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# Developmental stages identified in the trophozoite of the free-living Alveolate flagellate *Colpodella* sp. (Apicomplexa)

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**Summary.** In this study we performed light, immunofluorescent and transmission electron microscopy of *Colpodella* trophozoites to characterize trophozoite morphology and protein distribution. The use of Giemsa staining and antibodies to distinguish *Colpodella* life cycle stages has not been performed previously. Rhoptry and β-tubulin antibodies were used in immunofluorescent assays (IFA) to identify protein localization and distribution in the trophozoite stage of *Colpodella* (ATCC 50594). We report novel data identifying "doughnut-shaped" vesicles in the cytoplasm and apical end of *Colpodella* trophozoites reactive with antibodies specific to *Plasmodium* merozoite rhoptry proteins. Giemsa staining and immunofluorescent microscopy identified different developmental stages of *Colpodella* trophozoites, with the presence or absence of vesicles corresponding to maturity of the trophozoite. These data demonstrate for the first time evidence of rhoptry protein conservation between *Plasmodium* and *Colpodella* and provide further evidence that *Colpodella* trophozoites can be used as a heterologous model to investigate rhoptry biogenesis and function. Staining and antibody reactivity will facilitate phylogenetic, biochemical and molecular investigations of *Colpodella* sp. Developmental stages can be distinguished by Giemsa staining and antibody reactivity.

**Keywords:** *Colpodella* · Rhoptries · Trichocysts · Apical complex · *Plasmodium* RhopH3.

## Introduction

*Colpodella* species possess an apical complex with rhoptries, micronemes, a conoid and in some species polar rings [2, 4, 8]. Most species of *Colpodella* are free-living and feed on protist prey. The life cycle, phylogeny, morphology and ultrastructure of several *Colpodella* species (*C. pugnax*, *C. perforans*, *C. gonderi*, *C. edax*, *C. vorax*, *C angusta C. turpis* [2, 8], *C. pseudoedax, C. unguis, C. tetrahymenae* and *C. pontica* [4] have been described and include trophozoite and cyst stages. *Colpodella* proteins with a role in life cycle transformation and myzocytosis have not been identified. *Colpodella* species (ATCC 50594) maintained by the American Type Culture Collection (ATCC), has been used for phylogenetic analyses with data showing *Colpodella* branching closest to the apicomplexan clade [3]. *Colpodella gonderi* and *C. tetrahymenae* are ectoparasites of cilates [5]. In addition to the rhoptries, trichocysts are also present in several *Colpodella* species [4]. However, it is unclear what role trichocysts play in prey attacks and what type of proteins define *Colpodella* trichocysts. Knowledge of *Colpodella* biology and predatory behavior is crucial for understanding the development of intracellular parasitism among apicomplexans. The use of dyes and antibodies for routine morphological characterization and differentiation of *Colpodella* species has not been performed previously. To our knowledge, there are no antibodies to *Colpodella* rhoptry proteins. The purpose of this study was to investigate protein localization and distribution in *Colpodella* trophozoites and to characterize the different development stages identified by Giemsa staining. The fine structure

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of *Colpodella* sp. has been described in detail, showing the presence of apical complex organelles, cellular structures and life cycle stages [2, 4, 8]. However, proteins associated with the apical complex organelles, life cycle transformations and myzocytosis have not been identified. In this study we used antibodies prepared against whole rhoptries and the 110 kDa RhopH3 rhoptry protein of *Plasmodium* merozoites in immunofluorescence assay. The rhoptry specific antibodies are cross reactive among different *Plasmodium* species. RhopH3 has a role in nutrient uptake in *Plasmodium* species [7]. We employed the *Plasmodium* antibodies in the current study with the hypothesis that the antibodies would react with *Colpodella* rhoptry proteins. We report novel data showing anti-*Plasmodium* rhoptry antibody reactivity with *Colpodella* proteins in distinct organelles, providing further evidence in support of conservation of *Plasmodium* rhoptry proteins in *Colpodella* sp. The data further shows different developmental stages of trophozoites in *Colpodella* sp. and identifies the RhopH3 protein as a new marker to aid phylogenetic analyses.

## Materials and Methods

**Diprotist culture conditions.** *Colpodella* sp. (ATCC 50594) (Manassas, Virginia, USA) was cultured with prey species *Bodo caudatus* in *Enterobacter aerogenes* bacterized Hay medium (Wards Scientific) (Rochester, New York, USA). The diprotist culture was maintained in tissue culture flasks (Corning) containing 10 ml to 30 ml cultures. Cultures were incubated at 22-24ºC. Cells in tissue culture flasks were observed using an inverted microscope (Nikon TMS, Type 104) under phase microscopy to monitor cell density. Cultures were maintained aseptically.

**Staining and light microscopy.** For light microscopy, cells were fixed in absolute methanol for 1 min or fixed in 5% formalin (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at room temperature. Formalin fixed cells were smeared on glass slides, air-dried and stained with Giemsa stain (0.4% stock in buffered methanol, pH 6.8 diluted 1:20 in distilled water) (Sigma-Aldrich) for 1-2 min. Slides were rinsed with distilled water, air dried and the cell images were captured using an Olympus CX31 microscope with an Olympus SPOT IDEA U-TVO.5XC-3 camera attachment and analysis performed using SPOT imaging BASIC version 5.3, 2014 Software.

**Immunofluorescence and confocal microscopy.** Immunofluorescence and confocal microscopy was performed on *Colpodella* and *B. caudatus* diprotist cultures fixed in absolute methanol or 5 % formalin as described previously and incubated with rabbit and mouse polyclonal antibodies or mouse monoclonal antibodies; either individually or together in colocalization experiments [9]. Formalin fixed cells were permeabilized with 0.5% Triton X-100 before antibody incubation. *Plasmodium falciparum* infected human erythrocytes were used as a positive control for the antibodies. Antibodies specific for the 110 kDa high molecular weight *P. falciparum* and *P. berghei* RhopH3 protein and antibodies prepared against isolated whole rhoptries of *P. falciparum* and *P. yoelii* merozoites were used. The following antibodies were used: *Plasmodium* whole rhoptry specific antibodies were rabbit antiserum 676 (*P. falciparum*) and mouse antiserum PYSDS (*P. yoelii*); *Plasmodium* RhopH3 specific antibodies were rabbit antiserum 686 (recombinant *P. falciparum* RhopH3), mouse antiserum His-FLPbRhop-3 [FL] (recombinant *P. berghei* RhopH3), mouse monoclonal antibody 1B9 (native *P. falciparum* RhopH3); anti-β-tubulin mouse monoclonal antibody KMX-1 of *Physarum polycephalum* was also used in IFA. Secondary mouse or rabbit antibodies conjugated to Alexa 488, Alexa 633 and Alexa 647 diluted 1:1000 (Molecular Probes, ThermoFischer Scientific) were used. Normal (preimmune) mouse or rabbit serum (NMS and NRS, respectively), were used as negative controls for IFA. DAPI (4′, 6-diamidino-2-phenylindole) Fluoromount-G (Southern Biotech, Birmingham, AL, USA) was used to mount the slides. Images were collected using a Leica TCS-SP5II upright laser scanning confocal microscope (Leica Microsystems, GmbH, Wetzlar, Germany). SP8 True Scanning Confocal (TCS) on a DMI8 inverted microscope was used to generate differential interference contrast (DIC) images. Giemsa stained and confocal images were adjusted to 300 dpi using the CYMK color mode and RGB color mode on Adobe photo shop (CC). ImageJ was used to change the channel from magenta to red in the IFA images. Confocal microscopy was performed at the Cleveland Clinic, Lerner Research Institute Imaging Core, Cleveland, OH, USA.

**Transmission Electron Microscopy.** Diprotist culture containing *Colpodella* was centrifuged and the pellet was fixed in quarter strength Karnovsky fixative solution for 2 hours at room temperature. After washing, the specimen was post fixed for 2 hours in an unbuffered 1:1 mixture of 2% osmium tetroxide and 3% potassium ferrocyanide. After rinsing with distilled water, the specimen was soaked overnight in an acidified solution of 0.25% uranyl acetate. After another rinse in distilled water, they were dehydrated in ascending concentrations of ethanol, passed through propylene oxide, and embedded in a Poly/Bed 812 embedding media (Polysciences, Warrington, PA, USA). Thin sections (70 nm) were cut on a RMC MT6000-XL ultramicrotome. These were mounted on Gilder square 300 mesh nickel grids (Electron Microscopy Sciences, PA, USA) and then sequentially stained with acidified methanolic uranyl acetate and stable lead staining solution. These were coated on a Denton DV-401 carbon coater (Denton Vacuum LLC, NJ), and examined in an FEI Tecnai Spirit (T12) with a Gatan US4000 4kx4k CCD. Electron microscopy was performed at Case Western Reserve University, Electron Microscopy Core, Cleveland, OH, USA.

## Results and Discussion

Rhoptry protein identity and distribution in *Colpodella* sp. trophozoites is unknown. Investigations of the fine structure of *Colpodella* trophozoites using transmission electron microscopy has revealed the presence of apical complex organelles known as rhoptries, micronemes, conoid and polar rings at the apical end of *Colpodella* sp. similar to the organelles found in apicomplexan invasive stages [2, 4, 8]. In addition, wet mounts of *Colpodella* cultures observed with DIC [8] and phase contrast light microscopy [2, 4] allowed construction of the life cycle of *Colpodella* sp. However, specific proteins associated with the organelles of *Colpodella* and the transformation of the life cycle stages have not been identified, a necessity for biochemical and molecular investigations aimed at understanding cell development, cellular trafficking, biogenesis of the organelles and their role in myzocytosis. A major goal of this study was to identify *Colpodella* rhoptry proteins using antibodies specific to *Plasmodium* merozoite rhoptry proteins. A second goal was to investigate the distribution of rhoptry proteins among the different morphological stages observed by Giemsa staining. In this study, Giemsa staining of *Colpodella* cultures revealed additional developmental stages associated with the "swimming-feeding-dividing" stages of the trophozoite [2, 4, 8]. There are currently no antibodies specific for *Colpodella* rhoptry proteins. We therefore used antibodies against *Plasmodium* rhoptry proteins in IFA to identify trophozoites in the life cycle of *Colpodella*. Knowledge of specific proteins conserved between apicomplexans and *Colpodella* species provides additional markers for phylogenetic analyses and organelle isolation. Giemsa staining of the diprotist culture to distinguish *Colpodella* from *B. caudatus* revealed additional morphological stages not described previously [2, 4, 8]. Similarly, antibody reactivity with cells in the diprotist culture detected proteins distributed differently among morphological stages and among different cellular structures. We therefore investigated the identity of the morphological forms using antibodies against whole rhoptries of *P. falciparum* (antiserum 676), *P. yoelii* (antiserum PYSDS), the RhopH3 protein of *P. falciparum* (antiserum 686 and Mab 1B9) and *P. berghei* (His-FLPbRhop-3 [FL]) in IFA. These antibodies are cross reactive with rhoptry proteins among different *Plasmodium* species. Figure 1A-F shows representative Giemsa stained cells that were observed to have a pale pink cytoplasm, light blue nucleus with very few cytoplasmic inclusions. Additional cells that appeared to be mature forms showed more inclusions and increasingly darker cytoplasm with the presence of two flagella. Anti-*Plasmodium* rhoptry antibody reactivity with the different types of cells detected distinct "doughnut-shaped" vesicles that defined the apical end of the mature trophozoite. The vesicles were not detected in the "immature" stages (Figure 1, panels A and B, i-iv) following antibody reactivity with 676 (anti-*P. falciparum* rhoptries/green) and FL (anti-*P. berghei* RhopH3 protein/red). Initially, vesicles appeared in small numbers in the cells as shown in panel C, i-iv (FL/red and 676/green) and progressively accumulated at the apical end in mature cells as shown in panels D, i-iv (PYSDS/red and 686/green), E, i-iv (tightly colocalized 676/green and FL/red) and F, i-iv (tightly colocalized 686/green and FL/red). Antiserum 676 has antibody specificities to more than one protein and is therefore reactive with other proteins in addition to RhopH3. This accounts for the additional reactivity observed around the nucleus (Figure 1 A & B, iv) and in the

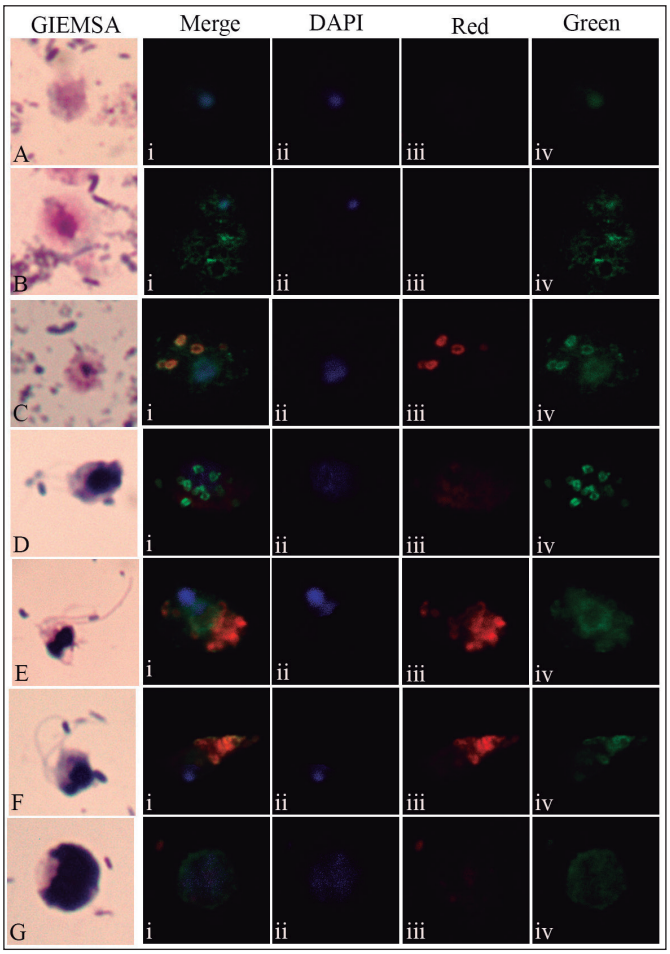


**Fig. 1.** Panels A and B; Giemsa stained immature *Colpodella* trophozoites; C-F, Giemsa stained *Colpodella* trophozoites showing the development of cytoplasmic structures and flagella. G, Giemsa stained *Colpodella* cyst. Immunofluorescence staining of *Colpodella* diprotist culture using antiserum 686 specific for *P. falciparum* RhopH3 [11], antiserum 676 specific for whole rhoptries of *P. falciparum* [6], antiserum PYSDS specific for whole rhoptries of *P. yoelii* treated with SDS and recombinant His-FLPbRhop-3 (FL) protein of *P. berghei* [10]. Cells were fixed with methanol alone or fixed with 5% formalin followed by permeabilization with 0.5% TritonX-100. Cells were incubated with antiserum 686 (1:100), 676 (1:100), PYSDS (1:200) and His-FLPbRhop-3 (1:200) followed by incubation with ALEXA-488 Goat anti-rabbit antibody (1:1000) and ALEXA 633 Goat anti-mouse antibody (1:1000). Smears were mounted with DAPI Fluoromount-G (Southern Biotech) for nuclear staining. Panels A-C, i-iv colocalization of 676 (green) and FL (red) antibodies; D, i-iv colocalization of PYSDS (red) and 686 (green) antibodies; E, i-iv 676 (green) and FL (red) antibodies; F and G, i-iv 686 (green) and FL (red).

cytoplasm (Figure 1C & E, iv). The observation of different morphological forms was suggestive of a sequence of trophozoite development in the *Colpodella* life cycle and we propose that the vesicles develop and become functional as the trophozoites mature. Our rationale for the selection of images representing stage development in *Colpodella* sp. life cycle was based on the appearance of the nucleus in the different stages observed and the appearance of the "doughnut-shaped" vesicles and flagella as the cells matured in later stages. We also based our rationale for stage development and differentiation in the trophozoite on knowledge of trophozoite stages among apicomplexans. Young blood stage trophozoites of *Plasmodium* do not possess rhoptries until the mature schizont stage when rhoptries develop in the invasive merozoite stage within schizonts. The images in Figure 1 were selected and organized to show the proposed developmental sequence based on the morphological forms observed in both Giemsa stained and IFA images. Antibodies against *Plasmodium* rhoptries were strongly colocalized with RhopH3 specific antibodies and reacted with *Colpodella* proteins. Giemsa stained cysts were also observed in the diprotist culture and the corresponding cysts also reacted with rhoptry specific antibodies by IFA (panel G). In Figure 2A-D, the doughnut-shaped vesicles were distributed throughout the cytoplasm in mature active *Colpodella* sp. trophozoites observed in close proximity. The vesicle distribution throughout the cytoplasm was typical of active cultures with increased predatory behavior in *Colpodella*. Negative control normal rabbit and mouse serum showed no reactivity with *Colpodella* proteins (Figure 2E-H). Antibody reactivity (686 and Mab 1B9) with trophozoites (white dashed circles) and schizonts of *P. falciparum* 3D7 strain are shown (Figure 2I-L) as a positive control for the rhoptry antibodies used. Anti-RhopH3 (686) and anti-β tubulin antibodies were reacted with diprotist culture samples containing actively feeding *Colpodella* trophozoites. Intense RhopH3 antibody reactivity (green) was observed around the anterior ends of *Colpodella* trophozoites containing ingested prey. In Figure 3A-D, *Colpodella* flagella were detected with anti-β tubulin antibody (red) (white arrow heads). Rhoptry specific antibody (green) also reacted with the flagella (Figure 3D). Three *Colpodella* cells were observed attached to one *B. caudatus* (Figure 3E-H). The red arrowhead shows the position of *B. caudatus* with no reactivity observed with the rhoptry antibody (green). Two *Colpodella* were observed attached to one *B. caudatus* (Figure 3I-L). The red arrowhead shows the position of *B. caudatus* sandwiched between two predators, with no reactivity observed with rhoptry protein specific antibodies. The location of *B. caudatus* was identified by the nucleus and kinetoplast stained by DAPI. Very faint anti-β-tubulin reactivity was observed with *B. caudatus* (Figure 3K, red). Cells shown in Figure 3 E-H and 3I-L were formalin fixed and permeabilized

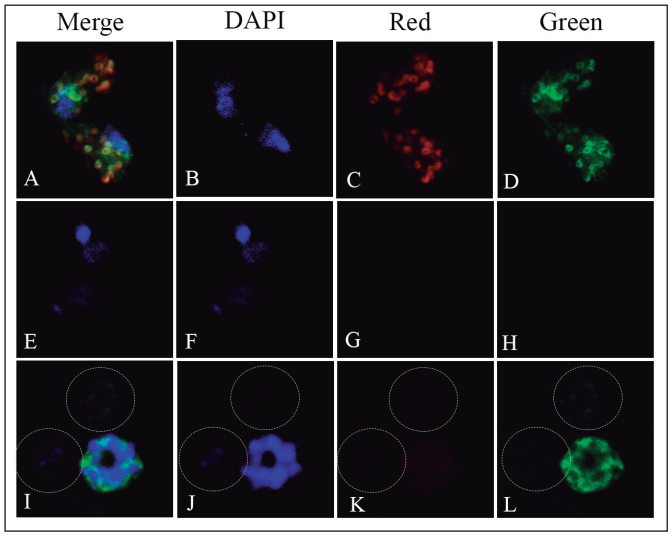


**Fig. 2.** Imunofluorescence staining of *Colpodella* and *P. falciparum* schizont infected erythrocytes with antiserum 686, 676, His-FLPbRhop-3 and monoclonal antibody 1B9 specific for *P. falciparum* RhopH3. A-D, Cytoplasmic "doughnut" shaped vesicles in trophozoites of *Colpodella*; E-H, No reactivity was observed on *Colpodella* with normal mouse and rabbit serum negative controls. I-L, Segmented *P. falciparum* schizont and trophzoite infected erythrocytes reacted with antiserum 686 and Mab 1B9.

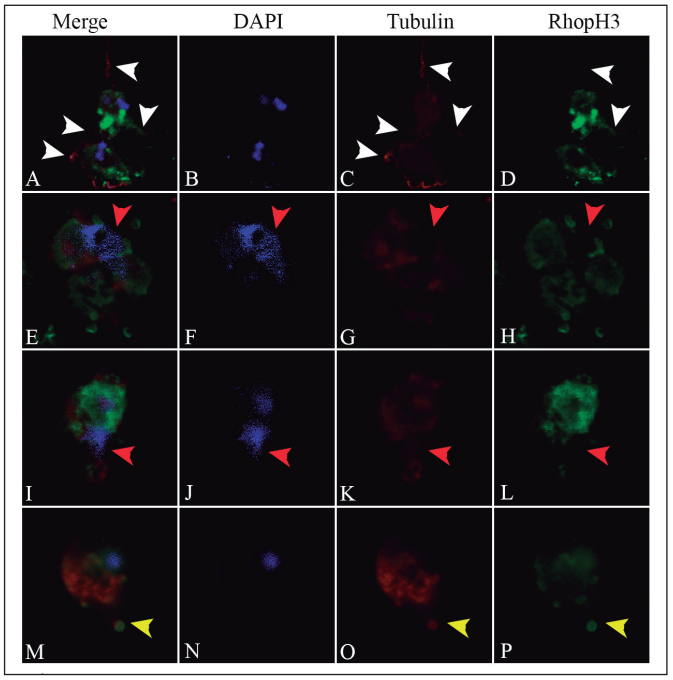


**Fig. 3.** Immunofluorescence staining of *Colpodella* diprotist culture with antiserum 686 (1:100 green) and anti-β-tubulin monoclonal antibody KMX-1 of *Physarum polycephalum* (1:1000, red) [1] followed by incubation with secondary antibodies as described. A-D, merge of antiserum 686, KMX-1 and DAPI showing cell body and flagella reactivity; E-H, merge of antiserum 686, KMX-1 and DAPI. *B. caudatus* (red arrowhead) surrounded by three *Colpodella*. DAPI staining identified nuclei; I-L, merge of antiserum 686, KMX-1 and DAPI. *B. caudatus* (red arrowhead) with two *Colpodella* attached; M-P, merge of 686 and His-FLPbRhop-3 (FL), "doughnut" shaped vesicles discharged at the apical region of a *Colpodella* trophozoite in tubular extensions projecting from the apical tip of the cell (yellow arrowhead).

for IFA and showed slight rounding of the cells following fixation. Cells shown in Figures 3A-D and 3M-P were fixed in absolute methanol for IFA. A doughnut-shaped vesicle appeared to be discharged at the apical tip of *Colpodella* trophozoites (Figure 3M-P, yellow arrowhead). Based on the observations of vesicles in the process of being discharged or extruded, it is unclear whether the vesicles represent rhoptries or trichocysts. Investigations of other *Colpodella* species using antibodies to rhoptries and other apical complex organelles will identify and confirm additional conserved proteins and the structures associated with the proteins. Immunofluorescence of *Colpodella* in diprotist culture was also observed with DIC microscopy. Two *Colpodella* trophozoites with ingested *B. caudatus* prey were observed in close proximity (Figure 4). RhopH3 antibody reactivity with vesicles was observed in the cytoplasm and both flagella were stained with anti-β-tubulin (Figure 4, A-E). A single *Colpodella* trophozoite completing the process of myzocytosis is shown in Figure 4, F-J. The remnant of *B. caudatus* is indicated by the red arrowhead (Figure 4 F, G and J) showing the nucleus of *B. caudatus*. Negative controls for IFA reactivity are shown with normal rabbit and mouse serum showing no protein reactivity with cells (Figure 4, K-O) and RhopH3 reactivity is shown with antiserum 686 and β-tubulin reactivity with KMX-1 (Figure 4, P-T) on *P. falciparum* schizont-infected human erythrocyte as positive control for the antibodies. A longitudinal section of a *Colpodella* trophozoite with cytoplasmic features is shown in Figure 5. The conoid (C), nucleus (NU), large food vacuole (FV), mitochondria (M) and Golgi (G) were observed. The pellicle (Pe), flagella (FL) and anterior flagella (aF) were also observed. The structural features were similar to those described for other *Colpodella* sp. [2, 4, 8]. Electron lucent and electron dense spherical vesicles at the apical end were observed and may represent the rhoptries (RH) (Figure 5). Immunoelectron microscopy will be used to confirm the trophozoite stages and structures reactive with antibodies at the apical end of *Colpodella* sp. Giemsa staining and antibody detection of *Colpodella* trophozoite proteins will facilitate routine identification of cells in diprotist cultures as well as differentiation of structures present in developmental stages of trophozoites and cysts. Identification of cell stages possessing apical complex organelles will also allow for enrichment of those stages for biochemical and molecular investigations. Important insights regarding the development of intracellular parasitism will be gained by continued biochemical analysis of *Colpodella* and related Colpodellid species. *Plasmodium* RhopH3 has a dual role in merozoite invasion and nutrient uptake [7]. Future studies will be aimed at investigations of RhopH3 involvement in myzocytosis. The novel data reported in this study represents the first use of Giemsa staining and antibodies to distinguish stages in the life cycle of *Colpodella*, and conservation of the 110 kDa RhopH3 protein in *Colpodella* sp. This data paves the way for biochemical and molecular analysis of *Colpodella* with the identification of additional molecules to aid phylogenetic analysis of *Colpodella* sp.

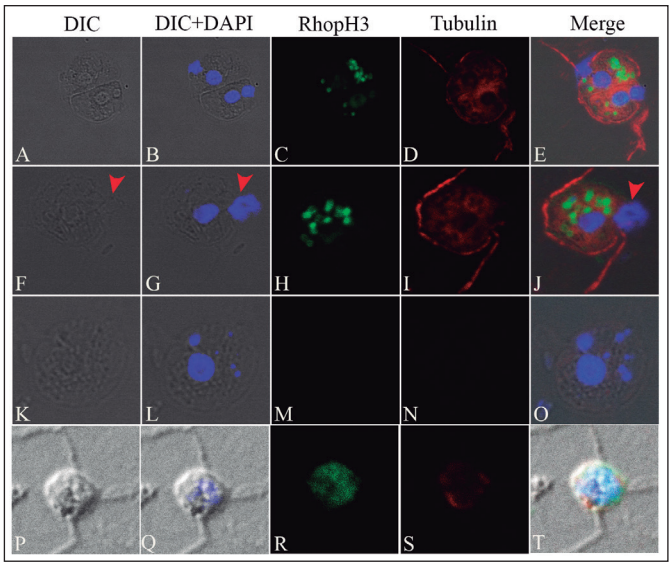


**Fig. 4**. Immunofluorescence staining of *Colpodella* diprotist culture and *P. falciparum* schizont-infected erythrocyte using RhopH3 and β-tubulin specific antibodies. DIC (A, F, K, P), DIC plus DAPI merge (B, G, L, Q), RhopH3 antibody reactivity (C, H, M, R), β-tubulin reactivity (D, I, N, S) and merge of both antibody signals with DAPI (E, J, O, T). A-E; Two *Colpodella* in close proximity, both with ingested prey. F-J; Single *Colpodella* in attack of single *B. caudatus* prey with cytoplasmic contents almost completely ingested (red arrowheads F, J and J). Vesicles are stained with anti-RhopH3 antibody and flagella with anti-β-tubulin. K-O; Negative control with normal rabbit and mouse serum showing no protein reactivity with cells. P-T; Positive control for *P. falciparum* specific antibody showing reactivity with *P. falciparum* schizont-infected human erythrocyte.

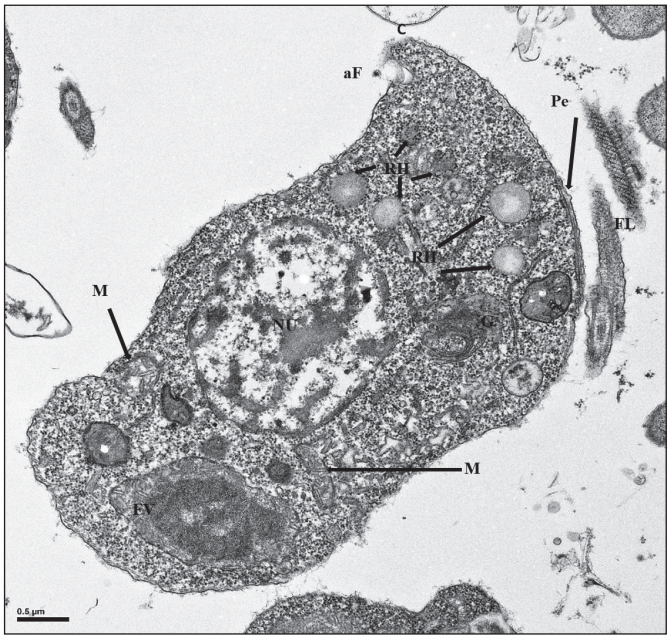


**Fig.5.** Transmission electron micrograph of longitudinal section of *Colpodella* (ATCC 50594) showing cytoplasmic organization. Conoid (C) in the rostrum, anterior flagellum (aF), flagella (FL), electron lucent and dense spherical vesicles (rhoptries, RH), mitochondria (M), Golgi (G), food vacuole (FV), nucleus (NU) and pellicle (Pe).

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**Competing interests.** None declared.

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