Variability and heritability of cell division pathways in *Toxoplasma gondii*

Ke Hu[1], David S. Roos[2], Sergio O. Angel[3] and John M. Murray[1,*]

[1]Department of Cell and Developmental Biology and [2]Department of Biology, University of Pennsylvania, 421 Curie Boulevard, Philadelphia, PA 19104, USA
[3]Laboratorio de Parasitologia Molecular, IIB-INTECH, UNSAM-CONICET, Chascomus, Pcia. de Buenos Aires, Argentina
*Author for correspondence (e-mail: murray@cellbio.med.upenn.edu)

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Summary

A histone 2b-YFP fusion protein stably expressed in *Toxoplasma gondii* has several advantages: it reveals previously hidden details of nuclear morphology; it makes it possible to observe cell-cycle events; it provides a basis for quantitative measurements of DNA content in living cells; and it enables sorting of live cells according to cell-cycle phase or ploidy. With this cell line it was possible to recognize and directly clone individual progeny arising from different patterns of cell division that produce two, three or four daughter cells. These experiments established that the progeny produced by all cell division pathways are viable and infective. Furthermore, the number of progeny produced by a mature parasite during cell division is not correlated with the number of its siblings. The complete repertoire of cell division pathways is therefore inherited by a single cell produced through any one of the individual paths. The results expand the range of what must be considered normal in *T. gondii* cell division and provide a useful tool for further study of nuclear structure and proliferation in this important human pathogen.

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Introduction

Toxoplasma gondii is a ubiquitous parasite, chronically infecting 10-90% of human populations worldwide. This protozoan belongs to the Apicomplexa, a phylum of more than 5000 protozoan parasite species (Levine, 1988), the best known of which is *Plasmodium falciparum*, responsible for the most lethal form of malaria, killing millions each year. Other important human and veterinary parasites in this phylum include species of *Eimeria* (a pathogen having great economic impact in the poultry and cattle industries), *Cryptosporidia* (an opportunistic human and animal pathogen, devastating for AIDS patients), *Babesia* and *Theileria* (cattle parasites) and many others.

In its asexual form, *T. gondii* can invade, proliferate and encyst in virtually any nucleated cell, though sexual differentiation occurs only in the cat (Bonhomme et al., 1992; Dubey, 1998; Dubey et al., 1998; Frenkel, 1973; Smith, 1995). Haploid tachyzoites invade host cells, establishing a parasitophorous vacuole whose membrane is derived from the host plasma membrane (Joiner et al., 1994; Mordue et al., 1999; Suss-Toby et al., 1996). Two parasites are typically produced in each mitotic cell cycle (~7-10 hours), and replication proceeds (synchronously at first) resulting in geometric expansion of clonal progeny until the host cell is lysed, ~48 hours post-infection. *T. gondii* infection in humans is frequently asymptomatic, but poses a severe risk in two situations. Primary infection during pregnancy often causes abortion or severe neurological birth defects. Reactivation of dormant parasite tissue cysts (bradyzoites) in a chronically infected host gives rise to rapidly replicating tachyzoites, which may be fatal in immunocompromised individuals.

Much of the pathogenesis associated with these Apicomplexan parasitic diseases is due to tissue damage caused by uncontrolled parasite proliferation. For example, malarial fevers and anemia are a consequence of red blood cell lysis, and Toxoplasma encephalitis is due to parasite-mediated destruction of neuronal tissue. Understanding the replication of these parasites is therefore essential for understanding the corresponding parasitic diseases.

In addition to the strong clinical incentive for studying cell division in the Apicomplexa, *T. gondii* provides unique opportunities to address basic mechanisms common to all dividing cells. Although their small size (~7×3 μm) and almost featureless appearance has delayed the study of cell division by conventional light microscopy, newer tools, in particular fluorescent protein reporters, now permit virtually all known subcellular structures to be visualized in living parasites. Unlike typical eukaryotic cells, every *T. gondii* has a reproducible shape during cell division, with its organelles arranged in predictable locations, each following a characteristic series of structural transitions as they are partitioned between daughters. The reproducible structure more than compensates for the small size of the cell, exposing fundamental processes with extraordinary clarity in these parasites.

In *T. gondii*, formation of new cells is initiated within an existing cell (Fig. 1) on a scaffold (the inner membrane complex, IMC) (Nichols and Chiappino, 1987) that consists of

cytoskeletal elements and flattened vesicles (Dubremetz and Torpier, 1978; Mann and Beckers, 2001; Porchet and Torpier, 1977; Snigirevskaya, 1969). Replicated (or replicating) chromatin and organelles become associated with the assembling scaffolds as cell division proceeds (Sheffield and Melton, 1968).

Previously we reported that in contrast to common belief, *T. gondii* in its tachyzoite form does not always produce just two new cells in a fixed pattern of cell division, but instead displays a remarkable versatility, splitting into three, four or even more new cells at once (Hu et al., 2002). In that study we were not able to monitor individual parasites over more than one generation, leaving unresolved two basic questions. Are multi-way splits a 'normal' event producing viable progeny, or are they mistakes or aberrations that yield defective cells? Is the number of new cells produced during cell division a constant for each lineage (i.e. an inheritable trait resulting from a permanent genetic change) or is this an epigenetic phenomenon?

To answer these questions we constructed a transgenic parasite that stably expresses a histone 2b-YFP fusion protein. In these cells, the morphological changes of karyokinesis are visible by fluorescence microscopy. In the present study, we assess the viability and heritability of cell divisions involving multi-way splitting in *T. gondii,* and explore factors that can stimulate the formation of multiple cells at once.

Materials and Methods

Histone 2b-YFP expressing parasites

The *T. gondii* expression vector ptubH2bYFP was constructed from ptubFNR-YFP/sagCAT, which in turn was derived from pBluescript KS$^+$ (Stratagene) as described earlier (Striepen et al., 2000; Striepen et al., 1998). The histone2b coding sequence was amplified from a plasmid containing *T. gondii* histone 2b cDNA using primers: 5′-GTCAagatctATGTCAGGGAAAGGTCCGGCACAG-3′ (sense) and 5′-ACTGcctaggTGCACCAGAAGTCGTGTACTTGGTCA-3′ (anti-sense), with restriction sites indicated by lower case letters.

The PCR product was digested with *Bgl*II and *Avr*II and ligated in place of FNR in a modified version of ptubFNR-YFP/sagCAT from which the N-terminal ATG of YFP had been removed (kindly provided by Omar Harb of the Roos laboratory). The resultant plasmid ptubH2bYFP, contains between the *Bam*HI and *Sma*I sites of pBluescript KS$^+$ (in reverse order), the selectable marker sagCATsag (Kim et al., 1993), the 5′-UTR of the *T. gondii* α-tubulin gene (Striepen et al., 1998), the histone2b coding region, a dipeptide linker (*Avr*II site, cctagg, coding for pro-arg), YFP minus its initial ATG codon and finally the 3′-UTR of the *T. gondii* DHFR-TS (Roos, 1993). 10^7 RH strain parasites were transfected with 50 μg plasmid DNA and inoculated into host cells as previously described (Roos et al., 1994). To produce stable transgenics, chloramphenicol was added 24 hours later to a final concentration of 6 μg/ml, and drug-resistant clones were isolated by limiting dilution after several rounds of selection.

T. gondii cultures for single cell cloning

Parasites were inoculated onto a gridded 35 mm culture dish (MatTek) plated with human foreskin fibroblast cells (HFF) and allowed to grow overnight in a 37°C CO_2 incubator. The culture medium (Roos et al., 1994) was replaced by pH-buffered medium (MEM without sodium bicarbonate; 1% FBS; 25 mM HEPES; 2 mM glutamine; 50 U/ml penicillin-streptomycin and 50 μg/ml gentamycin) immediately before moving the dish to a warmed microscope stage equipped with a micromanipulator (Narishige, Japan). Microinjection needles with 4 μm inner diameter (Eppendorf Scientific) were used as micropipettes to aspirate and transfer individual parasites to a new culture dish.

Identifying N-tuplets

Parasites that had been made as, or were making, multiple daughters ('N-tuplets') were identified by two criteria. (1) nuclear morphology (visualized by histone 2b-YFP), i.e. multi-lobed nucleus or more than two nuclei in the same cell or (2) by an aberrant number (i.e. not one, two or four) of parasites in a small vacuole (containing less than eight parasites). In the first case, the dishes were put back in the incubator until the parasites finished cell division. In the second case, the parasites were aspirated and transferred immediately. For vacuoles that had parasites making both twins and N-tuplets, only the N-tuplets were transferred. The N-tuplets are smaller than twins immediately after budding and can be distinguished visually by size in a side-by-side comparison. As a control, the twins in 'normal' vacuoles (i.e. containing two, four or eight parasites) were aspirated and transferred in the same way.

Single cell transfer

Under microscopic observation, the target vacuoles were first prodded with the micropipette to initiate active egress of the parasites from the host cell. The extracellular parasites were then aspirated into the micropipette and transferred to an identified grid square in a fresh culture dish of HFF cells in normal culture medium. For about two-thirds of the experiments, the culture dishes were immediately placed into a 37°C CO_2 incubator for later observation. For the other third of the experiments, transferred parasites were given up to 1 hour to invade on the microscope stage before being returned to the incubator. The dishes were kept in a 5% CO_2 chamber and maintained at 35-37°C while on the stage. The growth of the transferred parasites was then monitored over time.

Estimation of viability and multiple-daughter formation after transfer

The viability of the transferred parasites was assayed by counting the number able to proliferate after transfer. Small vacuoles that had 16 or fewer parasites were used to estimate the fraction of vacuoles making multiple daughters, defined as the number of vacuoles containing an aberrant number of parasites divided by the total number of vacuoles counted. The parasites in a small vacuole can be accurately counted and they divide in synchrony for at least the first four cycles after invasion. An aberrant number of parasites in a small vacuole is therefore a reliable indication of N-tuplet formation in a previous cell cycle.

Fluorescence microscopy

Confluent HFF cultures on glass coverslips were fixed in 3.7% paraformaldehyde ~18-24 hours after infection with parasites and permeabilized in 0.25% Triton X-100. Mouse anti-IMC1 monoclonal antibody (kindly provided by G. E. Ward, University of Vermont) was diluted 1:1000 in 2% BSA and detected using Alexa488- or Alexa594-conjugated goat anti-mouse antibody (Molecular Probes). Following antibody labeling, coverslips were incubated in 2.8 μM DAPI (Molecular Probes) for 5 minutes, followed by several brief washes in PBS. Coverslips were mounted with Fluoromount G (Southern Biotechnology Associates) or PBS. Live parasites were observed at 37°C in culture medium for short time periods in a chamber constructed from a coverslip and a thin spacer (Molecular Probes Secure-Seal™), and for longer times in a BiopTechs delta-T dish. Images were collected on a Zeiss Axiovert with a 100× N.A. 1.4 oil immersion objective or on an Applied Precision DeltaVision®

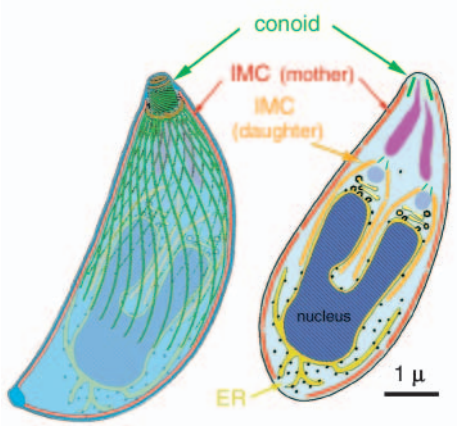

Fig. 1. Structure of *Toxoplasma gondii.* The left-hand transparent view of a cell, mid-way through mitosis with two daughter cells forming, shows the conoid (green), inner membrane complex (IMC, red), and subpellicular microtubules (green), which run along the cytoplasmic face of the IMC. The right-hand view shows a longitudinal section of this cell with subpellicular microtubules removed for clarity. Lobes of the dividing nucleus, bordered by ER (yellow-green), Golgi (yellow), and apicoplast (mauve) are surrounded by the developing daughter IMCs (orange). Two rhoptries (secretory organelles, purple) are shown just below the maternal conoid at the apical end of the cell.

Spectris™ deconvolution workstation using an Olympus IX70 inverted microscope with a 60× N.A. 1.2 water immersion lens.

FACS analysis and sorting

To prepare for flow cytometry and FACS, extracellular parasites were separated from host cells and debris by passage through a 3 µm filter (Nucleopore), centrifuged at 1000 ***g*** for 12 minutes, washed once with PBS and suspended in PBS at a concentration of ~5×10^7/ml. FACS sorting was carried out using either a Moflo® (Dako Cytomation) or Facsvantage® (Becton-Dickinson) instrument. Droplets containing single cells were identified by empirically determined values for amplitude and duration of the forward scattered light pulse (488 nm), amplitude of the side scatter pulse and intensity of fluorescence in the YFP channel. In some experiments, cells were stained for 30 minutes at room temperature with 10 µg/ml Hoechst 33342 in culture medium after initial centrifugation before washing with PBS, and the FACS analysis then included detectors for fluorescence emission from both YFP (488 nm excitation) and Hoechst (407 nm excitation).

Results

Monitoring daughter formation and DNA content with a fluorescent tag on chromatin

In contrast to most eukaryotic cells, stages of the *T. gondii* cell cycle cannot be recognized by light microscopy of living cells. To overcome this problem, a parasite with fluorescent chromatin was created by stably integrating into the genomic DNA a construct coding for histone 2b fused to the fluorescent protein YFP. These parasites grow and divide normally and their nuclei are easily visible by epifluorescence microscopy at all stages of the cell cycle.

Distinct chromosome-like strands of chromatin are clearly observed in mitotic cells, and local condensations of chromatin are resolved even in interphase cells (Fig. 2). The distribution

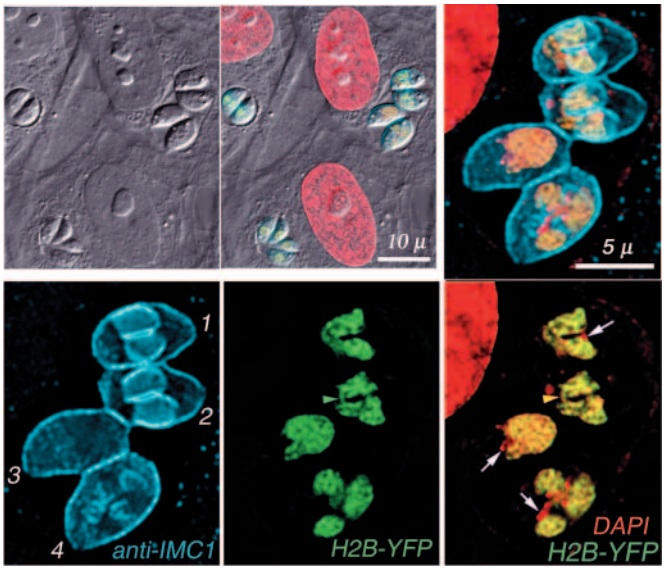

Fig. 2. Images of *T. gondii* expressing histone 2b-YFP fusion protein. The h2b-YFP appears green and parasites were also stained with anti-IMCI antibody to show the inner membrane complex (cyan) and DAPI to visualize DNA (red). The images are the sum of two optical sections close to the middle of the parasites, taken from a deconvolved 3D stack of 15 optical sections 0.3 µm apart. (Upper left panels) Overview DIC and DIC/fluorescence overlay images showing three parasite-containing vacuoles and portions of three host cells. (Upper right and lower panels) Enlarged views of the rightmost vacuole, containing four parasites, of which parasites 1 and 2 are both forming two daughters, parasite 3 is at the very beginning of daughter cell formation where only traces of daughter scaffold can be seen, and parasite 4 is assembling four daughters. The three seemingly separate nuclei of parasite 4 were found to be interconnected in adjacent optical sections (not shown). Chromosome-like aggregations of condensed chromatin (arrowheads) are clearly visible in the segregating nuclei. Apicoplast DNA is also visible (arrows) labeled with DAPI only (red), not with H2b-YFP. In all other regions, the H2b-YFP distribution corresponds precisely to the DNA distribution stained by DAPI.

of DNA revealed by DAPI staining is exactly matched by the distribution of H2b-YFP fluorescence except for a small patch of DNA that is stained by DAPI only (arrows, Fig. 2). This is the 35 kb circular genome of the apicoplast (Egea and Lang-Unnasch, 1995), known to be localized near the apical border of the nucleus in interphase cells, but unresolvable until now from the nuclear DNA during mitosis.

Fig. 3 shows several images from an extended series recording the nuclear division of eight parasites in a vacuole (see also Movie 1 in supplementary material). The parasites within this vacuole are dividing more or less synchronously. Nuclear division is finished by the 30-minute time point (not shown), approximately 15 minutes before cytokinesis (budding of the daughters) starts. There is a large-scale nuclear movement accompanying daughter cell budding. The sequence and timing of morphological changes observed in these living parasites matches exactly the sequence and timing inferred from observations of many static images of fixed and stained parasites (Hu et al., 2002).

In addition to making morphological changes visible, histone 2b-YFP fluorescence also provides a quantitative real-

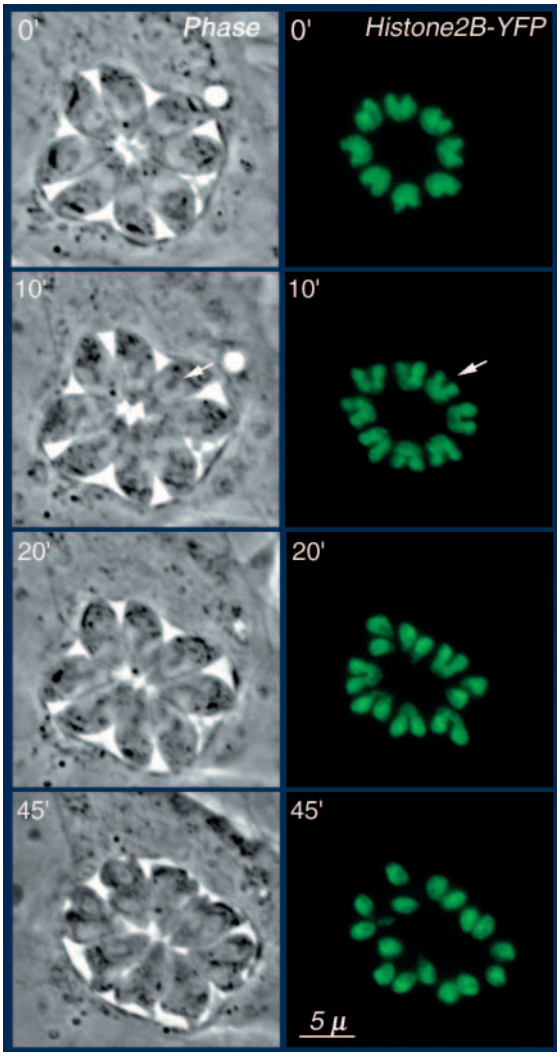

Fig. 3. Time-lapse microscopy of *T. gondii* expressing a histone 2b-YFP fusion protein. At time zero, eight parasites in one vacuole have synchronously begun karyokinesis, revealed as a lobulation of the chromatin in the epifluorescence image (also discernible by phase contrast, arrow). Nuclear division is completed in some nuclei by 20 minutes and in all by 30 minutes (not shown). Cytokinesis is discernible by phase contrast approximately 15 minutes after the completion of nuclear division (45 minute time point) and is completed within 15 minutes (not shown).

time measure of DNA content at the single cell level in living parasites. This is demonstrated in Fig. 4, which shows the results of fluorescence-activated cell sorter (FACS) analysis of a population of extracellular parasites expressing histone 2b-YFP that had also been stained with the cell permeant DNA specific fluorescent dye Hoechst 33342. Two populations account for the majority of cells, corresponding to 1N (haploid) (~94%) and 2N (~5%) parasites, with excellent proportionality between YFP and Hoechst fluorescence. A small number of parasites with a higher DNA content are also present (⩾3N, ~0.1%). Thus fluorescence of histone 2b-YFP provides both a light microscopic method of recognizing cell-cycle events and a signal that can be used to separate a bulk population of parasites according to DNA content.

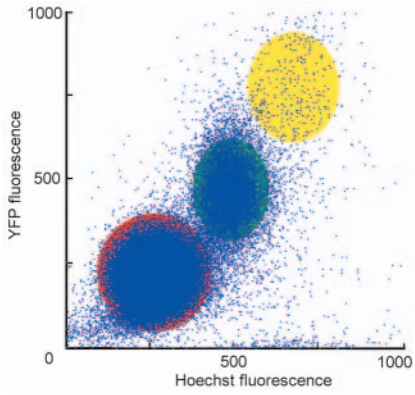

Fig. 4. Flow cytometry analysis of transgenic *T. gondii* expressing histone 2b-YFP and stained with Hoechst 3342. Two-dimensional scatter plot showing fluorescence in the YFP and Hoechst channels, on an arbitrary scale with logarithmic detection. Forward scatter intensity and pulse width, and side scatter intensity were used as gates to discriminate between intact single parasites (85.3% of 1.6×10^5 total events) and cell debris or multiple parasites in a cluster. Discrete peaks for haploid parasites are clearly observed: ~ 1N DNA content, 94% of the parasite population (red background region) and ~2N DNA content, 5.4% of the population (green background). A small number of parasites with DNA content ⩾3N (0.1%, yellow background) are also present.

Although easily measured, Hoechst fluorescence cannot itself be used for measuring DNA in living cells because of its severe toxicity. The use of other cell permeant fluorescent DNA probes for sorting living *Toxoplasma* is also prevented by toxicity and artifactual fluorescence resulting from binding to RNA.

Although multiple-daughter formation occurs in every strain of *T. gondii* examined (Hu et al., 2002), it might be a stable property of a small subset of cells in the population, perhaps resulting from some permanent genetic alteration. Alternatively, a homogeneous population of wild-type cells might give rise to multiple daughters at low frequency because of unknown environmental influences. To distinguish between these possibilities, an unsynchronized population of *T. gondii* expressing histone 2b-YFP was sorted into three different groups based on DNA content (1N, 2N and 3N or greater), which were then inoculated into separate dishes of fresh host cells. After ~24 hours, the dishes were examined to determine the proportion of vacuoles containing parasites that had made multiple daughters in a previous cell cycle (i.e. parasites per vacuole $\neq 2^N$) or were in the process of making multiple daughters in the current cell cycle. The results (Table 1) do not support the notion that different subsets of cells within the population give rise to different numbers of daughters.

The histone 2b-YFP transgenic parasites enable direct observation of the nuclear events leading to multiple-daughter formation in living *T. gondii*; phenomena that previously could only be inferred from static images of fixed cells (Hu et al., 2002). Fig. 5 shows a sequence of images taken from extended time-lapse microscopic observation. In vacuole number 1 at time zero, nine parasites are synchronously beginning karyokinesis. Note that the presence of nine parasites in this vacuole implies that at least three of them are progeny of a multiple-daughter event (see Discussion for elaboration of this

Table 1. Frequency of multiple-daughter formation in *T. gondii*

	% Multiple-daughter formation (s.d.)	Number of vacuoles counted
Pre-sort	8.9 (1.5)	360
DNA content=1N	12.3 (2.5)	170
DNA content=2N	13.3 (2.5)	188
DNA content≥3N	13.2 (4.7)	53

Frequency was measured before and after sorting into sub-populations based on DNA content. The numbers refer to the fraction of vacuoles that either had formed multiple daughters in a previous cycle (number of parasites$\neq 2^N$), or were currently making multiple daughters.

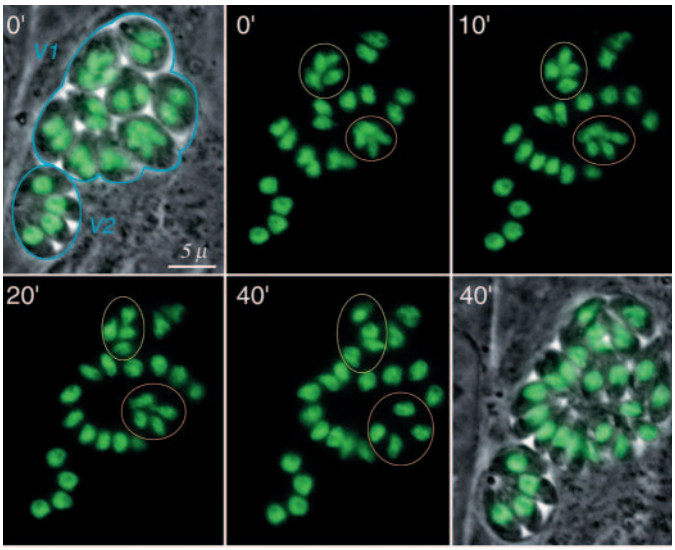

Fig. 5. Multiple-daughter formation in *T. gondii.* Phase and epifluorescence time-lapse images of a vacuole containing nine mitotic parasites (V1) and a second vacuole containing four interphase parasites (V2). Two parasites in the first vacuole are forming four daughters each (red and yellow ovals), leading eventually (40 minute time point) to a vacuole with 22 parasites.

point). Strict heritability of the multiple-daughter trait therefore seems unlikely, as only two of the nine are forming multiple daughters in the current cell cycle. This conclusion is consistent with the FACS sorting results in Table 1, and with images of fixed and stained wild-type parasites as shown in Fig. 6, which illustrates a seven-parasite vacuole with all seven producing twins, and a six-parasite vacuole in which four sets of twins and two sets of quadruplets are forming.

Is formation of multiple daughters a normal event or an aberration?

To determine if the progeny of multiple-daughter divisions are viable, and further analyze the pattern of inheritance of this trait, we directly cloned the individual daughter parasites. Parasites forming multiple daughters ('N-tuplets') were identified by epifluorescence and phase-contrast light microscopy of histone 2b-YFP transgenic parasites. These parasites were observed until completion of cell division and the newly formed daughter parasites were then individually aspirated into a micropipette under visual observation,

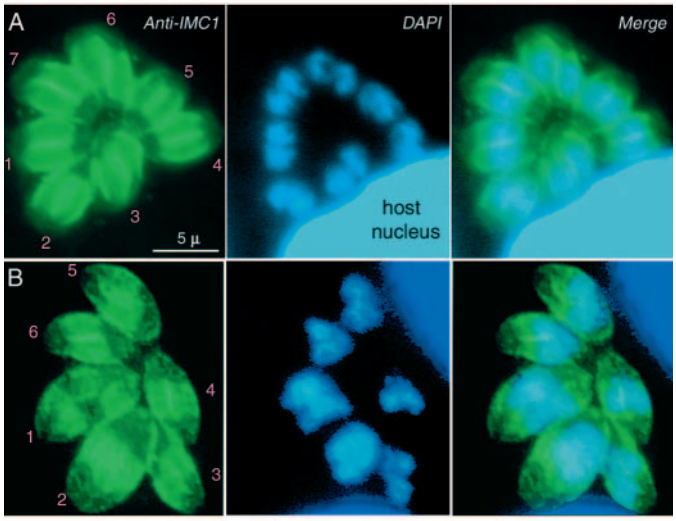

Fig. 6. Heritability of the multiple-daughter phenotype in *T. gondii*. Fluorescence images of parasites stained with anti-IMC1 antibody (green) and DAPI (blue). (A) A vacuole containing seven parasites (i.e. the product of at least one multiple-daughter event in previous cycles) all of which are forming two daughters in the current cycle. (B) A vacuole containing six parasites (i.e. at least one prior multiple-daughter event) two of which, numbered 1 and 2, are forming four daughters each. In both A and B, a portion of the host cell nucleus is visible in the DAPI image.

Table 2. Single-cell analysis of viability and heritability of the multiple-daughter forming trait

	% Viability (s.d.)	% Multiple-daughter formation (s.d.)
Pre-transfer	NA	6.5 (2.0)
Twins after transfer	65.5 (8.8)	18.2 (8.2)
N-tuplets after transfer	70.5 (6.9)	28.6 (9.9)

Parasites forming multiple daughters were identified in culture by observation of histone 2b-YFP fluorescence. Progeny from these multiple-daughter events ('N-tuplets') were aspirated into a micropipette, transferred to a new dish, and allowed to invade a new host cell. Vacuoles in the new host were monitored over time to assess their viability (i.e. successful proliferation in, and eventual lysis of the entire monolayer of host cells of the new dish) and formation of multiple daughters. Control parasites, progeny of a normal two-daughter formation event ('Twins'), were transferred and monitored in the same way. A total of 44 N-tuplets and 29 twins were transferred.

transferred to a fresh culture dish of uninfected fibroblasts and allowed to invade a new host cell. Subsequent development of parasites within each new vacuole was monitored for several rounds of cell division to assay viability and inheritance of the multiple-daughter phenotype. As a control, normal daughters (i.e. twins) were also transferred by micropipette and similarly monitored over time. Table 2 shows that there was no difference in viability between twins and N-tuplets (66% versus 70%; those counted as 'non-viable' include parasites that failed to invade or failed to proliferate after invasion). To our surprise however, the frequency of multiple-daughter events was increased three- to fourfold after transfer for both N-tuplets and control parasites (18% and 29% of vacuoles respectively, compared to 6.5% in the original population). Further experiments revealed that other mild environmental stimuli (e.g. decreased incubator CO_2 pressure) or laboratory

Table 3. Factors influencing the probability of multiple-daughter formation

	% Multiple-daughter formation (s.e.m.)	
	First infectious cycle	Second infectious cycle
1 hour	9.8 (1.3)	6.0 (1.0)
12 hours	18.0 (4.1)	11.0 (1.2)
24 hours	16.0 (3.1)	9.6 (1.1)

Histone2b-YFP parasites were harvested and used to inoculate fresh host cells according to three different protocols: (1) mechanically induced escape from host cells followed by immediate harvesting and inoculation (1 hour); (2) with a 12 hour delay after induced lysis of host cells before inoculation (12 hour); and (3) inoculation approximately 24 hours after spontaneous lysis of all the host cells in a dish (24 hour). The samples were subsequently monitored for formation of twins or N-tuplets over two generations. Between 800 and 2500 vacuoles were counted for each case.

manipulations (e.g. transfection with unrelated expression vectors) also increased the frequency of multiple-daughter formation. These effects are however temporary; the fraction of vacuoles containing multiple daughters returns to baseline after several cycles of invasion, proliferation, host cell lysis and re-invasion.

Table 3 shows the results of an experiment exploring the influence of a factor that may be a source of heterogeneity among individual parasites in a population: a variable delay in finding a new host cell. Parasites were collected by three protocols: (1) as extracellular parasites after spontaneous lysis of host cells and inoculated into a new dish after a delay of 24 hours; (2) by induced lysis followed by immediate inoculation into a new dish; and (3) with a 12 hour delay after induced lysis of host cells before inoculation. These three groups were allowed to infect fresh cells and subsequently monitored for formation of twins or N-tuplets. Spending 12-24 hours outside of a host cell seems to increase the frequency of subsequent N-tuplet formation approximately twofold, though the effect does not persist through the next cycle of invasion–lysis–re-invasion. On the other hand, by FACS sorting of these three extracellular populations (see Fig. 7), it was found that the cell-cycle stage of the parasite (1N versus 2N DNA content) at the time of invasion had no effect on the number of daughters formed in subsequent cell cycles (data not shown), in agreement with the previous experiment presented in Table 1.

Cell-cycle progression in extracellular parasites

Comparison of the flow cytometry profiles for the three parasite preparations used for the experiment in Table 3 revealed that the proportion of cells with DNA content greater than 1N decreased significantly (from ~18% to ~12%) while the parasites remained extracellular (Fig. 7). This was a surprising result, as it is generally believed that *T. gondii* does not progress through its cell cycle while it is outside a host cell. However, direct observation of histone IMC1-YFP parasites showed that cell-cycle progression continues extracellularly, as shown in Fig. 8. In this experiment, a suspension of extracellular parasites was monitored by time-lapse microscopy ~1 hour after they had lysed their host cells. During a 30 minute period, a few were observed to progress from late mitosis through completion of cytokinesis to form two daughter cells, as in the example shown (Fig. 8). The FACS analysis (Fig. 7) indicates that approximately one-third of the

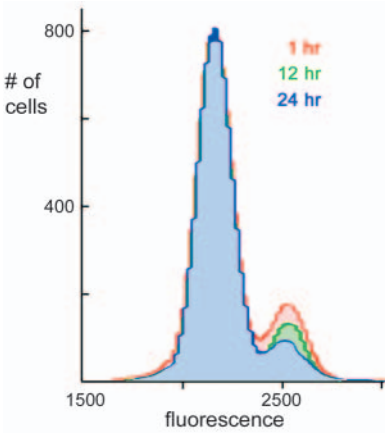

Fig. 7. Histograms of DNA content showing cell-cycle progression in extracellular parasites. Parasites were harvested and analyzed by flow cytometry according to three different protocols: (1) by mechanically inducing escape from host cells, washing to remove debris, staining with Hoechst 33342, followed by flow cytometry analysis (1 hr, red); (2) with a 12 hour delay after induced exit from host cells before washing, staining, and analysis (12 hr, green); and (3) harvested, washed, Hoechst stained and analyzed approximately 24 hours after spontaneous lysis of an entire culture dish of host cells (24 hr, blue). The graph shows superimposed histograms of fluorescence intensity (proportional to DNA content) of approximately 1×10^5 single cells from each of the three preparations, in arbitrary units on a logarithmic scale.

parasites with a DNA content of ~2N at the time of host cell lysis will go on to complete cell division if they remain extracellular for 24 hours, but DNA synthesis in the 1N extracellular population is retarded, thus decreasing the average ploidy of the population.

Discussion

Chromatin distribution visualized in living *T. gondii*

Time-lapse imaging of living cells has shown that histone 2b-YFP is an extremely useful marker for visualizing *T. gondii* nuclei in interphase and in mitosis. Detailed comparison with DNA distribution (visualized by DAPI staining) in fixed cells confirmed the fidelity of H2b-YFP as a marker for chromatin, and allowed localization of the 35 kb apicoplast genome, even when its distance from nuclear DNA decreased to less than the resolution limit of fluorescence microscopy with DAPI alone. The time-lapse images reported here show a sequence of morphological changes in the nucleus during karyokinesis that match the sequence inferred previously by examination of fixed, DAPI-stained cells. Furthermore, the flow cytometry measurements reported in Fig. 4 demonstrate that histone 2b-YFP fluorescence is a quantitatively reliable measure of DNA content in living cells, setting the stage for FACS-based separation of *T. gondii* cells according to ploidy or phase of the cell cycle. This line of stable transgenic parasites should be particularly valuable for future experiments analyzing structural changes of chromatin during cell division, for defining cell-cycle stages in analysis of differential gene expression, for establishing synchronized populations and for investigation of cell-cycle events in other life stages (e.g. encysted bradyzoites, sexual stages, sporozoites).

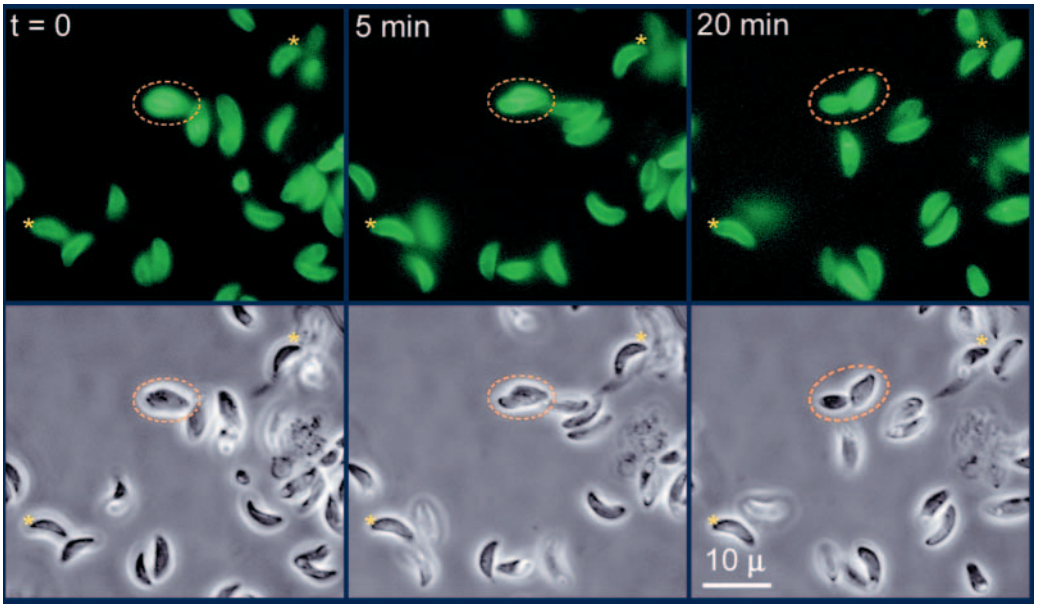

Fig. 8. Cell division in extracellular parasites. Parasites expressing IMC1-YFP were harvested after lysing out of host cells and suspended in culture medium. A droplet of parasite suspension was put on a coverslip, and a cell in mitosis (orange oval) was identified under epifluorescence observation. Other parasites in suspension moved in and out of the field of view during the observation period. For reference, two additional stationary parasites (interphase) are indicated by the yellow asterisks. Images were recorded every 5 minutes for 30 minutes, during which time the parasite in the orange oval progressed from mid-mitosis through completion of cytokinesis to form two daughter cells.

The multiple-daughter phenotype

Wild-type *T. gondii* tachyzoites do not follow a fixed pathway during cell division (Hu et al., 2002). In the predominant pattern, a mature parasite is partitioned into two daughter cells in the process referred to as endodyogeny. However, a variable but significant fraction of parasites follow alternate pathways that lead to formation of three, four, five or more daughters in a single cycle (up to eight have been observed). In the present paper these observations have been confirmed and extended to prove that the triplets, quadruplets, etc., produced by different multiple-daughter pathways are indistinguishable from and as viable as twins, capable of indefinite proliferation by repeated cycles of host cell invasion, multiplication, host cell lysis, and re-invasion. N-tuplets do sometimes bud out slightly later than twins in the same vacuole do, but not always, and the prolongation is not much greater than the variance of division times in an all-twin vacuole. Apparently the time required for division is not solely determined by metabolic demands.

Cell division producing multiple daughters at once is common, particularly during early development of many species (e.g. the 14th cell cycle of *Drosophila* embryogenesis when the syncytial blastoderm is cellularized) (Gilbert, 2003). In the Apicomplexan phylum, formation of multiple daughters at once during some part of the life cycle is routine for many species (Bannister et al., 2000; Chobotar et al., 1975; Dubey, 1998; Dubremetz and Elsner, 1979; Speer, 1983). What is novel in the current work is the demonstration that the number of daughters produced by *T. gondii* at a single stage of its life cycle is not fixed, but varies widely between clonally identical parasites, and between successive generations of the same clone. This has no measurable effect on the viability of the progeny (the background level of non-viable parasites among twins in laboratory cultures is ~1%) (Morrissette and Sibley, 2002).

Inheritance of cell division pathways

In principle these alternate pathways of cell division might result from either spontaneously arising somatic mutations or an infrequently displayed capability of the wild-type genome subject to control by environmental factors. Direct cloning of individual parasites arising as twins or as N-tuplets showed no evidence for inheritance of a fixed cell division pathway. Instead, the progeny from any single pattern proved capable of generating all other patterns in subsequent cell cycles. These observations on single living cells over the course of many cell cycles reinforce the conclusions tentatively drawn from images of many fixed cells at single time points. For example, the seven parasites in the vacuole shown in Fig. 6A must have arisen by one of four routes: (1) one cell producing seven daughters; (2&3) one cell produced twins, one of which produced either twins or triplets while the other produced quintuplets or quadruplets; (4) one cell produced triplets, which then produced two sets of twins and one set of triplets. Therefore at least three and perhaps all seven of the parasites in this vacuole were produced by a multiple-daughter event in the previous cell cycle, but all seven are producing twins. Similarly, there are four possible routes to the six parasites in the vacuole of Fig. 6B. The six parasites are either all twins (arising from triplets in the previous generation), all triplets, all sextuplets or a pair of twins and four quadruplets. Thus four or all six of the parasites are the direct product of a multiple-daughter event, or if not, then all six are derived from triplets in the previous generation, but two of the six are producing quadruplets in the current cycle. Previously it had been possible to argue that ‘stillbirths’ and subsequent ejection of non-viable parasites from the vacuole accounted for these discrepancies, though this had never been observed. An alternative explanation invoked asynchronous division to explain odd numbers of parasites in a vacuole,

though this too is almost never observed in vacuoles containing fewer than eight parasites. Both alternatives are excluded by the experiments reported here. *T. gondii* has a variable pattern of cell division. The variability is inherited and is the normal phenotype.

Factors that influence the choice of cell division pathways

In the first few rounds of cell division after invading a new host cell, the cell cycles of all parasites in a vacuole (i.e. the clonal progeny of a single invading parasite) are well synchronized. However, in later cycles the synchrony is lost, so that at the time of host cell lysis and release of vacuole contents (typically more than 256 parasites per vacuole) parasites at all stages of the cell cycle are present. After release, progress through the cell cycle is slowed but not completely blocked, at least for cells in mid- to late mitosis (Figs 7 and 8). All stages are competent to invade and establish a new vacuole, within which normal cell-cycle progression is resumed. Thus extracellular *T. gondii* is typically a heterogeneous mixture of parasites at different stages of the cell cycle. It would be reasonable to speculate that this heterogeneity in the population of invading parasites (i.e. heterogeneity in cell-cycle phase at the moment of invasion and vacuole formation) is somehow linked to the heterogeneity in number of daughters produced in a subsequent cell cycle. The data included in Tables 2 and 3 make this hypothesis both unlikely and unnecessary. Apparently the signals that direct *T. gondii* down one or the other of its cell division pathways are quite weak, and so easily perturbed by environmental 'noise' that heterogeneity is continuously maintained. Parasites derived from the same parental strain but transfected with different expression vector plasmids also often show differences in frequency of multiple-daughter formation. We conclude that the proportion of parasites producing multiple daughters instead of twins can be dramatically altered by diverse but quite mild experimental manipulations. None of these manipulations has a discernible effect on viability, raising the currently unanswerable question of what, if any, advantage accrues to *T. gondii* by maintaining this remarkable versatility in cell division pathways.

Compared to the stereotyped events of cell division in a typical eukaryote, the coexistence of multiple cell division pathways and seemingly inconsequential choice among them in *T. gondii* is astonishing. How is it possible to orchestrate all of the complex movements, organelle duplications and partitionings that comprise cell division when the number of progeny is not only variable, but seems to be indeterminate until well after the process is underway (Hu et al., 2002)? There appears to be no mechanism for counting progeny within the control sequence that regulates cell-cycle progression. Any checkpoints that regulate progression through sequential phases of the cell cycle in *T. gondii* are apparently able to monitor completion of each phase regardless of how many new copies are being produced (i.e. the checkpoint mechanisms may sense residual incomplete processes, rather than counting the number of completed events).

Cell-cycle checkpoints in *T. gondii*

Several main checkpoints have been identified in mammalian cells and yeast. The G1/S checkpoint monitors the environment and cell growth to decide whether to commit to DNA replication. The S/G2 checkpoint monitors the completion of DNA replication. The G2/M checkpoint monitors the amendment of DNA damage. The mitotic spindle checkpoint (M/G1) monitors the correct assembly of the mitotic spindle to ensure the proper segregation of chromosomes. Transitions from one phase to the next are catalyzed by different cyclin/cyclin-dependent kinase complexes (Beach et al., 1982; Culotti and Hartwell, 1971; Evans et al., 1983; Hartwell, 1971a; Hartwell, 1971b; Hartwell et al., 1974; Hartwell et al., 1970; Hoyt and Botstein, 1990; Masui and Markert, 1971; Murray and Li, 1991; Weinert et al., 1994).

A *T. gondii* cyclin gene has recently been identified using a yeast two-hybrid interactive screen and this gene can complement the function of CLN2 (a G1/S phase cyclin) in *Saccharomyces cerevisiae* (Kvaal et al., 2002). Parasite replication is blocked by p38 MAPK (mitogen-activated protein kinase) inhibitors (Wei et al., 2002) and members of the ERK family of MAP kinases have also been characterized in *T. gondii* (Roisin et al., 2000). Thus multiple components of the mammalian cell-cycle regulatory network are present, indicating that some cycle checkpoints are likely to be conserved in *T. gondii*. However, it is also clear that there must be differences (Radke et al., 2001). Some of the checkpoints identified in other eukaryotes do not function in exactly the same way in *T. gondii*. Blocking DNA synthesis or strand separation with DNA replication inhibitors does not completely prevent initiation of daughter scaffold assembly (Shaw et al., 2001), whereas these inhibitors do block or greatly delay cytokinesis in other eukaryotes. Furthermore, when daughter scaffold formation is blocked by microtubule depolymerizing drugs, parasites initiate multiple rounds of DNA replication (Morrissette and Sibley, 2002; Stokkermans et al., 1996). Therefore, although daughter scaffold formation and DNA replication normally appear to be coordinated, the coupling between them is easily disengaged. In contrast, organelle duplication appears to be rather tightly coordinated with scaffold formation (Hu et al., 2002; Shaw et al., 2001).

Presumably the relatively tenuous engagement between DNA replication and daughter scaffold assembly, as revealed by inhibitory drugs, underlies the ability of *T. gondii* to produce a variable number of progeny in a single cell cycle. In the absence of a checkpoint or strong 'licensing' mechanism (Lygerou and Nurse, 2000) to block initiation of a second round of DNA synthesis, a random delay in initiation of new apical complexes (the first step in daughter scaffold assembly) would occasionally be long enough to allow local re-initiation of DNA replication on one or both of the haploid genomes already synthesized in the first round. This scenario would account for the most common pattern of nuclear division in parasites forming three daughters, as reported previously (Hu et al., 2002). Each lobe of the nucleus with a segregated haploid genome and associated spindle pole normally, but not always, serves as the site of initiation of a new apical complex. However, a *T. gondii* checkpoint probably does come into play at this point, as continuation of daughter scaffold assembly seems to be globally blocked on all apical complexes until DNA replication is completed on every nuclear lobe (Hu et al., 2002). The block is not absolute: scaffold assembly eventually escapes after prolonged treatment with DNA replication inhibitors (Shaw et al., 2001).

In summary, the available evidence suggests that *T. gondii* lacks or has a comparatively weak checkpoint/licensing mechanism corresponding to the controls regulating initiation of DNA synthesis in mammalian cells and yeast, whereas the checkpoint corresponding to S/G2 appears to be more robust. The molecular identities of proteins involved in all of these putative checkpoint mechanisms remain to be established.

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