Title

Flagellar synchronization is a simple alternative to cell cycle synchronization for ciliary and flagellar studies

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Abstract:

The unicellular green alga, *Chlamydomonas reinhardtii*, is an ideal model organism for studies of ciliary function and assembly. In assays for biological and biochemical effects of various factors on flagellar structure and function, synchronous culture is advantageous for minimizing variability. Here, we have characterized a method in which 100% synchronization is achieved with respect to flagellar length but not with respect to the cell cycle. The method requires inducing flagellar regeneration by amputation of the entire cell population and limiting regeneration time, which results in a maximally homogeneous distribution of flagellar length at three hours post-amputation. We find that time-limiting new protein synthesis during flagellar synchronization limits variability in the unassembled pool of limiting flagellar protein and variability in flagellar length without regulating the cell volume. We also find that long and short flagella mutants that regenerate normally require longer and shorter synchronization times, respectively. By minimizing flagellar length variability using a simple method requiring only hours and no changes in media, flagellar synchronization facilitates the detection of small changes in flagellar length resulting from both chemical and genetic perturbations in *Chlamydomonas*. This method increases our ability to probe the basic biology of ciliary size regulation and related disease etiologies.

Introduction:

The unicellular, biflagellate alga *Chlamydomonas reinhardtii* is extensively used as a model organism for studying fundamental processes such as photosynthesis, cell motility, cell signaling, cell-cell recognition and regulation of ciliary assembly-disassembly [1]. This organism offers many advantages for molecular and biochemical studies of eukaryotic flagella as their flagellar structure and function is well conserved [2]. *Chlamydomonas* cells can be chemically or mechanically induced to shed their flagella (termed deflagellation). After amputation, they can regenerate flagella rapidly to pre-deflagellation lengths within 2 hours. During cell division, flagella are disassembled naturally as flagellar assembly and disassembly are precisely controlled throughout cell-cycle progression and cell division [3,4]. Flagellar resorption starts at the pre-prophase stage and continues about 30 minutes prior to mitotic cell division [5]. New flagella begin to form in the daughter cell after division [6,7]. During the sexual cycle, flagella begin to resorb a few hours after the fusion of gametes and proceed gradually as in vegetative growth [8].

As cell division plays a critical role in flagellar growth and resorption, cultures with a heterogeneous population of cells in different divisional stages vary in their flagellar length. In contrast, synchronous cultures which contain cells that are in the same growth stage have flagella with a comparatively homogeneous distribution of flagellar length. Thus, synchronous cultures provide advantages over non-synchronous cells for studying cellular or flagellar morphology and the effect of various chemical and genetic perturbations on flagellar length.

A wide range of physical and chemical methods have been applied to achieve synchronization for highly diverse cells or tissue types. Synchronization of bacteria can be carried out by single or multiple changes of temperature or light, single or multiple cycles of nutritional starvations, cell-cycle inhibitor block and size selection by filtration or centrifugation [9-12]. Fission yeast can be synchronized either by separating a subpopulation from an asynchronous culture using specialized centrifugation or by selecting cells from a lactose gradient [13]. Temperature sensitive cell cycle mutations or inhibitors are also used to block the cell cycle at different stages of growth, which allows cells to grow synchronously upon withdrawal of the block [14]. Common methods for mammalian cell cycle synchronization are either inhibition of DNA replication [15] or inhibition of mitotic spindle formation using different chemical inhibitors [16-18]. Non-chemical methods for cell cycle synchronization include amino acid and serum starvation [19]. Cells can also be mechanically

separated by physical methods such as flow cytometry, mitotic shake off or counter current centrifugal elutriation [18]. Hypoxic and hyperthermic shock have been used to synchronize the ciliate *Tetrahymena pyriformis* [20]. Photosynthetic algal cells are typically exposed to alternative light/dark cycles for synchronization [21,22].

In *Chlamydomonas reinhardtii*, like other photoautotrophic cells, the most common method used for cell synchronization is also the alternative light/dark cycles (12 hour/12 hour or 14 hour/10 hour) in minimal medium [23,24], though other methods such as periodic hypothermic conditions [25], selection by size [26] or variable wavelengths of light [27] have been applied. Synchronization can also be achieved by resuspending *Chlamydomonas reinhardtii* cells into low nitrogen medium for at least 15 hours [28]. *Chlamydomonas* cells undergo gametogenesis under nitrogen deprived conditions and cultures consist mostly newborn cells with smaller sizes [29-31]. During light-dark synchronization (L-D synchronization), cells can grow during the light phase to many times their original size [32]. In the dark phase, cells can undergo consecutive divisions to produce 2, 4, 8, 16 or even 32 daughter cells depending on the cell size [33]. In *Chlamydomonas reinhardtii*, cells divide in the middle of the dark cycle [23] whereas in *Chlamydomonas moewussi* cells, the division occurs during the late phase of darkness (23rd to 24th hour) [34]. Although cell division is restricted to each dark phase, the starting time of individual cell divisions varies from cell to cell. Thus, consecutive cell divisions take place throughout several hours of the dark period. As a result, the cells are always partially asynchronous in their division at any point in time [8]. In addition, cultures are maximally synchronized only after 3rd iteration of light-dark cycling since some population of the cells divide during 1st and 2nd iterations of the light phase [23]. Different factors such as light duration and intensity, temperature and culture density also have an effect on the degree of homogeneity [32,35]. For example, in *Chlamydomonas eugametos*, L-D synchronization can be achieved only if the culture is static without aeration [36].

While synchronization can be optimized, it is not possible to synchronize entire cell populations by any of the methods or techniques described above [37]. Traditional L-D synchronization or nitrogen starvation methods can only make partially synchronized *Chlamydomonas* cultures. As cells are not truly synchronized using these methods, high variabilities of flagellar length are still observed within the population. If the culture contains too much heterogeneity, it can be difficult to detect effects of flagellar length perturbations. As synchronized cells are ideal for assaying length related flagellar kinetics, here we outlined and characterized a method in which 100% of cells are synchronized with respect to their

flagellar length but not synchronized with respect to the cell cycle. We tested the utility of this method when evaluating chemical and genetic perturbations to flagellar length. Finally, we probed the basis of flagellar synchronization by probing the synthesized but unassembled pool of flagellar protein.

Materials and methods:

Strains and length altering chemical treatments:

Chlamydomonas reinhardtii wild type137c mt+ [CC125], *lf4-7* mt- [CC4534], *shf1-253* mt- [CC2348], *cnk2-1* [CC4689], *lf2-5* mt- [CC2287] and *lf3-2* mt- [CC2289] were obtained from the *Chlamydomonas* Resource Center at the University of Minnesota. All chemicals were purchased from Sigma (St. Louis, MO) and final concentrations of 0.4 mM 3-isobutyl-1-methylxanthine (IBMX), 10 mM sodium pyrophosphate (NaPPi), 10 µM latrunculin B (LatB), 25 mM lithium chloride (LiCl) and 10 µg ml^{-1} cycloheximide were used. Compounds were diluted to the indicated doses either with Tris-Acetate-Phosphate (TAP) medium or with 100% dimethyl sulphoxide (DMSO). For the chemical treatment, 1 or 2 ml of cells were treated with indicated concentration of chemicals with indicated controls and placed on a rotator for 90 minutes or 120 minutes.

Culture condition and different synchronization methods:

All cells were maintained on TAP plates containing 1.5% agar (Difco laboratories, Detroit, MI) [24]. For liquid cultures, cells were inoculated from TAP plates less than two weeks old.

Non-synchronous culture:

For non-synchronous culture, cells were grown in liquid TAP medium for 24 hours on a culture rotator drum at 25 °C under continuous illumination with an LED LumiBar with independent red and blue light control (LumiGrow, Inc.).

Light/Dark synchronization (L-D synchronization):

Cells were inoculated in minimal medium (M1 medium) from the TAP plates and kept in light for 12 hours and then dark for 12 hours alternating at 25 °C for at least three days. After each light/dark cycle (12 hour/12 hour), cultures were diluted to 2 x 10^5 cells ml^{-1} with fresh M1 medium. On the 4th day, after growing at light phase for 5 hours, cultures were immediately transferred to TAP medium prior to chemical treatment.

Synchronization by nitrogen starvation (M-N synchronization):

M-N synchronization was attained by inducing gametogenesis in nitrogen free minimal medium (i.e, M minus N) for 18-20 hours in continuous light at 25 °C under a LumiBar. These cells were then transferred to TAP medium for 4 hours prior to chemical treatment (Fig. S1).

Flagellar length synchronization (F-L synchronization):

For F-L synchronization, *Chlamydomonas* cells were grown in liquid TAP medium and then induced to regenerate flagella after acid-mediated flagellar excision [38]. 60 µl of 0.5 N acetic acid was added to 1 ml of cells for deflagellation (pH= 4.5). Immediately after 45 seconds, 70 µl of 0.5 N KOH was added to neutralize the medium which ultimately induced the flagellar regeneration. Wild type cells were grown for 3 hours for flagella-regeneration under continuous illumination with a LumiBar on a rotator drum.

Flagellar length and Cell volume measurement:

For flagella measurements, cells were fixed in 1% glutaraldehyde and kept at 4 °C. Cells were then centrifuged at 1000 x g for 1 minute and mounted between a glass slide and coverslip. Imaging was performed using Zeiss Axioscope differential interference contrast (DIC) microscope with a 40X objective lens and a Zeiss Axiocam 105 color camera. Flagellar length measurements were done by line segments and spline fitting using Image J software (NIH, USA). All flagella in a particular field were considered and at least 50 flagella were measured at each time point. For cell size determination, cell volumes were calculated using the ellipsoid equation $4/3\pi\ [L/2][W/2]^2$, where L is cell length and W is cell width [39]. Flagellar length distributions and cell volumes were plotted using GraphPad Prism software version 6 (GraphPad, USA).

Flagellar precursor pool determination:

For flagellar precursor pool determination, cells were allowed to regenerate their flagella in the presence of 10 µg ml^{-1} cycloheximide following deflagellation [40]. Cells from different synchronization methods were induced to regenerate flagella after acidic shock and then returned to neutral pH by adding KOH as described above and subjected to cycloheximide treatment immediately. For precursor pool determination in F-L synchronized cells, cells were allowed to regenerate their flagella for 2 hours, 3 hours and 5 hours after the 1st deflagellation and then subjected to a 2nd deflagellation prior to cycloheximide treatment. For all cases, cells were centrifuged at 1000 x g for 2 minutes after neutralizing and then

resuspended in TAP medium containing cycloheximide. Cells were placed on a rotator for 120 minutes and flagellar length measurements were carried out to determine the amount of unassembled limiting flagellar protein.

Statistical Analysis:

Statistical analysis was performed using GraphPad Prism software version 6. Descriptive statistics were expressed as mean and standard deviation (SD). Non parametric Mann-Whitney U test was performed for comparing two means. One-way analysis of variance (ANOVA) and Bonferroni's post hoc test were performed for multiple comparisons and to determine p values. For all datasets, $p<0.05$ was considered statistically significant. Frequency distribution and column statistics were carried out for determining mode and kurtosis. For curve fitting and slope determination, we performed non-linear regression with robust fit.

Results:

Flagellar length synchronization narrows the steady state flagellar length distribution:

Synchronous culture provides a better way to address cell cycle and related flagellar kinetics. However, cell cycle synchronization methods provide only partial synchronization and thus show high variance in flagellar length. To obtain 100% flagellar length (F-L) synchronization, we use an inherent property of *Chlamydomonas*, which is their ability to regenerate the flagella after amputation [41]. We first performed different synchronization methods and then compared their steady-state flagellar lengths to each other and to non-synchronized cells (Fig.1A, Table S1). For F-L synchronization, we tested different regeneration time intervals following deflagellation to determine the time point at which flagellar length shows minimal variability. We found that the pre-deflagellation flagellar length distribution was broad, which was expected as our starting culture was non-synchronous and contained a heterogeneous population of cells (Fig. 1B, red). After deflagellation, all flagella started to grow synchronously but the length distribution still remained broad after 2 hours of regeneration. At 2 and 2.5 hours, some cells were still in the 8-9 µm range and did not reach their original length (Fig. 1B, light green). The distribution narrowed and became highly homogeneous at 3 hours (Fig. 1B, green, Table S2). However,

the length-distribution remained narrow only for a short time, expanding again within 30 minutes and increasing with time (Fig. 1B, deep green). This timing was highly reproducible and Figure 1B is the combined result of three independent experiments. Based on the standard deviation graph of these distributions (Fig. 1B, lower panel), we selected 3 hours regeneration time post-deflagellation as the flagellar length synchronization time and the time at which to initiate further experiments. Likewise, after determining conditions of minimal variability for each synchronization method, we measured the steady state flagellar lengths for comparative analysis. The results revealed that the mean flagellar lengths at steady state were almost equivalent for all methods (Table S1). As expected, non-synchronous cells had larger variability compared with all other synchronized cells (Fig. 1A, red). The F-L synchronization method shows a remarkably narrow spread of measurements around the mean and the smallest variability of length across all synchronization methods (Fig. 1A, green and standard deviation bar graph in lower panel, Table S1). In contrast, conventional L-D synchronization and M-N synchronization have comparatively wider distributions (Fig. 1A, blue and purple respectively, Table S1) compared to F-L synchronization (green). Our findings suggest that the F-L synchronization is the most effective method for achieving maximum flagellar-length homogeneity.

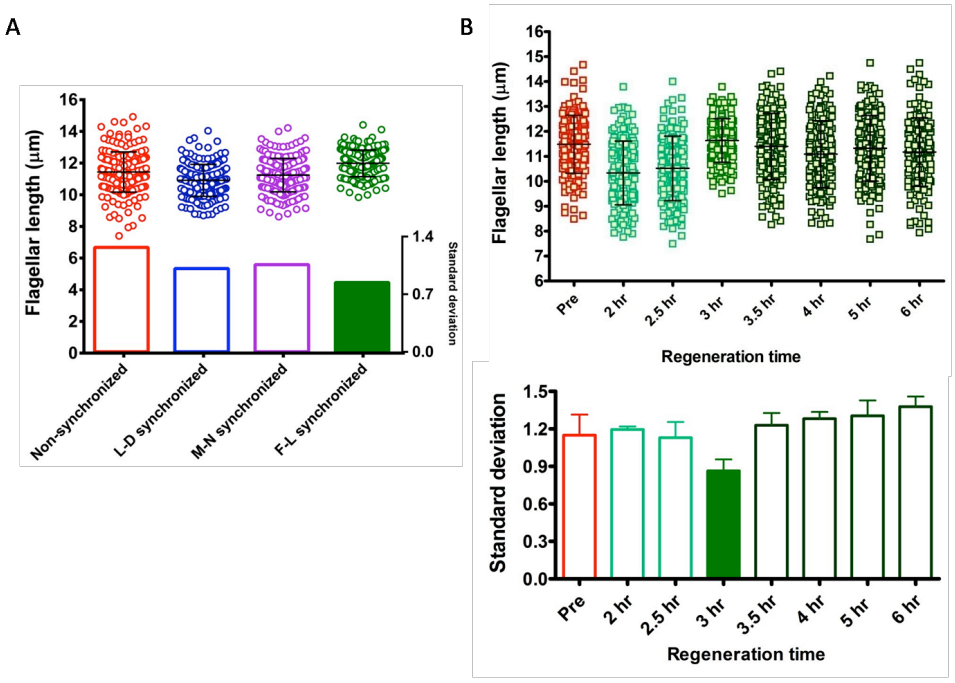

Figure 1: F-L synchronization narrows the flagellar length distribution compared to other synchronization methods. **A)** Distribution of steady state flagellar length after different synchronization methods. Non-synchronous cells were taken as control and steady state flagellar lengths were measured after each synchronization method as described in the text. Standard deviations of each distribution are shown below the individually plotted values. **B)** Wild type flagellar-length distribution at various time intervals during the regeneration after amputation. Pre-

deflagellation non-synchronous cells (pre) are shown in red. Regeneration was carried out for indicated times after deflagellation by pH shock (green). Lighter and darker green indicates before and after the time of F-L synchronization respectively. Combined data from three independent experiments are represented (N=50/each). Standard deviations are expressed as bar graphs in the lower panel. The filled SD bar represents F-L synchronization.

Effects of chemical perturbations are easily detected upon F-L synchronization:

Flagellar length can be perturbed chemically. If the perturbation has a small effect on flagellar length, high variance in the system may mask observed phenotypes. Our observations demonstrate that F-L synchronized cells have reduced variance in flagellar length compared to other methods (Fig.1A). Therefore, we tested the effects of several known flagellar length altering agents after reducing variability in the initial population through F-L synchronization and compared the results with other synchronization methods. When flagellar shortening was induced with 3-isobutyl-1-methylxanthine (IBMX) [42], latrunculin B (LatB) [43], and sodium pyrophosphate (NaPPi) [44], we observed more severe shortening in F-L synchronized cells compared to all the other methods (Fig. 2A-C, green, Table S3). Only L-D synchronization, which is a more time consuming synchronization method, demonstrates length reduction comparable with F-L synchronized cells (Fig. 2A-C, blue, Table S3). Effects on flagellar length are the most extreme in the case of NaPPi mediated length resorption (Fig. 2C). After the treatment, flagellar length distribution was significantly narrower in F-L synchronized cells compared to the others and produced a more dramatic shortening of the mean flagellar length (Fig. 2C, Table S3). L-D synchronized cells showed a reduced effect (~40% shortening) compared to F-L synchronized cells (~46% shortening) (Fig. 2C, lower panel). In addition to flagella shortening compounds, we also tested the effects of lithium chloride (LiCl), which is known to lengthen flagella [6]. The effect on flagellar length was the most dramatic in F-L synchronized cells after treatment of LiCl. While the variance and % change in mean flagellar length were comparable between L-D and F-L synchronized cells, each of the LiCl treated F-L synchronized cells had flagellar length longer than 13.5 μm with an average of 17 μm (Fig. 2D, green, Table S3). Broad distributions of lengths were observed in both non-synchronized and M-N synchronized cells ranging from 9 μm to 20 μm (Fig. 2D, red and purple respectively). As a result, the effect on flagellar length was less apparent. Taken together, all of these data demonstrate that the effect of each chemical is more prominent and detectable in F-L synchronized cells when the variance in starting flagellar distribution is minimized.

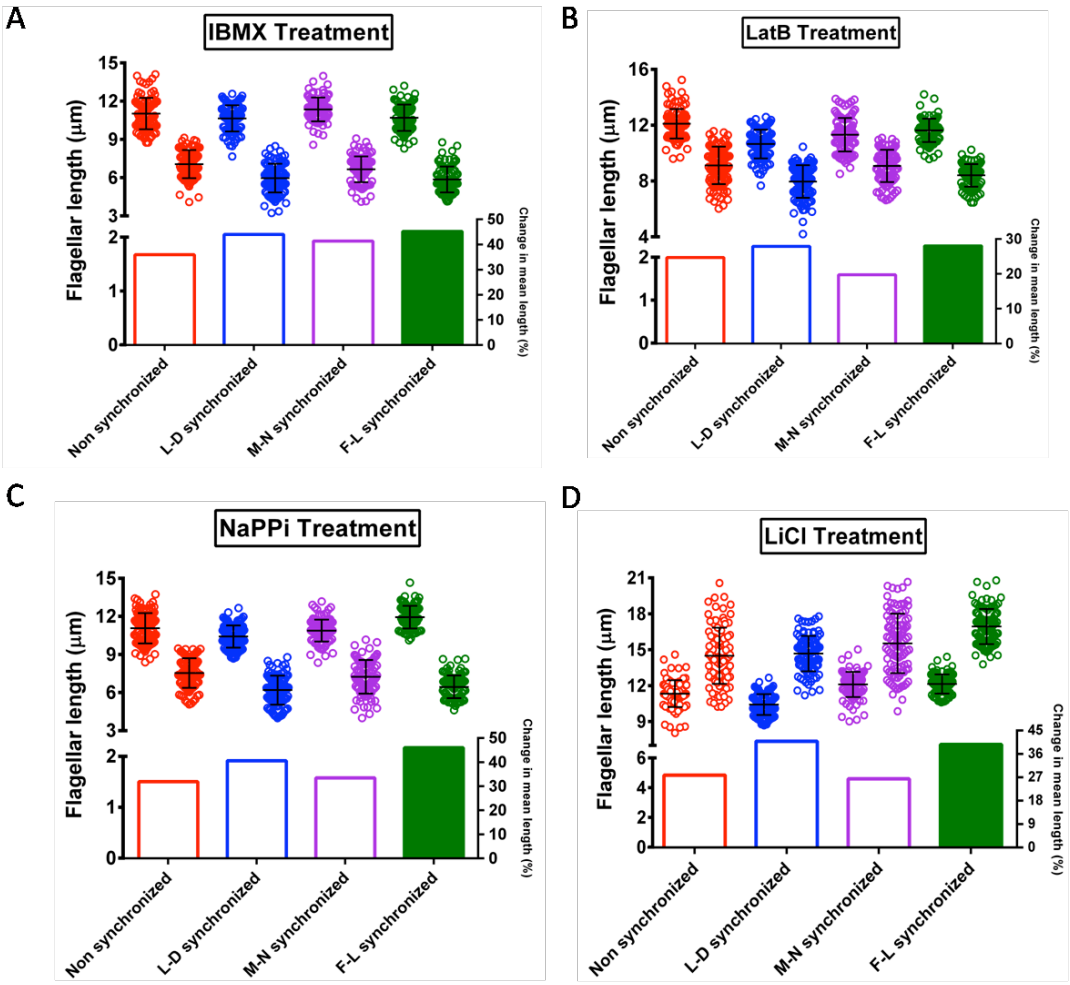

Figure 2*:* Effects of length-altering chemical treatments are more apparent after F-L synchronization. Flagella were measured from each cell after incubation with appropriate concentration of different chemicals for 90 minutes: **A)** 0.4 mM IBMX, **B)** 10 µM LatB, **C)** 10 mM NaPPi and **D)** 25 mM LiCl. For each pair: non-synchronized (red), L-D synchronized (blue), M-N synchronized (purple) and F-L synchronized (green), the first and second distributions represent control and treated respectively. N=100 cells (one flagellum per cell). Bars are mean and standard deviation (top half of panel). In each case, the percentage change in mean flagellar length is shown below individual plotted values.

Synchronization time varies in flagellar length mutants:

In *Chlamydomonas*, flagellar length mutants with both long and short flagella have been isolated [45-47]. Length distributions are reportedly wider in flagellar length mutants than wild type cells [46]. Therefore, we asked if F-L synchronization would increase our ability to detect length differences in these populations. Some long flagella mutants have defective regeneration kinetics after amputation [46] and therefore are not suitable for F-L synchronization. However, some mutants have regeneration kinetics comparable to wild type cells [46]. We first considered the *lf4-7* mutant cells which have the longest flagella of all identified long flagella (lf) mutants but can regenerate their flagella with wild type regeneration kinetics after amputation [47]. As expected, prior to deflagellation, the initial

population of *lf4-7* cells were distributed very widely (12 µm to 28 µm) (Fig. 3A, upper and lower panel, Table S4). Following amputation, we found at least four hours were required to regenerate flagella to their pre-deflagellation length. Like wild type cells, after reaching their original length it also takes extra time (6 hours of regeneration) to become homogeneously distributed (Fig. 3A, indicated by *). The tighter distribution could be maintained for only a short period of time. In the case of the short-flagella mutant *shf1-253* [45], flagella reached their pre-deflagellation length within 1.5 hours following deflagellation, but took an additional one hour (total 2.5 hours) to distribute more narrowly (Fig. 3B, upper and lower panel, * indicates synchronization time, Table S4). Finally, we studied *cnk2-1* mutant cells, which have only slightly longer flagella than wild type [48]. Like other length-mutants, these cells regenerated to normal length after 3.5 hours following amputation but distributed more narrowly at 5 hours of regeneration (Fig. 3C, upper and lower panel, indicated by *, Table S4). All of these findings suggest that all cells exhibit their tightest flagellar length distribution after they initially reach pre-deflagellation mean lengths and that the amount of time needed to reach minimum variance depends on the initial steady state flagellar length. In other words, mutants with longer flagella take longer and mutants with shorter flagella take less time to distribute more homogeneously. Thus, the optimal time for flagellar synchronization should be adjusted for individual strains.

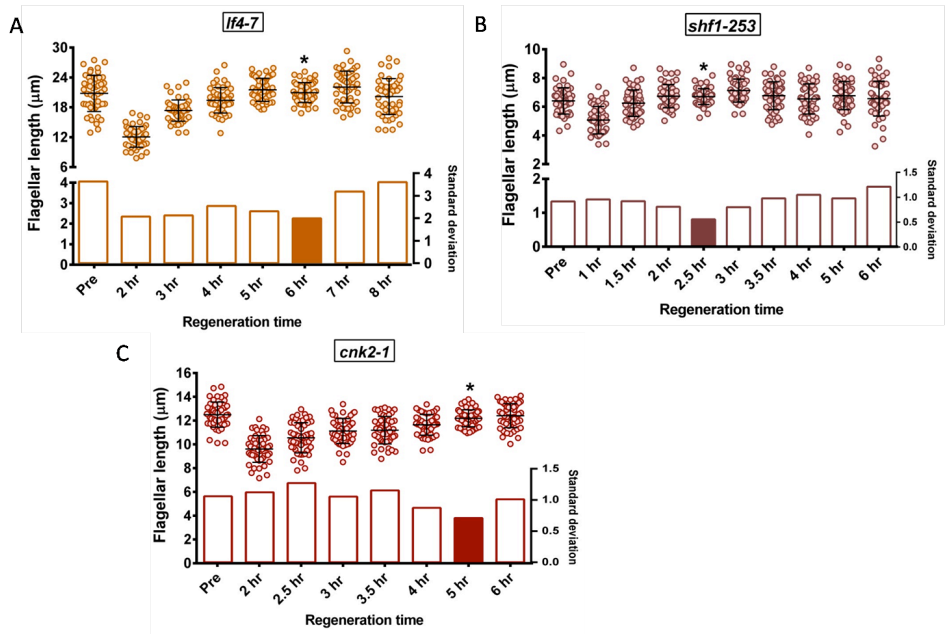

Figure 3: F-L synchronization time varies in different length mutants. For each mutant, distributions of flagellar length during regeneration are shown. **A)** long-flagella mutant, *lf4-7*, **B)** short-flagella mutant *shf1-253* and **C)** mildly long flagella mutant *cnk2-1*. 'Pre' represents the steady state length of the mutant pre-deflagellation. Bars are mean and standard deviation (top half of each panel). The asterisk indicates flagellar length synchronization time selected in each mutant on the basis of

minimal SD. N=50/ea. Standard deviations are represented by bar graphs in the lower half of each figure and the filled bar corresponds to the synchronization time for each mutant.

F-L synchronization may mask important outliers:

Some flagellar length mutants have a mean flagellar length comparable to wild type cells but have a positively skewed distribution that includes small numbers of extremely long flagella [46]. As, F-L synchronization reduces length variance, we asked if the informative long outliers would be lost after minimizing variability and thereby decrease our ability to appropriately phenotype this type of mutant. In order to test this, we chose two long-flagella mutants *lf2-5* and *lf3-2*, which were able to regenerate their flagella normally and had a large number of flagella in the wild type range [46]. When we induced regeneration for these two mutants up to 8 hours, we found that F-L synchronization time for both *lf2-5* and *lf3-2* was 4 hours (data not shown). The flagellar length distributions of *lf3-2* cells demonstrated that synchronized cells had a narrow distribution with flagella no longer than 20 µm and no shorter than 10 µm, which was expected. The synchronized distribution have a negative kurtosis (-0.3738) i.e, a distribution with short tails compared to the non-synchronized distribution which has a positive kurtosis (+0.0358) with relatively long tails. The average length of synchronized compared to the non-synchronized population was not changed significantly (Fig. 4A). The mode changed from 12.5 to 16 with the number of cells containing 13 µm to 16 µm long flagella increasing remarkably in case of F-L synchronized cells (Fig. 4A). Therefore, for *lf3-2,* F-L synchronization did not affect our ability to identify a mutant phenotype despite eliminating long outliers. In the case of *lf2-5*, F-L synchronization also removed outliers from the mutant population (Fig. 4B) but this time the average flagellar length between non-synchronized and synchronized cells differed markedly (Fig. 4C) by left-shifting the distribution. The mode changed from 16 to 13 and *lf2-5* showed average flagellar length value ~14 µm after F-L synchronization, which sometimes is seen in wild type populations (Fig. S2). Also, non-synchronized and synchronized kurtosis values (-0.6492 and -0.6753 respectively) are not significantly different. While F-L synchronization of *lf3-2* and *lf2-5* retains our ability to discriminate between wild type and mutant phenotypes by reducing the variance (Fig. 4C), the two mutants behave differently with respect to changes in descriptive statistics. Therefore, losing outliers during F-L synchronization has the potential obscure important information when evaluating genetic perturbations. We therefore recommend testing both synchronized and non-synchronized cells when characterizing new mutants.

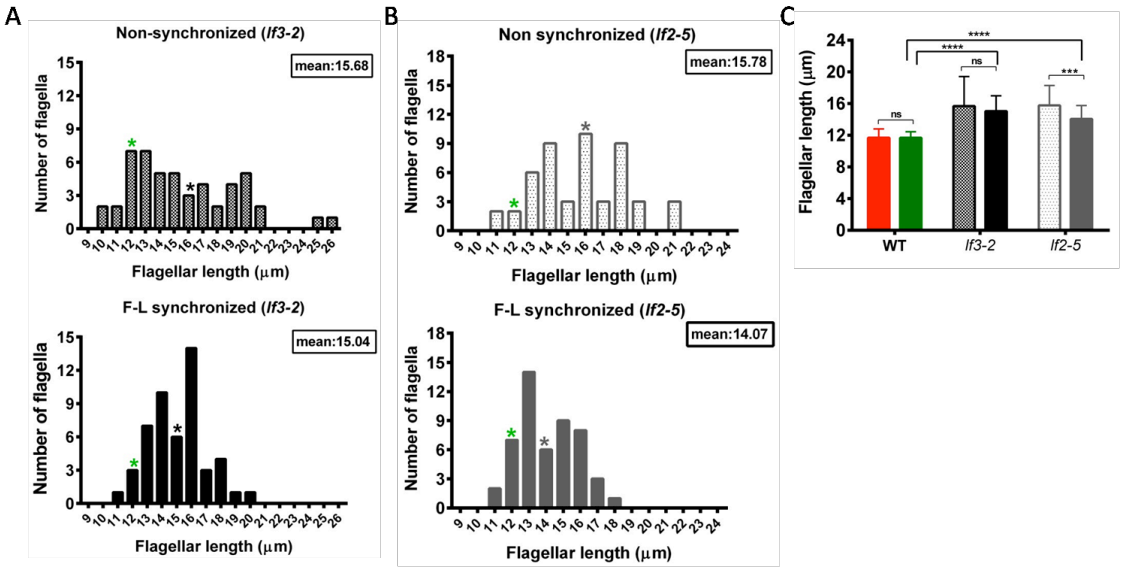

Figure 4: Distributions of flagellar length in long-flagella mutants before and after F-L synchronization. **A)** *lf3-2* and **B)** *lf2-5*. The upper panel of figure A and B represent the distribution of flagellar length in non-synchronous cells. Cells were then deflagellated by acidic shock and regenerated for 4 hours for F-L synchronization. Lower panel of figure A and B correspond to the distribution of flagellar length after F-L synchronization. N=50/ea (zeros excluded). The mean flagellar length of each mutant is marked by asterisk. Green asterisk indicates the mean flagellar length observed in wild type controls. The mean flagellar length of wild type and mutant cells were compared using Bonferroni's post hoc test and the data were represented in figure **(C).** The first and second bars within each pair represent mean flagellar length of non-synchronized and synchronized cells respectively. Mann-Whitney U test was performed for comparing two means in a pair and Bonferroni's post hoc test was performed for multiple comparisons. Asterisks indicate significant differences (**** $p \leq 0.0001$, *** $p \leq 0.001$, ns $p > 0.05$).

Flagellar length variability is related to precursor pool variability:

As flagellar synchronization time is highly reproducible within wild type populations, we hypothesized that there might be an internal regulator which is responsible for the narrow distribution pattern after 3 hours of regeneration. *Chlamydomonas* cells have a synthesized pool of unassembled flagellar proteins or at least a pre-existing pool of some protein that limits the rate of flagellar assembly (termed precursor pool). The size of this pool is sufficient to assemble flagella to half of their normal length if new protein synthesis is inhibited [40]. Limiting-precursor models of flagellar length control have been previously considered but flagellar length appears to be maintained independent of pool size or concentration [45]. However, completely blocking new protein synthesis can limit flagellar length so we asked if putting constraints on protein synthesis and incorporation might narrow the resulting flagellar length distribution. In this case, reduced variability during F-L synchronization would be due to synchronizing flagellar protein synthesis through deflagellation and time-limiting flagellar protein incorporation. In order to test our hypothesis, we determined the variance of

synthesized precursor pool size after different synchronization methods by deflagellating cells and allowing them to regenerate in the presence of cycloheximide. This allows existing flagellar protein to be incorporated into flagella but prevents the synthesis of new protein. To evaluate the relationship between flagellar variability and precursor pool variability, we compared flagella that had undergone 3 hours of regeneration (tightest distribution), and two other time points yielding increased variability: 2 hours and 5 hours (Fig.1B). We performed the same for non-synchronized, L-D synchronized and M-N synchronized cells. As expected, all cells after synchronization but prior to deflagellation for cycloheximide treatment recapitulated our previous findings (Fig. S3, Table S5). When we compared variance of precursor pools after different regeneration time intervals, we found the narrowest distribution of pool size at 3 hours of regeneration (green) but not in 2 hours (light green) or in 5 hours (deep green) (Fig. 5A, lower panel of 5B, Table S5). Non-synchronous cells have a precursor pool distribution comparable to 2 hours and 5 hours of regeneration (Fig. 5A, red). For all F-L synchronized cells, we observed that the variance of the precursor pool (Fig. 5B, lower panel) echoes the post-synchronization length variance (Fig. 5B, upper panel). We speculate that this is because we have limited the amount of time that the precursor pool can build up with F-L synchronization and that within this 3 hour time window, flagellar length is still more strongly influenced by deflagellation-induced protein synthesis than other factors. We suspect that the slower growing cells that produce shorter flagella after 2 hours of F-L synchronization also produce a smaller precursor pool. We also saw that L-D synchronized cells showed the minimum variance in cytoplasmic precursor pool of flagellar proteins (Fig. 5A, blue, Table S5) with M-N synchronized cells having a comparable precursor pool distribution (Fig. 5A, purple, Table S5). Because both L-D and M-N synchronization are cell cycle synchronization methods, we asked next if the narrow precursor pool variability in these cells correlated with the cell size distribution and if we were circumventing the cell size dependence of the flagellar precursor pool by time-limiting protein synthesis and incorporation.

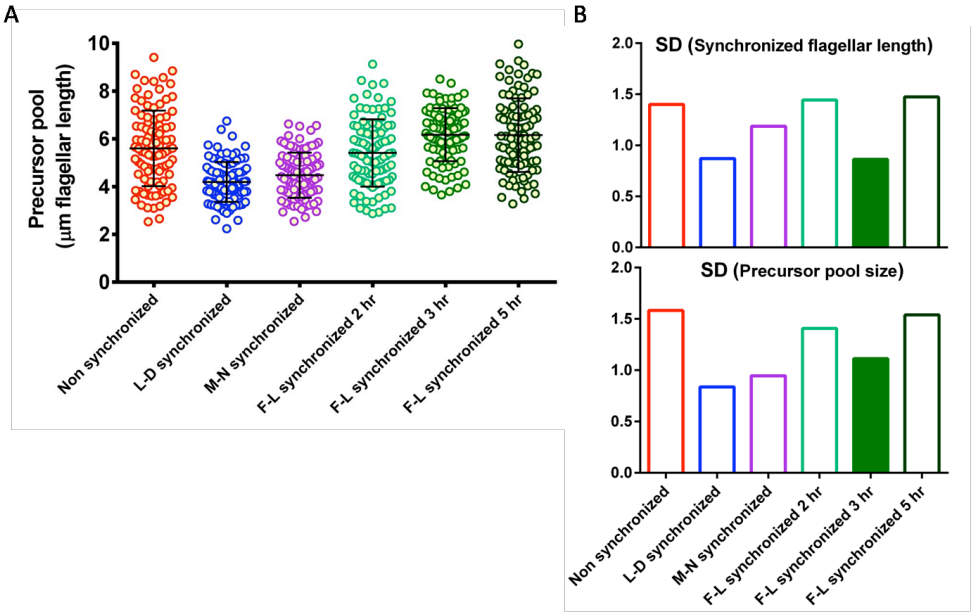

Figure 5: Relationship between flagellar length and precursor pool distribution. **A)** Flagellar precursor pool distributions (as determined by regeneration in cycloheximide after different synchronization methods). Non-synchronous (red), L-D synchronized (blue), M-N synchronized (purple), F-L synchronized for 2 hours (light green), F-L synchronized for 3 hours (green) and F-L synchronized for 5 hours (deep green) are compared. F-L synchronization for 3 hours shows minimal variability among F-L synchronized cells. N=100. Bars are mean and standard deviation. **B)** Post-synchronization standard deviations (top panel) and corresponding precursor pool standard deviations (bottom panel) of each distribution is represented by bars with matching colours. Cell cycle synchronized cells show the smallest variability in precursor pool distribution (blue, bottom panel). Of F-L synchronized cells (green), 3 hours synchronization shows the smallest variability in precursor pool distribution (filled bar, bottom panel).

Flagellar length synchronization breaks the relationship between cell size and precursor pool size:

It is reported that there is no simple relationship between cell size, flagellar length and precursor pool size [49]. However, we observed that variability of flagellar length is directly related to the variability in pool size (Fig. 5B). To better understand the role of the precursor pool in flagellar length variance, we tested the relationship between cell size and precursor pool size across synchronization methods. We regenerated the flagella for 2 hours in the presence of cycloheximide after different synchronization methods and measured flagellar length to determine the pre-existing precursor pool as before. This time, we also measured the corresponding cell volume. We found that non-synchronous cells which had variable cell sizes ranging from ~100 μm^3 to 900 μm^3, also showed a large range of precursor pool size with a flagellar length range of ~6.5µm (Fig. 6A). However, both in L-D and M-N synchronized cells, cell volumes were very restricted, ranging from ~100 μm^3 to ~400 μm^3 (Fig. 6B and C). This is unsurprising, as all cells are cell cycle synchronized. Since smaller

cells produce less protein [50], restricted cell volumes of cell cycle synchronized populations also limited the protein precursor pool. Thus, precursor pool in those cells was mostly distributed within a very narrow range (~4.5 μm flagellar length) (Fig. 6B and C). On the other hand, F-L synchronized cells, despite having a broad cell volume range like non-synchronized cells, retained a narrow range of precursor pool sizes as we have limited the amount of time the precursor pool can accumulate (Fig. 6D). In this case, flagellar lengths were mostly distributed within a small range (~4.5 μm) like cell cycle synchronized cells. The relation between cell volume and precursor pool distribution can be understood better if we compare the slopes of these graphs. Non-synchronous cells, where precursor pool variance increases with increasing cell volume have the largest slope among all (slope= 0.1076). L-D synchronized cells which have shown both restricted cell volume and precursor pool distribution demonstrate the smallest slope (slope= 0.0050). Despite having a narrow range of cell volumes, M-N synchronized cells have a comparatively wide flagellar length distribution, reflected in their comparatively higher slope (slope= 0.0079). Conversely, F-L synchronized cells with lower precursor pool variance showed equivalent slope to L-D synchronized cells despite of having wide range of cell volumes (slope= 0.0056). F-L synchronization, therefore, reduces the dependence of the precursor pool on the cell size distribution. In other words, by limiting regeneration to only 3 hours in F-L synchronized cells, we break the relationship between cell size and available unassembled flagellar protein.

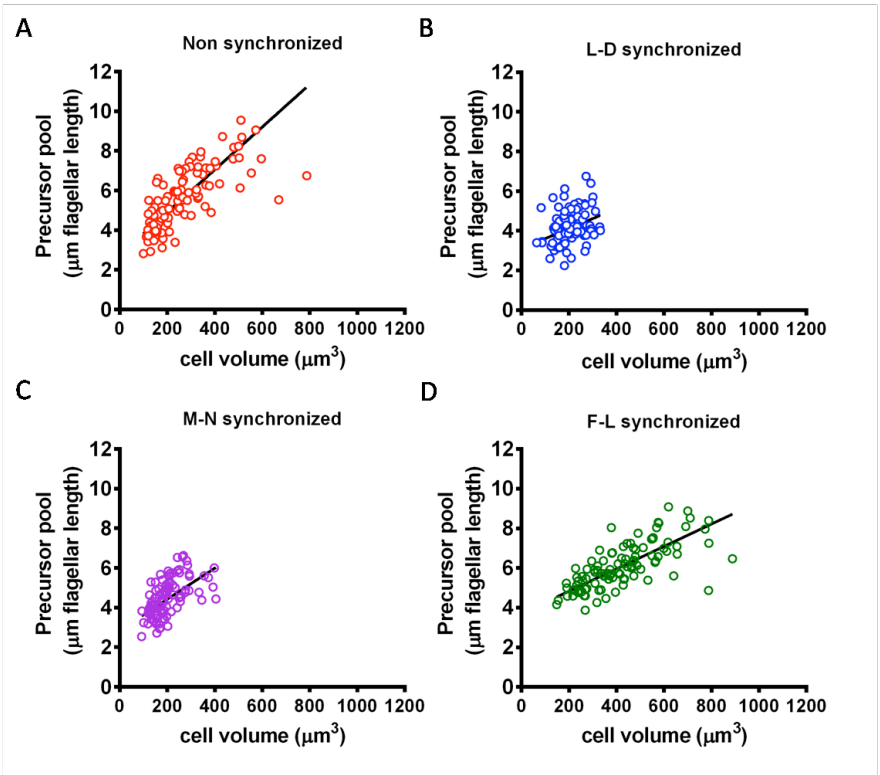

Figure 6: Cell size and flagellar precursor pool size are correlated. Flagellar length in cycloheximide, which corresponds to the precursor pool, is plotted along the Y axis. Matching cell volume is plotted along the X axis; **A)** Non-synchronized (red) **B)** L-D synchronized (blue), **C)** M-N synchronized (purple) and **D)** F-L synchronized (green). N=100. The straight line represents the best fitted line through the data point which was drawn after non linear robust fit regression. L-D and M-N synchronized cells have narrow cell volume and precursor pool ranges. F-L synchronized cells maintain a narrow precursor pool range without limiting cell volume.

Discussion:

Here we have shown a powerful new approach to understanding ciliary length-related biology by characterizing a synchronization method that minimizes flagellar length variability. It was previously known that flagellar length variability can be controlled by restricting the cell size (the basis of cell cycle synchronization). Our data suggests that the precursor pool size is also related to the cell size. Because cytoplasmic flagellar proteins in *Chlamydomonas* clearly exceed the amount assembled into flagella, unassembled flagellar precursor pool size does not correlate with the flagellar length during assembly and the length of flagella do not appear to be strongly dependent upon the number of flagella in mutants with variable flagellar number, precursor pool size was not considered a major regulator of flagellar length [44,49,51]. However, our novel finding is that we can circumvent the cell size dependence of flagellar length by limiting the amount of time the flagellar precursor pool can accumulate and incorporate into flagella. It is well known that mammalian cell ciliary studies

often initiate ciliogenesis by serum starvation of confluent cells [5]. By standardizing plating density and limiting serum starvation time prior to subsequent experimentation (thereby limiting the time window of assembly), the F-L synchronization method may be expandable to studies in mammalian cells.

Assembly of full length flagella requires pre-existing precursor pool and *de novo* synthesis of flagellar precursor proteins and also incorporation of those proteins into the flagellar structure [52]. The expression of genes encoding ciliary proteins is dramatically up-regulated after flagellar amputation to replenish the precursor pool and to provide proteins required for flagellar assembly [53]. We propose that F-L synchronization via deflagellation works by stimulating a highly regulated program of gene expression and flagellar protein incorporation so that all cells can regenerate their flagella synchronously regardless of their divisional phase. Synchronization is then achieved by limiting the time window that the cells are allowed to regenerate their flagella (three hours). The combination of simultaneous induction of the regeneration program along with imposing a restriction of the amount of protein synthesis and incorporation is reflected in a tighter length distribution pattern.

If time limiting protein synthesis and incorporation results in a narrow distribution of flagellar lengths, why is there an increased variability of flagellar lengths at an earlier flagellar synchronization interval (2 hours)? Rates of flagellar regeneration vary from cell to cell; some flagella are fast-growing and attain their original length within 2 hours of amputation whereas some slow-growing flagella can reach only 80% of their length within that period. We saw in measurements of unassembled flagellar protein that cells that have undergone 2 hours of F-L synchronization have a smaller precursor pool (mean: 5.4 μm flagellar length) compared to 6.2 μm for 3 hours F-L synchronization (Fig. 5B, lower panel, Table S5). The slow growth of some cells at 2 hours post-deflagellation may therefore be due to reduced protein synthesis and accumulation. We propose that, as we extend the regeneration time beyond two hours, slow-growing flagella finally catch up their original length and fast-growing flagella reach their maximum length by reducing their assembly rate to balance disassembly and reach equilibrium. To confirm this, we would need data on the individual cell level rather than at the population level, which we hope to perform in the future by trapping the motile cells in a microfluidic chamber [54].

While all cells must initiate a regeneration program upon deflagellation, with increasing time, the deflagellation-induced protein synthesis and incorporation program (which decreases as a function of time and flagellar length) may be overcome by other regulating factors such as disassembly, fragmentation, proteosomal degradation, feedback

control or autophagy [55-59]. Also, when the regeneration program is not tightly regulated at longer time points, cell cycle regulation may dominate the regeneration program resulting in the heterogeneous flagellar length and precursor pool distributions seen in non-synchronized cells.

The most commonly used method of cell cycle synchronization in *Chlamydomonas* is L-D synchronization, however, in L-D synchronized cells, natural variance of cells [37] prevents 100% synchronization of flagellar length. Using F-L synchronization, we can synchronize 100% of the population through deflagellation and produce a homogeneous distribution of length by 3 hours. Conventional L-D synchronization, by contrast, requires at least three days achieving comparable levels of homogeneity. F-L synchronization does not require a dark chamber with automated light switching. Moreover, the entire experiment can be performed in rich medium like TAP instead of minimal medium which is very sensitive to changes in pH. This facilitates the use of inhibitors that would otherwise dramatically affect pH of the medium. F-L synchronization demonstrated equivalent or stronger effects of length-altering chemicals on flagellar length compared to L-D synchronization.

The study presented in this article reveals the simplicity and usefulness of flagellar length synchronization, which can be coupled with the study of ciliary swimming, beating or chemical sensing. More broadly, it helps us to better understand both the basic biology of ciliary length regulation as well as ciliary length-related defects [60-63] by increasing our ability to detect small changes in cilia size.

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Author Contributions:

Experiments were designed by SD and PA. All experiments and flagellar/cell measurements were carried out by SD. Manuscript was written by SD and PA.

Conflicts of Interest:

None.

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