Published in final edited form as:

ACS Nano. 2017 December 26; 11(12): 12677–12686. doi:10.1021/acsnano.7b07200.

Hybrid Structured Illumination Expansion Microscopy Reveals Microbial Cytoskeleton Organization

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Abstract

Recently developed tissue-hydrogel methods for specimen expansion now enable researchers to perform super-resolution microscopy with ~65 nm lateral resolution using ordinary microscopes, standard fluorescent probes, and inexpensive reagents. Here we use the combination of specimen expansion and the optical super-resolution microscopy technique structured illumination microscopy (SIM) to extend the spatial resolution to ~30 nm. We apply this hybrid method, which we call ExSIM, to study the cytoskeleton of the important human pathogen *Giardia lamblia* including the adhesive disc and flagellar axonemes. We determined the localization of two recently identified disc-associated proteins, including DAP86676 which localizes to disc microribbons, and the functionally unknown DAP16263 which primarily localizes to dorsal microtubules of the disc overlap zone and the paraflagellar rod of ventral axonemes. Based on its strong performance in revealing known and unknown details of the ultrastructure of *Giardia*, we find that ExSIM is a simple, rapid, and powerful super-resolution method for the study of fixed specimens, and it should be broadly applicable to other biological systems of interest.

Keywords

super-resolution microscopy; expansion microscopy; structured illumination microscopy; giardia lamblia; cytoskeleton

Expansion microscopy (ExM) is a recently introduced super-resolution microscopy technique in which swellable polymer hydrogels are used to physically expand fixed biological specimens so that fine details can be resolved in the expanded specimen.¹ The method (Figure 1) is particularly attractive due to its simplicity and accessibility, its compatibility with conventional fluorescent probes and inexpensive off-the-shelf chemicals,

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ASSOCIATED CONTENT

Supporting Information. ExSIM, SMLM and confocal images of *Giardia lamblia* microtubules and DAPs, distortion analysis, chromatic registration, immunostaining conditions, and vector construction. The Supporting Information is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

and its ability to achieve a spatial resolution of ~65 nm using conventional (confocal) microscopes, or approximately four times better than that of the conventional microscope alone (~250 nm).^{2–4}

Several other super-resolution microscopy methods can achieve a higher spatial resolution. More mature methods, such as stimulated emission depletion microscopy (STED),^{5–7} and single-molecule localization microscopy (SMLM, also known as STORM, PALM, *etc.*),^{8,9} can routinely achieve a spatial resolution better than 50 nm but require substantial technical expertise to execute and often face considerable challenges when imaging multiple channels, densely labeled specimens, or thick specimens more than a few microns from the coverglass substrate.^{10–12} A second-generation hydrogel expansion method, termed iterative ExM (iExM), has very recently been reported to achieve an impressive resolution down to ~25 nm, but at the cost of substantially increased complexity, use of specialized custom fluorescent probes, and the introduction of significant distortions at small length scales.¹³

Here we use the all-optical super-resolution method structured illumination microscopy (SIM), which can achieve a modest ~twofold increase of resolution (to ~110 nm) over conventional microscopy,¹⁴ together with specimen expansion to achieve a comparable resolution (~30 nm) to that of STED, SMLM, and iExM.¹⁵ SIM is typically performed by recording a series of images with striped illumination patterns in order to capture previously undetectable spatial frequencies; the image series is then used to computationally reconstruct a high resolution image.¹⁶ Because of its generality, SIM is compatible with a wide range of fluorophores and is amenable to imaging of relatively thick,^{17,18} multicolor specimens, such as those generated by the ExM protocol. We refer to the use of SIM to image expanded specimens as ExSIM.

Several challenges exist in combining SIM with ExM. First, hydrogel specimens must be carefully immobilized since residual motion during acquisition can cause serious image reconstruction artifacts.¹⁹ Second, many popular commercial SIM instruments, including the one we use here, are equipped with oil immersion lenses such that imaging into water-based hydrogels incurs spherical aberration which must be carefully minimized through choice of immersion oil in order to avoid artifacts (*e.g.*, ghosting).¹⁹ Third, related to the previous, the hydrogel should be tethered closely to a coverglass substrate to help minimize spherical aberration resulting from imaging into water at depth. Fourth, sample preparation and data acquisition parameters should be adjusted to keep photobleaching artifacts to a minimum since expanded specimens are substantially dimmer than unexpanded ones and since 3D SIM requires 15 exposures of the sample per focal plane.¹⁹ We address all of these challenges here and note that many of our solutions would also be applicable when combining specimen expansion with other forms of optical super-resolution microscopy (*e.g.* STED or SMLM).²⁰

In this work, we selected the protozoan *Giardia lamblia* (Figure 1) as a specimen with which to develop and evaluate ExSIM because *Giardia*'s microtubule cytoskeleton contains highly ordered structures that have been well characterized by electron microscopy but are unresolvable with confocal ExM at ~65 nm resolution.²¹ Additionally, since giardiasis presently afflicts hundreds of millions of people worldwide, particularly in developing

nations, the study of recently identified novel *Giardia* proteins, termed disc-associated proteins (DAPs), may help identify badly needed targets for new therapies.^{22,23}

Results

The successful application of ExSIM to study *Giardia lamblia* required some modifications to the ExM protocol. *Giardia* cells are capable of quickly attaching to a range of surfaces including coverglass,²⁴ however, once fixed, specimens were typically only weakly adherent. Instead, live *Giardia* cells were first allowed to adhere to a poly-L-lysine-treated coverglass and were then fixed, yielding high confluency surfaces with predictable specimen orientation and sufficiently robust attachment to withstand numerous solution exchanges during sample processing. The subsequent application of our recently simplified ExM protocol with conventionally fluorescently labeled antibodies is described in detail elsewhere.² Briefly, the cells were immunostained with conventional primary and fluorescent secondary antibodies and then treated with the linker molecule methacrylic acid NHS ester to convert free amines on the specimen into polymerizable methacryloyl groups. These covalently attached methacryloyl groups enabled the covalent linkage of the antibody protein labels to the swellable acrylamide/acrylate matrix (Figure 1a). For this work, the hydrogel recipe was tuned to produce firmer hydrogels which still expanded isotropically, in this case with a 3.5× expansion factor. These hydrogels, which contain high concentrations of acrylamide, have the advantage of reliably detaching adherent gel-embedded specimens from their original substrates and are also easier to handle without incurring damage. After polymerization, the sample was homogenized by proteolytic digestion with Proteinase K and expanded by dialysis in water (Figure 1b). Once expanded, the hydrogel specimens were adhered to lysine-treated coverglass just prior to imaging. The strong adhesion between the negatively charged hydrogel and the positively charged surface was critical in eliminating drift or vibrations that would deteriorate the SIM image reconstruction results due to sample motion or “floating” of the specimen on top of a layer of water. In this work, all distances are reported in pre-expansion dimensions, unless otherwise noted.

The *Giardia* cytoskeleton contains several structures with features separated by ~65 nm, the resolution of confocal ExM), which we used to assess the improved optical resolution of ExSIM. At the core of the *Giardia* cell body (~10 μm × 15 μm) is the adhesive disc, a “suction-cup” composed of a tightly packed, right-handed semiconical-spiral array of microtubules and associated microribbons with a concentric spacing of ~40–70 nm (Figure 1).²¹ Many of the disc microtubules originate from a nucleation zone near to the bare region at the center of the disc where eight basal bodies are also located. Eight flagella flank the disc, and each flagellum contains an axoneme consisting of a canonical ~200–250 nm diameter array of nine microtubule doublets separated from each other by ~70 nm and surrounding a central pair of microtubules (“9 + 2” geometry, Figure 1f and Figure 4j).^{25,26} While the adhesive disc’s structural features have been previously studied by electron microscopy,^{21,27} and its components have been identified by proteomics methods and localized *via* immunofluorescence with lower-resolution methods,²³ these separate efforts were insufficient to localize components of the disc to specific substructures. Similarly, many key protein components of *Giardia* flagella and axonemes have not been localized.

As an initial test of ExSIM, we co-immunostained *Giardia* cells for acetylated tubulin and HA-tagged DAP86676, and we imaged the expanded hydrogel using SIM. We were able to capture the entire volume of an adhesive disc in one field of view ($41\ \mu\text{m} \times 41\ \mu\text{m} \times 15\ \mu\text{m}$ in post-expansion dimensions), and the tubulin channel revealed a clearly resolved and continuous, tightly wrapped right-handed spiral array of microtubules as expected (Figure 2a–b; Supporting Information Figure S1 and Movie 1). Individual microtubules could be tracked up to $12\ \mu\text{m}$ in length and over $1.5\ \mu\text{m}$ in height (Supporting Information, Figure S1). We were able to identify ~60 distinct microtubules on each side of the ventral groove (Figure 1b, VG), however, microtubules at the outer ~200–500 nm of the disc periphery or along the ventral groove were unresolvable due to their high density, curvature of the disc, and/or poor labeling (Supporting Information, Figure S1). Some microtubules were clearly observed to originate near the inner margin of the disc, rather than at the nucleation zone, and to terminate near the outer margin (Supporting Information, Figure S1), both of which have been previously observed by cryo-electron tomography.²¹

A co-immunostain of HA-tagged²⁸ DAP86676 (also known as δ -giardin) was used to localize the protein's distribution relative to adhesive disc microtubules. We observed that DAP86676 was also distributed in a right-handed spiral array (Figure 2c) that colocalized laterally with disc microtubules, but that DAP86676 was displaced $\sim 60 \pm 11\ \text{nm}$ (mean $\pm$ standard error of the mean (SEM)) above the microtubules of the disc (Figure 2d–h; Supporting Information, Figure S1). Control experiments with multi-band fluorescent beads were used to quantify the residual chromatic registration error to be $\sim 37 \pm 18\ \text{nm}$ (mean $\pm$ SEM, both laterally and axially) which corresponds to $\sim 11 \pm 5\ \text{nm}$ in pre-expansion dimensions (Supporting Information, Figure S2). Based on the good lateral colocalization with microtubules but a $\sim 60\ \text{nm}$ axial displacement, we assign DAP86676 to the ~ 30 – $100\ \text{nm}$ tall microribbon protrusions known from electron microscopy to emanate dorsally from disc microtubules (Figure 1d).²⁹ Fitting of the microtubule and DAP86676 cross-sectional profiles with Gaussian functions produced peak-to-peak separations of $\sim 54 \pm 1\ \text{nm}$ (mean $\pm$ SEM, Figure 2e–f), in good agreement with previous measurements by cryoelectron tomography²¹ ($53 \pm 2\ \text{nm}$, mean $\pm$ SEM), as well as SMLM measurements ($53 \pm 6\ \text{nm}$, mean $\pm$ SEM) from a similar region of the disc (Supporting Information, Figure S3). We analyzed ~800 different cross-sectional profiles for each of 18 microtubules, spanning a total length of $\sim 170\ \mu\text{m}$ and we determined the average full width at half maximum (FWHM) to be $33 \pm 3\ \text{nm}$ (mean $\pm$ SEM; Figure 2g), implying an ExSIM resolution of $\sim 30\ \text{nm}$ or better; this resolution is consistent with the nominal SIM lateral resolution of $110\ \text{nm}$ divided by the 3.5-fold expansion factor. Correlative pre- and post-expansion confocal imaging was used to confirm the 3.5 $\times$ expansion factor and to determine that the distortions were relatively minor, as with earlier ExM publications, at 2% or less (Supporting Information, Figure S4).^{1–4}

In a separate experiment, we co-stained for acetylated tubulin and HA-tagged DAP16263, a DIP13 homolog previously reported to localize to the supernumerary microtubules of the ventral disc.²³ An XY maximum intensity projection of the HA-tagged DAP16263 revealed that it primarily localized to a crescent-shaped parallel array of filaments (Figure 3a; Supporting Information, Figure S5). We conclude that disc-associated DAP16263 primarily localizes to the overlap zone where the two layers of microtubules of the adhesive disc

overlap (Figure 1c–d; Supporting Information, Figures S5–S6). This finding is in contrast to an earlier report²³ that DAP16263 localizes to short supernumerary microtubules which appear in a small patch positioned opposite the overlap zone, are not connected to the main disc microtubules, and lack associated microribbons.²⁵ From a careful examination of our data, it was apparent that DAP16263 tracked the dorsal layer of microtubules, rather than the ventral layer, both laterally (Figure 3b–d) and axially (Figure 3e–f), and that DAP16263 localized to a position between the two microtubule layers. These observations suggest a possible role of DAP16263 in securing the disc's dorsal and ventral layers together.

The flagellar axonemes of *Giardia* were also strongly stained by the acetylated tubulin antibody and revealed a wealth of detail by ExSIM (Figure 4; Supporting Information, Movie 2). In maximum intensity projections we resolved either four or five distinct parallel lines along the curvature of the axoneme, which we attributed to the individual microtubule doublets. These parallel lines were visible at a wide range of angles and heights throughout the image, and the images were void of edge ringing, ruling out their source as potential SIM artifacts, such as angle-specific stripes/hatching, honeycomb artifact, or “ghosting”.¹⁹ Axoneme transverse cross-sectional profiles were consistently hollow (Figure 4b–f) with a diameter of ~250 nm and we clearly resolved parallel bundles of microtubule doublet filaments within the axonemes with a separation of ~70 nm (Figure 4h–i), all in good agreement with prior measurements by electron microscopy (Figure 4j).²⁶ The features are somewhat elongated in the axial direction because the ~75 nm axial resolution of ExSIM is worse than the ~30 nm lateral resolution. We did not observe the central pair of microtubules, possibly due to the inability of the anti-acetylated tubulin antibody to access the axoneme core and/or a lack of acetylation on the central pair. Using 3D SMLM, we were able to visualize features in *Giardia* axonemes with approximately the same dimensions as we observed by ExSIM, although the SMLM images had an apparently poorer spatial resolution, perhaps due to the high density of the object, and a much more limited axial range <500 nm (Supporting Information, Figure S7).

Portions of the ventral axonemes of *Giardia* were also brightly stained by DAP16263 along a narrow filamentous structure that tracked both dorsal surfaces for distances of ~10 μm (Figure 4a). Occasionally, one of the ventral axonemes exhibited a second, shorter DAP16263 filament ~0.5–2 μm long near to the adhesive disc (Supporting Information, Figure S5). A side-on projection of flagella co-immunostained for acetylated tubulin and DAP16263 showed that the DAP16263 filaments at their distal tips approached within ~50 nm of the microtubules, but separated from the axoneme by up to ~500 nm near the adhesive disc (Figure 4g). Based on these observations, we believe that DAP16263 is a component of the paraflagellar rod, a dorsal protrusion of ventral flagella previously identified with electron microscopy and which is enclosed within the flagellar membrane (Figure 4j).^{25,30} A close inspection of our DAP16263/acetylated tubulin ExSIM data sets revealed that DAP16263 also displayed variable, often weaker localization patterns. These included punctate to broad labeling of: basal bodies at the disc center; short filaments extending from the basal bodies along the six posteriorly directed axonemes; and the cytoplasmic portion of the two anterior flagella (Supporting Information, Figure S5).

Discussion

The successful combination of specimen expansion and SIM required careful technique and attention to experimental procedure including: the use of a sturdy, high-acrylamide hydrogel for specimen expansion that is easier to handle and improves the ability to cleanly detach biological specimens from their substrate; a poly-L-lysine strategy for attaching hydrogels to coverglass substrates which minimizes hydrogel drift during data acquisition and which immobilizes the expanded hydrogel surface close to a coverglass substrate; and efforts to minimize spherical aberration through optimization of immersion oil and if necessary through post-acquisition deconvolution. We have documented the procedures for ExSIM in a detailed experimental protocol (Supplementary Protocol 1).

We found that adjacent microtubules of *Giardia*'s adhesive disc exhibited a spacing of 54 ± 1 nm by ExSIM, which agrees excellently with our SMLM measurements on unexpanded specimens (53 ± 6 nm) and cryoelectron tomography measurements (53 ± 2 nm)²¹ from similar regions of the disc. Based on Gaussian-fitting of adhesive disc microtubules, which had a FWHM of 33 ± 3 nm, we estimate a spatial resolution of ~ 30 nm, a value consistent with the ~ 110 nm resolution of SIM divided by the $3.5\times$ expansion factor. We observed $<2\%$ distortions over a range of length scales, in agreement with previously reported single-step expansion procedures.² Our high-acrylamide hydrogel consistently expanded $\sim 3.5\times$, with all experiments falling within the range of $3.4\text{--}3.6\times$, and they reliably detached specimens from the surface of the glass substrate. We were able to implement multi-color imaging over a range of over $2\text{ }\mu\text{m}$ (in pre-expansion dimensions) which is a larger thickness than is typically interrogated using established super-resolution microscopy methods such as SMLM at a similar ~ 30 nm spatial resolution.

We compared the performance of ExSIM and SMLM in imaging the microtubule arrays of *Giardia*'s adhesive disc and axonemes. While we measured similar dimensions for the structures by both methods, we found ExSIM to be superior in several ways. First, ExSIM could readily image much thicker samples in 3D with data sets of $\sim 2\text{ }\mu\text{m}$ (in pre-expansion units) compared to ~ 500 nm for SMLM. Second, SMLM excels at imaging relatively sparse specimens, such as sparse, thin layers of microtubules or nuclear pores,^{31,32} but is hindered by double-localization artifacts³³ in dense samples such as *Giardia*'s dense microtubule arrays. This underappreciated limitation of SMLM means that dense objects have worse spatial resolution since the maximum number of achievable localizations per detection volume, which is limited by the fluorophore photoswitching properties, will be divided among many objects.^{33,34} The increased volume of the astigmatic point spread function used in 3D SMLM³⁵ compounds this limitation. Third, the time to acquire a data set in ExSIM was much shorter than for SMLM (<1 min/channel compared to >20 min/channel), although this comes at the cost of substantially longer sample processing time for ExSIM. Finally, ExSIM is compatible with a much wider range of fluorophores than SMLM which is currently limited by fluorophore photoswitching performance particularly for multichannel imaging.³³

The ~ 33 nm FWHM that we observed for *Giardia*'s adhesive disc microtubules by both ExSIM and SMLM (Figure 2g; Supporting Information, Figure S3) was markedly narrower

than previous super-resolution microscopy measurements of microtubules in mammalian cells (~50–60 nm).^{36,37} Although microtubules are well-known to have a ~25 nm outer diameter,³⁸ microtubules labeled with primary and secondary antibodies are substantially broadened when using primary antibodies which bind the outer surface of microtubules (e.g., rat anti-tubulin YL1/2).^{36,37} In contrast, the anti-acetylated tubulin primary antibody that we used (mouse anti-acetylated tubulin, clone 6–11B-1) binds to an epitope on the inner surface of microtubules^{39,40} which likely leads to a narrower distribution. The narrow FWHM values may also be influenced by the unique structure of *Giardia*'s adhesive disc, where microtubules are bound on several sides by disc microribbons and other structures²⁹ that may help to confine immunolabeling to a narrow region. Some amount of punctate labeling of disc microtubules is evident in our data for both ExSIM and SMLM. This may result from an uneven distribution of the acetylation post-translational modification in the lumen of microtubules, from imperfect labeling of the specimen by the antibody, fixation artifacts, or (for ExSIM only) minor expansion artifacts. In comparison with the 50–60 nm microtubule FWHM typically observed by superresolution microscopy, however, the ~33 nm FWHM values observed here are considerably smaller and would be expected to lead to more punctate signals simply due to the smaller number of labels able to bind the specimen per unit length.

Using ExSIM, we were able to resolve several key features of *Giardia*'s adhesive disc and flagellar axonemes which were not previously known. First, we localized the protein DAP86676 to adhesive disc microribbons, rather than crossbridge interstices, in agreement with suggestions from a previous immunoelectron microscopy study.⁴¹ Second, we found that disc-associated DAP16263 proteins localize to the overlap zone, not super-numerary microtubules as had been previously reported.²³ Further, DAP16263 localized between the two layers of microtubules in the disc overlap zone but laterally and axially tracked the dorsal microtubule layer, suggesting a possible role for DAP16263 in regulating the conformational dynamics of the disc that are hypothesized to facilitate attachment to the host.²⁴ Third, we found that DAP16263 localized strongly to the ventral axonemes at a position which, in the distal portion of the DAP16263 filament, corresponds to location of the small paraflagellar rod in *Giardia* that was previously identified by electron microscopy but was of unknown composition (Figure 1; Figure 4j).^{25,30} The ventral flagella exhibit a sinusoidal motion unique among the eight flagella of *Giardia*, and the paraflagellar rod and fin contributes to the ability of the ventral flagella to generate propulsive force.⁴² Fourth, ExSIM also revealed that DAP16263 displayed weaker, but notable localization patterns on basal bodies and proximal regions of the axonemes. In particular, the appearance of filaments along the anterior surface of the anterior flagella suggests that DAP16263 is likely involved in the network of proteins observed by scanning electron microscopy to link the anterior axonemes to the marginal plate.⁴³ The multiple instances of DAP16263 localizing to junctions between distinct structures (dorsal/ventral disc microtubules, ventral axoneme/paraflagellar rod, and anterior flagella/marginal plate) are intriguing, especially considering that the protein lacks the microtubule-binding domain of its better-known homolog DIP13.²³ Future work will be necessary to uncover the function of DAP16263 for these dynamic tubulin-based organelles. More broadly, gaining new insights into *Giardia*'s biology through a detailed localization of key proteins will be valuable in understanding the mechanism of

ventral disc functions and maintenance of infection as well as uncovering new drug targets for a human parasite that actively infects ~4% of the world's population.²³

Previous work using SMLM was unable to resolve the individual microtubule doublets of mouse sperm axonemes,⁴⁴ although SMLM and STED have been able to reveal the 9-fold symmetry of peripheral centriole components (~300 nm diameter *versus* 200–250 nm for axonemes).^{45,46} Here, we were able to resolve axoneme microtubule substructure using both ExSIM and SMLM, however, ExSIM consistently yielded superior data. Thus, ExSIM emerges as a promising method for studying nanoscale protein distributions on flagella, cilia, and related structures which impact an ever growing range of biological processes and diseases.⁴⁷

Finally, we would like to add that having SIM and ExM capabilities offer a versatile pair of super-resolution microscopy methods which offer several advantages alone or in combination. First, SIM is able to attain a modest ~twofold increase in resolution over confocal microscopy (~110 nm lateral resolution), is rapid, has a relatively low exposure dosage compared to STED and SMLM, is compatible with nearly all fluorescent probes and fixed or living specimens, and requires minimal sample processing. Second, ExM can achieve robust ~65 nm lateral resolution in fixed, thin or thick specimens using conventional fluorophores and widely available confocal microscopes.^{1–4} Third, the combination of SIM and ExM achieve a lateral spatial resolution of ~30 nm, with a demonstrated separation of features here of ~50 nm.

During review of this work, Cahoon *et al.* published an account of the use of SIM to study synaptonemal complexes in expanded *Drosophila* ovaries.¹⁵ While the work faced some challenges with inconsistently expanding hydrogels and residual SIM artifacts, the results nonetheless demonstrate the power of combining specimen expansion and SIM for nanoscale imaging of biological specimens. Additionally, similar improvements in performance may be obtainable by combining specimen expansion with other super-resolution modalities including SMLM and STED.^{20,48}

Our work was somewhat limited by the use of a popular, commercial SIM microscope which is only configured for operation with an oil-immersion objective lens. This constraint can lead to serious SIM artifacts when imaging deep into aqueous hydrogels due to refractive index mismatch, and we therefore imaged only modestly thick specimens with it (~8 μ m, post-expansion dimensions). The problem could be largely bypassed, however, by either: 1) developing an index matching solution¹⁸ for use with highindex oil immersion lenses which is compatible with expansion hydrogels (*i.e.*, minimal hydrogel shrinkage and minimal fluorophore quenching); or 2) using water-immersion objective lenses for SIM microscopy and optimizing their performance. Either of these approaches, if successful, would greatly extend the useable depth of ExSIM, and the method could potentially become only limited by objective lens working distance and fluorophore bleaching rates.

Methods

Reagents

Unconjugated donkey anti-rat (712-005-151), donkey anti-mouse (715-005-151) and rabbit anti-mouse (315-005-003) secondary antibodies, conjugated Alexa Fluor 488 donkey anti-rat antibody (712-545-150), and unconjugated streptavidin (016-000-084) were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Conjugated Alexa Fluor 568 goat anti-rat antibody was purchased from Thermo Fisher (Waltham, MA, USA). Primary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA) including mouse anti-acetylated tubulin (clone 6–11B-1, T7451) and rat anti-HA (clone 3F10, 11867423001). Bovine serum albumin (BSA) was purchased from Santa Cruz Biotechnology. NHS-functionalized (amine-reactive) dyes and biotin were obtained from (ATTO 488) Sigma-Aldrich or (Alexa 546, Alexa 568, Alexa 647, EZ-link NHS-PEG-4-Biotin) Thermo Fisher. Dyes were obtained in 1 mg aliquots from the suppliers, dissolved at a concentration of ~100 mg/mL in anhydrous DMSO, sub-aliquoted into anhydrous DMSO at 10 mg/mL, and stored at –20 °C. NAP-5 size-exclusion chromatography columns were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom). Methacrylic acid N-hydroxy succinimidyl ester (MA-NHS), anhydrous dimethyl sulfoxide (DMSO), sodium bicarbonate, PIPES salt (for buffer), Ethylene Glycol Tetraacetic Acid (EGTA), magnesium chloride, Triton X-100 (TX100), poly-L-lysine solution (0.1% w/v), cold water fish skin gelatin, and sodium acrylate were obtained from Sigma-Aldrich. MA-NHS was dissolved in anhydrous DMSO at a concentration of 1 M and stored at –20 °C until used. Guanidine hydrochloride was purchased from MP Biomedicals (Irvine, CA, USA). PBS and TAE (Tris Acetate EDTA) 10×stock solutions, and Proteinase K (>600 U/mL, EO0491) were obtained from Thermo Fisher. Acrylamide (40%), N,N'-methylenebisacrylamide (bis-acrylamide, 2%), ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were purchased from Bio-Rad (Hercules, CA, USA). Paraformaldehyde (32%) and glutaraldehyde (50%) were obtained from Electron Microscopy Sciences (Hatfield, PA, USA). TetraSpeck 0.1 μm fluorescent beads were purchased from Thermo Fisher.

Preparation of fluorophore-labeled antibodies and streptavidin

Fluorophore-conjugated antibodies or streptavidin were prepared as follows. To 80 μL of unconjugated protein (~1.3 mg/mL IgG, or 1 mg/mL streptavidin) was added 10 μL of aqueous 1 M sodium bicarbonate (pH ~8.3) and 1 μL of NHS-dye stock in DMSO. These reagents were allowed to react at room temperature (22 °C) for ~30 min. For singly labeled antibody, typically ~5–10 μg of NHS-dye was added, and for dually-labeled antibodies containing fluorophores and biotin, 10 μg of NHS-PEG-4-Biotin was added after the first reaction. During the reaction, a NAP-5 size-exclusion chromatography column, for purification of labeled antibody from free dye, was equilibrated by flowing ~10 mL of PBS through each column. The ~100 μL reaction was loaded onto the column followed by flowing through and discarding 600 μL of PBS and flowing through and keeping 400 μL eluate. The eluate was characterized by absorption spectroscopy by measuring the average concentration of dye and average concentration of antibody according to the instructions provided by the dye manufacturers. Care was taken to avoid adding more than ~5 % DMSO

to the antibody solution to avoid disturbing the antibody in all antibody-labeling reactions. The obtained dye to protein ratios are listed in Supporting Information, Table 1.

Preparation of poly-L-lysine coverglass

For plating of *Giardia lamblia*, 12 mm round #1.5 coverglass was purchased from Electron Microscopy Sciences, and soaked in 5 M KOH overnight. After copious rinsing with water, the glass was dried before 1 μ l of poly-L-lysine solution was evenly spread on the surface and heated on a hotplate at 90 °C for 10 min. The slides were then rinsed with water and dried. For immobilization of hydrogel specimens for SIM imaging, 25.4 mm $\times$ 50 mm #1.5 coverglass (Fisher) were exposed to an air plasma for 60 s to render the glass hydrophilic, then 3 μ l of poly-L-lysine was evenly spread on the surface and allowed to dry. Excess water from hydrogel specimens was gently removed using a paper towel or compressed air, and the gel was transferred onto the lysine coated glass to form a drift-free adhesion just before imaging. Refer to Supplemental Protocol for additional information about sample mounting.

Giardia Culturing and Immunostaining

Giardia lamblia strain WBC6 (ATCC 50803) with integrated expression of HA-tagged DAP86676 or HA-tagged DAP16263 (see below) were cultured in TYI-S-33 medium supplemented with 10% bovine serum, 0.05 mg/ml bovine bile, and 35 μ g/ml puromycin in tightly capped 15 mL polystyrene culture tubes. At 70–90% confluency, the cells were iced for 10 min, gently mixed, and 2 mL of cells were added to each well of a 24-well plate containing a 12mm round coverglass coated with poly-L-lysine as described above. The cells were allowed to recover in an atmosphere-controlled incubator containing 95% N₂ at 37 °C for 1–2 h until they were densely adhered to the coverglass. For fixation, the cell media was quickly aspirated and 1 mL of warm 3.2% PFA in 1 $\times$ PEM buffer (100 mM PIPES, 1 mM EGTA, 1 mM MgCl₂, pH 6.9) was immediately added to each well. The fixation was allowed to proceed for 20 min at 37 °C, then the samples were washed and stored in PBS containing 3 mM NaN₃ at 4 °C. For immunostaining, *Giardia* were incubated in PEMBALG (50 mM PIPES, 5 mM EGTA, 10 mM MgSO₄, 1% BSA, 0.1 M lysine, 0.5% gelatin, 3 mM NaN₃ and 0.1% TX100) for 30 min, followed by overnight incubation with primary antibodies in PEMBALG. The sample was washed, incubated with secondary antibodies for 1 hr, washed, and if necessary, incubated with tertiary antibody, or labeled streptavidin for 1 hr. All washes involved a minimum of three PBS exchanges, with the last step being allowed to rest for 15 min. Antibody dilutions and additional details are summarized in Supporting Information, Table 1.

Vector Construction and Giardia Transformation

HA-tagged DAPs for integrated expression in *Giardia* were constructed by amplifying 86676 (8-Giardin) or 16263 from WBC6 genomic DNA and inserted into pKS_3HA_Pur (linearized by NotI and AflIII) by Gibson Assembly. After construction, 86676_3HA_Pur or 16263_3HA_Pur were linearized for homologous recombination by PstI or NsiI, respectively and ~10 μ g of plasmid was electroporated into 300 μ l of *Giardia* in a 0.4 cm cuvette using a GenePulser XCell (BioRad) set to 375 V, 1000 μ F and 750 ohms. After overnight recovery in growth media without antibiotics, selection began with 15 μ g/ml

puromycin and ramped up to 35 $\mu\text{g/ml}$ over 10 days. See Supporting Information, Table 2 for primers.

Polymerization, Digestion and Expansion

After immunostaining, the sample was washed and incubated in a solution containing 25 mM solution of MA-NHS in PBS for 30 min. The sample was then washed and incubated in monomer solution (8.6% sodium acrylate, 20% acrylamide, 0.075% bis-acrylamide, 2 M NaCl, 1 $\times$ PBS) for 5 min. Fresh stocks of 10% APS (w/w) and 10% TEMED (w/w) in water were diluted in monomer to a final concentration of 0.2% (w/w) to initiate polymerization. Excess incubation monomer was immediately wicked off the sample and it was inverted onto a 70 μL droplet of the initiated monomer and allowed to polymerize for 20 min. The polymerized sample on the coverglass was then digested overnight in 1 mL of digestion buffer (0.8 M guanidine hydrochloride, 1 $\times$ TAE, 0.5% TX100, pH 8.3) containing ~ 6 U/mL of Proteinase K at 37 $^{\circ}\text{C}$. The sample released from the coverglass after digestion and was transferred to a dish containing ~ 50 mL of water. The sample was allowed to expand for ~ 2 hr in deionized water, with water exchanges every ~ 30 min until complete. See Supplementary Protocol for additional details.

Fluorescence Microscopes

3D Structured-Illumination microscopy was performed using a GE Healthcare DeltaVision OMX with an Olympus 60 $\times$ 1.42 NA oil objective. Raw images were acquired from 20–100 ms per frame at 95 MHz CMOS readout. Two-channel data spanned 10–15 μm in post-expansion depth and required acquisition times of ~ 2 minutes, however the specimen signal was typically limited to the central < 8 μm of the stack in order to avoid edge ringing artifacts during reconstruction. Immersion oil (Laser Liquid 5610, Cargille Labs, Cedar Grove, NJ, USA) was selected from range of $n = 1.514$ to $n = 1.530$ and was carefully optimized to compensate for spherical aberration. This was accomplished by taking short z-stacks to observe the axial profile of approximately diffraction-limited objects near the plane of interest in the specimen. The oil was then adjusted to yield symmetric intensity profiles above and below the plane of interest. Image reconstruction was performed in SoftWorks (GE Healthcare).

Confocal microscopy was performed on a Leica SP5 inverted confocal scanning microscope at the UW Biology Imaging Core using a 63 $\times$ 1.2 NA water lens (Leica, Nussloch, Germany).

Localization microscopy was performed on a homebuilt Nikon Ti-U system using a Nikon CFI Plan Apo Lambda 100 $\times$ 1.45 NA objective and a 647-nm diodepumped solid-state laser source (MPB Communications, Pointe-Claire, QC, Canada). A 405-nm solid state laser (Obis, Coherent) was used for activation to increase the rate of fluorophore blinking. Localization images were acquired on an EMCCD (iXon Ultra 897, Andor) operating at 200 frames per second. For 3D localization microscopy, a 1000 mm cylindrical lens was placed in the imaging path to create an astigmatic point spread function.

Expansion Factor and Distortion Analysis

Correlative pre- and post-expansion images of the same specimen were recorded by confocal microscopy, with the pre-expansion images being obtained prior to the MA-NHS treatment step. Registration of pre- and post-expansion images using rigid (translation, rotation and uniform scaling in 3-dimensions), and nonrigid (B-spline) transformations were performed to determine the physical expansion factor and analyze distortions, respectively. Detailed protocols of image registration and distortion analysis using the open source software *Elastix*⁴⁹ have been reported previously.² For routine work, a ruler was used to measure the macroscopic gel size before and after expansion to confirm it was in the range of 3.4–3.6× for every specimen.

Image Processing

After SIM reconstruction, all images were subjected to custom lateral and axial chromatic registration. To generate the registration transforms, 0.1 μm TetraSpeck beads were immobilized on glass coverslips, as well as in an acrylamide matrix (5% acrylamide, 0.25% bis-acrylamide), and were imaged using widefield illumination on the SIM microscope. The subpixel bead positions were determined for the 488 nm and 568 nm channels using 3D Gaussian fitting. The lateral XY positions of the beads were used to find and apply a similarity transform (translation, rotation and uniform scaling) to map the 568 nm channel to the 488 nm channel. Axial chromatic registration was performed by resampling the 568 nm channel with a cubic polynomial that mapped it to the 488 nm channel using the surface-adsorbed immobilized beads as a reference frame. After registration, the residual 3D chromatic error was $\sim 37 \pm 18$ nm in physical dimensions which corresponds to $\sim 11 \pm 5$ nm in pre-expansion dimensions. For samples containing adhesive disc structures traversing many microns in depth (>5 μm post-expansion dimensions), the 568 nm channel exhibited significant “ghosting” that could not be compensated for using index oil alone; we applied a depth-dependent axial deconvolution to the 568 nm channel using an axial point-spread function determined from the axial profile of the beads obtained by SIM. To computationally straighten curved features in this work, structures were first isolated using a binary mask to determine the direction of propagation in 2D or 3D, then resampled in 40 nm increments in the planes perpendicular using cubic interpolation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

This work is supported by the University of Washington (J.C.V.), a Burroughs-Wellcome Career Award at the Scientific Interface (J.C.V.), NIH grants R01MH115767 (J.C.V) and R01AI110708 (A.R.P.), and an NSF Graduate Research Fellow-ship DGE-1256082 (T.J.C.).

The authors would like to thank J. Scott and L. Wordeman (University of Washington, Seattle, WA) for access to a SIM microscope; S. Dawson (University of California, Davis, CA) for helpful discussion and advice; and the Biology Imaging Facility at the University of Washington.

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Figure 1. Schematic diagram of specimen expansion and microtubule cytoskeleton of *Giardia lamblia*. Structures containing microtubules are displayed in green and disc-associated proteins (DAPs) are displayed in magenta. (a) Fixed *Giardia* specimens were immunostained, treated with a linker group, embedded in a swellable acrylamide/acrylate polymer, and digested with Proteinase K (inset below). (b) Dialysis in water expanded the specimen by 3.5 $\times$. Key landmarks are also shown in **b**, including structures of the adhesive disc (microtubules, DAP86676, overlap zone (OZ), and ventral groove (VG)), the eight flagella of *Giardia* (axonemal microtubules, anterior flagella (AF), and DAP16263 associated with the two ventral flagella (VF)), and the $\sim 10\ \mu\text{m} \times 15\ \mu\text{m}$ cell body (dashed line). (c,d) Dorsal and ventral layers of adhesive disc microtubules in the overlap zone with plus and minus ends of microtubules as indicated. DAP16263 is shown in its assigned location from this work in **c**. (e) Transverse view of adhesive disc microtubules and their associated microribbons containing DAP86676. (f) Transverse view of ventral flagella with canonical “9 + 2” microtubule axoneme geometry, and localization of DAP16263 to the paraflagellar rod as determined in this work. The long fin of the ventral flagellum, opposite to the paraflagellar rod, is truncated as shown.

Figure 2.

ExSIM image of *Giardia* adhesive disc microtubules and DAP86676. (a) Maximum intensity projection of *Giardia* stained for acetylated tubulin with the z-dimension position color-coded according to the color scale bar. (b,c) Zoomed-in view of boxed area in a showing acetylated tubulin and DAP86676. (d) Cross-section projection of acetylated tubulin (green) and DAP86676 (magenta) of the boxed in region of b and c. (e) Line profiles and Gaussian fits (solid lines) from boxed in regions of d (arb., arbitrary units). (f) Distribution of microtubule-microtubule spacing and (g) microtubule full width at half maximum (FWHM) as determined from transverse line profiles fit to a series of Gaussian functions. (h)

Distribution of DAP86676-tubulin distance along the axial direction. All distances and scales are in pre-expansion units. Scale bars, 1 μm (a), 200 nm (b–d).

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Figure 3.

ExSIM of *Giardia* adhesive disc microtubules (magenta) and DAP16263 (green). (a) Maximum intensity projection of *Giardia* adhesive disc stained for acetylated tubulin and HA-tagged DAP16263, showing that DAP16263 localizes to the crescent-shaped overlap zone where the ventral and dorsal disc microtubule layers overlap. (b–d) Straightened maximum intensity projection of dorsal microtubules (MTs), DAP16263, and ventral microtubules, respectively, from the boxed area in panel **a**, indicating that DAP16263 laterally tracks the dorsal microtubules. (e–f) Transverse x-z views of the adhesive disc and DAP16263 along the dashed lines in **b–d**, indicating that DAP16263 tracks the dorsal layer

but localizes between the dorsal and ventral microtubule layers. (g) Histogram of DAP16263 displacement from the dorsal layer of microtubules determined for the region highlighted in **a**. All distances and scale bars are in pre-expansion units. Scale bars, (**a**) 1 μm , (**b–f**) 200 nm.

Figure 4.

ExSIM images of *Giardia* flagella. (a) Maximum intensity projection of *Giardia* flagella stained for acetylated tubulin (green) and HA-tagged DAP16263 (magenta). (b–f) End-on projections of axoneme and DAP16263 from the solid lines marked in **a**. (g) Side-on cross section of the boxed axoneme in **a**. A weak axial “ghost” artifact is visible in the DAP16263 channel due to spherical aberration resulting from index of refraction mismatch for the longer wavelength fluorophores. Top-down maximum intensity projection of the dorsal half (h) and ventral half (i) of the axoneme marked in **a** and **g**. The projections were laterally smoothed parallel to the axoneme direction by a 45 nm wide moving average. (j) Electron

micrograph of a transverse section of a ventral flagellum showing the electron-dense paraflagellar rod. All distances and scale bars correspond to pre-expansion dimensions. Scale bars, (a) 1 μm , (b–f, h, i) 200 nm, (g) 500 nm, (j) 100 nm. Panel **j** was adapted from Parasitology Research, “Triton-labile antigens in flagella isolated from *Giardia lamblia*”, 74, 1988, 415–423, Clark J. T. and Holberton D. V. With permission of Springer.