Proximity Staining Using Enzymatic Protein Tagging in Diplomonads

Ásgeir Ástvaldsson,a Kjell Hultenby,b Staffan G. Svärd,a* Jon Jerlström-Hultqvist**

aDepartment of Cell and Molecular Biology, BMC, Uppsala University, Uppsala, Sweden
bDepartment of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden

ABSTRACT The diplomonads are a group of understudied eukaryotic flagellates whose most prominent member is the human pathogen Giardia intestinalis. Methods commonly used in other eukaryotic model systems often require special optimization in diplomonads due to the highly derived character of their cell biology. We have optimized a proximity labeling protocol using pea ascorbate peroxidase (APEX) as a reporter for transmission electron microscopy (TEM) to enable the study of ultrastructural cellular details in diplomonads. Currently available TEM-compatible tags require light-induced activation (1, 2) or are inactive in many cellular compartments (3), while ascorbate peroxidase has not been shown to have those limitations. Here, we have optimized the in vivo activities of two versions of pea ascorbate peroxidase (APXW41F and APEX) using the diplomonad fish parasite Spironucleus salmonicida, a relative of G. intestinalis. We exploited the well-known peroxidase substrates, Amplex UltraRed and 3,3′-diaminobenzidine (DAB), to validate the activity of the two tags and argue that APEX is the most stable version to use in Spironucleus salmonicida. Next, we fused APEX to proteins with established localization to evaluate the activity of APEX in different cellular compartments of the diplomonad cell and used Amplex UltraRed as well as antibodies along with superresolution microscopy to confirm the protein-APEX localization. The ultrastructural details of protein-APEX fusions were determined by TEM, and we observed marker activity in all cellular compartments tested when using the DAB substrate. Finally, we show that the optimized conditions established for S. salmonicida can be used in the related diplomonad G. intestinalis.

IMPORTANCE The function of many proteins is intrinsically related to their cellular location. Novel methods for ascertainment of the ultrastructural location of proteins have been introduced in recent years, but their implementation in protists has so far not been readily realized. Here, we present an optimized proximity labeling protocol using the APEX system in the salmon pathogen Spironucleus salmonicida. This protocol was also applicable to the human pathogen Giardia intestinalis. Both organisms required extraneous addition of hemin to the growth medium to enable detectable peroxidase activity. Further, we saw no inherent limitation in labeling efficiency coupled to the cellular compartment, as evident with some other proximity labeling systems. We anticipate that the APEX proximity labeling system might offer a great resource to establish the ultrastructural localization of proteins across genetically tractable protists but might require organism-specific labeling conditions.

KEYWORDS APEX, DAB, Giardia, proximity labeling, Spironucleus salmonicida

The diplomonads are a diverse group of unicellular flagellated microorganisms that are binucleated and lack some typical eukaryotic features, e.g., Golgi and mitochondria capable of oxidative phosphorylation, although mitochondrial remnant organelles (MROs) have been identified, e.g., mitosomes in Giardia spp. and hydrogeno-
somes in *Spiroplasma salmonicida* (4–6). The highly derived cell biology of the binucleated and tetraploid diplomonad cells as well as high proportion of hypothetical proteins uncovered in genome sequencing efforts present special experimental challenges. Furthermore, even related lineages, like *Giardia* and *Spiroplasma* spp., contain thousands of lineage-specific genes whose functions cannot easily be inferred by homology (7). There has been steady progress in developing diplomonad genetic tools, including successful demonstrations of genetically encoded ultrastructural labeling in *Giardia intestinalis* (8) and in *S. salmonicida* (9), but the methods have not been evaluated and optimized in a systematic way in any diplomonad.

Horseradish peroxidase (HRP) has been used as a genetically encoded fusion partner, but a number of shortcomings, such as sensitivity to the redox state of a cell compartment and the necessity to coordinate Ca$^{2+}$ ions, have limited its use (10). HRP is also sensitive to inactivation by aldehyde-based fixatives and does not exhibit robust activity after fixation optimal for the preservation of cell ultrastructure. The class 2 peroxidases, including ascorbate peroxidase (APX) from legumes, lack disulfide bonds, have no need to coordinate Ca$^{2+}$ ions, and are active even after harsh chemical fixation (11). Recently, the Ting lab developed the pea ascorbate peroxidase (APEX), a mutated version of APX, which is monomeric and active in all cellular compartments tested (11). APEX is active with a range of different substrates, including 10-acetyl-3,7-dihydroxyphenoxazone (Amplex UltraRed) and 3,3′-diaminobenzidine (DAB). When transfected cells expressing the peroxidase are immersed in a solution of Amplex UltraRed and hydrogen peroxide (H$_2$O$_2$), the peroxidase oxidizes the substrates to generate a precipitate called resorufin at the location of the tagged protein, which can be imaged by fluorescence microscopy (11). Using DAB is analogous to the reaction with Amplex UltraRed, but here, peroxidase catalyzes the oxidation of the DAB substrate in the presence of H$_2$O$_2$, generating a dark brown alcohol-insoluble precipitate at the location of the protein of interest (11). The precipitate is visible by light microscopy, and the osmiophilic nature of the precipitate enables enhanced contrast by transmission electron microscopy (TEM) (see Fig. S1 in the supplemental material) (11).

Here, we describe an optimized protein proximity labeling protocol in the diplomonad *S. salmonicida* using the pea version of APEX as a fusion partner (11), thereby expanding the method toolkit already established in this organism (12). We also demonstrate that the optimized protocol is applicable to the diplomonad model organism *G. intestinalis*, the most studied diplomonad parasite. The comparative ease of use of the APEX system will make high-resolution ultrastructural labeling more tractable and not exclusively reliant on antibody staining. It will also permit high-resolution comparative ultrastructure localization studies of shared diplomonad traits.

**RESULTS**

**Initial optimization of ascorbate peroxidase activity.** We generated transfected strains carrying episomal vectors that expressed protein fusions to two mutated variants of pea ascorbate peroxidase, W41FAPX and APEX, to evaluate and optimize their activities in *S. salmonicida*. We tagged a well-characterized structural protein, annexin 5 (SS50377_10477), which has a very distinct localization in the anterior end of the cell, resembling a hat-like structure of unknown function (9). Initial experiments showed no clear evidence of W41FAPX or APEX-derived peroxidase activity in the transfected cells when grown in the standard liver digest-yeast extract-iron (LYI) growth medium, even though we were able to demonstrate the presence of the expected full-length protein and its correct subcellular localization (see Fig. S2A). We reasoned that a lack of peroxidase activity might be connected to low bioavailability and incorporation of the heme cofactor since diplomonad cells do not synthesize their own heme (13). Interestingly, *Giardia* spp. are still able to scavenge sufficient heme to accommodate at least five heme-incorporating proteins (13). Homologues to those very same proteins are also present in the genome of *S. salmonicida*, indicating that it may also be able to scavenge heme from the extracellular milieu. To boost APEX activity, we grew our transgenic cell lines in LYI medium supplemented with 200 μM hemin. Under these
growth conditions, we noticed pronounced in vivo APEX activity, validating our suspicions of low bioavailability of hemin as the reason for a lack of APEX activity.

**Optimization of hemin and hydrogen peroxide concentrations.** Excessive hemin concentrations are known to be detrimental to cells due to the production of reactive oxygen species or membrane peroxidation (14, 15). In line with these observations, we noticed poor growth and changes to cell morphology at 200 \( \mu \)M hemin supplementation. We sought to minimize the concentration of added hemin to establish a balance between cell viability and APEX activity. The transfectants were grown in five concentrations of hemin (0 to 200 \( \mu \)M), and the optimal hemin concentration was then estimated using the readout from both DAB and Amplex UltraRed substrate staining (see Fig. S3A). DAB and Amplex UltraRed were reacted with 10 mM and 8.5 mM \( \text{H}_2\text{O}_2 \) for 15 and 30 min, respectively. At the lowest hemin concentration, 40 \( \mu \)M, we observed healthy cells with the typical pyriform appearance, but the DAB signal was reduced compared to that with higher concentrations. The optimal hemin concentration was estimated to be between 80 and 120 \( \mu \)M since at these concentrations, the cells displayed pyriform morphology, could be maintained by weekly passage using an inoculum similar to that in in hemin-free medium, and showed easily detectable peroxidase activity with distinct localization. Using Amplex UltraRed as the substrate yielded similar results, but at higher hemin supplementation concentrations, the fluorescent signal became more diffusely localized (see Fig. S3B).

To establish further the optimal conditions for the peroxidase activity while maintaining cell survival and integrity, we used the DAB substrate and titrated the \( \text{H}_2\text{O}_2 \) concentration in a range of 0 to 3 mM while keeping the hemin concentration constant at 100 \( \mu \)M. With no addition of \( \text{H}_2\text{O}_2 \), the signal is effectively quenched, although we noticed some residual signal that might represent endogenous peroxidase activity. The signal is strongly stimulated by a low concentration of \( \text{H}_2\text{O}_2 \), and it does not significantly change with concentrations of 200 \( \mu \)M or above. High \( \text{H}_2\text{O}_2 \) concentrations negatively affected the cells, and the signal became more delocalized, even if the cells were fixed with glutaraldehyde or paraformaldehyde prior to labeling with the substrates (Fig. 1A). The above-mentioned observations were also found to hold true for the Amplex UltraRed substrate (Fig. 1B). A comparison between APEX and \( \text{W41FAPX} \) during the hemin and \( \text{H}_2\text{O}_2 \) titration experiments showed that the activity of APEX is much higher than that of \( \text{W41FAPX} \) at low hemin and \( \text{H}_2\text{O}_2 \) concentrations. The signal generated by \( \text{W41FAPX} \) is also more prone to be delocalized, while the APEX signal is more distinct and localized to the expected cellular structures (Fig. 1 and S3).

APEX is functional in different cell compartments of the diplomonad cell. We evaluated the broad functionality of APEX in diplomonads by constructing a collection of C-terminal protein fusions (13 proteins in *S. salmonicida* and 11 in *G. intestinalis*) (Table 1 and Fig. S4). The expression was provided by the native promoter of each gene from the context of an episomal plasmid vector. The localizations of most of these proteins were known a priori to allow us to inspect the activity level of APEX in different cell compartments. However, we also included two proteins with uncertain or previously unknown localization to demonstrate the utility of APEX in the characterization of novel proteins (Table 1). We appended a C-terminal V5 epitope tag to all the constructs to be able to independently confirm the localization and expression of the APEX fusion using immunofluorescence and Western blotting. APEX activity was then investigated using Amplex UltraRed and DAB, as described previously. We were unable to generate viable transfectant lines for histone H3 (SS50377_17319) and histone H3B (SS50377_17654) in *S. salmonicida* and fibrillarin (GL50803_97219), Cen H3 (GL50803_20037), and histone H3 (GL50803_135231) in *G. intestinalis*. We were able to confirm the expression of most protein-APEX-V5 fusions in *S. salmonicida* transfectants by Western blotting, and the observed molecular weights were in line with the predicted in silico values. The exception was the nuclear protein histone H3 variant (SS50377_10544), where no protein expression was observed (see Fig. S2A). In the transgenic *G. intestinalis* strains, we only detected the expected protein species expressed for three proteins, striated
S. salmonicida transfectants expressing Anx5-APEX or Anx5-APX were grown in LY1 medium supplemented with 100 μM hemin. Transfectants were washed with HBSS-G and spotted on a poly-lysine-coated microscopy slide. (A) Cells were fixed with 2% glutaraldehyde in 100 mM cacodylate buffer with 2 mM CaCl₂ before being reacted with 0.5 mg/ml DAB and 0 to 3 mM H₂O₂ for 15 min. Cells were washed with cacodylate buffer and PBS, mounted with VectaShield, and viewed in a phase-contrast microscope. Cells with white deposits in the anterior of the cells are positive for Anx5-APEX or Axn5-APX staining. (B) Cells were fixed with 2% paraformaldehyde in PBS and reacted with 50 μM Amplex UltraRed and 0 to 8.5 mM H₂O₂ for 30 min. The cells were washed extensively, mounted with VectaShield with DAPI, and imaged in a fluorescence microscope. Cells with red deposits in the anterior of the cells are positive for Anx5-APEX or Anx5-APX staining. Scale bars = 10 μm.

FIG 1 H₂O₂ titrations. S. salmonicida transfectants expressing Anx5-APEX or Anx5-APX were grown in LY1 medium supplemented with 100 μM hemin. Transfectants were washed with HBSS-G and spotted on a poly-lysine-coated microscopy slide. (A) Cells were fixed with 2% glutaraldehyde in 100 mM cacodylate buffer with 2 mM CaCl₂ before being reacted with 0.5 mg/ml DAB and 0 to 3 mM H₂O₂ for 15 min. Cells were washed with cacodylate buffer and PBS, mounted with VectaShield, and viewed in a phase-contrast microscope. Cells with white deposits in the anterior of the cells are positive for Anx5-APEX or Axn5-APX staining. (B) Cells were fixed with 2% paraformaldehyde in PBS and reacted with 50 μM Amplex UltraRed and 0 to 8.5 mM H₂O₂ for 30 min. The cells were washed extensively, mounted with VectaShield with DAPI, and imaged in a fluorescence microscope. Cells with red deposits in the anterior of the cells are positive for Anx5-APEX or Anx5-APX staining. Scale bars = 10 μm.
TABLE 1 Full list of S. salmonicida and G. intestinalis proteins used in this study with GiardiaDB accession numbers, sizes, and localizations

| Hypothetical protein | Organism        | GiardiaDB accession no. | Size (bp) | Localization (reference) |
|----------------------|-----------------|-------------------------|-----------|--------------------------|
| 10316                | S. salmonicida  | SSS0377_10316           | 3,242     | Two foci in anterior end of the cell (4) |
| Acid phosphatase     | S. salmonicida  | SSS0377_10140           | 1,082     | Endoplasmic reticulum-predicted localization |
| Annexin 5            | S. salmonicida  | SSS0377_10477           | 932       | Unknown structure in anterior of the nuclei (12) |
| BiP                  | S. salmonicida  | SSS0377_11120           | 1,925     | Endoplasmic reticulum (12) |
| Fibrillarin          | S. salmonicida  | SSS0377_13348           | 971       | Nuclei (12) |
| Histone H3           | S. salmonicida  | SSS0377_17319           | 431       | Nuclei (predicted localization) |
| NADH oxidase         | S. salmonicida  | SSS0377_12178           | 1,133     | Perinuclear region around the nuclei (4) |
| Histone H3B          | S. salmonicida  | SSS0377_17654           | 440       | Nuclei (predicted localization) |
| Histone H3var        | S. salmonicida  | SSS0377_10544           | 401       | Nuclei (predicted localization) |
| IFT46                | S. salmonicida  | SSS0377_16623           | 818       | Flagella (12) |
| IscS                 | S. salmonicida  | SSS0377_17654           | 1,197     | Hydrogenosomes (4) |
| IscU                 | S. salmonicida  | SSS0377_11862           | 445       | Hydrogenosomes (4) |
| PFORS                | S. salmonicida  | SSS0377_10765           | 3,140     | Hydrogenosomes (4) |
| SHMT                 | S. salmonicida  | SSS0377_17865           | 1,241     | Hydrogenosomes (4) |
| Acid phosphatase     | G. intestinalis | GL50803_7556            | 1,202     | Acid phosphatase activity in peripheral vesicles (29) |
| Alpha-14 giardin     | G. intestinalis | GL50803_15097           | 1,010     | Local slabs in flagella, and plasma membrane (18, 19) |
| Alpha-19 giardin     | G. intestinalis | GL50803_4026            | 1,316     | Ventral flagellum pair (30) |
| BiP                  | G. intestinalis | GL50803_17121           | 2,033     | Endoplasmic reticulum (31) |
| Cen H3               | G. intestinalis | GL50803_20037           | 470       | Nuclei (32) |
| Fibrillarin          | G. intestinalis | GL50803_97219           | 938       | Nucleolus (33) |
| Histone H3           | G. intestinalis | GL50803_135231          | 440       | Nuclei (32) |
| Histone H3B          | G. intestinalis | GL50803_3367            | 479       | Nuclei (32) |
| IscS                 | G. intestinalis | GL50803_14519           | 1,301     | Mitosomes (5) |
| IscU                 | G. intestinalis | GL50803_15196           | 638       | Mitosomes (5) |
| SALP-1               | G. intestinalis | GL50803_4410            | 764       | Ventral disc (16, 17) |
|                      | S. salmonicida  | SS50377_10316           | 3,242     | Two foci in anterior end of the cell (4) |
| Fiber assemblin-like protein 1 (SALP-1; GL50803_4410), alpha-14 giardin (GL50803_15097), and IscU (GL50803_15097). Additional N-terminal degradation products were detected for binding immunoglobulin protein (BiP; SSS0377_11120) in S. salmonicida and alpha-14 giardin in G. intestinalis (see Fig. S2B).

Next, we used structured illumination superresolution microscopy (SIM) to determine the subcellular localization of the protein-APEX-V5 constructs. Amplex UltraRed was used to initially assay the activity of APEX in different cellular compartments, and we found that the resorufin and V5 signals were colocalized in all transfectant strains. In S. salmonicida, the V5 antibody stain is observed in a more specific pattern and can be resolved with more detail, while the resorufin signal is more diffuse (Fig. 2A to H). In G. intestinalis, the V5 antibody does not produce efficient labeling; instead, the resorufin signal is stronger. Again, the patterns of the antibody staining and the resorufin precipitates are closely matched (Fig. 2I to K). The expressed proteins in both S. salmonicida and G. intestinalis display localizations (see descriptions below) that correspond well with their previously published subcellular localization or the expected localization based on data from other eukaryotes (Table 1).

In determination of the localizations of two of the proteins in S. salmonicida, acid phosphatase and BiP, show labeling around the two nuclei stretching to the cell posterior along the recurrent flagellar axis. The area posterior to the nuclei showed an especially prominent stain. This labeling pattern is consistent with previous work that localized proteins to the endoplasmic reticulum (ER) in S. salmonicida (Fig. 2A and B) (12).

Fibrillarin was found to be localized to distinct subregions in both nuclei. The stain was often found in a diffuse pattern at the nuclear periphery in regions not overlapping the genomic DNA stain. The focus of the stain was often in the anterior part of the nucleus, but it was not uncommon to find parts of the signal at other nuclear locations as well. This localization is consistent with previous localization data in S. salmonicida (Fig. 2C) (12).

SIM localization of intraflagellar transport 46 (IFT46) protein revealed punctuate localization along the flagella and the basal bodies. There was a clear enrichment of signal in the distal ends of the flagella. Again, this localization is consistent with previous localization data in S. salmonicida (Fig. 2D) (12).
The hydrogenosome is a type of MRO previously described in *S. salmonicida* (4). We determined the localizations of four hydrogenosomal proteins (IscU, IscS, serine hydroxymethyltransferase [SHMT], and pyruvate ferredoxin oxidoreductase [PFOR5]) using APEX fusions. All four proteins showed a typical hydrogenosome staining pattern with tens of round to ovoid foci distributed in the cytosol (Fig. 2E to H) (4).

Last for *S. salmonicida*, we determined the localization of two previously uncharacterized proteins. SS50377_12178 is protein with homology to NADH:flavin oxidoreductases that has potentially been laterally transferred from prokaryotes.

**FIG 2** Superresolution microscopy (SIM) of *S. salmonicida* (A to H) and *G. intestinalis* (I and J) transfectants. Transfectants expressing APEX-V5 were grown in LYI medium (*S. salmonicida*) and TYDK medium (*G. intestinalis*) supplemented with 100 μM hemin. Cultures were fixed with 2% paraformaldehyde in PBS and treated with 50 μM Amplex UltraRed (Red) and 200 μM H₂O₂ for 30 min. V5 epitope (green) was detected using a primary monoclonal mouse anti-V5 antibody and a secondary polyclonal goat anti-mouse Alexa Fluor 488-conjugated antibody. The cells were stained with 2 μg/ml DAPI solution (blue) for 10 min and mounted with VectaShield. Imaging was done by a Zeiss LSM710 microscope with a SIM module. Scale bars = 1 μm.
Localized distinctly to the perinuclear region around both nuclei (Fig. 3A). In a previous localization effort attempting to identify novel hydrogenosomal proteins, SS50377_10316 was serendipitously localized to two foci of uncertain identity in the anterior end of the cell (4). SS50377_10316 is a hypothetical protein with no recognizable homologs outside diplomonads. Our SIM localization data confirmed the previous localization and were able to further resolve each focus as two elongate structures positioned in the anterior end of the cell in close proximity to the nuclei (Fig. 3B).

**FIG 3** SIM and TEM images of two previously uncharacterized proteins. Transfectants expressing 10316-APEX-V5 and 12178-APEX-V5 were grown in LYI medium supplemented with 100 μM hemin. (A and B) SIM images. Transfectants were fixed with 2% paraformaldehyde in PBS and treated with 50 μM Amplex UltraRed (red) and 200 μM H₂O₂ for 30 min. V5 epitope (green) was detected using a primary monoclonal mouse anti-V5 antibody and a secondary polyclonal goat anti-mouse Alexa Fluor 488-conjugated antibody. The cells were stained with 2 μg/ml DAPI solution (blue) for 10 min and mounted with VectaShield. Imaging was done by a Zeiss LSM710 microscope with a SIM module. Scale bars = 1 μm. (C to E) TEM images. Transfectants were fixed with 2% glutaraldehyde in 100 mM cacodylate buffer with 2 mM CaCl₂ and labeled with 0.5 mg/ml DAB and 300 μM H₂O₂ for 15 min. Samples are postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer following dehydration in ethanol and acetone. Samples were embedded in LX-112 resin, and 50- to 60-nm sections were cut. Samples were viewed at 80 kV on a Hitachi HT 7700 lens and imaged with a Veleta camera. APEX-catalyzed DAB deposition appears as higher contrast areas at the site of the tagged protein. Some areas with labeling are indicated by arrows. Abbreviation: N nucleus. Scale bars are indicated in the images.

**SALP-1** is a *Giardia*-specific ventral disc protein with homology to striated fiber assemblins (16, 17). SALP-1 tagged with APEX displayed a distinct localization to the ventral disc. V5 localization of SALP-1 suffered from poor signal, possibly due to difficulties in antibody accessibility (Fig. 2I).

In diplomonads, the annexins (in *Giardia* spp. also known as alpha-giardins) constitute an expanded family of proteins associated with the membrane and cytoskeleton.
Alpha-14 giardin has previously been reported to localize to the flagella (18, 19). We obtained a clear localization of Amplex UltraRed to the flagella, whereas the V5 localization was only faint but primarily seen in the flagella (Fig. 2J).

Finally, we fused APEX to the mitosome protein IscU. Characteristically, SIM and Amplex UltraRed signals were seen to colocalize in a linear arrangement between the nuclei and at peripheral cellular sites typical of mitosome localization in Giardia spp. (Fig. 2K) (5).

In summary, we were able to obtain reliable protein localizations using the Amplex UltraRed substrate for all strains where an APEX fusion resulted in a demonstrable expression of a full-length protein. The resorufin signal generated by APEX was found to be less distinctly localized than the V5-tag derived signal when viewed using SIM. We were more successful in generating effective APEX fusions in S. salmonicida than in Giardia spp.

Ultrastructural localization using APEX-catalyzed DAB deposition. Finally, we investigated the ultrastructural localization of the tagged proteins in the successful APEX transgenic strains. The transfectants were grown according to the optimized protocol, being fixed in glutaraldehyde in cacodylate buffer before the DAB substrate is added to the samples. Directly after the DAB treatment, the cells were viewed with a transmitted light microscope to validate the labeling efficiency before TEM sample preparation (see Fig. S5).

In determination of the localizations of S. salmonicida ER-localized fusion proteins, acid phosphatase and BiP, display prominent labeling in TEM micrographs. The perinuclear area showed a clear signal, and the nuclear-posterior area displayed an interconnected ER network. The ER label was found to track as layered sheets along the recurrent flagellar axes outside the striated lamina that enclose the recurrent flagella. In cross-sections of tapering cell ends, these sheets were bridged in an apparent S shape. The ER sheets tapered but were always present toward the cell posterior where the recurrent flagella exit the cell body (Fig. 4A to F). The labeled regions are consistent with the distribution of ER in S. salmonicida, as described by Jørgensen and Sterud (20).

Labeling of the fibrillarin-APEX fusion strain revealed darkened nuclei, with the anterior ends showing a particularly intense stain. In some cells, there were lobes of intense stain in other parts of the nucleus. These were most of the time seen as connected at the anterior ends. The organization of the nucleolus in S. salmonicida appears to be reminiscent of the diffuse nucleolar organizing regions seen in Giardia trophozoites (21), although they appear to occupy a proportionally larger volume of the nucleus. We did not observe any distinct granular nucleoli similar to those recently demonstrated in Giardia spp. (Fig. 4G to I) (22).

The IFT46 protein fusion is detected as a general darkening of the flagella with highly localized foci on the flagella, expected to be IFT particles. Even though we were able to easily visualize the DAB stain by light microscopy, we were unable to detect any labeled structures by TEM. We attribute this failure to be due to the comparative rarity and minute size of these stained cellular features.

IscU is clearly detectable as intensely staining dark vesicles that are bounded by double membranes. In some micrographs, it was possible to see intensely stained granulated areas in the matrix. The organelle sizes and their cellular distribution are fully consistent with previous immuno-EM and TEM data for S. salmonicida hydrogenosomes (Fig. 4J to L) (4).

SS50377_12178 shows a clear perinuclear labeling in DAB-stained whole cells (Fig. S4F). Surprisingly, we were unable to demonstrate any convincing DAB-derived deposits in the nuclear area by TEM.

TEM micrographs of the SS50377_10316 APEX fusion showed intense staining of the nucleus-abutting section of the basal body pockets. In dividing cells with duplicated cellular structures, the labeled basal body pockets were found to be paired up before nuclear division (Fig. 3C and D). Future studies attempting to understand cell division
Transmission electron microscopy (TEM) images of DAB-stained S. salmonicida transfectants and WT. (A to C) Acid phosphatase-APEX; (D to F) BiP-APEX; (G to I) fibrillarin-APEX; (J to L) IscU-APEX; (M to O) WT. Cultures were grown in LY1 medium containing 100 μM hemin. Cells are fixed with 2% glutaraldehyde in 100 mM cacodylate buffer with 2 mM CaCl₂ and treated with 0.5 mg/ml DAB and 200 μM H₂O₂ for 15 min. Samples were postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer following dehydration in ethanol and acetone. Samples were embedded in LX-112 resin, and 50- to 60-nm sections were cut. Samples were viewed at 80 kV on a Hitachi HT 7700.
dynamics in *S. salmonicida* would be able to use SS50377_10316 as a marker to track basal body nuclear dynamics.

DAB staining of *G. intestinalis* alpha-14 giardin transfectants results in very prominently labeled flagella. TEM sections revealed that the DAB stain was primarily associated with the flagellar plasma membrane (Fig. 5A to C). A lower intensity of staining was also present in the plasma membrane. This labeling is consistent with that seen by alpha-14 giardin antisera (19).

Whole cells with SALP-1 labeling displayed dark outlines of the ventral disc. TEM imaging of the cells showed a clear ventral disc labeling which increased at the disc periphery. At higher magnification, the SALP-1 signal is found to be associated with the ventral disc microribbons, as previously postulated (Fig. 5D to F) (17).

The *Giardia* IscU signal was seen in membrane-bounded vesicles distributed in the cytosol (Fig. 5G to I). This localization is fully consistent with mitosome localization in *Giardia intestinalis* (5).

**DISCUSSION**

In this study, we describe the successful optimization of a proximity labeling system based on the APEX in the diplomonad *S. salmonicida*. The method was also showed to be applicable to *G. intestinalis*. The flexibility of APEX allowed us to use both fluorescent- and osmiophilic-yielding substrates with the same transfectant strains. We further expect that the APEX toolkit is expandable by utilization of the well-developed commercial substrate catalog available for HRP. As an example, recent work has seen the development of spatially resolved in vivo proximity labeling using APEX in combination with the novel substrate biotin-phenol (23).

Robust APEX activity in both diplomonads required the addition of exogenous hemin to the growth media. This likely reflects the low abundance of the heme cofactor, which is needed for APEX activity, in the basal growth media of the two diplomonads. We determined that the optimal conditions to load the cells with heme for APEX activation were well below toxicity levels of the cells and that signal could be further modulated by H$_2$O$_2$ titration. One substantial benefit to APEX is the lack of disulfides and calcium-binding sites which render it more versatile as a fusion partner than HRP. In line with this, we have been able to demonstrate that APEX is active in a broad selection of cellular compartments, including the ER, nucleus, plasma membrane, the perinuclear region, cytoskeleton, and the MROs. The observed signal strength for some constructs, especially in TEM, was sometimes weak and difficult to find. For example, even though *S. salmonicida* IFT46 and SS50377_12178 yielded robust signal using Amplex UltraRed and in whole DAB-stained cells, we were unable to determine the corresponding labeled regions in TEM. For IFT46, we believe that the signal is spatially confined to highly specific regions, presumed to be complex B of IFT particles (24), so we did not chance a productive cross-section. There are flagellar isolation protocols developed for use in *Giardia* spp. (25) which if adapted to *Spironucleus* spp. would increase the chances of observing stained IFT particles.

Recently, a further evolved version of ascorbate peroxidase called APEX2 was shown to offer labeling with increased sensitivity and robustness for weakly expressed proteins (8, 26). This protein was previously successfully employed by Zumthor et al. to demonstrate the ultrastructural localization of proteins in *G. intestinalis* (8). We have performed preliminary experiments using APEX2 derived from soybean in our diplomonad systems using the conditions we optimized for APEX in the present study. We observed that APEX2 fusions display increased activity but also yielded increased background staining (see Fig. S6). Based on this, we recommend the use of APEX rather...
FIG 5 Transmission electron microscopy (TEM) images of DAB-stained *G. intestinalis* transfectants and WT. (A to C) Alpha 14-giardin-APEX; (D to E) SALP-1-APEX; (G to I) IscU-APEX; (J to L) WT. Cultures were grown in TYDK medium supplemented with 100 µM hemin. The cells were fixed with 2% glutaraldehyde in 100 mM cacodylate buffer with 2 mM CaCl₂ and labeled with 0.5 mg/ml DAB and 300 µM H₂O₂ for 15 min. Samples were postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer following dehydration in ethanol and acetone. Samples were embedded in LX-112 resin, and 50- to 60-nm sections were cut. Samples were viewed at 80 kV on a Hitachi HT 7700 lens and imaged with a Veleta camera. Scale bars are 2 µm in panels D and K; 1 µm in A, B, C, E, F, H, J, and L; and 500 nm in G and I.
than APEX2 in *Spironucleus salmonicida*, at least until reaction conditions can be optimized. The previous demonstration of APEX2 function in *G. intestinalis* (8) indicates that the use of either APEX or APEX2 should be considered depending on the expression level or cellular location of the protein under study. This could be relevant to compensate for the apparent lower labeling efficiency we observed for non-membrane-bounded proteins in *Giardia* spp.

It is clear from our data that using the Amplex UltraRed substrate in combination with APEX fusions yield reliable localization information. However, we were clearly able to achieve higher localization resolution by antibody-based labeling using the V5 tag than using the Amplex UltraRed substrate. We attribute this to the tendency of resoruﬁn to leach from its immediate site of generation. Thus, presently, the best combination for simultaneous high-resolution localization using ﬂuorescence microscopy and TEM would be offered by an APEX-epitope tag combination.

Our approach was able to conﬁrm the ultrastructural localization of 9 diplomonad proteins, including a protein in *S. salmonicida* with previously unclear or unknown localization. As might be expected from a larger protein tag, we had some issues making fusions to some of our target proteins. For a few proteins, we were unable to recover transfected cells despite several attempts, indicating issues with toxicity of the fusion protein. Several additional proteins showed no expression or a wrongly sized polypeptide in the recovered transfectants. However, we did not observe any evidence of tag-induced mistargeting, and we believe that most of the toxicity issues are not speciﬁcally connected to APEX and can be resolved by switching the tagging termini, by integration on the chromosome, or by using an inducible expression system. The difﬁculties in making fusions were especially pronounced in *Giardia intestinalis*, which agree with our previous experiences that protein tagging is more likely to be more successful in *S. salmonicida* than in *Giardia intestinalis*. In this sense, *S. salmonicida* can be utilized as a viable comparative model for localization in case the protein is refractory to localization in *Giardia intestinalis* and an ortholog exists in *S. salmonicida*.

We believe that the APEX proximity labeling system will be of great use in the study of the intricate cell biology and cytoskeletal systems present in diplomonad cells and many other protists with transfection possibilities. Our anticipation is that future developments to the APEX system might be able to extend its use in diplomonads to include spatially resolved enzymatic tagging and enable compartment-resolved proteomics.

**MATERIALS AND METHODS**

**Cell culture.** Cultures were grown in axenic cultures in tightly closed slanted polypropylene tubes (Nunc). *S. salmonicida* (ATCC 50377), obtained from Atlantic salmon, was grown in modiﬁed LVI medium (12) at 16°C, while *G. intestinalis* WB clone 6 (ATCC 50803) was grown in TYDK (Diamond’s TYI-S-33 supplemented with bile according to the methods of Keister) culture medium at 37°C (27).

**Vector constructions.** The pea APEX, APEX<sup>W41F</sup>, and the soybean APEX2 genes were PCR ampliﬁed from the pUC57 Apex plasmid (Addgene identiﬁer [ID] 40306), the pCAG APX W41F plasmid (Addgene ID 40307), and the pCDNA3 Connexin43-GFP-APEX2 plasmid (Addgene ID 49385), respectively, using corresponding primers. The C-terminal primers contained overhangs of either the V5 or the 3× hemagglutinin (3×HA) epitope tags to generate fusions (see Table S1). The products were inserted into the pSPiro-PAC (12) and pPAC (28) vectors using restriction digestion for transfections in *S. salmonicida* and *G. intestinalis*, respectively. The sizes of the inserts are as follows: APEX<sup>W41F</sup>-3×HA and APEX-3×HA, 828 bp and 30.28 kDa, respectively; APEX-V5 and APEX2-V5, 789 bp and 28.3 kDa, respectively. Coding sequences of target genes were ampliﬁed from genomic DNA using PCR and included a 100- to 400-bp upstream putative promoter region. The primer sequences can be found in Table S1. Primer sequences used for amplification of the *S. salmonicida* genes NADH, SS50377_10316, IscU, IscS, SHMT, and PFORS can be found in reference 4; those for IFT46, BiP, and ﬁbrillarin can be found in reference 12, and those for annexin 5 can also be found in reference 12. Restriction digestion was used to insert the genes into the expression vectors. All constructs were veriﬁed using Sanger sequencing at SciLifeLab, Uppsala, Sweden. Transfection of *S. salmonicida* was done according to reference 12, while *G. intestinalis* was transfected as described in reference 4. Transfectants were selected using 50 μg/ml puromycin and grown under constant selective pressure.

**Immunofluorescence assay.** Preparations of *S. salmonicida* and *G. intestinalis* transfecteds for immunofluorescence assay mostly followed the guidelines in reference 4. Cultures were pelleted by centrifugation and washed with Hanks balanced salt solution with glucose (HBSS-G) (*S. salmonicida*) or phosphate-buffered saline (PBS) (*G. intestinalis*). Cells were spotted on poly-lysine-coated microscope
slides (Thermo Fisher Scientific catalog no. ER-2088-CE24) and fixed with 2% paraformaldehyde (Sigma-Aldrich catalog no. 344198) in PBS for 20 min at 37°C. Fixative was quenched with 0.1 M glycine for 10 min at room temperature (RT). Cells were permeabilized with 0.2% Triton X-100 in PBS for 20 min at RT and blocked with 2% bovine serum albumin (BSA) in 0.1% Triton X-100 in PBS for 1 h at RT or overnight (O/N) at 4°C. V5 epitope-tagged proteins were detected using anti-V5 monoclonal antibody SV5-Pk1 and diluted 1:750 (Abcam catalog no. AB27671) in block solution at RT for 1 to 2 h. 3×HA-tagged proteins were detected using either an Alexa Fluor 488-conjugated anti-HA monoclonal antibody HA.11, diluted 1:250 (Nordic BioSite catalog no. 901509) in block solution or primary rabbit anti-HA monoclonal antibody C29F4, and diluted 1:1,600 (Cell Signaling catalog no. 37245) in block solution at RT for 1 to 2 h. Primary antibodies were detected using Alexa Fluor 488-conjugated goat anti-mouse polyclonal antibody, diluted 1:800 (Life Technologies catalog no. A11029) in block solution at RT for 1 h or Alexa Fluor 488-conjugated goat anti-rabbit polyclonal antibody, and diluted 1:350 (Life Technologies catalog no. A11008) in block solution at RT for 1 h. Slides were mounted using VectaShield containing 4,6-diamidino-2-phenylindole (DAPI); Vector Laboratories catalog no. H-1200). Cells were viewed using a Zeiss Axioscope 2 fluorescence microscope (Carl Zeiss GmbH), and images were processed using Zen lite 2012 (blue edition) version 1.1.2.0, ImageJ-Fiji version 1.51d, and Adobe Illustrator CC.

**Western blotting.** Preparations of cell lysates for expression analyses followed the guidelines in reference 28. Cultures were pelleted by centrifugation and washed with HBSS-G (S. salmonicida) or PBS (G. intestinalis). Cells were normalized to 0.05 optical density (OD) units/10 μl at 600 nm in a spectrophotometer. Samples were boiled for 10 min in 1× Laemmli buffer with 100 mM dithiothreitol (DTT). Protein separations were performed using precast polyacrylamide gels (Bio-Rad Mini--Protein Any-kD TGX stain free, catalog no. 456-8125) and transferred to polyvinylidene difluoride (PVDF) (Bio-Rad catalog no. 162-0177). Membranes were blocked with 5% dry milk (Semper) in 0.05% dithiothreitol (DTT). Protein separations were performed using precast polyacrylamide gels (Bio-Rad catalog no. H9262). Slides were mounted using Vectashield without DAPI and processed the same way as for the immunofluorescence assay. The remaining pellet was pelleted once more before resuspending in 2% glutaraldehyde and 1% formaldehyde in assay buffer.

**TEM.** Bulk samples for TEM were prepared using the same procedure as on slides. Pellets were fixed in 2% glutaraldehyde in assay buffer with 2mM CaCl2, pH 7.4) and spotted on a poly-lysine-coated slide (Thermo Fisher Scientific catalog no. ER-2088-CE24). Cells were fixed with 2% glutaraldehyde (Sigma-Aldrich catalog no. G5882) in assay buffer for 60 min, washed with assay buffer, and quenched with 20 mM glycine in assay buffer for 5 min. After washing, the cells were labeled for 15 min with 3,3'-diaminobenzidine (DAB) labeling solution containing 0.5 mg/ml DAB (Sigma-Aldrich catalog no. D5905) and 300 μM H2O2, if not stated otherwise. Slides were mounted using VectaShield without DAPI and processed the same way as the immunofluorescence assay images. Images were processed using Bio-Rad Image Lab version 4.0 and Adobe Illustrator CC.
SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/mSphereDirect.00153-19.

FIG S1, PDF file, 0.1 MB.
FIG S2, PDF file, 0.5 MB.
FIG S3, PDF file, 1.8 MB.
FIG S4, PDF file, 0.2 MB.
FIG S5, PDF file, 0.2 MB.
FIG S6, PDF file, 0.4 MB.
TABLE S1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS
The project was supported by the Swedish Research Council Formas. We acknowledge Elin Einarsson for her help and insights on the project, and we thank the BioVis facility at SciLifeLab in Uppsala, Sweden, for assistance during the SIM imaging.
S.G.S. and J.J.-H. conceived the study. Å.Å. and J.J.-H. designed and constructed all strains. All experimental work and imaging were done by Å.Å., except K.H. performed electron microscopy analyses. Å.Å. and J.J.-H. drafted the manuscript. All authors contributed to the manuscript and read and approved the final version.

REFERENCES
1. Gaietta G, Deerinck TJ, Adams SR, Bouwer J, Tour O, Laird DW, Sosinsky GE, Tsien RY, Ellisman MH. 2002. Multicolor and electron microscopic imaging of connexin trafficking. Science 296:503–507. https://doi.org/10.1126/science.1068793.
2. Shu X, Lev-Ram V, Deerinck TJ, Qi Y, Ramko EB, Davidson MW, Jin Y, Ellisman MH, Tsien RY. 2011. A genetically encoded tag for correlated light and electron microscopy of intact cells, tissues, and organisms. PLoS Biol 9(10):e1001041. https://doi.org/10.1371/journal.pbio.1001041.
3. Hopkins C, Gibson A, Stinchcombe J, Futter C. 2000. Chimeric molecules employing horseradish peroxidase as reporter enzyme for protein localization in the electron microscope. Methods Enzymol 327:35–45. https://doi.org/10.1016/S0076-6879(00)27265-0.
4. Jerlström-Hultqvist J, Einarsson E, Xu F, Hjort K, Ek B, Steinhauf D, Hultenby K, Bergquist J, Andersson JO, Svärd SG, Jerlström-Hultqvist J, Einarsson E, Xu F, Hjort K, Ek B, Steinhauf D, Hultenby K, Bergquist J, Andersson JO, Svärd SG. 2013. Hydrogenosomes in the diplomonad Spironucleus salmonicida. Nat Commun 4:2493. https://doi.org/10.1038/ncomms3493.
5. Tovar J, León-Avila G, Sánchez LB, Sutak R, Tachezy J, Van Der Giezen M, Hernández M, Müller M, Lucocq JM. 2003. Mitochondrial remnant organelles of Giardia function in iron-sulphur protein maturation. Nature 426:172–176. https://doi.org/10.1038/nature01945.
6. Marti M, Li Y, Scharner EM, Wild P, Köhler P, Hehl AB. 2003. The secretory apparatus of an ancient eukaryote: protein sorting to separate export pathways occurs before formation of transient Golgi-like compartments. Mol Biol Cell 14:1433–1447. https://doi.org/10.1091/mbc.e02-08-0467.
7. Xu F, Jerlström-Hultqvist J, Einarsson E, Åstvaldsson Á, Svärd SG, Andersson JO. 2014. The genome of Spironucleus salmonicida highlights a fish pathogen adapted to fluctuating environments. PLoS Genet 10(10):e100453. https://doi.org/10.1371/journal.pgen.100453.
8. Zurnthor JP, Cernikova L, Rout S, Kaech A, Faso C, Hehl AB. 2016. Static clathrin assemblies at the peripheral vacuole—plasma membrane interface of the parasitic protozoan Giardia lamblia. PLoS Pathog 12: e1005756. https://doi.org/10.1371/journal.ppat.1005756.
9. Einarsson E, Åstvaldsson Á, Hultenby K, Andersson JO, Svärd SG, Jerlström-Hultqvist J. 2016. Comparative cell biology and evolution of annexins in diplomonads. mSphere 1:e00032-15. https://doi.org/10.1128/mSphere.00032-15.
10. Laberge M, Huang Q, Schweitzer-Stenner R, Fidy J. 2003. The endogenous calcium ions of horseradish peroxidase C are required to maintain the functional nonplanarity of the heme. Biochim Biophys Acta 1607:573–581. https://doi.org/10.1016/S0005-2736(02)0044-5.
11. Martell JD, Deerinck TJ, Sancak Y, Poulos TL, Mootha VK, Sosinsky GE, Ellisman MH, Ting AY. 2012. Engineered ascorbate peroxidase as a genetically encoded reporter for electron microscopy. Nat Biotechnol 30:1143–1148. https://doi.org/10.1038/nbt.2375.
12. Jerlström-Hultqvist J, Einarsson E, Svärd SG. 2012. Stable transfection of the diplomonad parasite Spironucleus salmonicida. Eukaryot Cell 11:1353–1361. https://doi.org/10.1128/EC.00179-12.
13. Rafferty SP, Dayer G. 2015. Heme proteins of Giardia intestinalis. Exp Parasitol 159:13–23. https://doi.org/10.1016/j.exppara.2015.08.001.
14. Kumar S, Bandypadhyay U. 2005. Free heme toxicity and its detoxification systems in human. Toxicol Lett 157:175–188. https://doi.org/10.1016/j.toxlet.2005.03.004.
15. Schmitt TH, Frezzatti WA, Schreier S. 1993. Hemin-induced lipid membrane disorder and increased permeability: a molecular model for the mechanism of cell lysis. Arch Biochem Biophys 307:96–103. https://doi.org/10.1063/1.461515.
16. Palm D, Weiland M, McArthur AG, Winiecka-Krusnell J, Cipriano MJ, Kim, the diplomonad parasite Spironucleus salmonicida. Eukaryot Cell 11:1353–1361. https://doi.org/10.1128/EC.00179-12.
17. Rafferty SP, Dayer G. 2015. Heme proteins of Giardia intestinalis. Exp Parasitol 159:13–23. https://doi.org/10.1016/j.exppara.2015.08.001.
18. Kumar S, Bandypadhyay U. 2005. Free heme toxicity and its detoxification systems in human. Toxicol Lett 157:175–188. https://doi.org/10.1016/j.toxlet.2005.03.004.
19. Vahrmann S, Sterud E. 2006. The marine pathogenic genotype of Spironucleus barnahus from farmed salmonids redescribed as Spironucleus salmonicida n. sp. J Eukaryot Microbiol 53:531–541. https://doi.org/10.1111/j.1550-7408.2006.00144.x.
20. Jiménez-García LF, Zavala G, Chávez-Munguía B, Ramos-Godínez MDP, García R, Ortega-Pierres G. 2008. Identification of nucleoli in the early nucleus salmonicida n. sp. J Eukaryot Microbiol 53:1353–1361.https://doi.org/10.1128/EC.00179-12.
21. Martell JD, Deerinck TJ, Sancak Y, Poulos TL, Mootha VK, Sosinsky GE, Ellisman MH, Ting AY. 2012. Engineered ascorbate peroxidase as a genetically encoded reporter for electron microscopy. Nat Biotechnol 30:1143–1148. https://doi.org/10.1038/nbt.2375.
chondrial intermembrane space in live cells via ratiometric APEX tagging. Mol Cell 55:332–341. https://doi.org/10.1016/j.molcel.2014.06.003.

24. Hou Y, Qin H, Follit JA, Pazour GJ, Rosenbaum JL, Witman GB. 2007. Functional analysis of an individual IFT protein: IFT46 is required for transport of outer dynein arms into flagella. J Cell Biol 176:653–665. https://doi.org/10.1083/jcb.200608041.

25. Clark JT, Holberton DV. 1988. Triton-labile antigens in flagella isolated from Giardia lamblia. Parasitol Res 74:415–423. https://doi.org/10.1007/BF00535140.

26. Lam SS, Martell JD, Kamer KJ, Deerinck TJ, Ellisman MH, Mootha VK, Ting AY. 2015. Directed evolution of APEX2 for electron microscopy and proximity labeling. Nat Methods 12:51–54. https://doi.org/10.1038/nmeth.3179.

27. Keister DB. 1983. Axenic culture of Giardia lamblia in TYI-S-33 medium supplemented with bile. Trans R Soc Trop Med Hyg 77:487–488. https://doi.org/10.1016/0035-9203(83)90120-7.

28. Jerlström-Hultqvist J, Stadelmann B, Birkestedt S, Hellman U, Svärd SG. 2012. Plasmid vectors for proteomic analyses in Giardia: purification of virulence factors and analysis of the proteasome. Eukaryot Cell 11:864–873. https://doi.org/10.1128/EC.00092-12.

29. Feely DE, Dyer JK. 1987. Localization of acid phosphatase activity in Giardia lamblia and Giardia muris trophozoites. J Protozool 34:80–83. https://doi.org/10.1111/j.1550-7408.1987.tb03137.x.

30. Šaric M, Vahrannon A, Niebur D, Klumpers V, Hehl AB, Scholze H. 2009. Dual acylation accounts for the localization of α19-giardin in the ventral flagellum pair of Giardia lamblia. Eukaryot Cell 8:1567–1574. https://doi.org/10.1128/EC.00136-09.

31. Luján HD, Mowatt MR, Conrad JT, Nash TE. 1996. Increased expression of the molecular chaperone BiP GRP78 during the differentiation of a primitive eukaryote. Biol Cell 86:11–18.

32. Dawson SC, Sagolla MS, Cande WZ. 2007. The cenH3 histone variant defines centromeres in Giardia intestinalis. Chromosoma 116:175–184. https://doi.org/10.1007/s00412-006-0091-3.

33. Narcisi EM, Glover CVC, Fechheimer M. 1998. Fibrillarin, a conserved pre-ribosomal RNA processing protein of Giardia. J Eukaryot Microbiol 45:105–111.