

Assessing the *Toxoplasma* Tachyzoite Cell Cycle Phases Using Fluorescent Ubiquitination-Based Cell Cycle Indicator

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Abstract

Toxoplasma gondii is an apicomplexan parasite that infects a wide variety of eukaryotic hosts and causes toxoplasmosis. The cell cycle of *T. gondii* exhibits a distinct architecture and regulation that differ significantly from those observed in well-studied eukaryotic models. To better understand the tachyzoite cell cycle, we developed a fluorescent ubiquitination-based cell cycle indicator (FUCCI) system that enables real-time visualization and quantitative assessment of the different cell cycle phases via immunofluorescence microscopy. Quantitative immunofluorescence and live-cell imaging of the *ToxoFUCCI*^S probe with specific cell cycle markers revealed substantial overlap between cell cycle phases S, G₂, mitosis, and cytokinesis, further confirming the intricacy of the apicomplexan cell cycle.

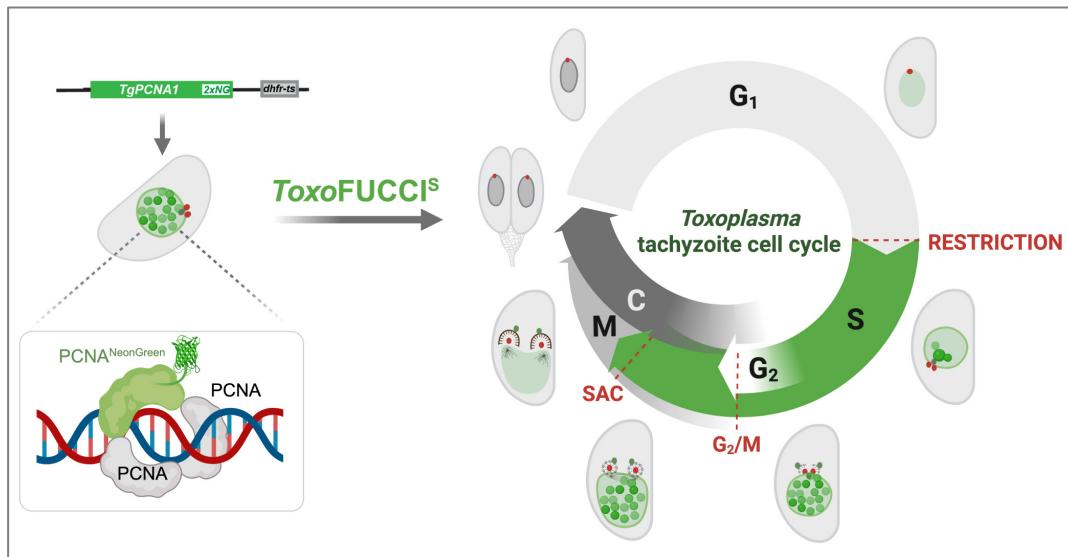
Key features

- This protocol describes the development of the transgenic lines capable of detecting individual cell cycle phases and processes of the *Toxoplasma* tachyzoite cell cycle.
- Quantitative immunofluorescence analysis and real-time microscopy enable the measurement of each cell cycle phase.
- The *ToxoFUCCI*^S probe helps to gain new insights into the highly flexible, overlapping nature of cell cycle organization in apicomplexan parasites.

Keywords: Apicomplexa, *Toxoplasma gondii*, Cell cycle, FUCCI, PCNA1

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Graphical overview



Background

Fluorescent ubiquitination-based cell cycle indicator (FUCCI) is a powerful technology that enables real-time visualization of the cell cycle [1–6]. FUCCI technology exploits the phase-specific proteolysis of critical cell cycle regulators. Sequential activation of major E3 ligases, APC/C and SCF^{Skp2}, orchestrates the precise regulation of DNA replication factors. This temporal machinery controls the production of Geminin accumulation during S and G₂ phases to prevent DNA re-replication. On the contrary, Cdt1 (chromatin licensing and DNA replication factor 1) is expressed in M and G₁ phases and assists in licensing the origins of replication [7–9]. The original FUCCI probes invented by Sakaue-Sawano et al. in 2008 used fluorescent protein fusions of Geminin and Cdt1 and accurately detected cell cycle progression in mammalian cells in real time [2]. This technology has revolutionized cell cycle analysis. It provided a new dimension for studying cellular dynamics, enabled the assessment of drug effects on cell replication, visually distinguished proliferating and quiescent cells, and allowed the development of various stem cell populations [3,5]. Due to the lack or altered expression of the conventional FUCCI markers, the FUCCI adaptation to the unicellular apicomplexan parasite *Toxoplasma gondii* required technical adjustments [10,11]. To satisfy FUCCI marker requirements, a candidate protein should be abundant and localized in distinguishable cellular compartments such as the nucleus and cytoplasm, and its cell cycle expression should be controlled. According to these criteria, a DNA replication factor, TgPCNA1 (proliferating cellular nuclear antigen 1), was identified as a suitable FUCCI marker and successfully used to determine the organization of the *T. gondii* tachyzoite cell cycle [10]. While the *ToxoFUCCI^S* cell line alone is sufficient to detect major cell cycle phases such as G₁, S, and mitosis concurrent with cytokinesis (budding), the combination of *ToxoFUCCI^S* with quantitative immunofluorescence microscopy analysis (qIFA) using a set of cell cycle markers facilitates the identification of the intervals within the composite cell cycle phase [10]. Previous studies validated cell cycle-dependent expression and localization of the suggested markers, including basal complex component TgBCC0 [12], MORN (membrane occupation and recognition nexus) repeat-containing protein TgMORN1 [11,13–15], and inner membrane complex protein TgIMC1 [16,17].

Materials and reagents

Biological materials

1. Human foreskin fibroblasts (HFF) (ATCC SCRC-1041)
2. *Toxoplasma gondii* RH TIR1 strain (genotype: RHΔhxgprtΔku80; *TUB1:TIR1-3xFLAG, SAG1:CAT*) [18]
3. *Toxoplasma gondii* RH Tati Δku80 strain [19]

Reagents

1. Dulbecco's modified Eagle's medium (DMEM) (Millipore Sigma, catalog number: D6429)
2. Fetal bovine serum (FBS) (Millipore Sigma, catalog number: F0926)
3. Antibiotic antimycotic solution (PSA) (Millipore Sigma, catalog number: A5955)
4. Hank's balanced salt solution (Millipore Sigma, catalog number: H9394)
5. Mycoplasma Detection PCR kit (MP biomedicals, catalog number: 93050201)
6. Dimethyl sulfoxide (DMSO) (Fisher Scientific, catalog number: BP231)
7. 0.5% Trypsin EDTA solution (Fisher Scientific, catalog number: 15400054)
8. Adenosine triphosphate (ATP) (New England Biolabs, catalog number: P0756S)
9. Reduced L-glutathione (RPI, catalog number: 70188)
10. 6× gel loading dye (New England Biolabs, catalog number: B7025)
11. dNTPs (New England Biolabs, catalog number: N0447S)
12. Ethanol (Fisher Scientific, catalog number: BP28184)
13. DNA oligomers (Integrated DNA technologies, IDT, 25 nmole)
14. DNA Extraction kit (Monarch Genomic DNA purification Kit, NEB, catalog number: T3010S)
15. Miniprep kit (Qiagen, catalog number: 27106)
16. PCR Cleanup kit (Macherey Nagel, catalog number: 740609.50)
17. Phusion DNA Polymerase (New England Biolabs, catalog number: M0535S)
18. 10× Tris-Borate-EDTA (TBE) buffer (Bio-Rad, catalog number: 1610770)
19. Orange G powder (Fisher Scientific, catalog number: AC416550100)
20. 1 kb+ DNA ladder (New England Biolabs, catalog number: N3232L)
21. Gibson Assembly master mix (New England Biolabs, catalog number: E2611S)
22. *E. coli* NEB5 α chemically competent cells with SOC media (New England Biolabs, catalog number: C2987H)
23. Agarose (Millipore Sigma, catalog number: A9539-500)
24. LB agar (Fisher Scientific, catalog number: BP1425-500)
25. LB broth (Fisher Scientific, catalog number: BP1426-500)
26. Ampicillin sodium salt (Fisher Scientific, catalog number: BP-1760-25)
27. 7.5 M ammonium acetate solution (Millipore Sigma, catalog number: A2706)
28. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Millipore Sigma, catalog number: MBD0015)
29. Rabbit anti-myc antibodies (clone 71D10) (Cell Signalling, catalog number: 2278)
30. Rabbit anti-NeonGreen antibodies (Cell Signaling Technology, catalog number: 53061S)
31. Mouse anti-Centrin antibodies (clone 20H5) (Millipore Sigma, catalog number: 04-1624)
32. Rabbit anti-TgMORN1 serum (provided by Dr. Marc-Jan Gubbels, Boston College, MA)
33. Rabbit anti-TgIMC1 serum (provided by Dr. Gary Ward, University of Vermont, VT)
34. Sodium azide (NaN₃) (Fisher Scientific, catalog number: S227I-500)
35. Proteinase K (Fisher Scientific, catalog number: EO0491)
36. Anti-rabbit, cross-adsorbed secondary antibody (Invitrogen, catalog number: A11012)
37. Anti-mouse, cross-adsorbed secondary antibody (Invitrogen, catalog number: A11032)
38. Potassium chloride (KCl) (Fisher Scientific, catalog number: P217-500)
39. Monopotassium phosphate (KH₂PO₄) (Fisher Scientific, catalog number: BP362-500)
40. Sodium phosphate dibasic (Na₂HPO₄) (Fisher Scientific, catalog number: S374-500)
41. Sodium chloride (NaCl) (Fisher Scientific, catalog number: BP358)
42. Calcium chloride (CaCl₂) (ThermoFisher Scientific, catalog number: 10035)
43. Magnesium chloride (MgCl₂) (Fisher Scientific, catalog number: 7791-18-6)
44. HEPES (RPI, catalog number: 7365)
45. Tween-20 (Millipore Sigma, catalog number: P1379)
46. Pyrimethamine (Millipore Sigma, catalog number: SML3579)
47. Triton X-100 (Fisher Scientific, catalog number: AAA16046AE)
48. PacI endonuclease (New England Biolabs, catalog number: R0547S)
49. NsiI endonuclease (New England Biolabs, catalog number: R0127S)
50. EcoRV endonuclease (New England Biolabs, catalog number: R3195)
51. 4% paraformaldehyde solution (PFA) (Fisher Scientific, catalog number: AAJ19943K2)
52. 10 mg/mL ethidium bromide solution (Millipore Sigma, catalog number: E1510)

Laboratory supplies

1. Microcentrifuge tubes (1.7 mL) (Fisher Scientific, catalog number: 14222-171)
2. PCR tubes (Millipore Sigma, catalog number: AXYPOR02D)
3. T-25 and T-75 culture flasks (Fisher Scientific, catalog numbers: FB012936, FB012938)
4. 96-well, 24-well, and 6-well plates (Fisher Scientific, catalog numbers: FB012931, FB012929, FB 012927)
5. 15 mL conical centrifuge tubes (VWR, catalog number: 21008-212)
6. 50 mL conical centrifuge tubes (Fisher Scientific, catalog number: 12-565-270)
7. Snap cap tubes (Fisher Scientific, catalog number: 07-000-212)
8. 10, 200, 1,000 μ L pipette tips (USA Scientific, catalog numbers: 1120-3710, 1120-8710, 1122-1730)
9. 5 mL, 10 mL, 25 mL serological pipettes (Fisher Scientific, catalog number: 13-678-11)
10. 10 mL Luer lock syringes (Fisher Scientific, catalog number: 14-955-459)
11. Blunt end needles 23G (Grainger, catalog number: 5FVK7)
12. Stericup filter units 500 and 1,000 mL (Fisher Scientific, catalog numbers: S2GPU05RE, S2GPU10RE)
13. 3.0 μ m pore size filter membrane (Millipore Sigma, catalog number: TSTP02500)
14. Cell scrapers (Fisher Scientific, catalog number: 07-000-553)
15. 0.22 μ m sterile syringe filters (Fisher Scientific, catalog number: 50-209-3153)
16. Glass pipettes (Fisher Scientific, catalog number: 13-678-20C)
17. Nitrocellulose/filter sandwich (Bio-Rad, catalog number: 1620213)
18. Petri dishes (VWR, catalog number: 25384-342)
19. 2 mm electroporator cuvette (Fisher Scientific, catalog number: FB102)
20. MyFugeTM Minispin (Alkali Scientific, catalog number: 50-233-6060)
21. 15 mm, 22 mm glass coverslips (Fisher Scientific, catalog numbers: 501215160, 12541016)

Solutions

1. Tachyzoite medium (see Recipes)
2. Pyrimethamine selection media (see Recipes)
3. 10 \times phosphate-buffered saline (PBS) (see Recipes)
4. Cytomix electroporation buffer pH 7.6 [20] (see Recipes)
5. 3% IFA block buffer (see Recipes)
6. IFA permeabilization buffer (see Recipes)
7. 0.8% agarose gel (see Recipes)

Recipes

1. Tachyzoite medium

DMEM supplemented with 3% FBS and 1% PSA. Store at 4 °C.

2. Pyrimethamine selection media

Tachyzoite medium (Recipe 1) with 1 μ M pyrimethamine. Store at 4 °C.

3. 10 \times PBS

1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, and 20 mM KH₂PO₄ in a final volume of 1 L. Adjust pH to 7.4. Store at room temperature.

4. Cytomix electroporation buffer pH 7.6 [20]

10 mM KPO₄ (pH 7.6), 120 mM KCl, 0.15 mM CaCl₂, 5 mM MgCl₂, 25 mM HEPES, 2 mM EDTA. Adjust the solution to the final volume of 1 L. Filter-sterilize and store at 4 °C.

5. 3% IFA block buffer

500 mL of 1 \times PBS and 15 mL of FBS. Run through a Stericup filter and add 500 μ L of 2 mM NaN₃. Store at 4 °C.

6. IFA permeabilization buffer

900 mL of nanopure water, 100 mL of 10× PBS, 1 mL of 2 mM NaN₃, and 5 mL Triton X-100.

7. 0.8% agarose gel

0.8 g of agarose in 100 mL of 1× TBE buffer. Microwave until clear, cool down, and add 5 µL of 10 mg/mL ethidium bromide solution.

Equipment

1. Autoclave (Steris, model: Amsco Lab 250)
2. Biological safety cabinet (Labconco, model: 302411000)
3. Benchtop centrifuge with 15 and 50 mL conical vial holders (Eppendorf, model: 5910R)
4. Water bath at 37 °C (Shel Lab, model: SWB15)
5. Cell culture incubator at 34 and 37 °C with 5% CO₂ (Eppendorf, catalog number: 6731010015)
6. Incubator at 37 °C (Fisher Scientific, model number: FFCO300DABB)
7. Bacterial shaker at 37 °C (New Brunswick Scientific, model: I26R)
8. Sterile Pyrex borosilicate glassware for solutions and reagents (test tubes, beakers, storage vials)
9. Amaxa Nucleofector II electroporator (Lonza, catalog number: 40700053)
10. Hemacytometer (Fisher Scientific, catalog number: 2671154)
11. Inverted-phase contrast microscope (Nikon, model: TMS-F and Olympus, model: CKX53SF)
12. Zeiss Axio Observer inverted microscope equipped with filters for multichannel fluorescence imaging (green, DAPI, and red)
13. Gel imaging system (Foto dyne Incorporated, model: 1-1430)
14. Microcentrifuge (Eppendorf, catalog number: 2231001179)
15. Microwave (Black+Decker, catalog number: 810004819726)
16. Pipettes and carousel (Fisher, catalog numbers: 13684251, 3116000015)
17. Motorized pipette controller (Eppendorf, catalog number: 4430000018)
18. Protein transfer apparatus (Bio-Rad, model: Trans-Blot Turbo System)
19. SDS-PAGE electrophoresis apparatus (Bio-Rad, model: Mini-Protean Tetra Cell)
20. Thermocycler (ThermoFisher Scientific, catalog number: 4484073)
21. Refrigerator (LG, model: LRTLS2403S)
22. Freezers -20 and -80 °C (Fisher Scientific, catalog number: FBH07CFSA and Eppendorf, model: CryoCube F740hi)
23. Liquid nitrogen storage (Thermo Scientific, catalog number: 7400)
24. Nanodrop spectrophotometer (Thermo Scientific, catalog number: 13400519)
25. Vacuum aspirator (Barnant Company, catalog number: 400-3910)
26. Heat block (Fisher Scientific, catalog number: 88-860-023)

Software and datasets

1. Google Chrome (Google), Safari, or any other web browser
2. SnapGene (Version 8.0)
3. Zen 3.2 (Zeiss)
4. NEB ligation calculator (New England Biolabs) (nebiocalculator.neb.com)

Procedure

A. Creating *ToxoFUCCI*^S strain

1. Retrieving gene information from the ToxoDB database (VEuPathDB)

- a. Genomic sequences and coding regions of *T. gondii* genes of interest were obtained from the ToxoDB database (www.toxodb.org).
- b. Find the nucleotide sequence of the coding 5' and 3' UTR regions of *TgPCNA1* on the TGME49_247460 gene page.
- c. From the drop-down menu, located on the left, select “Sequences” to access and download the predicted genomic sequence.

2. Primer design and PCR amplification

- a. Use a forward and reverse primer with ligation-independent cloning (LIC) overhangs. The PCNA1 forward primer includes a 5' LIC overhang for vector annealing, fused to the *TgPCNA1* 5'UTR sequence. The 5'end of the reverse primer includes a LIC overhang for the vector annealing, followed by the last 27 nucleotides of *TgPCNA1* ORF excluding the stop codon.

PCNA1_For: tacttccaaatccaatttaatgcacgaaatccggcggcacgc

PCNA1_Rev: tcctccacttcaattttagccatccatcatagagtcgtccatgcc

- b. Purchase primers from Integrated DNA Technologies (www.idtdna.com).

- c. Use a DNA Extraction kit to isolate genomic DNA from the parental *T. gondii* RH TIR1 strain [21]. Culture and collect parasites as described in section D below.

- d. Set up a PCR reaction consisting of 100 ng of genomic DNA, 2 μ L of 10 mM dNTPs, 5 μ L of 10 μ M PCNA1_For primer, 5 μ L of 10 μ M PCNA1_Rev primer, 5 μ L of DMSO, 20 μ L of 5 \times Phusion buffer, and 1 μ L of Phusion DNA polymerase, and adjust the volume to 100 μ L with deionized water.

- e. PCR thermocycling conditions: one cycle of denaturation at 98 °C for 1 min; 32 cycles of denaturation at 98 °C for 10 s, annealing at 62 °C for 15 s, and extension at 72 °C for 50 s; one cycle of extension at 72 °C for 5 min.

- f. Confirm amplification of the 3.1 kb DNA fragment by mixing 5 μ L of PCR mixture with 2 μ L of Orange G dye and resolving the PCR product on a 0.8% agarose gel with a 1 kb+ DNA ladder. Purify the amplified DNA fragment using a PCR Cleanup kit.

- g. Use a nanodrop to measure the DNA concentration of the purified PCR product.

3. Gibson assembly cloning and *E.coli* plasmid transformation

- a. Vector preparation: Linearize 5 μ g of pLIC-2 \times NeonGreen-DHFR [10] vector DNA with endonuclease PacI following the manufacturer's recommendation. Confirm the vector linearization by agarose gel electrophoresis, purify linear DNA using a DNA Extraction kit, and measure DNA concentration using a nanodrop spectrophotometer.

- b. Use the NEB ligation calculator to determine the DNA amount required for a 1:3 (vector: insert) molar ratio.

Optional: Use SnapGene software to simulate Gibson Assembly and generate the pLIC-TgPCNA1-2 \times NeonGreen-DHFR plasmid map.

- c. Gibson assembly reaction: mix on ice 100 ng of PacI-treated pLIC-2xNeonGreen-DHFR plasmid DNA, 3 \times molar excess of purified TgPCNA1 DNA fragment, and 5 μ L of 2 \times NEBuilder HiFi DNA assembly master mix. Adjust the volume to 10 μ L with nuclease-free water. Incubate the reaction in a thermocycler at 50 °C for 15 min.

- d. Mix 50 μ L of chemically competent *E.coli* NEB5 α bacteria with 2 μ L of the annealed Gibson assembly mixture and incubate on ice for 30 min. Heat-shock bacteria for 30 s in the heat block set to 42 °C. Chill the reaction on ice for 2 min. Add 950 μ L of SOC media and recover transformed cells in a bacterial shaker at 250 rpm for 60 min at 37 °C. Spread 100 μ L of the transformed *E.coli* NEB5 α cells onto LB agar plates with 100 μ g/mL ampicillin. Incubate the plates overnight at 37 °C. Set up overnight liquid cultures of 6–8 individual colonies in 5 mL of LB broth with 100 μ g/mL ampicillin. Grow cultures in the bacterial shaker for 16–18 h at 250 rpm at 37 °C.

- e. Pellet *E.coli* NEB5 α cells expressing pLIC-TgPCNA1-2xNeonGreen-DHFR plasmid in a tabletop centrifuge at 6,800 \times g for 3 min. Extract plasmid DNA using QIAGEN Prep Spin Miniprep kit. Measure DNA concentration by spectrophotometry. The expected yield range is 300–800 ng/ μ L.

- f. Validate the correct plasmid assembly by Sanger sequencing using the following primers:

pLIC_For: gtggataaccgttattaccgccttg

NeonGreen_Rev: accatcggtgggttaccagttac

- g. Linearize 50 μ g of pLIC-TgPCNA1-2xNeonGreen-DHFR plasmid with endonuclease EcoRV according to the manufacturer's recommendation. Confirm plasmid linearization by agarose gel electrophoresis. Precipitate DNA in a 70% ethanol solution with 0.75 M ammonium acetate. Store at -20 °C until further use.

4. Parasite transfection, cloning, and selection of transgenic clones

- a. Culturing parasites: Infect a T-25 flask of confluent HFF cells with 10 6 freshly egressing RH TIR1 parasites. Grow parasites until they lyse 75%–80% of the host monolayer for 36–48 h at 37 °C. Release parasites from the host cells by passing the scraped monolayer through a blunt-end 23G needle 3–4 times. Separate parasites from the host cell remnants

using a 3.0 μm pore-size filter and pellet them in a 1.7 mL conical tube using a tabletop centrifuge at $1,800 \times g$ for 15 min at 4°C . Aspirate the supernatant and resuspend the parasite in 300 μL of Cytomix buffer supplemented with 0.2 M ATP and 0.5 M reduced L-glutathione (complete Cytomix).

b. Pellet 50 μg of endonuclease-digested and ethanol-precipitated pLIC-TgPCNA1 2xNeonGreen-DHFR in the microcentrifuge at $15,000 \times g$ for 10 min. Aspirate and air dry the DNA pellet. Rehydrate the DNA pellet in 100 μL of parasite suspension in complete Cytomix and transfer the mixture into a 2 mm electroporation cuvette.

c. Electroporate parasites with a single 1.7–1.8 kV/176–200 μs pulse using Amaxa Nucleofector II set on program T016. Immediately transfer parasites into a tachyzoite medium in a T-75 flask with a confluent HFF monolayer and recover them for 24 h at 37°C with 5% CO_2 .

d. Replace growth medium with pyrimethamine selection medium. Pass cultures 2–3 times in selection media until a stable drug-resistant population is established.

e. Check the plasmid integration efficiency by live fluorescence microscopy of the polyclonal population. Infect HFF monolayers on coverslips with 10^3 tachyzoites under selection. After 24 h of growth in the tachyzoite medium, fix slides with 4% PFA (5 min, room temperature), incubate in IFA permeabilization buffer (10 min, room temperature), followed by IFA blocking buffer (30 min, room temperature), and stain with DAPI (nuclear dye). Visualize parasites expressing TgPCNA1^{NG} in a Zeiss Axio Observer inverted microscope using ET-GFP block.

f. Clone parasites by limiting dilution. Scrape HFF monolayers infected with a pyrimethamine-resistant population of tachyzoites, pass parasites through a G23 blunt-end needle, and separate from ruptured host cells using a 3 mm filter. Count parasites using a haemocytometer according to the manufacturer's recommendations. Dilute the parasite suspension in the tachyzoite medium to a final concentration of 250/mL. Using an 8-channel pipette, perform serial 1:1 parasite dilutions in a 96-well plate to achieve a final concentration of 1–2 parasites per well. Grow cloned cultures at 37°C with 5% CO_2 for 10 days to allow plaque development.

g. Identify wells with individual plaques under an inverted light microscope (Nikon TMS-F). Expand clonal parasites by growing in 24-well and T-25 plates.

h. Confirm live TgPCNA1^{NG} expression. Grow clonal parasites in the tachyzoite medium on glass coverslips for 18–20 h at 37°C with 5% CO_2 . Fix slides with 4% PFA (5 min, room temperature), incubate in IFA permeabilization buffer (10 min, room temperature), and then incubate in the IFA blocking buffer (30 min, room temperature) and stain with DAPI (nuclear dye). Visualize parasites expressing TgPCNA1^{NG} in a Zeiss Axio Observer inverted microscope using ET-GFP block.

i. Confirm the plasmid integration in individual clones by PCR. Prepare a crude genomic DNA sample of individual clones. Pellet parental and transgenic clonal parasites from 100 μL of parasite suspension in the growth medium in 200 mL PCR tubes using a Minispin centrifuge for 5 min at room temperature. Remove the supernatant and resuspend the parasites in 42 μL of PBS with 0.5 μL of Proteinase K solution, 2.5 μL of DMSO, and 5 μL of 5 \times HF-Phusion buffer. Incubate the reaction in the thermocycler at 37°C for 1 h, followed by 50 $^\circ\text{C}$ for 2 h and 95 $^\circ\text{C}$ for 15 min.

j. Prepare three diagnostic PCR primers. A forward Primer 1 is nested in the *TgPCNA1* gene. A reverse Primer 2 is complementary to *TgPCNA1* 3'UTR. A reverse Primer 3 is complementary to the *NeonGreen* sequence. The combination of Primer 1 and Primer 2 amplifies a 1.3 kb locus of the native *TgPCNA1*, while the combination of Primer 1 and Primer 3 will amplify a 1.1 kb locus of *TgPCNA1* fused with *NeonGreen*. Note that published *ToxoFUCCI*^S parasites carry *TgPCNA1*^{NG} as a second copy [10].

Primer 1: GAAGCCAAAGCCTTGCAGAT

Primer 2: CGAAGGAAACCATCGATGCCA

Primer 3: ACCATCGTGGGGTACCAAGTAC

k. Set up four PCR reactions. Test parental and transgenic parasite genomes using Primer 1/Primer 2 and Primer 1/Primer 3 combinations. The thermocycling conditions are as follows: one cycle of denaturation at 98 $^\circ\text{C}$ for 1 min; 32 cycles of denaturation at 98 $^\circ\text{C}$ for 10 s, annealing at 60 $^\circ\text{C}$ for 15 s, and extension at 72 $^\circ\text{C}$ for 25 s; and one cycle of extension at 72 $^\circ\text{C}$ for 5 min. Confirm DNA amplification by mixing 5 μL of PCR mixture with 2 μL of Orange G dye and resolving PCR products on a 0.8% agarose gel with a 1 kb+ DNA ladder.

l. Confirm the TgPCNA1^{NG} protein expression in the transgenic clones by western blot using the anti-mNeonGreen antibody as previously described [10]. Expand and cryopreserve at least three clonal populations of RH TIR1 expressing TgPCNA1^{NG} protein for future use.

B. Endogenous C-terminal tagging of *TgBCC0*

1. Retrieve the *TgBCC0* genomic sequence (TGME49_294860) from ToxoDB database (www.toxodb.org).
2. Primer design and PCR amplification

a. Use BCC0_For and BCC0_Rev primers to amplify the 3'end of *TgBCC0* ORF. The forward primer includes a 5'LIC overhang for the vector annealing and *TgBCC0*-specific sequence. The 5' end of the reverse primer includes LIC overhang for the vector annealing, followed by the last 24 nucleotides of *TgBCC0* ORF, excluding the stop codon. Order the *TgBCC0* tagging primers from IDT DNA (www.idtdna.com).

BCC0_For: tacttccaatccaaatttaatgcagggtgcgtccaggaaacagagag

BCC0_Rev: tcctccacttcaatttttagcgtaaaacgtcacttgcgtcccccag

b. Amplify and purify a 2.4 kb *TgBCC0* genomic fragment as described in step A2 using proper primer annealing temperature and PCR extension.

3. Cloning via Gibson assembly and transformation in *E.coli*

a. Follow the steps described in step A3 and clone the *TgBCC0* gene fragment into the pLIC-SM-3xmyc-CAT vector [10].

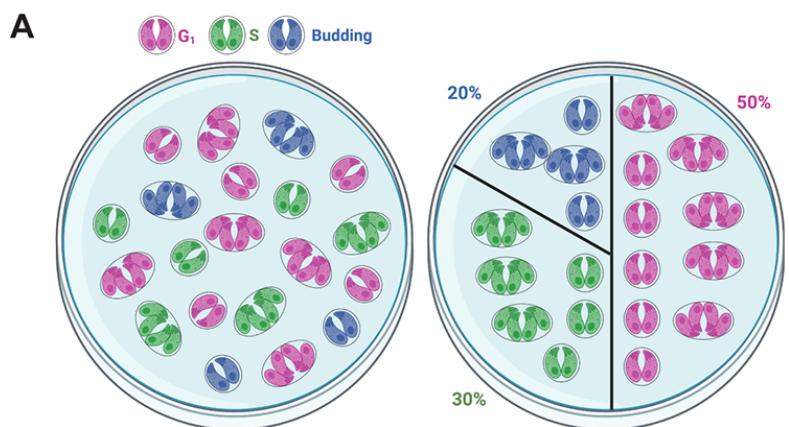
4. Parasite transfection, cloning, and selection of clones

a. Create an alternative *ToxoFUCCI* model in RH Tati *Δku80* strain using the protocol described in section A [10,19].

b. Electroporate 50 mg of NsiI-linearized and ethanol-precipitated pLIC-*TgBCC0*-SM-3xmyc-CAT plasmid in the RH Tati *Δku80* *ToxoFUCCI* tachyzoites following the procedure described in step A4. Select a chloramphenicol-resistant parasite population. Obtain and verify individual RH Tati *Δku80* *ToxoFUCCI* clones expressing *TgBCC0*^{myc} by IFA, PCR, and western blot.

C. Measurement of tachyzoite cell cycle phases

1. Use *ToxoFUCCI*^S strain alone or expressing cell cycle markers or co-stained with antibodies against cell cycle markers to identify the individual cell cycle phases. In the absence of a reliable synchronization technique, the durations of the cell cycle phases can be determined using a combination of quantitative live and immunofluorescent microscopy (Figure 1A). The live *TgPCNA1*^{NG} fluorescence detects *ToxoFUCCI*^S parasites actively replicating DNA [10].



B Cell cycle landmarks

cell cycle phase compartment	mitosis/budding						Marker (technology)
	G ₁	S	G ₂	meta-	ana-	telo-	
centrosome	1	2	2	2	2	2	Centrin (IFA)
chromosome	1	1-2	2	2	2	2	DAPI (FACScan)
centrocone	1	1	1	1+2R	2+2R	2+2R	TgMORN1 (IFA)
bud scaffold	R - basal rings						TgIMC1, TgBCC0 (IFA)

Figure 1. Quantitative immunofluorescence microscopy (qIFA) approach to examine tachyzoite cell cycle. (A) A cartoon representation of the asynchronously growing tachyzoite vacuoles randomly distributed in the microscopic field on the left and organized according to the cell cycle phases on the right. The color represents a vacuole in a specific cell cycle

stage. (B) A matrix designed to distinguish cell cycle phases based on the presence and the number of various mitotic structures (compartments). R: basal (MORN) ring.

2. Supplement *ToxoFUCCI^S* live fluorescence with anti-Centrin antibody staining to quantify centrosomes [10,22,23].
3. Use anti-MORN1 serum to visualize spindle morphology and basal rings [10–12,14,24].

4. Use anti-TgIMC1 antibodies to identify tachyzoites undergoing cytokinesis (budding) [10,17,25].

Note: Tachyzoites maintain relative cell cycle synchrony within the vacuole. Yet, parasite populations are asynchronous, and the fraction of vacuoles in each cell cycle stage reflects the time parasites spend in each stage (Figure 1A). For example, the tachyzoite cell cycle is composed of ~50% G₁, 30% S, and 20% of vacuoles undergoing overlapping mitosis (M) and budding (C, cytokinesis) [10,26,27]. Therefore, a random 100 vacuoles will represent a combination of ~50 vacuoles of parasites with a single centrosome (G₁), ~50 vacuoles of parasites with duplicated centrosomes (S + G₂ + M/C), of which ~15–20 vacuoles of parasites will have variable size buds (M/C), and ~10 vacuoles will have parasites with basal rings attached to a single or a duplicated centrocone (meta- and anaphases of mitosis) (Figure 1B).

5. Quantitative immunofluorescence

- a. Scrape infected HFF monolayers, pass three times through a G23 blunt-end needle, and separate freshly egressing parasites using a 3 mm filter (*ToxoFUCCI^S* strain or derivatives). Infect coverslips with confluent HFF monolayers with 10³ filtered parasites. **Important:** Incubate for 15–20 min at 37 °C with 5% CO₂ and wash infected monolayers 2–3 times with Hank's buffer to remove non-invaded parasites. Grow parasites at 34 °C with 5% CO₂ for 18 h. It allows parasites to complete two replication cycles. The resulting population primarily consists of cells at the optimal stage for cell cycle evaluation: vacuoles of four synchronously dividing parasites [10].
- b. Perform IFA. Fix slides with 4% PFA (5 min, room temperature), incubate in IFA permeabilization buffer (10 min, room temperature), incubate in the IFA blocking buffer (30 min, room temperature), and stain with appropriate antibodies and DAPI (nuclear dye). Visualize TgPCNA^{NG} in green, markers in red, and DAPI in blue channels using a Zeiss Axio Observer inverted microscope.

- c. **Important:** Choose microscopic fields without bias. Count all parasites within each vacuole according to parameters defined in the experiment. Perform three biological replicates of each experiment. Use three people to do counts.

6. Cell cycle phase measurement

- a. G₁ phase: Quantify 500 random *ToxoFUCCI^S* parasites and determine how many of them exhibit diffused nuclear TgPCNA^{NG} staining and a single centrosome visualized with anti-Centrin antibodies (Figure 2, G₁ panel).
- b. S phase: Quantify 500 random *ToxoFUCCI^S* parasites and determine how many of them exhibit aggregated nuclear TgPCNA^{NG} staining that corresponds to the active replication foci (Figure 2, S-phase panel).
- c. S/C (budding) interval: Use a RH Tati *Δku80* *ToxoFUCCI* line expressing one of the earliest markers of cytokinesis TgBCC0^{myc} [10]. Stain parasites with anti-myc antibodies. Quantify 500 parasites with aggregated nuclear TgPCNA^{NG} (active DNA replication) and determine the fraction that also expresses TgBCC0^{myc} (Figure 2, S/C panel).
- d. S/M/C interval: Stain *ToxoFUCCI^S* parasites with anti-TgMORN1 antibodies. The TgMORN1 localizes to the spindle compartment centrocone and basal complex, representing the parasite cytoskeleton. Quantify 500 parasites with aggregated nuclear TgPCNA^{NG} (active DNA replication) and determine the fraction of the parasites that have a single centrocone with two MORN1 rings (basal complexes of the future buds), likely representing a prophase of the mitosis (Figure 2, S/M/C panel).
- e. M/C interval: Stain *ToxoFUCCI^S* parasites with anti-TgIMC1 antibodies. Quantify 500 parasites with diffused nuclear and bright conoidal TgPCNA^{NG} building TgIMC1 positive internal buds (Figure 2, M/C panel).
 - i. To distinguish metaphase and anaphase, stain *ToxoFUCCI^S* parasites with anti-TgMORN1 antibodies. Metaphase parasites have diffused nuclear and bright conoidal TgPCNA^{NG} and a single centrocone associated with two MORN1 rings. Anaphase parasites have diffused nuclear and bright conoidal TgPCNA^{NG} and a duplicated centrocone, each associated with a MORN1 ring.
 - ii. To distinguish telophase, stain *ToxoFUCCI^S* parasites with anti-TgIMC1 antibodies. Telophase parasites have two nuclei (use DAPI stain), bright conoidal TgPCNA^{NG}, and two nearly mature internal buds within the recognizable mother cytoskeleton.

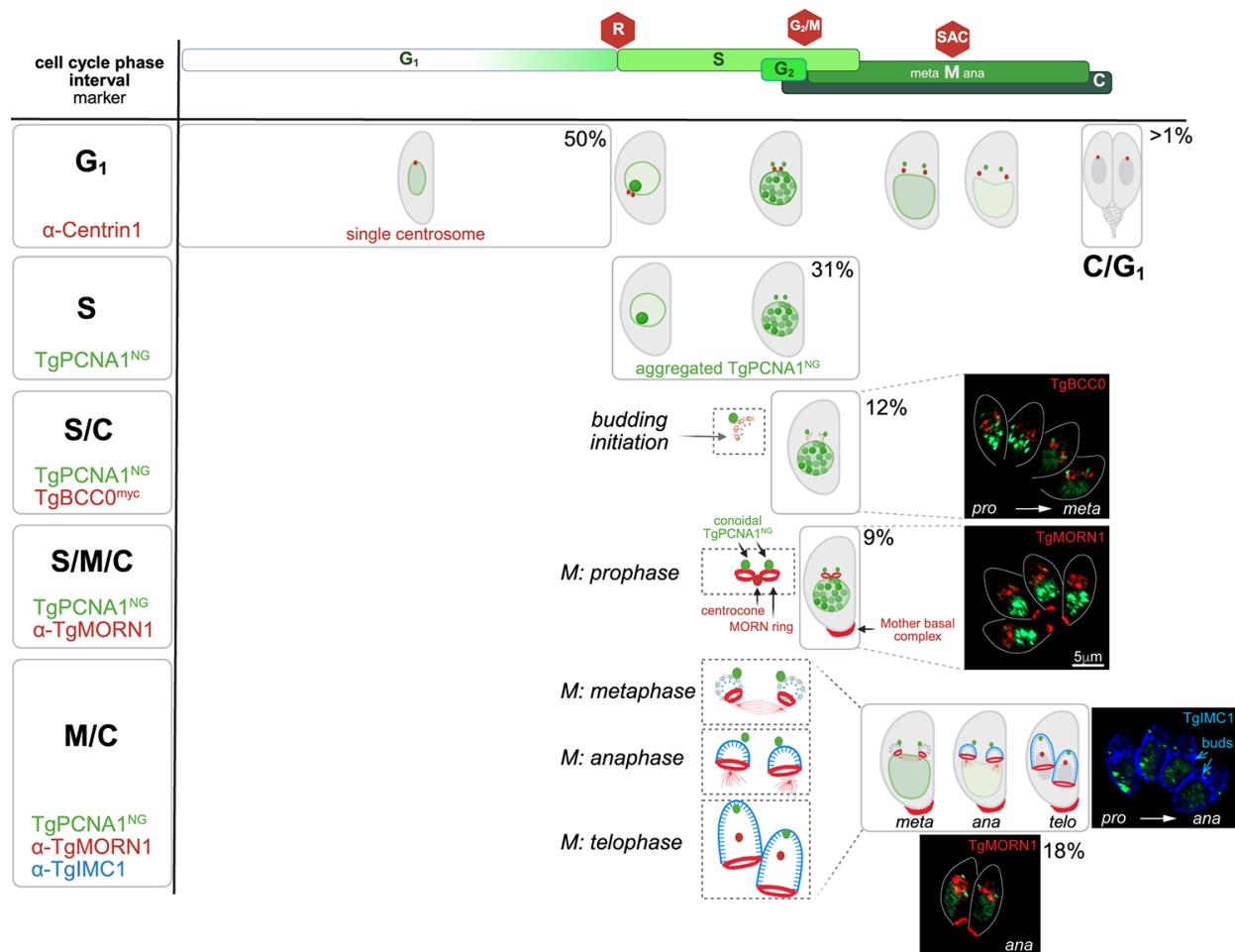


Figure 2. Measurement of the tachyzoite cell cycle phases using *ToxoFUCCIS* strain. The upper panel shows *T. gondii* cell cycle organization. Cell cycle phases and their relative durations are indicated. The red stop signs mark three major checkpoints. The left vertical panel lists cell cycle phases or intervals and markers used to determine a phase duration or an overlap of the indicated phases. The main window shows cartoons of the tachyzoites undergoing cell cycle progression and depicts the phase of interest. The corresponding live/immunofluorescence microscopy images of the selected intervals are shown on the right. The percentage is the time that the tachyzoite spends in the indicated cell cycle frame. As a reference, the first panel shows all major morphological transitions of the tachyzoite, including the transition to the next division cycle (C/G₁).

Data analysis

We used a T-test in our quantifications to determine whether the difference between the means of two groups was statistically significant. For example, we quantified data from three independent experiments to compare the proportions of replicating and non-replicating parasites. Statistical differences between groups were assessed using an unpaired two-tailed t-test in Excel. The formula used to calculate the t-value is as follows:

$$t = (X_1 - X_2) / \sqrt{(s_1^2/n_1 + s_2^2/n_2)}$$

where X₁ and X₂ are means of the two samples (replicating and non-replicating), s₁² and s₂² are the variances of the two samples, and n₁ and n₂ are the sample sizes (three biological replicates, n = 3). A value <0.05 was considered statistically significant.

Validation of protocol

This protocol was used to build the *ToxoFUCCI^S* reporter system expressing the green fluorescent DNA replication factor TgPCNA1, as described in Batra et al. [10]. This transgenic line and its derivatives were instrumental in establishing the cell cycle organization of replicating *T. gondii* tachyzoites. Live-cell and immunofluorescent microscopy validated the *ToxoFUCCI^S* probe. In addition, flow cytometry analysis confirmed the reproducibility of the *ToxoFUCCI^S* signal distributed across the asynchronous population. Furthermore, the strain was capable of detecting cell cycle checkpoints, including TgCrk6-mediated spindle assembly checkpoint and TgCrk4-regulated G₂/M transition [11,14].

General notes and troubleshooting

General notes

1. Attempts to attach NeonGreen to endogenous TgPCNA1 were unsuccessful, likely because the TgPCNA1^{NG} cannot form the functional trimer ring on the replicating DNA. Therefore, TgPCNA1^{NG} was introduced as a second copy, which did not alter tachyzoite survival or cell cycle organization (see Graphical abstract) [10].
2. The *ToxoFUCCI^S* strain may lose TgPCNA1^{NG} expression after 50 passages; therefore, earlier passages should be preserved and used in experiments.
3. The S-phase starts with the emergence of a single TgPCNA1^{NG} aggregate, followed by amplification and spread of aggregates throughout the nucleus, and ends with a few or a single aggregate [10]. Therefore, the S-phase duration should account for a variable number of TgPCNA1^{NG} aggregates. Note that TgPCNA1^{NG} accumulates in conoids of the daughter buds after 60% of S phase is completed [10]. The conoidal accumulation remains post-S phase and should not be mistaken for DNA replication foci. Please use co-staining with DAPI to distinguish nuclear and cytoplasmic TgPCNA1^{NG} aggregates.
4. The *ToxoFUCCI^S* approach can be used to study the effects of specific proteins on cell cycle progression by creating an auxin-induced degradation model of the gene of interest in the *ToxoFUCCI^S* background. For example, we validated *Toxoplasma* checkpoint kinases by creating and examining the *ToxoFUCCI^S* strains co-expressing TgCrk6^{AID-HA} or TgCrk4^{AID-HA} [10].

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This protocol was used in Batra et al. [10].

Competing interests

The authors declare that they have no competing interests.

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