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Flow Cytometric Characterization of Encystation in *Entamoeba invadens*:

SHORT TECHNICAL REPORT

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Abstract

Entamoeba histolytica causes dysentery and liver abscess mostly in countries that lack proper sanitation. Infection is acquired by ingestion of the cyst form in contaminated food or water. *E. histolytica* does not encyst *in vitro*; thus, *E. invadens*, a reptilian parasite that encysts *in vitro*, has been used as a surrogate. Cysts are small and possess chitin-rich walls. These are characteristics that may be exploited by flow cytometry. We stained encysting *E. invadens* cells with a fluorescent chitin stain, and analyzed fluorescence and forward scatter by flow cytometry. We demonstrate that flow cytometry can be used to track differentiation, reveal unique cell populations, and evaluate encystation inhibitors.

Graphical Abstract

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Keywords

Chitin; encystation; *Entamoeba histolytica*; *Entamoeba invadens*; flow cytometry; forward scatter

Entamoeba histolytica is a protozoan pathogen that causes amoebic dysentery and amoebic liver abscess in human and non-human primates (reviewed in [1]). It is transmitted by the cyst form of the parasite in fecally-contaminated food and water, making it prevalent in the developing world where sanitation practices are substandard. According to the World Health Organization, greater than 800 million people defecate in the open and at least 10% of the world's population consumes food from crops irrigated with wastewater. Thus, there is considerable risk for transmission of *E. histolytica*.

After ingestion, cysts exit the stomach and enter the small intestine, where unknown cues trigger excystation. Excystation is characterized by the emergence of motile, amoeboid trophozoites, which move to and establish disease in the large bowel (reviewed in [2]). Here, infection follows one of two, non-mutually exclusive, routes. The parasite may invade and cross the gut epithelial layer, enter the circulatory system, and establish an extra-intestinal infection, the most common of which is liver abscess. Alternatively, as a result of undefined signals, trophozoites may form infective cysts (encystation) which are released from the host. *E. histolytica* does not readily encyst in axenic culture; thus, less is known about encystation than invasive disease. Therefore, *E. invadens*, a reptilian parasite that encysts *in vitro*, has been used as a model [3–7]. *E. invadens* exhibits efficient stage conversion in culture after simultaneous reduction of osmolarity, glucose, and serum in the medium [6].

Studies using *E. invadens* have revealed that cysts are typically smaller and rounder than trophozoites [8], quadrinucleated, and possess walls that are rich in chitin [9], chitosan fibrils [10] and chitin-binding proteins (e.g., [11]). The unique cyst wall confers desiccation-, acid-, heat-, and detergent-tolerance to the structure (reviewed in [2]).

Encystation also depends on a heterotrimeric protein complex, the Gal/GalNAc lectin, which is found on the surface of the parasite [6]. The Gal/GalNAc lectin binds galactose (Gal) and N-acetylgalactosamine (GalNAc)-terminated glycoconjugates found in the host mucin layer and on the surface of host cells. An early step in encystation is self-aggregation of the trophozoites. The Gal/GalNAc lectin is thought to facilitate this aggregation event because free Gal inhibits self-aggregation and encystation [6]. Researchers have used a variety of ways to track the progress of encystation. These methods have included microscopic analysis of cells stained with fluorescently-labeled chitin-binding reagents (e.g., wheat germ agglutinin) [12], tracking encystation-specific enzyme activity [13], or RT-PCR [14,15]. The latter is complemented by the published transcriptome of encysting *E. invadens* cells [3]. Another method of tracking encystation efficiency is incubation of cells in detergent, such as sarkosyl, followed by manual counting using a hemacytometer [7]. Since detergent lyses trophozoites and immature cysts (pre-cysts), the surviving detergent-resistant cells (DRC) are thought to be terminally-differentiated mature cysts.

Here, we describe a novel way to track encystation in *Entamoeba* using flow cytometry. Flow cytometry can simultaneously measure multiple physical characteristics (e.g., shape, size, fluorescence) of individual cells. Given the changes in cell shape and size that occur during encystation of *Entamoeba*, and given the commercial availability of fluorescent chitin-binding reagents, we envisaged that this technology could be used to track encystation-specific attributes in a temporal fashion. This would provide a rapid method for assessing encystation progress. Flow cytometry has been used to track DNA content during encystation in *E. invadens* [16] and *Giardia intestinalis* [17] and chitin accumulation during encystation in *Acanthamoeba castellanii* [18]. To the best of our knowledge, this is the first report describing a flow cytometry-based method to assess *Entamoeba* encystation by simultaneously tracking changes in cell size/shape and chitin.

E. invadens trophozoites were incubated in nutrient-rich medium (control) or encystation medium as described [6,7]. Cells were collected over time, stained with the fluorescent chitin stain, Congo Red [18], fixed, and analyzed by flow cytometry by collecting ten-thousand individual events. In addition to fluorescence, flow cytometers measure the light scattered by single particles at right angles to the laser beam (side scatter, SSC) and in the forward direction (forward scatter, FSC). SSC is most affected by the optical homogeneity while FSC is most affected by cell size and shape. Therefore, to evaluate information about cell size/shape and chitin simultaneously, all data were analyzed using FSC versus fluorescence density plots.

Control trophozoites, which were not induced to encyst, formed a single population (P1) of cells (Fig. 1Aa). The parameters of this control population were then used to gate populations of cells throughout stage conversion. During encystation, a second population (P2) of cells arose (Fig. 1Ab–c). This population exhibited higher fluorescence and lower FSC than control trophozoites (Fig. 1Aa). The size of this population increased and the cells became more uniform in size and fluorescence in a time-dependent fashion (Fig. 1Ab–c; Fig. 1B). Reduced FSC and increased fluorescence may be interpreted as decreased size and increased chitin content, respectively, which is consistent with the changes that occur in *E. invadens* cells during stage conversion. We obtained similar results with a second fluorescent

chitin-binding stain, Alexa Fluor® 488-wheat germ agglutinin (data not shown). This supports the notion that we are authentically tracking changes in chitin with the Congo Red stain.

To further characterize P1 and P2, we induced encystation, treated cells with a detergent, sarkosyl, and again analyzed the population by flow cytometry. Few cells were found in the P1 “boundary” after treatment with sarkosyl (Fig. 1Ad), supporting the identity of P1 cells as detergent-sensitive trophozoites. On the other hand, cells were detected in P2 after detergent-treatment (Fig. 1Ad). This strengthens the inference that the P2 population contains detergent-resistant cysts. Treatment with detergent also allowed us to “manually” estimate encystation efficiency, which is defined as the percent of cells that have acquired detergent-resistance. Specifically, detergent-resistant cells were enumerated using a hemacytometer. This manual counting revealed that $45.9 \pm 5.5\%$ and $70.6 \pm 10.8\%$ of cells acquired detergent-tolerance at 48 h and 72 h, respectively. Interestingly, the percent of cells that were detergent-resistant (determined by counting) was lower than the percent of cells in the P2 population (Fig. 1B). This indicates that some of the cells in P2 are still detergent-sensitive. The identity of detergent-sensitive P2 cells has not been discerned; however, they may represent cells that are in an intermediate phase of encystation, having acquired some of the characteristics of cysts even though they are not yet refractory to detergent. Others have shown that *E. invadens* can react with chitin-binding dyes, produce encystation-specific transcripts [14], and begin to alter their DNA content even within the first 24 h of encystation [16] when few cells have acquired detergent-resistance. The current study demonstrates that flow cytometry can also be used to efficiently track populations of early differentiating cells.

By 48 h, the encysting population acquired a significantly reduced mean FSC ($P<0.001$) and a significantly increased mean fluorescence ($P<0.001$) compared to un-encysted trophozoites (Fig. 2Aa,b, B, C). Therefore, to further assess the value of flow cytometry in tracking stage conversion in *E. invadens*, we analyzed populations of cells that were induced to encyst for 48 h in the presence of several known inhibitors. As described above, an initial step in encystation is the formation of multicellular aggregates [6]. Free Gal is a potent inhibitor of encystation presumably by blocking this aggregation event [6]. Gal also inhibits the expression of at least three encystation-specific transcripts [6]. Another sugar, N-acetylglucosamine (GlcNAc), can inhibit encystation albeit by a different molecular mechanism [6]. GlcNAc does not inhibit the initial aggregation step, nor does it have the same effect on encystation-specific gene expression. Instead, GlcNAc is thought to block encystation by interfering with the activity of chitin synthase, which uses UDP-GlcNAc as a substrate. Another ligand of the Gal/GalNAc lectin, N-acetylgalactosamine (GalNAc), does not seem to inhibit stage conversion in this parasite [6]. As expected, the formation of a P2 population was hampered in the presence of Gal (Fig 2Ac, Fig. 2B, C) or GlcNAc (Fig 2Ad, Fig. 2B, C), but not in the presence of GalNAc (Fig. 2Ae, Fig. 2B,C). We also treated cells, which had been encysted in the presence of sugars, with detergent and manually estimated encystation efficiency using a hemacytometer. Only $7.1 \pm 5.3\%$ and $5.2 \pm 3.7\%$ of cells acquired detergent-tolerance in the presence of Gal or GlcNAc, respectively. These were not significantly different from each other but were significantly lower ($P<0.001$) than the percent of cells acquiring detergent-resistance during encystation in the absence of sugars

($43.3 \pm 1.5\%$). Although manual counting suggests that Gal and GlcNAc are equally potent inhibitors of terminal encystation, flow cytometry revealed additional information. For example, prior to detergent treatment, the percent of cells in P2 was significantly higher ($P < 0.05$) in Gal-treated populations ($41.1 \pm 22.7\%$) than in GlcNAc-treated populations ($20.1 \pm 6.0\%$). This shows that progress toward terminal differentiation varied in the presence of inhibitory sugars. In other words, by 48 h into encystation, more cells acquired the characteristics of the P2 population in the presence of Gal than in the presence of GlcNAc. We also observed fewer aggregates by microscopy during encystation in the presence of Gal (data not shown). Despite this, cells seem to be differentiating (acquiring P2 attributes) in the presence of Gal albeit at a slower rate than control cells.

Flow cytometry revealed that there were two distinct populations of detergent-tolerant cells in the GlcNAc-treated cultures (Fig. 2Ah). Distinct subpopulations of detergent-resistant cells were not observed in any of the other conditions (Fig. 2Af,g,i). When compared to each other, the two detergent-resistant subpopulations observed in the GlcNAc-treated cultures seemed to differ in their mean fluorescence but not in their mean FSC, suggesting they were dissimilar in their chitin content and/or the ability to bind Congo Red, but not in their size. This is in accordance with the predicted mode of action of GlcNAc as an inhibitor of chitin synthase. It is interesting that there is a population of cells that possesses less chitin but is still detergent-tolerant.

In addition to sugar inhibitors of encystation, we also analyzed populations of cells that were induced to encyst in the presence of a chemical inhibitor, DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid). DIDS is a commercially-available small molecule inhibitor of human Rad51 [19], a DNA recombinase that regulates homologous search and strand exchange during homologous recombination. We have previously shown that DIDS decreases the activity of *ehRad51*, the *Entamoeba* ortholog of Rad51, and inhibits encystation [20]. *E. invadens* cells were induced to encyst in the absence or presence of DIDS (30 μ M or 150 μ M) for 48 h. Manual counting of detergent-resistant cells confirmed that DIDS inhibits terminal differentiation (Fig. 2D); however, flow cytometry showed that the formation of P2 was not inhibited (Fig. 2E).

In summary, we have shown that flow cytometry can be used to track terminal differentiation of *Entamoeba* cells without a detergent-treatment step. Furthermore, combining flow cytometry with detergent-treatment uncovers additional information that is not revealed by simple counting of detergent-resistant cells. Of particular interest were the differences in the formation of P2 in the presence of inhibitors. The formation of P2 was most significantly inhibited by GlcNAc, less significantly inhibited by Gal, and not inhibited at all by DIDS. Given the predicted modes of action of these inhibitors, one interpretation of the data is that chitin synthase, the Gal/GlcNAc lectin, and *ehRad51* play roles at increasingly later stages in encystation. It is interesting that DIDS allows cells to acquire all of the characteristics of the P2 population, while inhibiting the acquisition of detergent-resistance. Although homologous recombination between nuclei in the quadrinucleated cyst has never been demonstrated, perhaps the function of *ehRad51* is necessary at the latest stages of differentiation after nuclear division.

In the future, it may be possible to use flow cytometry to assess the potency of new encystation inhibitors or track encystation in genetically-modified *E. invadens* cells. Although we used a cytometer, which analyzes cells without sorting them, it may also be possible to collect subpopulations with a cell sorter, using parameters such as those employed in this study (e.g., chitin-staining and FSC). Conducting transcriptomic, translational, and/or proteomic analyses on these sorted subpopulations will augment our understanding of encystation in the *Entamoebae*. Given the versatility of modern cell sorters, it would be interesting to add additional sorting parameters, such as DNA-staining [16]. This would further refine the subpopulations and provide insight into the connection between cell cycle and encystation. Finally, given that this technique allows for the visualization of encystation without application of detergents, it may also be possible to use this technique to discover conditions (both external and genetic) that allow the human pathogen, *E. histolytica*, to advance, even partially, toward terminally differentiated cysts *in vitro*.

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References

- Petri WA Jr, Haque R, Mann BJ. The bittersweet interface of parasite and host: lectin-carbohydrate interactions during human invasion by the parasite *Entamoeba histolytica*. *Annu. Rev. Microbiol.* 2002; 56:39–64. [PubMed: 12142490]
- Eichinger D. Encystation in parasitic protozoa. *Curr. Opin. Microbiol.* 2001; 4:421–426. [PubMed: 11495805]
- Ehrenkaufer GM, Weedall GD, Williams D, Lorenzi HA, Caler E, Hall N, Singh U. The genome and transcriptome of the enteric parasite *Entamoeba invadens*, a model for encystation. *Genome Biol.* 2013; 14:R77. [PubMed: 23889909]
- Singh N, Ojha S, Bhattacharya A, Bhattacharya S. Stable transfection and continuous expression of heterologous genes in *Entamoeba invadens*. *Mol. Biochem. Parasitol.* 2012; 184:9–12. [PubMed: 22426570]
- Field J, Van Dellen K, Ghosh SK, Samuelson J. Responses of *Entamoeba invadens* to heat shock and encystation are related. *J. Eukaryot. Microbiol.* 2000; 47:511–514. [PubMed: 11001149]
- Coppi A, Eichinger D. Regulation of *Entamoeba invadens* encystation and gene expression with galactose and N-acetylglucosamine. *Mol. Biochem. Parasitol.* 1999; 102:67–77. [PubMed: 10477177]
- Hendrick HM, Welter BH, Hapstack MA, Sykes SE, Sullivan WJ Jr, Temesvari LA. Phosphorylation of eukaryotic initiation factor-2 α during stress and encystation in *Entamoeba* species. *PLoS Pathog.* 2016; 12:e1006085. [PubMed: 27930733]
- Herrera-Martinez M, Hernandez-Ramirez VI, Lagunes-Guillen AE, Chavez-Munguia B, Talamas-Rohana P. Actin, RhoA, and Rab11 participation during encystment in *Entamoeba invadens*. *Biomed. Res. Int.* 2013; 2013:919345. [PubMed: 24175308]
- Chatterjee A, Ghosh SK, Jang K, Bullitt E, Moore L, Robbins PW, Samuelson J. Evidence for a "wattle and daub" model of the cyst wall of *Entamoeba*. *PLoS Pathog.* 2009; 5:e1000498. [PubMed: 19578434]

10. Das S, Van Dellen K, Bulik D, Magnelli P, Cui J, Head J, Robbins PW, Samuelson J. The cyst wall of *Entamoeba invadens* contains chitosan (deacetylated chitin). *Mol. Biochem. Parasitol.* 2006; 148:86–92. [PubMed: 16621070]
11. Ghosh SK, Van Dellen KL, Chatterjee A, Dey T, Haque R, Robbins PW, Samuelson J. The Jacob2 lectin of the *Entamoeba histolytica* cyst wall binds chitin and is polymorphic. *PLoS Negl. Trop. Dis.* 2010; 4:e750. [PubMed: 20652032]
12. Aguilar-Diaz H, Diaz-Gallardo M, Laclette JP, Carrero JC. *In vitro* induction of *Entamoeba histolytica* cyst-like structures from trophozoites. *PLoS Negl. Trop. Dis.* 2010; 4:e607. [PubMed: 20169067]
13. Das S, Gillin FD. Chitin synthase in encysting *Entamoeba invadens*. *Biochem. J.* 1991; 280(Pt 3): 641–647. [PubMed: 1764027]
14. Ghosh SK, Field J, Frisardi M, Rosenthal B, Mai Z, Rogers R, Samuelson J. Chitinase secretion by encysting *Entamoeba invadens* and transfected *Entamoeba histolytica* trophozoites: localization of secretory vesicles, endoplasmic reticulum, and Golgi apparatus. *Infect. Immun.* 1999; 67:3073–3081. [PubMed: 10338523]
15. Segovia-Gamboa NC, Chavez-Munguia B, Medina-Flores Y, Cazares-Raga FE, Hernandez-Ramirez VI, Martinez-Palomo A, Talamas-Rohana P. *Entamoeba invadens*, encystation process and enolase. *Exp. Parasitol.* 2010; 125:63–69. [PubMed: 20045689]
16. Singh N, Bhattacharya S, Paul J. *Entamoeba invadens*: dynamics of DNA synthesis during differentiation from trophozoite to cyst. *Exp. Parasitol.* 2011; 127:329–333. [PubMed: 20727884]
17. Einarsson E, Troell K, Hoepfner MP, Grabherr M, Ribacke U, Svard SG. Coordinated changes in gene expression throughout encystation of *Giardia intestinalis*. *PLoS Negl. Trop. Dis.* 2016; 10:e0004571. [PubMed: 27015092]
18. Connell C, Rutter A, Hill B, Suller M, Lloyd D. Encystation of *Acanthamoeba castellanii*: dye uptake for assessment by flow cytometry and confocal laser scanning microscopy. *J. Appl. Microbiol.* 2001; 90:706–712. [PubMed: 11348429]
19. Ishida T, Takizawa Y, Kainuma T, Inoue J, Mikawa T, Shibata T, Suzuki H, Tashiro S, Kurumizaka H. DIDS, a chemical compound that inhibits RAD51-mediated homologous pairing and strand exchange. *Nucleic Acids Res.* 2009; 37:3367–3376. [PubMed: 19336413]
20. Kelso AA, Goodson SD, Chavan S, Say AF, Turchick A, Sharma D, Ledford LL, Ratterman E, Leskoske K, King AV, et al. Characterization of the recombination activities of the *Entamoeba histolytica* Rad51 recombinase. *Mol. Biochem. Parasitol.* 2016; 210:71–84. [PubMed: 27678398]

Highlights

- Cysts of *Entamoeba* spp. have attributes that may be tracked by flow cytometry
- Flow cytometry was used to track chitin and cell size in encysting cells
- A distinct subpopulation of cells arose during encystation
- Flow cytometry also provided insight into the mechanism of encystation inhibitors

Fig. 1.

Encystation of *E. invadens* evaluated by flow cytometry. *Entamoeba invadens* (strain IP-1) was cultured axenically in TYI-S-33 medium in 15 mL glass screw cap tubes at 25°C [4]. Stage conversion was induced by incubating *E. invadens* trophozoites (5×10^5 cells/ml) in 47% LYI-LG, a standard encystation medium with reduced glucose, osmolarity and serum [6]. Trophozoites (control) or cells that had been encysting for 48 h or 72 h were harvested by scraping followed by centrifugation ($500 \times g$, 5 min). For staining, pellets were resuspended in Congo Red (57 μ M in serum free medium) and incubated at room temperature for 15 min with rotation. After staining, cells were pelleted by centrifugation and fixed in 4% (v/v) paraformaldehyde in PBS for 15 min at room temperature. Fixed cells were resuspended in PBS and analyzed by flow cytometry using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). Red fluorescence emission signals were collected using a 585/40 filter. Ten-thousand events were collected for each sample. Data were analyzed by plotting forward scatter (FSC) versus fluorescence (FL). In some cases, mature detergent-resistant cysts (DRC) were purified from 72 h encysting cell populations by incubation in 0.01% (v/v) sarkosyl in PBS on ice for 10 min. Purified DRCs were enumerated by counting using a hemacytometer or stained and analyzed by flow cytometry as described above. (A) Typical flow cytometry density plots for trophozoites (Control), cells encysting for 48 h or 72 h, or DRCs. Control trophozoites formed a single population (P1) of cells (a). During encystation, a second population (P2) of cells arose (b,c). After

treatment with detergent, cells were detected in P2 but not in the P1 “boundary” (d). **(B)** Percent of total cells found in P2 (obtained from flow cytometry data) or the percent of cells that had acquired detergent resistance (DRC) (obtained from manual counting) accumulating over time during encystation. Data represent the mean $\pm$ standard deviation (S.D.) of 4 experiments. P2 and DRC populations increased over time. However, at each time point, the percent of cells that were detergent-resistant was lower than the percent of cells in the P2 population indicating that some of cells in P2 are still detergent-sensitive.

Fig. 2.

Flow cytometric analysis of *E. invadens* cells encysting in the presence of inhibitors. Trophozoites (Control) were induced to encyst for 48 h in the presence of Gal (10 mM [6]), GlcNAc (10 mM [6]), GalNAc (10 mM [6]) or DIDS (30 µM or 150 µM [20]). Cells or DRCs were stained and analyzed as described in Fig. 1. All values are given as means $\pm$ S.D. (n=4). In all cases, statistical analyses of means compared to the mean of control cells were performed using GraphPad Instat V.3 with an unpaired t test, Welch corrected (two-tail *P* value). *P* values of less than 0.05 (*) were considered statistically significant. *P* values less than 0.001 (***) were considered highly statistically significant. (A) Typical flow cytometry

density plots for control trophozoites or cells encysting in the presence of sugar inhibitors before (a–e) and after (f–i) treatment with detergent. The mean percent of cells found in P2 and the mean percent of cells that acquired detergent-resistance (DRC) are given. Formation of a P2 population and DRCs was hampered in the presence of Gal or GlcNAc but not in the presence of GalNAc. **(B and C)** Mean FSC (B) or FL (C) of cells encysted in the presence of sugar inhibitors. By 48 h, the encysting population acquired a significantly reduced mean FSC and a significantly increased mean fluorescence compared to control trophozoites. Gal and GlcNAc prevented the acquisition of such population attributes but GalNAc did not. **(D and E)** Percent of cells acquiring detergent resistance (D) or the percent of cells found in P2 (E) during encystation in the presence of DIDS. DIDS inhibits the formation of DRCs (D); the decrease approaches significance. DIDS did not inhibit the acquisition of P2 characteristics (E).