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Novel structural labeling method using cryo-electron tomography and biotin–streptavidin system

Toshiyuki Oda, Masahide Kikkawa *

Department of Cell Biology and Anatomy, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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ABSTRACT

There are a number of large macromolecular complexes that play important roles in the cell, and identifying the positions of their components is a key step to understanding their structure and function. Several structural labeling methods have been applied to electron microscopy in order to locate a specific component within a macromolecular complex, but each method is associated with problems in specificity, occupancy, signal intensity or precision. Here, we report a novel method for identifying the 3D locations of proteins using biotin–streptavidin labeling and cryo-electron tomography. We labeled a biotinylation-tagged intermediate chain of an axonemal dynein by streptavidin within the *Chlamydomonas* axoneme and visualized the 3D positions of the labels using subtomogram averaging. Increase of the density attributed to the bound streptavidin was validated by Student's *t*-test. In conclusion, the combination of the biotin–streptavidin system and cryo-electron tomography is a powerful method to investigate the structure of large macromolecular complexes.

1. Introduction

There are numerous large macromolecular complexes that play important roles in various cells, but investigation of their nature and function has been difficult due to their structural complexity. For example, the nuclear pore complex is composed of 30 different proteins (Hoelz et al., 2011) and forms a 66- to 112-MDa molecular complex. The axoneme, which is the core structure of cilia and flagella, is composed of >300 proteins (Merchant et al., 2007; Pazour et al., 2005) and the total molecular weight of its 96-nm repeat is estimated to be a least 500 MDa. To understand the structure and function of these large macromolecular complexes, it is important to locate their components by specific labeling for electron microscopy (EM). However, the existing labeling techniques each have their own strengths and weaknesses (Table 1). When evaluating a labeling method, there are four factors to be considered: specificity, degree of labeling (occupancy), signal intensity and precision of localization.

In this study, we report a novel technique to localize a specific protein within the axoneme using cryo-electron tomography (cryo-ET) and a biotin–streptavidin system. The small biotinylation tag did not interfere with the assembly of the axonemal structures. The streptavidin tetramer penetrated the meshwork of the axonemal structures and made strong signals with high precision. The specificity and degree of streptavidin labeling were sufficient enough to give significant densities on averaged subtomograms.

2. Materials and methods

2.1. Strains and media

Chlamydomonas reinhardtii strain 137c and *oda6* were kindly provided by Dr. Kamiya (Gakushuin University). *oda6-ic2-n-bccp* (*ic2N*) was isolated as described previously (Oda et al., 2013). Briefly, the IC2-deficient mutant *oda6* was transformed and rescued with an expression plasmid containing the IC2 gene fused with a fragment of acetyl-CoA carboxylase biotin carboxyl carrier protein (Fig. 1). The resulting *ic2N* mutant cells expressed IC2 harboring a biotinylation tag at the amino terminus. Cells were then grown in Tris–acetate–phosphate (TAP) media.

2.2. Preparation of axonemes

Chlamydomonas cells were deflagellated with dibucaine-HCl (Wako Pure Chemical Industries, Osaka, Japan) and axonemes were collected by centrifugation (Witman et al., 1978). Flagella were demembranated with 1% Nonidet P-40 in HMDEK (30 mM Hepes-NaOH pH 7.2, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT),

Abbreviations: Cryo-ET, cryo-electron tomography; Cryo-EM, cryo-electron microscopy; ODA, outer dynein arm; IC2, intermediate chain 2; OID linker, outer–inner dynein linker.

* Corresponding author. Fax: +81 3 5841 3339.
E-mail address: mkikkawa@m.u-tokyo.ac.jp (M. Kikkawa).

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Table 1
Comparison of EM structural labeling methods.

	Specificity	Occupancy	Signal intensity	Precision/resolution	References
Immuno-EM	Often Low	Low	High (Colloidal gold)	Low (~40 nm[a])	1
Antibody/Fab labeling	Varies	Varies	High (Fab 50 kDa)	High ~ Very High (Fab: 7 nm)	2, 3, 4
Maleimide-gold conjugation	High	Varies	Low (11Au, 5 kDa)	Very High (0.8 nm[b])	5, 6
Cryo-ET Mutant analysis	–	–	High (large structural defects)	Low	7, 8, 9,10,11,12
Metallothionein tagging	High	High	Low (7 kDa + ~16Au or 18Cd)	High (Monomer: ~2 nm)	13, 14
Cryo-ET streptavidin labeling	High	High	High (69 kDa[c])	High (Tetramer: ~4 nm)	This study

References: 1, D'Amico and Skarmoutsou (2008); 2, Prasad et al. (1990); 3, Samsó and Koonce (2004); 4, Wu et al. (2012); 5, Hainfeld and Powell (2000); 6, Safer (1999); 7, Bui et al. (2008); 8, Bui et al. (2012); 9, Carbajal-González et al. (2013); 10, Heuser et al. (2009); 11, Heuser et al. (2012); 12, Pigino et al. (2011); 13, Mercogliano and DeRosier (2007); 14, Nishino et al. (2007).

[a] Assuming that the label is composed of primary and secondary antibodies (14.2 nm × 2) plus colloidal gold (~10 nm).

[b] The diameter of undecagold.

[c] The total molecular weight of BCCP tag (9 kDa) and streptavidin (60 kDa).

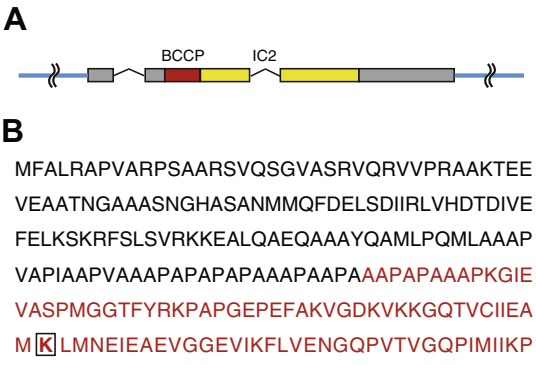

Fig.1. Expression construct of IC2 tagged with BCCP at the amino terminus. (A) Diagram of the expression construct. Blue: upstream and downstream non-coding sequences of *Chlamydomonas* IC2 gene; gray: 5′- and 3′-untranslated regions of IC2 gene; red: BCCP tag sequence; yellow: exons of IC2 gene; and kinked black lines: introns of IC2 gene. (B) Amino acid sequence of *Chlamydomonas* acetyl-CoA biotin carboxyl carrier protein (sequence ID: XP_001700442). Amino acids 141–228 (red) were used for biotinylation-tagging of IC2. Boxed lysine residues indicate the biotinylation site (Cronan, 1990).

1 mM EGTA, 50 mM CH_3COOK, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) buffer.

2.3. Fluorescence microscopy of axonemes

Demembranated axonemes were attached to glass slides and blocked with 1 mg/ml BSA in HMDEK buffer. Axonemes were incubated with 1 μg/ml Alexa Fluor 546-conjugated streptavidin (Invitrogen, Carlsbad, CA) for 1 min. Labeled axonemes were washed three times with HMDEK buffer and observed using a fluorescence microscope (IX70, Olympus, Tokyo, Japan). Images were recorded using a CCD camera (ORCA-R2; Hamamatsu Photonics, Hamamatsu, Japan).

2.4. Cryo sample preparation

Demembranated axonemes were incubated with 0.1 mg/ml streptavidin (Wako Pure Chemical Industries) for 1 h at 4 °C in HMDEK buffer. Axonemes were separated from unbound streptavidin by centrifugation and were resuspended in HMDEK buffer at a concentration of 0.04 mg/ml. Home-made holey carbon grids were glow-discharged and coated with 15-nm colloidal gold (BB International, Cardiff, UK). Suspended axonemes (5 μl) were loaded onto the grids, which were washed once with HMDEK containing a 10-fold diluted 15-nm colloidal gold suspension conjugated with bovine serum albumin (Aurion, Wageningen, The Netherlands). Grids were then plunge-frozen in liquid ethane with an automated plunge-freezing device, EM GP (Leica Microsystems, Wetzlar, Germany).

2.5. Image acquisition

Grids were transferred into a JEM-3100FEF transmission electron microscope (JEOL, Tokyo, Japan) with a Gatan 914 high-tilt liquid nitrogen cryo-transfer holder (Gatan Inc., Pleasanton, CA). Tilt series images were recorded using a 4096 × 4096-pixel TemCam-F416 CMOS camera (TVIPS, Gauting, Germany) and automated acquisition software, "Recorder" (System in Frontier Inc., Tokyo, Japan). The angular range of the tilt series was from −61° to 61° with 1.0° to 2.0° increments. The tilt angles were determined according to the cosine rule (Saxton et al., 1984). Total electron dose was limited to approximately 100 $e^-/Å^2$. Images were recorded at 300 keV, with 13-μm or 15-μm defocus, at a magnification of ×25,700 and a pixel size of 6 Å. The in-column energy filter was not used because we had not established the optimal conditions for recording with the omega filter at the time of data collection.

2.6. Image processing

The tilt series images were aligned and back-projected to reconstruct 3D tomograms using software "Composer" (System in Frontier Inc.). Tomograms of intact axonemes with a high signal-to-noise ratio were selected and used for subtomogram averaging of the 96-nm repeats of the outer doublet microtubules (Fig. 2A and B). Alignment and averaging of subtomograms were conducted using custom Ruby-Helix scripts (Metlagel et al., 2007) and the PEET software suite (Nicastro et al., 2006). The center of the subtomogram was set to the midpoints between the microtubule-attaching sites of the radial spoke pairs (RS midpoint, Fig. 2C) as follows. First, we manually determined the centers of the axoneme in the cross section of the tomogram based on the positions of the central pair microtubules. Second, we calculated the central 3D spline curve (Fig. 2D, broken bending line) that passes through these center points. Third, we manually located one of the RS midpoints near the origin of the tomogram and calculated the coordinates of the rest of the RS midpoints assuming that the radial spoke pairs form a 4.5-start helix with a repeat of 96 nm (i.e., 48-nm axial rise between doublets) based on our tomographic observations, as well as previous reports (Goodenough and Heuser, 1985; Nicastro et al., 2005). As the actual position of the radial spoke does not faithfully follow this helical path, the coordinate of the RS midpoint was searched translationally along the filament axis with a wide search range (±48 nm). Cross-correlations between the subtomogram and reference point were maximized using the "alignSubset" function of the PEET program suite. Initially,