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Short technical report

Flow cytometric characterization of encystation in *Entamoeba invadens*

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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.

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

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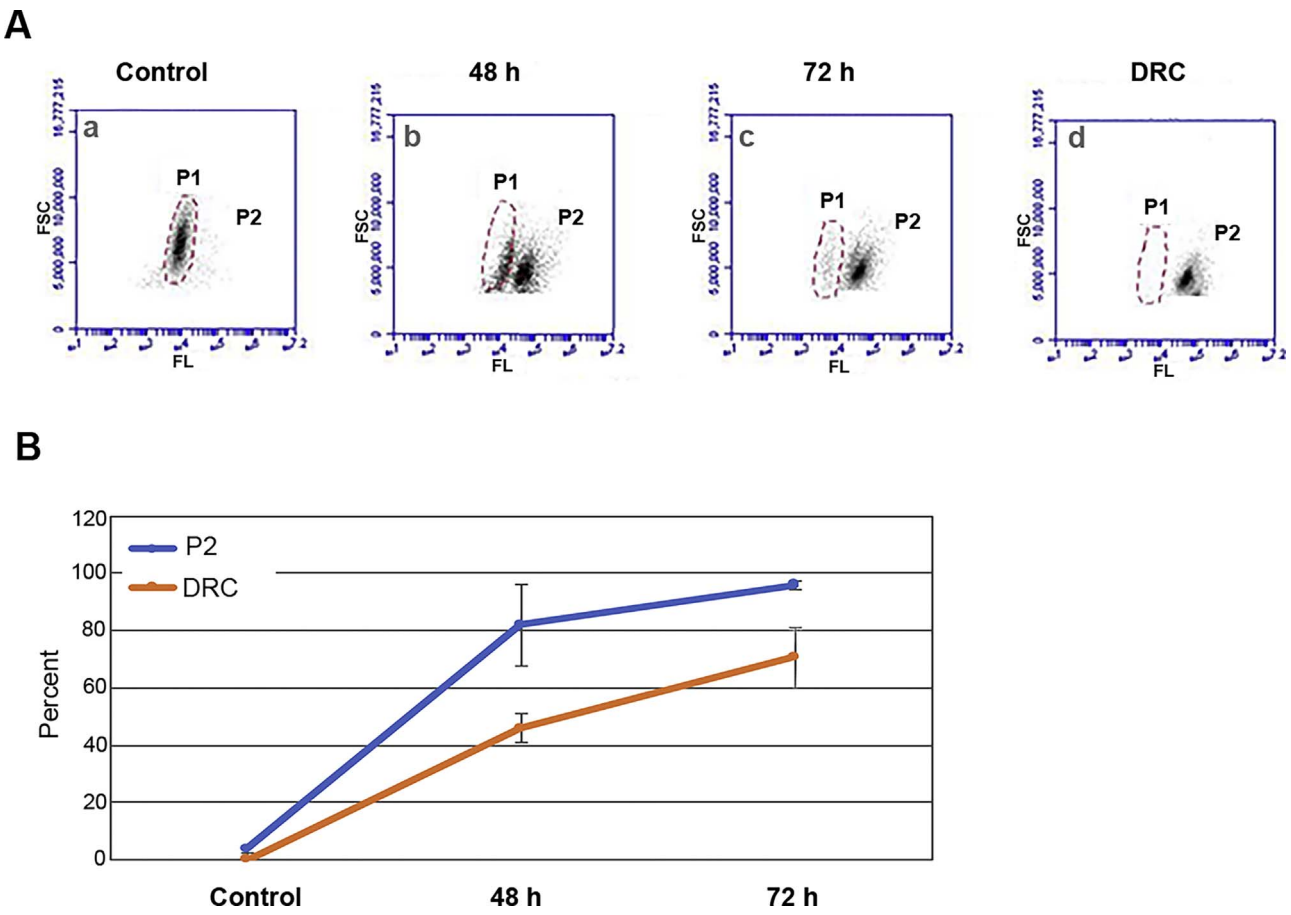

Fig. 1. Encystation of *E. invadens* evaluated by flow cytometry. *Entamoeba invadens* (strain IP-1) was cultured axenically in TYI-S-33 medium in 15 mL glass screw cap tubes at 25 °C [4]. Stage conversion was induced by incubating *E. invadens* trophozoites (5×10^5 cells/ml) in 47% LYI-LG, a standard 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 × *g*, 5 min). For staining, pellets were resuspended in Congo Red (57 μM 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 0.1% (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 ± 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.

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 castellani* [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; B). 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