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Organelle size equalization by a constitutive process

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Summary

The spatial organization of a cell arises in part from the precise control of organelle size, but how a cell achieves this control remains an elusive problem. Two predominant models for size control persist, (i) induced control, where organelle genesis, maintenance, and disassembly are three separate programs that are activated in response to size change [1, 2], and (ii) constitutive control, where stable size results from the balance between continuous organelle assembly and disassembly [3, 4]. The problem has been studied for over 50 years in the bifflagellate *Chlamydomonas reinhardtii* because the flagella are easy to measure, their size changes only in the length dimension, and the genetics are comparable to yeast [5]. Length dynamics in *Chlamydomonas* flagella are quite robust: they maintain a length of about 12 μm and recover from amputation in about 90 min with a growth rate that decreases smoothly to zero as the length approaches 12 μm [6]. Despite a wealth of experimental studies, existing data are consistent with both induced and constitutive control models for flagella. Here we developed novel microfluidic trapping and laser microsurgery techniques in *Chlamydomonas* that allow us to distinguish between length control models by measuring the two flagella on a single cell as they equilibrate after amputation of a single flagellum. The results suggest that cells manage ciliary/flagellar length by constitutive control with a single, tunable cytoplasmic pool parameter.

Results and Discussion

Induced versus constitutive control over flagellar length

Flagella are dynamic structures that maintain a stable length. Axonemal microtubules, the structural core of the flagellum, undergo constant assembly and disassembly while maintaining the same overall length [3]. In the unicellular, biflagellate green alga *Chlamydomonas reinhardtii* the two flagella are of equal length within each cell. What regulates assembly and disassembly to determine the length and make sure the two lengths are equal? In considering possible models for flagellar length control systems, we can distinguish two broad classes of models for flagellar length equalization, which we call “induced” and “constitutive”. Induced control means that a cell responds to unequal flagellar lengths, such as when a single flagellum is amputated, by differentially triggering a program

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(e.g. activity of a shortening enzyme in the remaining flagellum) to restore correct lengths. Constitutive control means that these programs are always active in both flagella and not specifically modified when length is perturbed, so that while energy-consuming pathways may be subcomponents of the control system, these pathways naturally correct for length perturbations.

If the induced control model is correct, the key factors to identify and characterize are the signaling networks that are specifically activated in one flagellum at a time. By contrast, for constitutive control, it is critical to understand how the natural length-dependence of intraflagellar transport (IFT) [7], the length-independent rate of axonemal microtubule disassembly [3], and the amount of available flagellar precursor protein in the cytoplasm are controlled. Current experimental evidence cannot rule out either type of model, so better methods are needed to quantitatively probe the length control system in a manner that would allow us to differentiate between the models.

A classic experimental paradigm for probing flagellar length control is the equalization of flagellar lengths following flagellar amputation in *Chlamydomonas* 6]. If both flagella are detached, they regrow in approximately 1.5 hours. If only one of the two flagella is detached, it also grows back, but while it grows back, the other flagellum shortens, and continues to shorten until the two flagella reach equal lengths. This behavior, known as the "long-zero response", presents an opportunity to acquire time-lapse quantitative measurements of flagellar growth and shortening simultaneously in single cells, with the potential to discriminate among different quantitative models for flagellar length control. Thus, we developed methods to measure and analyze this long-zero response with a particular focus on comparing predictions of constitutive versus induced control models.

Microfluidic trapping keeps motile cells immobile and healthy for many hours

Previous studies used paralyzed strains or compression with a coverslip to hold cells in place for viewing. However, paralyzed mutant strains have structurally altered axonemes and are shorter than normal flagellar lengths. Moreover, in our hands *Chlamydomonas* cells often fail to divide when compressed by a coverslip, suggesting poor gas exchange and/or mechanical damage affects cell physiology.

To facilitate long term imaging, we designed a microfluidic trapping system that allows simultaneous imaging of up to 100 motile, wildtype *Chlamydomonas* cells in polydimethylsiloxane (PDMS) wells, without compression and under continuous fluid flow of fresh, aerated media (Figure 1A–C). In tests, the cells remained healthy, able to regenerate flagella, maintain flagellar length, and undergo cell division, for over 18 hours.

Laser microsurgery induces cells to sever their flagella at the base

Induction of the long-zero response requires amputation of one of the two flagella. Prior studies used finger-pressure tapping on the coverslip or fluid shearing with blenders or syringes prior to mounting under the coverslip to induce flagellar detachment. With such methods the experimenter cannot choose which flagellum will be amputated, raising potential concerns that the flagellum that happened to pop off might be somehow different compared to the other flagellum. As an alternative approach, we used a femtosecond infrared (IR) laser at 110 mW of power after the objective (Figure S1) to cut individual flagella on trapped cells. After laser severing, the remaining stump immediately detached regardless of where the laser cut the flagellum along its length (Figure 1D). The *fa1* mutant, which prevents flagellar excision during pH shock [8], retained the injured flagellar stump after laser severing of the distal portion of the flagellum (however, these cells died without any regeneration), suggesting the same pathway that mediates pH induced deflagellation

also mediates laser-induced deflagellation. Targeting the cell body with the same laser power induced cell death, whereas targeting anywhere outside the cell had no effect on the cell or flagella.

When we observed cells trapped in the microfluidic chamber following severing of one flagellum, we found that the long-zero response was easily observed (Figure 2A, B). We can thus reproduce the basic phenomenon that was previously observed in paralyzed cells trapped under coverslips but with several important advantages: the cells are genetically wild-type, they are grown under continuous fluid flow, they are not mechanically compressed, and we can choose which flagellum to target with the laser.

Overshoot behavior does not occur in microfluidically trapped cells

With the laser-based method for measuring the long-zero response, we next investigated a key result in the existing literature -- the ability of the shortening flagellum to become shorter than the growing flagellum, a phenomenon variously described in the prior literature as "overshoot" and "undershoot" [6, 9]. These previous reports indicated that while most cells with an amputated flagellum directly equalized the lengths of the regenerating and uninjured flagella to roughly 2/3 full length, some fraction of the cells with an amputated flagellum shortened their uninjured flagellum to a length significantly shorter than the regenerating flagellum “overshooting” the length before eventually recovering [6]. Overshoot requires that the two flagella be in distinct operational states when their lengths become equal, since one continues to shorten and one continues to grow after reaching equal lengths. An induced shortening program can explain this observation because a cell could independently activate shortening in one flagellum and not the other. If not stopped in time, the shortening would cause the long flagellum to overshoot the length of the regenerating flagellum. But in a constitutive model, two flagella of the same length should have the same net growth or shortening rate. Hence, when both flagella reach the same length, their behavior must be the same. Thus, overshoot cannot be explained in a constitutive model. We therefore checked carefully to observe this important experimental behavior in our improved long-zero method.

In 100 cells examined by our methods, we never saw a single example of overshoot (Figure 2C,D; $p<0.001$, Binomial statistic, probability of overshoot = 0.1), indicating that the overshoot behavior does not occur in microfluidically trapped cells and may have been an unintended consequence of the previous experimental setup, which involved compressing cells and depriving them of fresh media or gas exchange. When we grew cells compressed under a coverslip and applied laser ablation to remove one flagellum, we observed behaviors that resemble those previously reported [6], including cases where flagella of identical length show opposite changes in growth (Figure S2). However, coverslip-compressed cells failed to divide on a normal time scale, showed enlarged vacuoles not seen in healthy cells suggesting a stressed state, and in some cases died during observation. They also tended to resorb and/or sever their flagella even when not amputated. Thus, the shortening of long flagella past the point of flagellar length equalization (overshoot) seen under these conditions seems less likely to reflect a legitimate mode of flagellar length control than a response of flagella to cellular stress.

Overshoot behavior was the only experimental evidence contradicting a constitutive model for length control. Constitutive models can explain all other known phenomena of length control including length equalization during the long-zero response [3] but could never explain overshoot for the reasons outlined above. Because overshoot behavior never occurred in healthy cells grown in the microfluidic plate conditions, our results are consistent with the idea that, in a single cell, flagella that are of equal length always have the same rate of length change, which supports the constitutive control model.

We note that our distinction of constitutive versus induced specifically refers to the states of the two flagella, and our conclusion of constitutive control simply means that the same control pathways are at work in both flagella, as opposed to the shortening flagellum turning on a different control pathway than the elongating flagellum. Flagellar severing induces transcription of genes encoding flagellar proteins but this is a common effect felt by both flagella. Transcription induced by severing is necessary for both flagella to reach the wild-type length [6], presumably because it is necessary to re-synthesize the flagellar protein precursors lost when flagella are severed. However, transcription and translation are not necessary to achieve equalization of the lengths of the two flagella [9]. Thus, induced pathways play a role in overall flagellar length determination, but not in the specific process of ensuring equal lengths between the flagella.

Long-zero response in cells with separated basal bodies

One explanation for the long-zero response is that the two flagella share components from a common cytoplasmic pool that is depleted when the amputated flagellum regenerates [3]. Since short flagella have more efficient IFT [3, 4, 9], they grow faster and hence consume precursor more quickly than long flagella. This depletes the precursor pool below its steady state value, causing the longer flagellum to shorten. Simulations confirm this model can explain the long-zero response [3]. However, it has been reported that *vfl* mutants, which lack the fibers connecting the basal bodies, cannot equalize flagellar length [10], suggesting activation of shortening requires physical communication between basal bodies. This result argues against simple competition for a common cytoplasmic precursor pool.

However, with our new methods, when we analyzed the long-zero response in mutants with separated basal bodies (*vfl2* 11] and *asq2* 12]) we observed length equalization in all cases (N=10 *vfl2* cells; N=10 *asq2* cells) (Figure 3), although sometimes with a longer lag time. The same result was seen in cells containing two non-associated flagellar pairs due to incomplete cytokinesis (N=4). These results are consistent with the competition model.

A mathematical model of the flagellar growth and maintenance system accurately fits the observed dynamics with a single parameter set

To further test whether a single consistent mechanism acting in both flagella could explain equalization kinetics, we formulated a mathematical model incorporating constant disassembly at the flagellar tip [3,4], known IFT rates [7], stochasticity in IFT [13, 14], and competition for a dynamic cytoplasmic precursor pool (see Supplemental Data Appendix 1). We tested the model response to long-zero amputation with and without protein synthesis as well as dual flagellar amputation (Figures 4 and S3). The model could account for all experimentally observed dynamics, thus induced states are not needed to explain the long-zero response [6].

A key parameter in the previously proposed "balance point" model for length control was the rate of axonemal disassembly at its distal tip [3, 15–17]. Previously, this parameter was estimated at ~0.01 μm/min from the shortening rate of flagella following inactivation of IFT using temperature-sensitive mutants [3]. However, retrograde IFT, which requires anterograde IFT, may play a role in flagellar disassembly [1]. Hence, prior studies may have under-estimated the disassembly rate.

Our model allows us to compute the disassembly rate from long-zero kinetics (see Supplemental Data Appendix 2), yielding a mean disassembly rate of 0.50 μm/min (Figure 4), 50-fold greater than estimates based on shortening in IFT mutants [3]. We hypothesize that in the absence of IFT, tip-disassembly products cannot be transported away from the assembly site at the flagellar tip and could therefore reincorporate into the axoneme.

Implications for organelle size control

All cells face the problem of organelle size control. In order to operate with optimal metabolism and behavior, the size of the organelles must be modulated to meet the instantaneous environmental conditions that a cell is experiencing. Our results show that cells use a constitutive method to maintain correct flagellar length by using constant turnover of flagellar components so that length is sensitive to the available pool of precursor, which is regulated in the cytoplasm.

Materials and methods

Strains and cell culture

lf1, *lf2-1*, *lf2-5*, and *lf3* were a generous gift of Pete Lefebvre. The KAP-GFP rescue of *fla3* was a generous gift of Mary Porter. *lf4*, *ptx2*, and *ptx8* strains were a generous gift of George Witman. *eye2* and *eye3* were a generous gift of Carol Dieckmann. *asq2*, *vfl1*, *vfl2*, *pf16*, *pf18, CC124, CC125*, and *CC1009* strains were acquired from the *Chlamydomonas* stock center (University of Minnesota).

Cells were grown on 1% agarose plates made with TAP media and transferred to M1 liquid media for 36 hours under continuous illumination prior to amputation (see *Chlamydomonas* Sourcebook).

Microfluidics

Microfluidic chambers were fabricated by CellASIC Inc, using a custom design based on the yeast Y04 template (Part# C04 for the Chlamydomonas custom plate). The device is made of PDMS glued to a No. 1.5 coverslip. Cells were held in the plate using a pressure of roughly 2 psi (varied from plate to plate).

Microscopy

Light microscopy was performed on a Zeiss Axiovert 200 using the custom configuration shown in Fig. S1 with a tunable (710 – 990 nm), 76 MHz, 200 femtosecond Spectra Physics Mai Tai laser, which uses a titanium-sapphire crystal (Newport Corporation, Irvine, CA). We used a 730 nm wavelength and a laser power of 110 mW after the objective, which corresponds to 157mW before the objective. The irradiance was 33×10^6 W/cm^2 in the focal plane. Imaging was performed using a 40× 1.4 NA lens, Phase contrast 3 filters and a Hamamatsu CCD camera with 6 pixels per micron. Movies were recorded at 1 frame per second for the duration of the experiment.

Measurement of flagella

Flagella were measured by hand-tracing in ImageJ64. Measurements were made whenever the flagellum was not moving and in focus, thus the time points were initially unevenly spaced.

Analysis and mathematical modeling

All analysis and mathematical modeling was carried out using custom software in MATLAB. For calculation of the shortening rate, the regeneration curves for each cell were first cropped to the time where the lengths of the unamputated and regenerating flagellum had not yet finished equilibrating. The regeneration curves were then smoothed using a 3-point running average filter. For each cell, the disassembly rate was calculated for each pair of time points in the regeneration curve, and then the calculated values were averaged to produce a disassembly rate for each cell.

Simulations of amputation were run with specified parameters in Figure 4. In order to account for the nubs of flagella that are not severed by our deflagellation method, we started amputated flagella at a length of 2 microns and unamputated flagella at 15 microns in the simulations. In the display, we subtract the two microns for concordance with the actual cellular measurements, where the length of the nub is set to 0 μm. In order that flagella never obtain negative lengths, the shortening rate was set to approach zero for very short flagellar lengths by setting the shortening rate equal to $k_z{*}^L/(L{+}0.1)$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Microfluidic device allows laser severing flagella in motile *Chlamydomonas* cells
- Flagella equalize length after severing one flagellum without overshoot
- Modeling and measurement together determine the rate of flagellar disassembly
- Length equalization can be modeled using a single set of invariant parameters

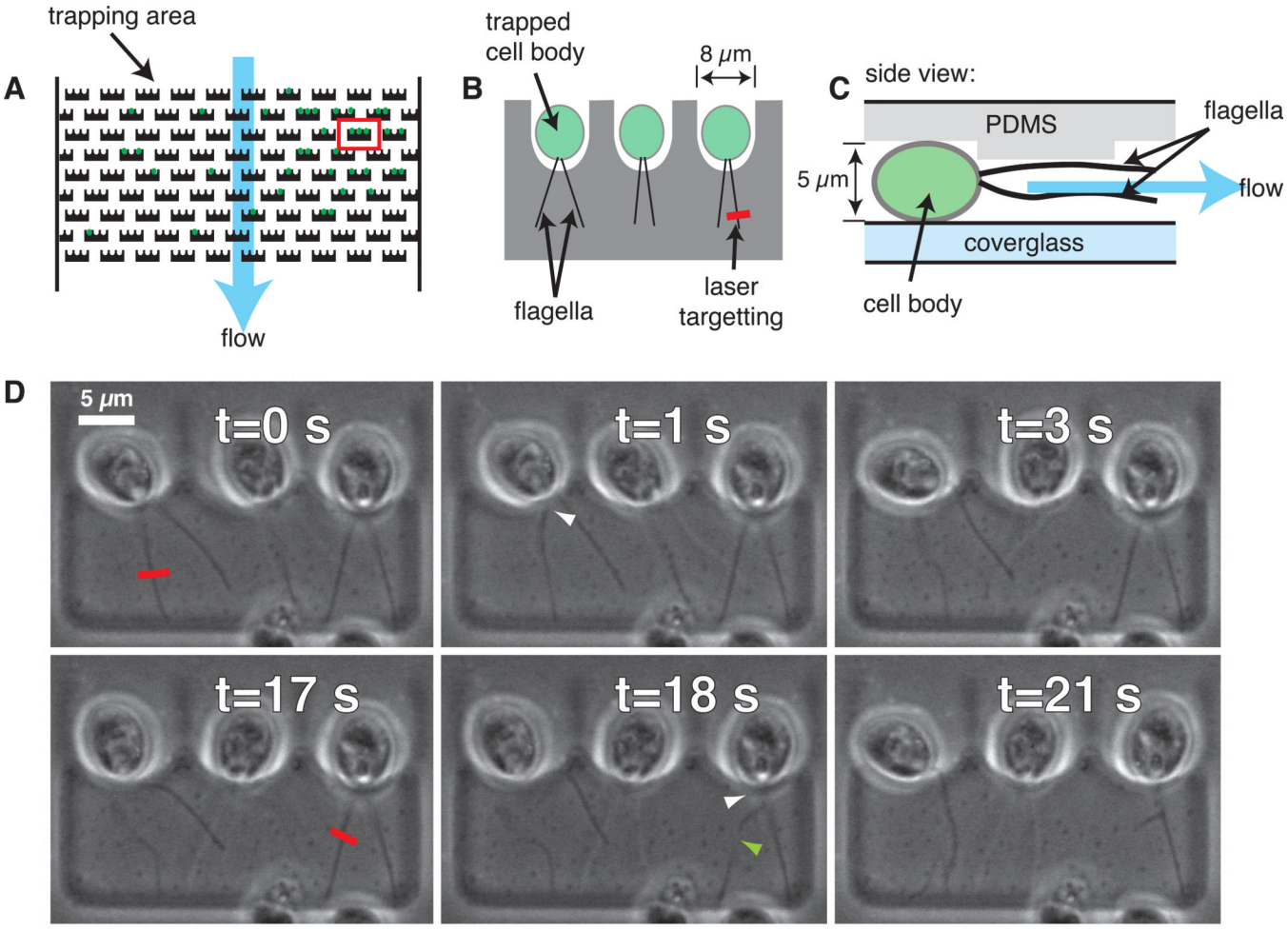

Figure 1.

Microfluidic trapping and laser-microsurgery allow selective deflagellation of *Chlamydomonas* cells. (A) A custom-designed microfluidic chamber traps motile cells and holds them in place using continuous flow of fresh media at 7mm/s. (B) Zoom view of the red box in (A). (C) Side view of one cell in (B). (D) Laser microsurgery on individual flagella induces cells to eject injured but not uninjured flagella. Red dashed line indicates the laser-targeting site. White arrowheads indicate where the flagellum was ejected from the cell body at the flagellar base. Green arrowhead indicates where the laser cut the flagellum in two. Cell diameter is ~5 μm.

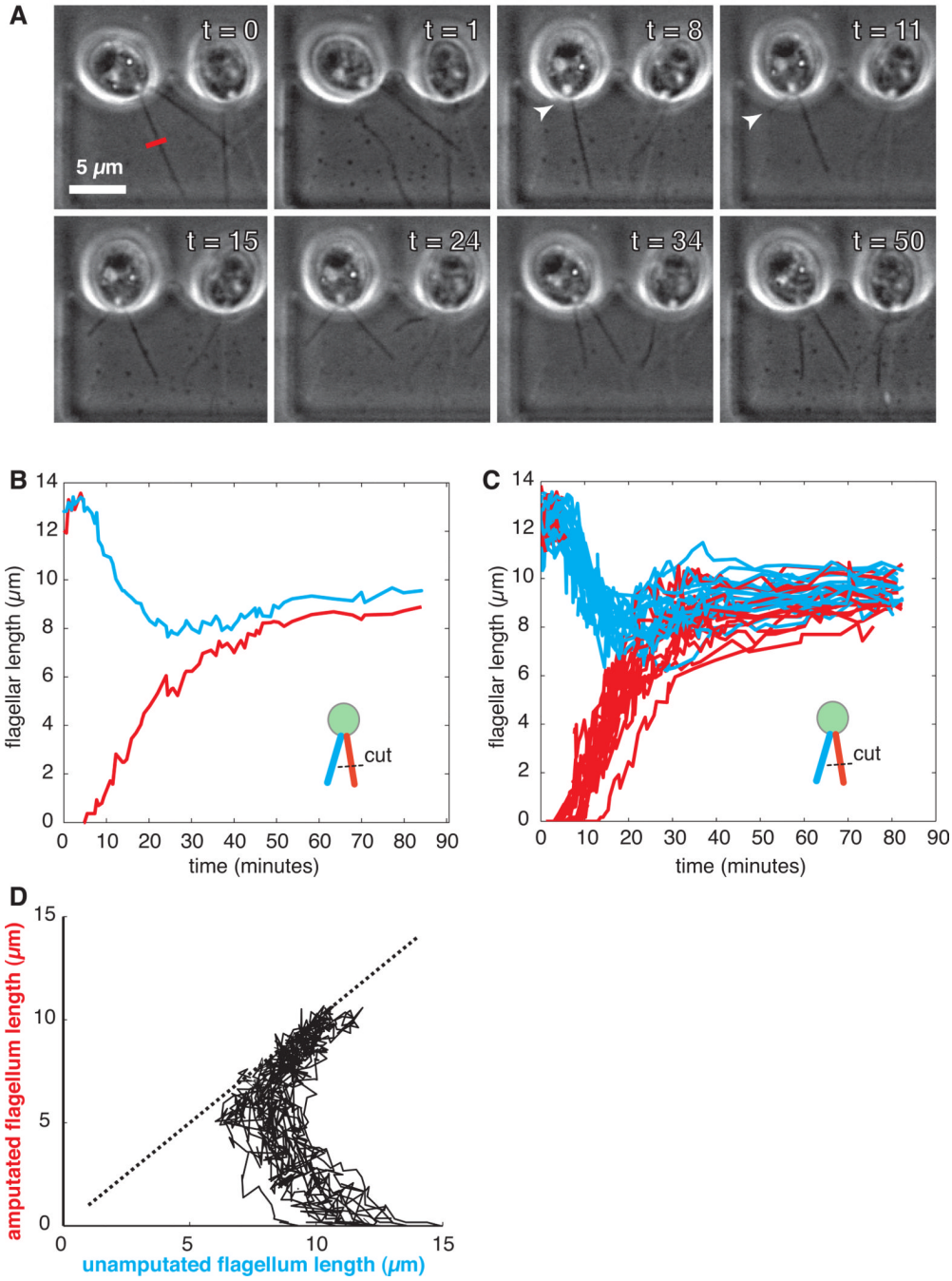

Figure 2.

Biflagellate wildtype cells exhibit a single, characteristic behavior in response to single deflagellation. (A) After laser-induced deflagellation of a single flagellum, the remaining long flagellum shortens while the ejected flagellum regrows. Once the two flagella reach roughly equal length, they then both begin to grow back to near-wildtype length. Times are in minutes. Red line indicates laser targeting. White arrowheads indicate the tip of the regenerating flagellum where it is more difficult to see in these still images. (B) The flagellar length kinetics measured over time for a single cell, shown in (A). (C) The flagellar length kinetics for 20 cells that underwent laser-induced single deflagellation at the same time in the same chamber show a characteristic regeneration response. (D) The regenerating

flagellum never became longer than the unamputated flagellum, as seen by plotting the regenerating flagellum's length against the unamputated flagellum's length for the cells plotted in (C). The dotted line indicates where the two flagella on a single cell are equal length.

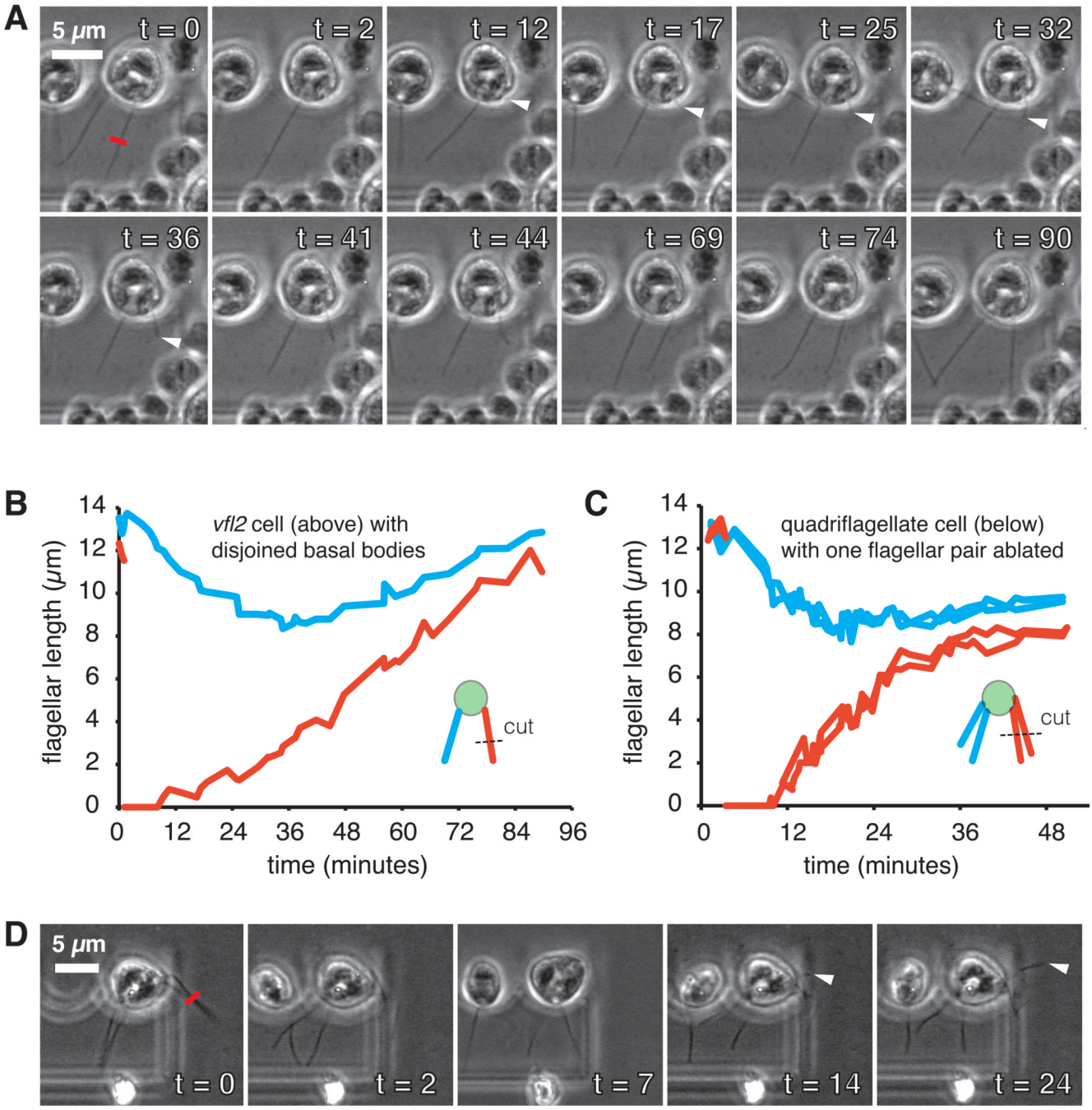

Figure 3.

Cells with flagella that are not connected at their base show long-zero flagellar regeneration behavior typical of wildtype cells. (A) Pictures and (B) graph of long-zero regeneration in an example *vfl2* cell with two flagella that are separated by about 4 microns at their bases shows regeneration behavior typical of wildtype cells. However, note that the minimum length reached by the long flagellum is reached at roughly 35 minutes post-deflagellation rather than the typical 20 minutes of wildtype cells. (C) Graph and (D) pictures of long-zero regeneration in a wildtype cell that has two unconnected pairs of flagella rather than the typical one pair. The pair on the right was amputated (red line). Note that the pair of unamputated flagella reaches its minimum length at the 20 minutes mark, typical of

wildtype cells with a single flagellar pair. White arrowheads indicate regenerating flagellar tips.

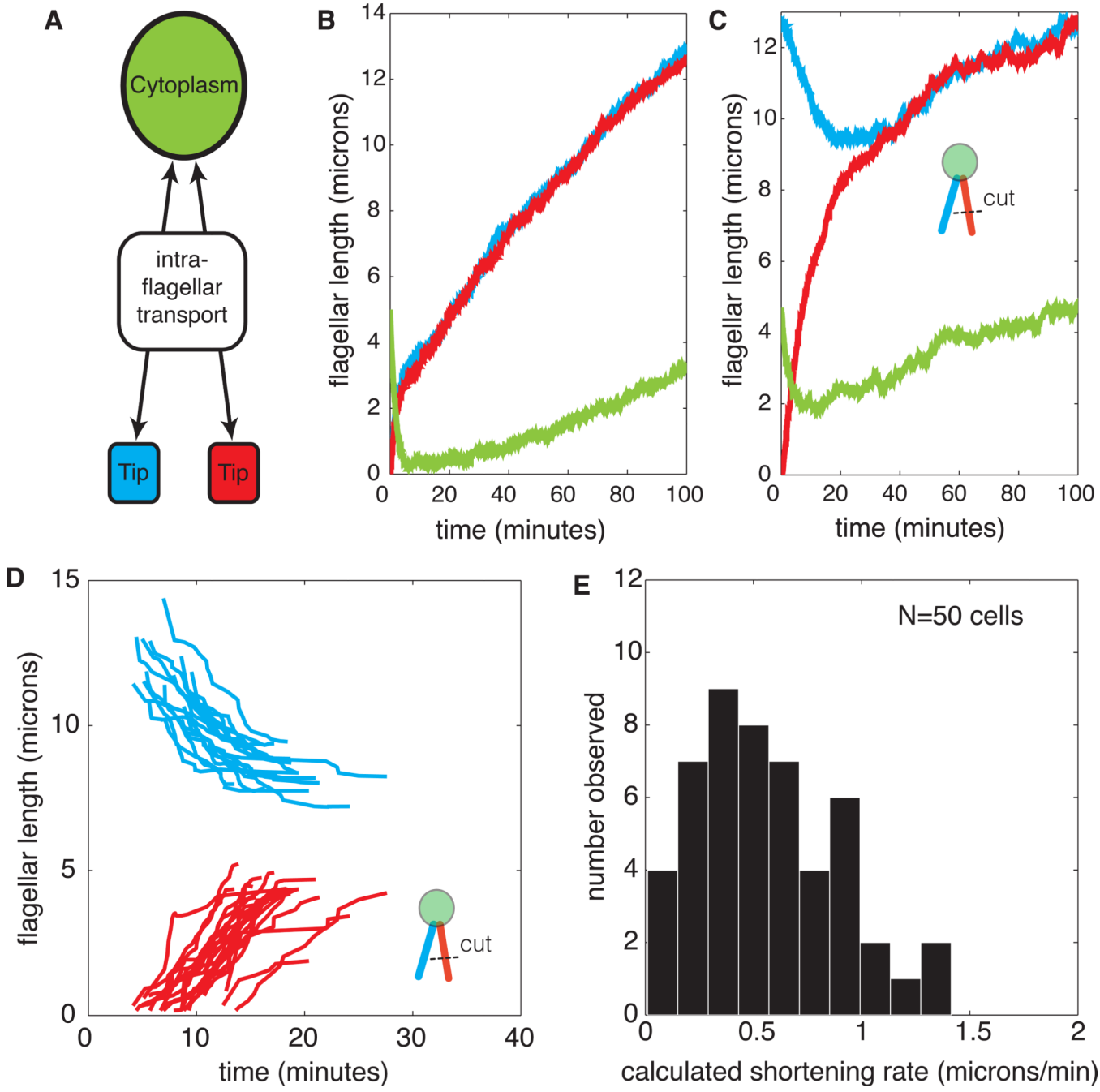

Figure 4.

A model of the flagellar length control system accurately reproduces the flagellar length dynamics and allows calculation of the disassembly rate. (A) Schematic of the model (refer to Supplemental Data Appendix 1 for derivation). (B) Model dynamics for dual flagellar regeneration. (C) Model dynamics for long-zero flagellar regeneration. red=amputated flagellum. blue=unamputated flagellum. green=cytoplasmic pool. Parameters for the model are ***k1*** =0.001, ***k2*** =0.5, and ***k3*** =0.05. (D) Length data that were used to calculate the disassembly rate using the scheme outlined in the text and Supplemental Data Appendix 2. Traces were truncated to the initial part of the response in which the lengths were highly unequal, in order to avoid pathological behavior of the equations when the difference in

length becomes small. (E) Calculation of the disassembly constant using this scheme for 50 cells yielded a range of disassembly constants with a mean of 0.50 μm/min.