanding the function and mechanisms of the CBMs and other biomolecules[11], and their interaction with cellulose and other constituents of biomass.



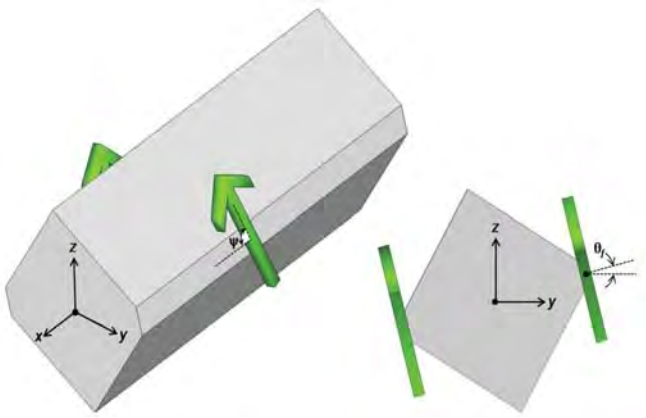


Figure 3: (left): Statistical summary of orientation data for *Ct*CBM3: Histograms of polar angle θ_f and azimuthal angle Ψ, and (right): Model showing these angles relative to the crystalline facets of cellulose.

3. PHOTO-ACTIVATED LOCALIZATION MICROSCOPY OF SINGLE CBMS

The most recent innovation in single molecule techniques extends single molecule localization methods to systems which are not necessarily sparse. The diffraction limit is avoided by simply imaging one molecule at a time, recording its position with nanometer precision, and repeating this process *on ad infinitum*[11-12]. By sequentially imaging sparse subsets of single molecules, and localizing their centroids with molecular precision, composite optical images can be constructed with up to two orders of magnitude higher spatial resolution than conventional methods. The technique relies on photo-activation of fluorescent proteins, followed by photo-bleaching or photo-switching, such that only a sparse subset of molecular tags are excited in a given time window. If molecular fluorophores are strategically attached to relevant cellular structures, structural and chemical information of the cell and its constituents may be obtained with nanometer resolution.

In this study, *Ct*CBM3 was genetically engineered to tag with a *C*-terminal photoactivatable mCherry fluorescent protein, PAmCherry (PDB 3KCS/3KCT),[13,14] and a short linker peptide, to form *Ct*CBM3-PAmCherry. The detailed protocol for CBM cloning, expression, and purification was described in previous reports. [8,9] *Valonia* nanocrystals were then exposed to purified *Ct*CBM3-PAmCherry in aqueous buffer, and imaged using a computer controlled photo-activated localization microscopy (PALM) system. Results of these experiments, showing PALM imaging of cellulose microfibril bundles, and their subsequent PALM analysis, are shown below in figure 4. The figure shows a composite image (sum of all raw fluorescence images), a PALM image, which consists of the centroids of all localized molecules plotted with a radii equal to the uncertainty in their position (which was, on average, ~ 10 nm), and zoomed-in areas of both images. The scale bars are 2 μm and 250 nm, in upper and lower images, respectively.

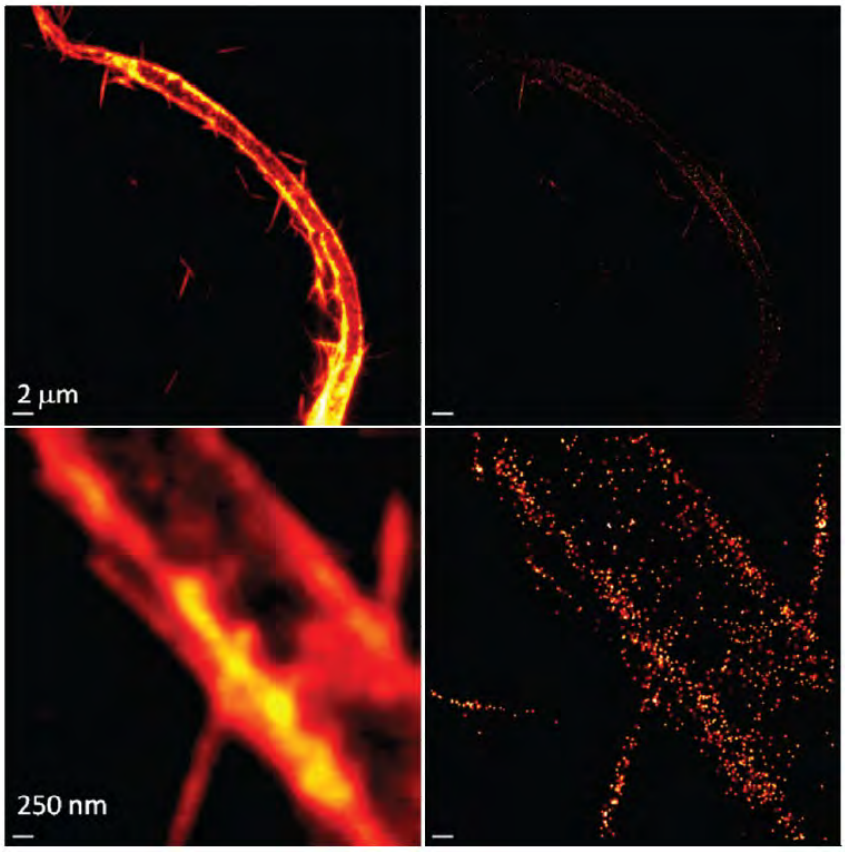


Figure 4: (upper left): Composite (summed) raw fluorescence image of *Valonia* microfibril bundle, (upper right): deconvolved PALM image, (lower left): zoomed-in composite fluorescence image, (lower right): zoomed-in PALM image (see text).

Figure 5 below shows a correlation analysis of the CBM binding sites observed in these images. The nearest neighbor distributions give information about the frequency of binding sites on the cellulose crystal. The figure shows that when considering all binding sites along the fiber axes (yellow line), the nearest neighbor distribution shows there is a minimum spacing of roughly 20nm, consistent with previous TEM measurements [9]. Analysis perpendicular to the fiber axis (green line) similarly shows peaks roughly 20nm apart, consistent with the expected size of the microfibrils and their close-packing. Averaging over many parallel lines (blue line) similarly shows a minimum spacing. These observations provide *in situ* observations of CBM-Cellulose interactions with nanometer spatial resolution.

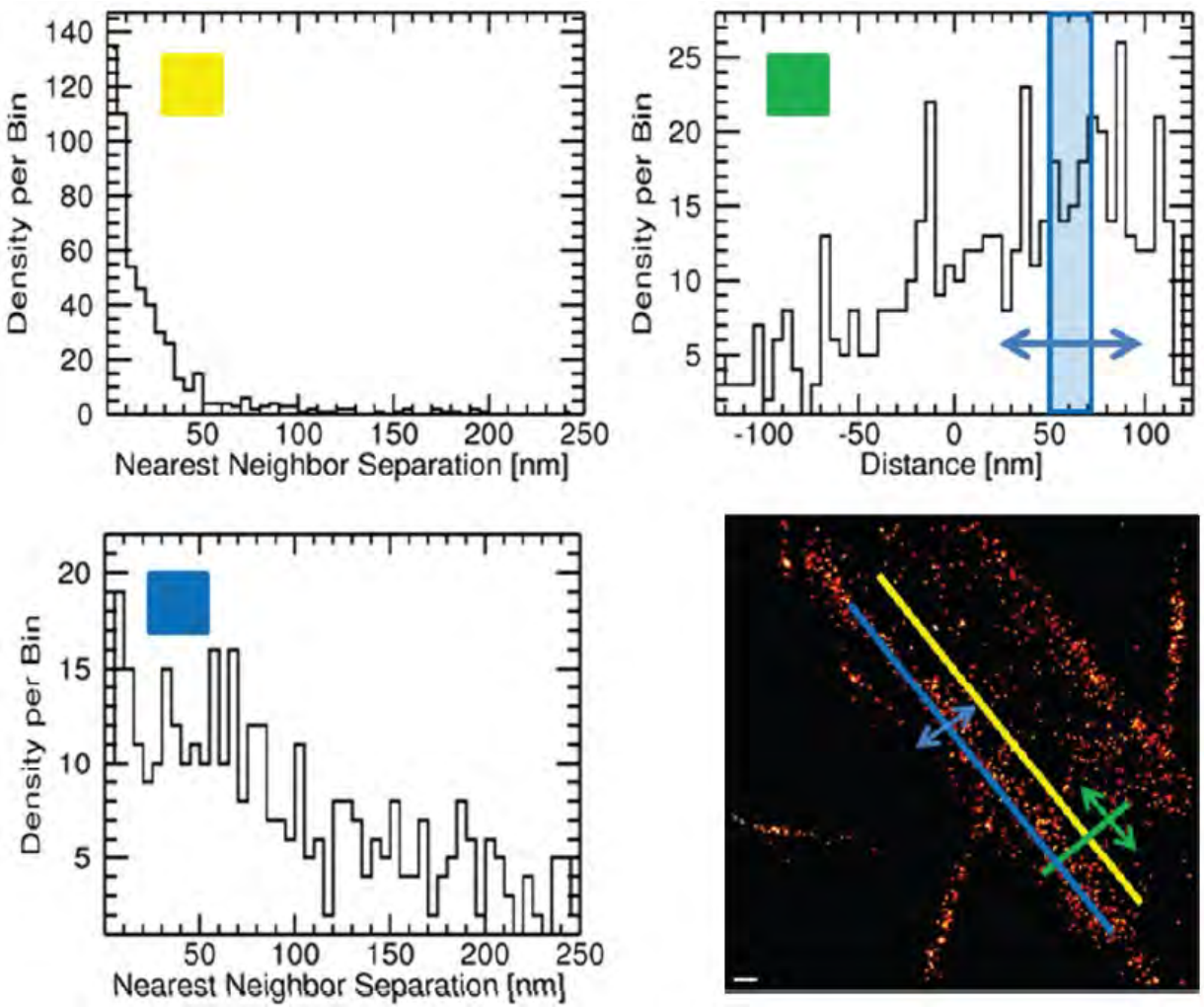


Figure 5: (Clockwise from upper left): Histogram along the axis for *Valonia* cellulose nanocrystal bundle, (upper right): Distribution perpendicular to the long-axis for nanocrystal bundle, (lower left): nearest neighbor distribution averaging over a 20nm window (see text).

4. CONCLUSIONS

We adapted the single molecule methods defocused orientation and position imaging (DOPI) and photo-activated localization microscopy (PALM) towards the study of single carbohydrate binding modules (CBMs). The CBM, an important component of most cellulase systems, serves to locate the enzyme on the cellulose surface, and facilitates hydrolyzing the constituent polysaccharides which make up the plant cell wall. Using DOPI, we observed the

preferential binding of the CBMs to the <110> facets of cellulose microfibrils, with a well-defined cross orientation relative to the microfibril long-axis. These observations are consistent with previously published works regarding the specificity of the CBMs to the two hydrophobic faces of the cellulose microfibril, and provide new insight into the degree to which the CBMs are constrained to the cellulose surface. Using PALM, we demonstrated the successful incorporation of the photo-activated fluorescent protein PAmCherry with family 3 CBMs, and characterized their site-specific binding with nanometer spatial resolution. The measurements again are consistent with previously reported TEM measurements of quantum dot labeled CBMs on individual cellulose nanocrystals, but also provide high resolution localization in aqueous buffer. Indeed, all measurements were performed *in situ*, thus removing any ambiguity due to drying and sample preparation. Thus, the forementioned studies demonstrate significant potential to study the binding of the CBMs, and the action of cellulases on cellulose substrates *in situ*, with nanometer spatial resolution.

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