Supporting Information
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Fig. S1. Development of low-fluorescence SB5050. (A) Spectral scan of TYDK components. The fluorescence from each TYDK component was analyzed over the typical spectra used in fluorescence microscopes. Note that autofluorescence decreases at higher wavelengths. Approximately 90% of the TYDK autofluorescence comes from N-Z Case and Yeast Extract (Dataset S1 includes tested formulations). (B) The table indicates fluorescence (FL) background normalized to TYDK (excitation, 475/28 nm; emission, 523/36 nm), the percentage of cells dividing within 1 h of being released from the microtubule inhibitor albendazole used to partially synchronize the cell cycle. (*All division in HBS occurred within 5 min of cells being transferred from TYDK + albendazole, which precludes the use of agarose overlay to inhibit cell motility.) Measurements are from three independent experiments. (C) SB5050 medium reduces autofluorescence and aids in time-lapse imaging. All images were acquired and scaled identically except for inset. TYDK background fluorescence is problematic, eGFP is unusable, and signal-to-noise quickly becomes an impediment as imaging bleaches out mNeonGreen. mNeonGreen is a superior fluorescent protein for imaging in Giardia as a result of fast fold times (less than 5 min) and higher intrinsic brightness. (Inset) eGFP-Tubulin scaled for optimal viewing to demonstrate that eGFP was given sufficient time to fold (40 min). (Scale bar: 10 μm.)
Fig. S2. Timing of mitosis and cytokinesis in live Giardia. (A) A representative division event as captured with 4D DIC time-lapse microscopy. Mitosis starts at 00:00, signified by rearrangement of anterior flagella exit points (white arrows). Blue numbers correspond to magnified views. At 07:49, daughter cell ventral discs are nearly fully formed, marked by dashed ellipses. The path of the cleavage furrow was predicted by the position of the internal axonemes of the caudal flagella (cf; dashed lines). At 07:54, the cleavage furrow began to ingress between the two daughter discs and between the intracytoplasmic axonemes of the caudal flagella. White dimension line denotes path and distance to scission. Cytokinesis was completed by 8:54 (time in min:s). (Scale bar: 5 μm.) (B) Histogram describes the time cells (n = 93) spent in mitosis (median, 6 min 28 s ± 52 s). Dataset collected from more than three independent movies. (C) Histogram describes time cells (n = 130) spent in cytokinesis, median time 50 s, "∞" indicates cells did not complete cytokinesis during the recording (imaged at least 15 min). Note that 89% of cells completed cytokinesis within 2 min. (D) Cleavage furrow measurements for individual cells with smoothed LOESS trajectories. (E) Bootstrapped LOESS curves within the 95% CI.
Fig. S3. Individual trajectories from PF16-KD, Actin-KD, and BFA treatment. (A) Western blot showing ~69% reduction in PF16 24 h after morpholino treatment. (B) Individual trajectories of PF16-KD cells. (C) Western blot showing ~72% reduction of actin 24 h after morpholino treatment. (D) Individual trajectories of actin-KD cells. (E) PDI2 and HA-Rab11 localization 60 min after DMSO and BFA treatment. (Scale bar: 5 μm.) Note cyan dimension bars showing swelling after BFA treatment. (F) Individual trajectories of BFA-treated cells. Cells for analysis were selected from three independent experiments.
Fig. 5A. Statistical analysis of experimental manipulations. (A) Frequency plots showing percentage of cells dividing over time. (B) Kaplan–Meier survival analysis. Experimental treatments resulted in reduced probability of completing cytokinesis compared with control treatments. PF16-KD, \( P = 3.17 \times 10^{-11}, n_{PF16} = 56, n_{ctrl} = 141 \); Actin-KD, \( P \approx 0, n_{actin} = 191, n_{ctrl} = 141 \); Rab11-KD, \( P = 3.07 \times 10^{-9}, n_{Rabll} = 80, n_{ctrl} = 141 \); BFA, \( P \approx 0, n_{BFA} = 83, n_{DMSO} = 106 \). All data were acquired from at least three independent experiments except for the DMSO control and Rab11, which came from two independent experiments.
Fig. 55. Actin is cleared ahead of the advancing furrow. (A) Average projections of cells early and midway through cytokinesis with plot of actin intensities along the furrow of 10 cells (black line is the mean). Note low levels in the first 1 μm of the furrow. (B) Average projections of cells early and late in cytokinesis with plot of actin levels across the furrow of five cells (black line represents the mean). Note that, in contrast to cells with a contractile ring, actin levels dip precisely at the midpoint of the cell. (C) Projected images of three central optical sections for cells at progressive stages of cytokinesis. Note that actin is cleared just ahead of the advancing furrow (yellow arrows). (Scale bars: 5 μm.)
Fig. S6. Rab11 traffics on intracytoplasmic axonemes and dominant mutants block cytokinesis. (A) Another example of Rab11 and actin being enriched at the ends of nascent flagella. This cell is from an experiment in which the culture was partially synchronized by using the microtubule inhibitor albendazole. This treatment presumably caused the observed flagella misorientation. However, this orientation allows the nascent posterolateral flagella to be more easily observed and demonstrates that Rab11 and actin tracked the position of the flagella tips. Boxed region is magnified, and regions of Actin and Rab11 colocalization are shown in green with tubulin in magenta. (B) Fixed pTet::HA-Rab11DN (dominant negative) expressing cells accumulated HA-Rab11DN between arrested daughter cells. (C) pTet::HA-Rab11CA (constitutively active) expressing cells accumulated HA-Rab11CA between daughter cells and failed in cytokinesis. (Scale bars: 5 μm.) Note the abnormal placement of the cleavage furrow in B and C. (D) Quantification of cells in the process of cytokinesis (active or arrested) for the indicated Rab11 mutant fixed 24 h after Tet-induced expression. At least 1,400 cells from three independent experiments were counted for each condition (*P < 0.05). (E) Western blot showing that the Rab11 antisense translation blocking morpholino effectively depletes Rab11.
**Movie S1.** Selected focal planes from 4D movie of mNG-Tub corresponding to the cell in Fig. 1. Movie is 15 min 56 s in real time.

**Movie S2.** Time-lapse movie of mNG–β-tubulin showing flagella flexion and positioning during cytokinesis. Note that the caudal flagella flex to push daughter cells apart at the initiation of cytokinesis, and, when the flagella are no longer juxtaposed, the cells swim apart to complete cytokinesis. Movie is 87 s in real time.

**Movie S3.** DIC time-lapse movie of a *Giardia* trophozoite progressing from interphase to cytokinesis. Movie is 9 min 40 s in real time.
Movie S4. PF16-HA depletion results in failed cytokinesis. Movie is 23 min 47 s in real time.

Movie S5. Actin-KD cell fails to divide as a result of physical blockage of furrow progression. Movie is 20 min 30 s in real time.
Movie S6. Actin-depleted cell stalls at the tail-to-tail phase and completes cytokinesis after 60 min. Movie is 16 min 10 s in real time.

Movie S7. Representative BFA-treated cell that arrested in cytokinesis. Movie is 17 min 58 s in real time.
Movie S8. A 4D movie of mNG-Rab11. Movie is 56 s in real time.

Movie S9. A 3D stack of fixed cells at different stages of cytokinesis: HA-Rab11 (red), actin (green), tubulin (grayscale), and DNA (blue). Note that actin and Rab11 colocalize at the ends of the forming posterolateral and ventral flagella.

Dataset S1. Alternative media formulations

More than 40 different medium formulations were tested for their ability to support growth and reduce autofluorescence compared with TYDK media. Cell counts and fluorescence levels for experimental media formulations are reported as mean values from three technical replicates.

Dataset S2. Oligo sequences