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.  
  
5) Please disregard the comment below if all of your figures are original.  
If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."   
  
**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. 121ºC 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 it's 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/predivisional 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 antibody 10 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 (KH2PO4, 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 (Na2HPO4, MW 141.96, providing 245 mM phosphate in 20X M2) or the heptahydrate (Na2HPO4\*7H2O, 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 KH2PO4 and 12.2 mM Na2HPO4). 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 CaCl2 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.