

Journal of Visualized Experiments

Synchronization of *Caulobacter crescentus* for investigation of the bacterial cell cycle --Manuscript Draft--

Manuscript Number:	JoVE52633R2
Full Title:	Synchronization of <i>Caulobacter crescentus</i> for investigation of the bacterial cell cycle
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	<i>Caulobacter crescentus</i> , cell cycle, asymmetric cell division
Manuscript Classifications:	1.20.812: Chromosomes, Bacterial; 2.3.440: Gram-Negative Bacteria; 2.3.660.50: Alphaproteobacteria; 7.4.299.134: Cell Cycle; 7.4.299.134.220: Cell Division; 7.5.355: Genetic Processes; 7.5.355.105: Cell Division; 7.5.355.315: Gene Expression Regulation; 7.5.355.600.164: Chromosome Duplication; 7.6.590.110: Bacterial Processes
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Abstract:	The cell cycle is important for growth, genome replication, and development in all cells. In bacteria, studies of the cell cycle have focused largely on unsynchronized cells making it difficult to order the temporal events required for cell cycle progression, genome replication, and division. <i>Caulobacter crescentus</i> provides an excellent model system for the bacterial cell cycle whereby cells can be rapidly synchronized in a G0 state by density centrifugation. Cell cycle synchronization experiments have been used to establish the molecular events governing chromosome replication and segregation, to map a genetic regulatory network controlling cell cycle progression, and to identify the establishment of polar signaling complexes required for asymmetric cell division. Here we provide a detailed protocol for the rapid synchronization of <i>Caulobacter</i> NA1000 cells. Synchronization can be performed in a large-scale format for gene expression profiling and western blot assays, as well as a small-scale format for microscopy or FACS assays. The rapid synchronizability and high cell yields of <i>Caulobacter</i> makes this organism a powerful model system for studies of the bacterial cell cycle.
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Dear Sephora,

Please find our revised manuscript. We thank you and the reviewers for your thorough review of the manuscript. We have addressed the major concerns by correcting an error in the M2 salts recipe present in Table 1. Our responses to each comment are in red, and each change in the manuscript was done with the track-changes feature in Microsoft Word as requested. We believe you will find our revised version of the manuscript to be improved.

**Sincerely,
Jared Schrader**

TITLE: *Synchronization of Caulobacter crescentus for investigation of the bacterial cell cycle*

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KEYWORDS:

Cell cycle, cell biology, systems biology, synchronization, *Caulobacter*, asymmetric cell division

SHORT ABSTRACT:

Synchronization of bacterial cells is essential for studies of the bacterial cell cycle and development. *Caulobacter crescentus* is synchronizable through density centrifugation allowing a rapid and powerful tool for studies of the bacterial cell cycle. Here we provide a detailed protocol for the synchronization of *Caulobacter* cells.

LONG ABSTRACT:

The cell cycle is important for growth, genome replication, and development in all cells. In bacteria, studies of the cell cycle have focused largely on unsynchronized cells making it difficult to order the temporal events required for cell cycle progression, genome replication, and division. *Caulobacter crescentus* provides an excellent model system for the bacterial cell cycle whereby cells can be rapidly synchronized in a G0 state by density centrifugation. Cell cycle synchronization experiments have been used to establish the molecular events governing chromosome replication and segregation, to map a genetic regulatory network controlling cell cycle progression, and to identify the establishment of polar signaling complexes required for asymmetric cell division. Here we provide a detailed protocol for the rapid synchronization of *Caulobacter* NA1000 cells. Synchronization can be performed in a large-scale format for gene expression profiling and western blot assays, as well as a small-scale format for microscopy or FACS assays. The rapid synchronizability and high cell yields of *Caulobacter* make this organism

a powerful model system for studies of the bacterial cell cycle.

INTRODUCTION:

The bacterial cell cycle controls both the replication of the genome and the division of daughter cells. Importantly, as antibiotic resistance is a growing threat to public health, the bacterial cell cycle presents an untapped target for antibiotic development.

In the bacterium *Caulobacter crescentus*, each cell cycle leads to an asymmetric division, yielding two daughter cells of different fates (Fig. 1A) ^{1,2}. One daughter cell inherits a flagellum and is motile while the other daughter inherits a stalk and is sessile. An integrated genetic circuit controls cell cycle progression and cell fate by transcriptional regulation, phospho-signaling, and regulated proteolysis ³. In addition, chromosome replication and concurrent segregation yield daughter cells that contain exactly one copy of the chromosome ⁴. Importantly, these two cell types can be rapidly separated by colloidal silica particle density centrifugation in the synchronizable NA1000 strain ⁵⁻⁷ allowing the isolation of the swarmer cells from the rest of the population with high yields (Fig. 1B). Isolated swarmer cells then proceed synchronously through asymmetric cell division. Here, we detail the protocol used for synchronizing *Caulobacter* strain NA1000. We provide protocols and common troubleshooting tips for both large- and small-scale synchronizations. This experimental procedure provides a powerful tool to interrogate the spatiotemporal control of the *Caulobacter* cell cycle and cell fate.

PROTOCOL:

1. Large-scale synchrony - Optimal for western blot, microarray/RNA-seq, and other material intensive assays

1.1) From a freezer stock or a plate, grow a 5mL overnight culture of strain NA1000 by shaking at 28 °C in PYE medium.

1.2) Inoculate 0.5 mL of the cells from step 1.1 in 25 mL of M2G (Tables 1-2) and shake at 28 °C until the culture reaches an OD₆₀₀ between 0.5 and 0.6.

1.3) Inoculate the cells into 1 L of M2G and shake at 28 °C.

1.4) Once OD₆₀₀ reaches 0.5 to 0.6, confirm the presence of swarmer cells using liquid mounted phase microscopy. Spot 1 µL of cells on a glass slide, cover with a cover slip, and image by phase microscopy. Confirm the presence of swarmer cells by visualizing rapidly swimming cells in the population.

1.5) Spin cells for 15 min at 7 k x g at 4 °C in a JA-10 rotor.

1.6) Discard the supernatant and add 180 mL of cold M2 (Tables 1-2) and gently resuspend all the cells using a serological pipet. Discard loosely pelleted cells; they are predivisional and

stalked cells.

1.7) Add 60 mL of cold Colloidal silica solution (be sure to mix the Colloidal silica suspension well before adding to the cells) and mix the cell suspension well.

1.8) Pour the cell suspension into eight 30 mL tubes and spin for 30 min at 6.4 k x g at 4 °C in a JA-20 rotor. Note: One should see two distinct bands; the swarmer band is the lower band while stalked/predivisinal cells are in the top band (Fig. 1B).

1.9) Carefully aspirate the top band off and remove the liquid to ~1 cm above the swarmer band (the lower band).

1.10) (Critical) Using a Pasteur pipet, carefully remove the swarmer band and place into a clean tube. To wash away the Colloidal silica, top the tube off with cold M2 and spin for 10 min at 6.4 k x g at 4 °C in a JA-20 rotor.

1.11) Carefully discard the supernatant and resuspend the cells in 20 mL of cold M2 and spin for 10 min at 6.4 k x g at 4 °C in a JA-20 rotor.

1.12) Resuspend all the pellets into 30 mL of cold M2 and measure the OD₆₀₀ using a spectrophotometer and blank using cold M2 medium. Save 1 µL for phase imaging to check for swarmer cells; 90-95% of cells should be swarmers.

1.13) Spin down the cells for 5 min at 6.4 k x g at 4 °C in a JA-20 rotor. Resuspend cells in 28 °C M2G medium so that the A₆₀₀ is ~0.3-0.4 and begin shaking at 28 °C. Note: Typical yields are between 30 and 60mL of swarmer cell culture from 1L of unsynchronized cells.

1.14) Begin taking time points (wild type culture will take approximately 135-140 minutes to divide)^{8,9}. At each time point, measure the OD₆₀₀. Check that the OD₆₀₀ after division is approximately 2X the initial OD₆₀₀.

1.15) For western blot or gene expression assays, remove 1mL aliquots of the culture at the desired time points, spin down at max speed in a tabletop centrifuge for 30 seconds, rapidly decant or aspirate the medium, and flash freeze the cell pellet in liquid nitrogen. Store the cells at -80 °C until downstream analysis.

2. Small-scale synchrony – Optimal for microscopy

2.1) From a freezer stock or a plate, grow a 5 mL overnight culture shaking at 28 °C in M2G.

2.2) Dilute in 15 mL of M2G (Tables 1-2) and grow until mid-log (OD₆₀₀ = 0.5-0.6).

2.3) Spin at 6.4 k x g for 10 minutes at 4 °C in a JA-20 rotor, and resuspend in 1 mL cold M2 (Tables 1-2) and transfer to a 2 mL microcentrifuge tube.

- 2.4) Spin at 15 k x g for 3 min in a microcentrifuge tube to pellet cells, aspirate off the supernatant, put the pellet on ice, and resuspend in 900 μ L of cold M2.
- 2.5) Add 900 μ L of cold PVP coated colloidal silica and spin for 20 minutes at 15 k x g at 4 °C in a microcentrifuge tube.
- 2.6) (Critical) Aspirate or pipet off the top stalked/predivisional cell band and collect the bottom swarmer band into a new microcentrifuge tube.
- 2.7) Wash the swarmer cells two times in 1 mL of cold M2 while centrifuging at 15 k x g for 3 minutes.
- 2.8) Before the final spin, move the cells into a pre-chilled 1 mL glass test tube and measure the OD₆₀₀ of the cells compared to a blank of M2.
- 2.9) Resuspend the final cell pellet into 28 °C M2G at an OD between 0.3 – 0.4 and shake/roll cells at 28 °C. Note: Typical yields are between 2 and 4 mL of swarmer cell culture.
- 2.10) For microscopy experiments, at the desired time points place 1 μ L of cells onto an M2G agarose pad for imaging.

REPRESENTATIVE RESULTS:

Synchronization typically yields two bands of cells (Fig. 1B): the swarmer band, which has a higher density, and a stalked/predivisional cell band of lower density. To ensure efficient synchronization common controls include monitoring the OD₆₀₀ and measuring the levels of CtrA protein by western blot at distinct cell cycle time points. The OD₆₀₀ should increase by approximately 2 fold during the course of the cell cycle (Fig. 2). The western blot for the cell cycle master regulator CtrA is a useful control to verify a good synchrony (Fig. 2). CtrA is utilized in the swarmer cell to block DNA replication and is degraded upon the onset of DNA replication¹⁰. CtrA is then synthesized later in the cell cycle and activates transcription of a host of developmental genes including many components of the flagellum^{11,12}. A successful synchrony will have this oscillating pattern of CtrA protein levels.

Figure Legends:

Figure 1: The cell cycle of *Caulobacter crescentus*. (A.) A cartoon schematic of the swarmer cell cycle. The swarmer cells differentiate into replication competent stalked cells by retracting pili, ejecting flagella, and initiating DNA replication. The circles and theta structures shown inside the cell outlines represent quiescent and replicating chromosomes. Cells then progress through the cell cycle building a single flagellum at the pole opposite the stalk. Upon division, two unique cell types are generated, a replication blocked motile swarmer cell and a replication competent stationary stalked cell. (B.) Representative results for density centrifugation. Lower density stalked and predivisional cells float near the top of the gradient while dense swarmer

cells end up toward the bottom of the tube. Scale bars are 1 μm in phase microscopy images.

Figure 2: Representative results for a successful swarmer cell synchrony. The cell mass measured by OD₆₀₀ should slowly increase and ultimately double throughout the course of the assay. 1 mL aliquots were pipetted into plastic cuvettes from a large cell synchrony and the OD₆₀₀ measured using a spectrophotometer at the indicated time points. Additionally, the CtrA cell cycle master regulatory protein should be present in the swarmer cell to block DNA replication, followed by a rapid degradation coincident with the initiation of DNA replication. CtrA is then regenerated later in the cell cycle to activate expression of many developmental genes including critical flagellar and pili components. Western blots for cell cycle master regulatory protein CtrA were performed by taking 1 mL aliquots of a large-scale synchrony. The cells were resuspended in 250 μL Laemmli sample buffer per OD₆₀₀, separated on a 10% TRIS-GLY PAGE, transferred to PVDF, and blotted with anti-CtrA antibody. Failed synchrony procedures lead to CtrA western blots with no change in protein levels. α -CtrA antibody¹² was incubated at a 1:10,000 dilution for 1.5 hours in 3% milk TBST and washed 3 times in TBST. Goat- α -rabbit secondary was then added at 1:10,000 dilution in 3% milk TBST for 1 hour, washed 3 times with TBST, and imaged on film using a chemiluminescent detection kit.

Table 1. 20X M2 Salts Recipe.

Table 2. M2 and M2G recipe.

DISCUSSION:

The bacterial cell cycle is a fundamental process in life and is important for the study of growth and as a target for next generation antibiotics. Here, we detailed the rapid synchronization procedures for *C. crescentus* NA1000, a model organism for the study of the bacterial cell cycle and asymmetric cell division. This method is amendable to western blot, gene expression profiling, and fluorescence microscopy assays to investigate the spatiotemporal regulation of the bacterial cell cycle.

The protocol is quick and yields healthy synchronized cells. Other synchronization methods require long starvation of cells and rely on the stringent response to arrest the cell cycle in the same state, leading to cells that are dissimilar to cultures in logarithmic growth¹³. While “baby machines” and other surface attachment methods have been used successfully^{14,15}, they typically have low yields of synchronized cells. The density centrifugation protocols presented here allow higher purity and yields of synchronized *C. crescentus* NA1000 swarmer cells.

Additional complications can lead to potential problems throughout the synchrony procedure. Have a liquid mount phase microscope handy to optically check the purity of cells before synchronization and to check the purity of swarmers. A good synchrony will yield >95% swarmer cells. Check the OD₆₀₀ on a spectrophotometer to ensure the cells are doubling during the time course of the synchrony.

Cells don't synchronize as well in the presence of antibiotics or some mutations. If the strain does not carry a replicating plasmid, try to remove the antibiotics from the growth medium to improve swarmer cell yield. Cells grown in the presence of antibiotics or mutant strains will also have a slower cell cycle, so it is important to check the division time by microscopy for comparison to healthy strains.

Avoid vortexing the cells, as this will shear off the flagella and lower the quality of the synchrony. In preparation for the synchrony protocol don't grow the cells above OD₆₀₀ 0.5. This ensures healthy growth that isn't altered by high cell density. Be careful when aspirating/pipetting off supernatants containing the stalked and predivisional cell populations. In particular, do not touch the swarmer band which may lead to contamination and/or a loss in yield of the swarmer cells. The cell pellet will be very loose in the first wash of cells after removing them from the Colloidal silica. Be careful when removing the supernatant not to disrupt the pellet.

Occasionally NA1000 cells can lose synchronization capacity due to a loss of DNA in the prophage region of the genome⁷. In this case, no swarmer band is observed or the swarmer band intensity is dramatically reduced. If possible, collect cells if present in the swarmer band and streak onto a PYE plate to re-isolate the synchronizable cells. If working with a mutant strain, it is often useful to reintroduce the mutation into a fresh batch of NA1000. Conversely, mutation of critical cell cycle regulatory proteins can, in some cases, disrupt the ability of cells to synchronize¹⁶.

M2G medium was initially described with a higher 20 mM phosphate concentration^{8,17}; however, current studies use a lower 10 mM concentration as presented in Table 1^{18,19}. Alternative to the standard M2G medium, it is possible to use richer PYE medium. Here the unsynchronized growth and growth after synchronization can be substituted for PYE.

C. crescentus NA1000 provides a valuable experimental tool to study the bacterial cell cycle. Additionally, due to the single copy of the chromosome in the swarmer cell, this bacterium has also become a powerful model to study the structure of the bacterial chromosome.

ACKNOWLEDGMENTS:

The authors thank members of the Shapiro lab and Erin Schrader for comments on the manuscript. The authors acknowledge financial support from: NIH postdoctoral fellowship F32 GM100732 to JMS and NIH grants R01 GM51426 and R01 GM32506 to LS.

DISCLOSURES:

The authors have nothing to disclose.

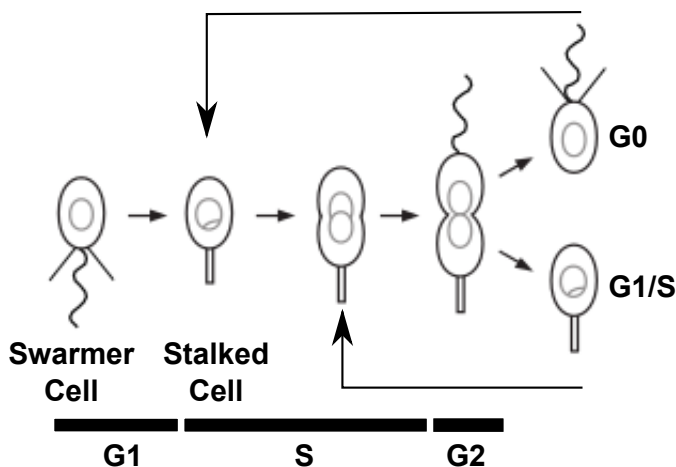
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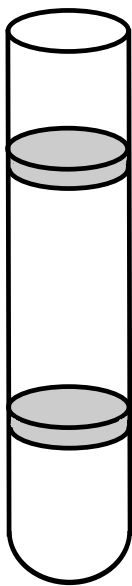
A. Figure 1

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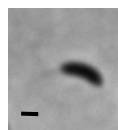
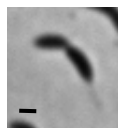


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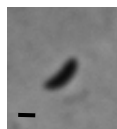
Large Scale



Stalk/Predivisional



Swarmer



Small Scale

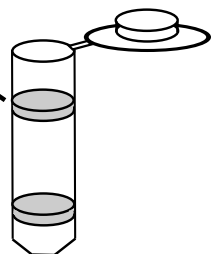
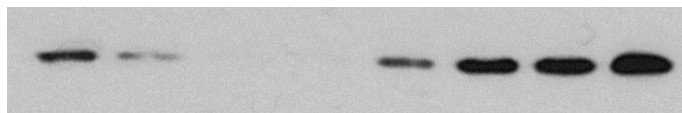
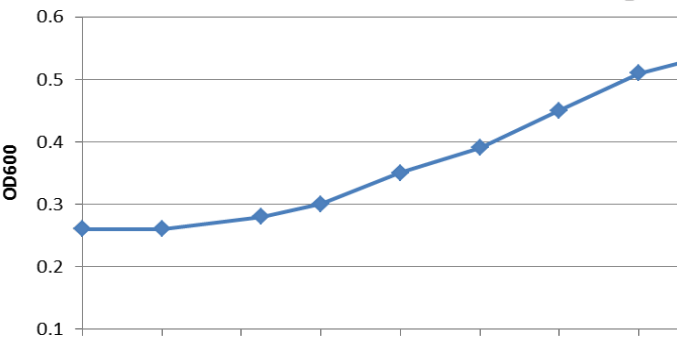
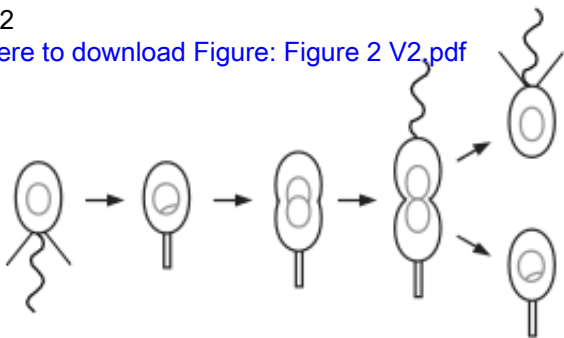


Figure 2

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α -CtrA

0 20 40 60 80 100 120 140

Time after synchrony (Minutes)

Table 1. 20X M2 Salts Recipe.	
Na ₂ HPO ₄	17.4 g
KH ₂ PO ₄	10.6 g
NH ₄ Cl	10 g
H ₂ O	Resuspend in 1 L & autoclave

Table 2. M2 and M2G recipe.		
20X M2 Salts	50 mL	
0.5 M MgSO ₄	1 mL	
20% Glucose	10 mL	Substitute for H ₂ O in M2
Ferrous Sulfate Chelate Solution	1 mL	
0.1 M CaCl ₂	5 mL	Add last to avoid precipitation
H ₂ O	Fill up to 1 L	
Sterile filter with 0.22 µM filter		

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
PVP Coated Colloidal Silica (Percoll)	Sigma-Aldrich	P4937	
Colloidal Silica (Ludox AS-40)	Sigma-Aldrich	420840	
JA10 Rotor	Beckman-Coulter	369687	
JA20 Rotor	Beckman-Coulter	334831	
Ferrous Sulfate Chelate Solution	Sigma-Aldrich	F0518	
30 mL Centrifuge Tubes	Corning	8445	
Na ₂ HPO ₄	EMD	SX0720-1	
KH ₂ PO ₄	VWR	BDH9268-500G	
NH ₄ Cl	Amresco	0621-500g	



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Article Title:

Synchronization of *Carlobacter crescentus* for Investigation of the Bacterial Cell Cycle

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Date:

8/21/14

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Editorial comments:

- 1) All of your previous revisions have been incorporated into the most recent version of the manuscript. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document. Please use this updated version for any future revisions.
- 2) Editor modified the formatting of the Discussion section to comply with the JoVE format. Specifically, section sub-headings "Troubleshooting" and "Modifications" were removed and the text re-organized to paragraph style. The third paragraph of the Discussion was moved to the end of the section for better flow.
- 3) Please name the cell type in step 1.1.
We now refer to strain NA1000 in this step.
- 4) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.
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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Synchronization is an important procedure for Caulobacter research. The manuscript does a good job of describing a very reliable procedure.

Major Concerns:

None

Minor Concerns:

none

Reviewer #2:*Manuscript Summary:*

In this manuscript, the authors describe a technique that has been essential in elucidating the steps and regulatory control of the bacterial cell cycle. The synchronization procedure is used in countless laboratories and the original procedure described in the literature (ref. 5 from this manuscript, Evinger & Agabian, 1977) has been modified in the last 35 years, but a new reference and protocol have not been published. In this manuscript, the authors describe two versions of the procedure that are useful for different downstream applications. They also discuss some of the important variables that can affect the success of a synchrony.

Overall, I am enthusiastic about the publication of this manuscript and the availability of a video protocol for such an important technique.

Major Concerns:

In the introduction, the authors discuss the importance of using the non-adhesive NA1000 strain. This description is a bit misleading because the non-adhesive nature of the NA1000 strain is not the phenotype that allows synchrony (see the discussion section of this manuscript (lines 232-236), Marks et al. 2010 (ref. 7), and/or Abel S, Bucher T, Nicollier M, Hug I, Kaever V, Wiesch zur PA, Jenal U. 2013. Bi-modal Distribution of the Second Messenger c-di-GMP Controls Cell Fate and Asymmetry during the Caulobacter Cell Cycle. PLoS Genet 9:e1003744.) This point should be clarified as it could be misleading.

We have changed the description of the NA1000 strain from non-adhesive to synchronizable for clarity.

There seems to be some disagreement in the literature regarding the 20X M2 salts recipe. Many papers cite this one [Ely B. 1991. Genetics of *Caulobacter crescentus*. Meth. Enzymol. 204:372-384.] when referring to the M2 recipe. The Ely paper reports a 10X M2 base with 17.4 g Na₂HPO₄, 10.6 g KH₂PO₄, and 5 g NH₄Cl. It is the same recipe as reported in this manuscript. However, most published *Caulobacter* literature reports these concentrations: M2 salts: 6.1 mM Na₂HPO₄, 3.9 mM KH₂PO₄, 9.3 mM NH₄Cl or this recipe for 20X M2 salts: 17.4 g Na₂HPO₄, 10.6 g KH₂PO₄, 10 g NH₄Cl. Both of these are different than reported here. It seems that this paper would be a good place to clarify or reconcile any of these differences. Also, it isn't clear if these are the anhydrous or hydrate forms of the chemicals? Additionally, specific autoclave conditions are not provided, but would be useful (i.e. 121°C for 30 minutes...).

Our Excel table contained an error to double the weight of the Na₂HPO₄ and KH₂PO₄. We have corrected this error and the table now reads: 17.4 g Na₂HPO₄, 10.6 g KH₂PO₄, 10 g NH₄Cl. To clarify the exact chemicals used we included them in the Materials table.

It would be helpful to see what the data presented in Figure 2 would look like if the culture were not synchronized. What is the shape of the growth curve of the non-synchronized cells at the same starting OD? What does the anti-CtrA Western look like for a non-synchronous population at similar time points? These may be obvious to people who work with synchronized *Caulobacter* already, but would be helpful for someone learning the procedure for the first time. It can be very helpful to have examples of both "failure" and "success" with which to compare one's own results. Because this is a methods paper with the intention of teaching this technique, it would seem important to include some additional panels in Figure 2.

A failed synchrony would show that the CtrA levels did not change as all the cells are growing at different phases of the cell cycle. To reflect this we have added the following sentence to Figure legend 2:

"Failed synchrony procedures lead to CtrA western blots with no change in protein levels."

Some specific comments regarding the steps in the procedure:

In some places, the timing is discussed, in others it is not. For example, how long is the incubation between steps 1.3 and 1.4? For planning purposes, this seems important. Are modifications in the timing possible? Can step 1.2 be done overnight and step 1.3 done in the morning? Because some of the steps are discussed with specific suggested timing, for consistency, timing estimates should be provided for the remaining steps.

We have removed the timing in the day and made the protocol more universal by judging based on the OD₆₀₀ of the cells. The sentence now reads:

"Inoculate 0.5 mL of the cells in 25 mL of M2G (Tables 1-2) and shake at 28 °C until the culture reaches an OD₆₀₀ between 0.5 and 0.6."

Step 1.7 - It would be helpful to mention that Colloidal silica in this step is Ludox.

[Editorial comment: According to the JoVE format, commercial language should not be included in the protocol. You may disregard the above comment.]

1.12 - This step could be articulated more clearly. Is each pellet resuspended individually in 30 mL or are all 8 pellets combined in the 30 mL?

All cells should be resuspended in cold M2 for spectrophotometric analysis as written. This will be well suited for the video protocol.

2.5 - it would be helpful to mention that the PVP coated colloidal silica is Percoll

[Editorial comment: According to the JoVE format, commercial language should not be included in the protocol. You may disregard the above comment.]

2.9 - what is the typical yield (volume) for the small-scale procedure? This information is provided for version 1 of the protocol, it would be helpful to have it for version 2 as well.

We have added the following sentence to section 2.9: “Note: Typical yields are between 2 and 4 mL of swarmer cell culture.”

2.10 - It would be helpful to have a reference to or explanation of how to prepare the agarose pad for microscopy. Again, because this is a methods paper with the intention of teaching this technique, it seems particularly important to provide some guidance here.

Overall, I think the procedure is well suited to presentation in a JoVE video. The writing in the protocol should be cleaned up to be more consistent and cleaner (there are some confusing steps and some inconsistencies in the level of detail provided in each step).

While this would be well adapted for JoVE, articles on imaging bacteria cells on agarose pads already exist. We have focused this article on the synchronization of *Caulobacter* cells and not on the downstream experiments.

Minor Concerns:

Regarding the materials and equipment list, it would be helpful if the ordering information for the bottles/tubes required for the JA-10 rotor were included. In addition, ordering information for the M2 salts reagents (sodium phosphate dibasic, potassium phosphate, ammonium chloride, glucose) would be helpful, especially for students, new investigators or laboratories learning to work with *Caulobacter* for the first time.

We have added the ordering information for the 20X M2 salts in the Materials section. In a previous version of the manuscript we included ordering information for the tubes but it was removed due to trade names.

It is a bit confusing/unclear that in Figure 1 the colloidal silica is identified by its trade name (Ludox or Percoll), but in the procedure it is described as colloidal silica or PVP coated silica. It would be helpful if the trade names were also provided in the step by step procedure.

[Editorial comment: For consistency, please remove the trade names from the figure.]

We have removed the trade names from the figure.

Because this procedure will be accompanied by a video, this may not be necessary, but it would be helpful to have images of the tubes with stalked/predivisinal and swarmer cell bands - the cartoons are informative, but a photo would be more useful.

In the discussion, it might be relevant to mention that perturbations of certain cell cycle genes can affect the expression of the genes that allow for synchrony (Abel S, Bucher T, Nicollier M, Hug I, Kaever V, Wiesch zur PA, Jenal U. 2013. Bi-modal Distribution of the Second Messenger c-di-GMP Controls Cell Fate and Asymmetry during the Caulobacter Cell Cycle. PLoS Genet 9:e1003744.). While these are unlikely to cause major effects for most users, they are important to know about.

We have now included the following sentence in the discussion of mutant strains: "Conversely, mutation of critical cell cycle regulatory proteins can, in some cases, disrupt the ability of cells to synchronize¹⁴."

Reviewer #3:

Manuscript Summary:

This protocol highlights the key feature of Caulobacter—cell cycle synchronization—that makes this organism such a powerful tool for dissecting bacterial cell development. Importantly, this protocol establishes the benefits of this system over bacterial synchronization methods used for other bacteria, and it includes all of the key steps in synchronizing Caulobacter.

The manuscript is pertinent to the field because it outlines this powerful and easy technique, and a video of how to conduct this method will be of great utility to researchers just getting in to this field. For instance, Caulobacter is easily cultured and as a non-pathogen is safe to manipulate, making this system ideal for study at the undergraduate level. This technique would facilitate teaching pedagogies such as the "flipped classroom," where students view a video of the technique on their own before a laboratory experience, thereby providing them an opportunity to arrive at lab well prepared.

For the most part, the manuscript is clear. There are, however, issues that the authors should address before publication. But these outstanding issues, I feel, are easily addressed. They include:

Major Concerns:

* The level of detail for the described procedures needs to be increased. The procedures lack clarity for describing how to accomplish each step without prior knowledge. In other words, my belief is that one in the Caulobacter field would easily interpret this protocol, but I suspect that novices would have a challenging

time interpreting how to, for instance, conduct the appropriate controls for CtrA production. With some additional explanation, these techniques should be easily accessible to anyone with basic skills in culturing bacteria.

Our article describes the synchronization and focuses on the synchronization and not the downstream experiments utilized to study the cell cycle. We have included more detail for the protocol of CtrA western blots in the figure legend. The figure legend now contains the following sentences:

“ α -CtrA antibody¹⁰ was incubated at a 1:10,000 dilution for 1.5 hours in 3% milk TBST and washed 3 times in TBST. Goat- α -rabbit secondary was then added at 1:10,000 dilution in 3% milk TBST for 1 hour, washed 3 times with TBST, and imaged on film using a chemiluminescent detection kit.”

* The manuscript is an ideal opportunity to clarify the recipes for M2G. The components of M2G need to be updated to reflect the actual salt forms and/or the molar concentrations of each ionic species used. Also, I believe there is a discrepancy with the M2 recipe reported here and the one typically used: the recipe reported here comes from Bert Ely's early papers (I believe the first report is here: Isolation of Spontaneously Derived Mutants of *Caulobacter Crescentus*. Genetics 1977, 86, 25-32. And an excellent review of these techniques is here: Ely, B. Genetics of *Caulobacter Crescentus*. Meth Enzymol 1991, 204, 372-384.), and I believe current practice uses Na₂HPO₄ and KH₂PO₄ at half the concentration reported by Ely.

We made an error in the original Excel file that doubled the amount of materials in the 20X M2 Salts. We have corrected this error and the table now reads: 17.4 g Na₂HPO₄, 10.6 g KH₂PO₄, 10 g NH₄Cl. To clarify the exact chemicals we have included them in the Materials table. This is half the original concentration, and we have noted this by adding the following sentence to the discussion:

“M2G medium was initially described with a higher 20mM phosphate concentration^{15,16}; however, current studies use a lower 10 mM concentration as presented in table 1^{17,18}. ”

Minor Concerns:

* Finally, this manuscript needs some additional minor copyediting.

* I also suggest some changes to the Figures to help better illustrate the unique morphologies of *Caulobacter*: why settle for just a cartoon of *Caulobacter* when actual microscopy images are easily collect.

Additional Comments to Authors:

Below are specific comments describing issues to be addressed:

1. The abstract describes the swarmer cells as *Caulobacter* in the G0 phase of the cell cycle. I imagine that this is intended to draw parallels to the eukaryotic cell cycle, which is an important point to highlight. But in this case, I suggest that the cartoon schematic in Fig. 1A should annotate/illustrate the stages of the cell: swarmer cells in G0/G1 phase, stalked cells in S phase and predivisional cells in G2 phase.

The phases of the cell cycle are now explicitly indicated in Figure 1A.

2. For Fig. 1B, I suggest including a description of each tube size (eg. 30 mL centrifuge tube for large scale, 2 mL microcentrifuge tube for small scale) in the figure legend.

We do not feel the tube size needs to be included in the figure legend.

3. Table 1. needs additional description for the salt forms used. For instance, what is the final phosphate concentration in 1X M2G? Potassium phosphate monobasic is most often obtained as the anhydrous salt (KH_2PO_4 , MW 136.09), so 21.2 g per liter used for 20X M2 salts yields 156 mM of phosphate. But what about the sodium phosphate dibasic, which is often sold as the anhydrous salt (Na_2HPO_4 , MW 141.96, providing 245 mM phosphate in 20X M2) or the heptahydrate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, MW 268.07, providing 130 mM phosphate in 20X M2). These numbers are based on classic preparations by Bert Ely's lab (Genetics 1977, 86, 25-32. & Meth Enzymol 1991, 204, 372-384.), but neither describes whether the salts are anhydrous or hydrates. My guess is that the anhydrous salts are used, which would make 20 mM phosphate (7.8 mM KH_2PO_4 and 12.2 mM Na_2HPO_4). One additional clarification is necessary for whether 10 mM or 20 mM phosphate constitutes M2G. A clarification here is important considering that nearly every *Caulobacter* paper cites Ely's Meth Enzymol 1991, 204, 372-384 paper (eg. Nucleic Acids Research, 35(20), e137; PLoS Biology, 3(10), e334; Molecular Cell, 43(4), 550-560.), which reports 20 mM phosphate, but I know of at least one report of using M2G that is defined as 10 mM phosphate (Molecular Microbiology, 80(3), 695-714.). I suggest including the salt form used for each component and perhaps the catalog number and brand, too. In addition, including a table of the final concentrations of each component found in 1X M2G would also be helpful for those new to *Caulobacter* cell biology.

To avoid confusion on the salt forms used we have included the product information for each chemical in the M2 salts in the Materials section.

4. Table 2 would benefit from including the salt form used for each component and perhaps the catalog number and brand. In particular, many labs use Sigma's ferrous sulfate chelate solution (Sigma #F10518), which is a convenient source for this component that those new to *Caulobacter* should know. I know this is included in the Table of Materials/Equipment, but it would be beneficial to have

these. Finally, in our hands, order of addition in making M2G is important. Including a more detailed recipe would be helpful.

We have included the product information only in the Materials table to avoid commercial language as per the JoVE journal policy. We have stated to add the CaCl_2 last to avoid precipitation in Table 2.

5. Regarding the culture media used, consider citing the early methods papers (Poindexter, J. S. (1964). Bacteriological Reviews, 28, 231-295; and Ely's two papers: Genetics 1977, 86, 25-32; Meth Enzymol 1991, 204, 372-384.).

We have included citations to both of the Ely papers which describe the 20mM phosphate M2G recipe in detail.

6. At several points in the protocol, visual checks of the Caulobacter culture are needed. Consider including a general description of the type of microscope needed (eg: power of the objective? is it a dissecting microscope?).

We currently state that one should use a "liquid mount phase microscope" to image the cells. We routinely image the cells on different microscopes.

7. To give researchers a realistic expectation of representative synchrony, Fig. 2 would benefit from showing clear images from a time lapse of synchronized Caulobacter progressing through the cell cycle. DIC or phase contrast microscopy images would be ideal here.

We have included phase microscope images of Caulobacter cells in each of the major stages of the cell cycle in Figure 1B.

8. Language could be more precise in the protocol some suggestions include:
a. Line 72 (also, Line 127). "From a freezer stock or a plate of Caulobacter, inoculate 5 mL of PYE and grow shaking overnight at 28 °C to obtain an overnight culture.

We have changed this sentence to state: "From a freezer stock or a plate, grow a 5mL overnight culture of strain NA1000 by shaking at 28 °C in PYE medium."

b. Line 75. "The following morning, inoculate 25 mL of M2G with 0.5 mL of the overnight culture in step 1.1 and shake at 28 °C until the afternoon.

We have now indicated that the cells are from step 1.1

c. Line 85. "7 K" should read "7 k". SI units for 1000's is lower case. Also, JA-10 rotors hold 500 mL centrifuge bottles, so this implies that the 1 L culture from step 1.3 should be split. Be explicit about this split.

We have changed the K to small k throughout the manuscript. This splitting will be viewed in the video and can be done into 2 or 4 500mL bottles. Users with different rotors/centrifuges may have to split into different sized aliquots, so we have not included any explicit information on the split sizes.

d. Line 87. Followup to above comment for Line 85: should 180 mL of cold M2 should be added to each 500 mL bottle?

We have changed this sentence to emphasize that all the cells should be resuspended in 180mL.

e. Line 88. "predivsional" typo.

We have corrected this typo.

f. Line 94. "8 30 mL tubes" should read "eight 30 mL tubes" or clarify in a similar manner.

We have made this change.

g. Line 114. State that the 30-60 mL typical yields come originally from a 1 L culture (step 1.3).

We have added a statement that they come from 1L of cells.

h. Line 117. Citation for doubling times? Perhaps include somewhere the typical doubling times for NA1000 in M2G and PYE, and include a citation.

We have added a citation for the doubling time.

i. Consider including references to your tables for M2G, PYE recipes and Percoll/Ludox beads

We cannot use trade names such as ludox and percoll in the article. Tables 1 and 2 comprising the M2G recipe is referenced in section 1.2.

j. Line 153. Consider including a description of the agarose pad (% agarose, media)

We have focused this article on the synchronization of Caulobacter and not the downstream applications. We do not wish to include agarose pad making as a part of this article.

k. Line 161. It is unclear how these western blot data were collected. Are the aliquots normalized to the optical density? If so, please describe.

No, they were not. In section 1.15 we explain that they are each 1mL aliquots of the synchronized culture.

l. Line 164. Consider including the number of developmental genes controlled ("at least XXX genes) rather than the general "a host of different...".

It is difficult to define what a developmental gene is, therefore, we have left this sentence the same.

m. Line 188. Describe how the western blot controls were prepared.

As described previously, we have added two sentences to the Figure 2 legend.

n. Line 190. Because the authors suggest CtrA protein levels as an appropriate control for assessing the synchrony, they should include a description and/or citation for the anti-CtrA antibody. How does one obtain this antibody? Is it commercially available?

We have included a citation for the antibody and the dilution and procedure for western in the Figure 2 legend.

o. Line 200-201. Remove "as noted...protocols" and just start the sentence at "This method is amenable...".

We have made this change.

p. Line 204. Make clear that the "other synchronization methods" are for bacterial other than Caulobacter.

We have left this the same, as these methods could be applied to *Caulobacter*.

q. Line 212. This paragraph should be rewritten. This paragraph feels out of place with very little connection to the preceding discussion. Consider additional transitional language. A further description of what type of phase microscope is needed (see point 6 above). Line 213: consider rewriting so that this sentence does not end in a preposition. Line 214: include how one can assess (or how the authors assessed) whether >95% of the cells are swarmer. Was this visually scored? Or was a software package such as Jacob-Wagner's MicrobeTracker (<http://www.yale.edu/jacobswagner/software.htm>) used? Is there a citation for this efficiency?

This was visually scored by manual counting. A software package may also be used. We have corrected the sentence ending in a preposition.

r. Line 217. Consider beginning this paragraph is a transitional statement such as, "This protocol for *Caulobacter* synchronization is not without its faults." Also, is there a citation for cells not synchronizing well in antibiotics? Consider listing a representative mutant or two that are poor at synchronization.

We added the following sentence to begin this paragraph:

"Additional complications can lead to potential problems throughout the synchrony procedure."

s. Line 219. Citation for slower cell cycle? Is there an illustrative example of an antibiotic that slows growth?

As antibiotics are well known to inhibit growth we have not included a citation.

t. Line 223. The tone of this paragraph seems too casual. In particular, "make it look like they aren't swimming". And include an additional explanation for why shearing the flagella is problematic. Does it, for instance, affect the overall quality or yield of the synchrony?

We have changed the sentence to say "Avoid vortexing the cells, as this will shear off the flagella and lower the quality of the synchrony."

u. Line 228. Regarding the care needed for the "loose cell pellet": if this additional level of care is vital, then this description should be included in the detailed protocol itself.

We feel the video will highlight this step and have not moved the sentence.