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Peer Review Information

Journal: Nature Methods
Manuscript Title: Engineering of bidirectional, cyanobacteriochrome-based light inducible dimers (BICYCL)s
Corresponding author name(s): Matias Zurbriggen , Maruti Uppalapati, Andrew Woolley

Editorial Notes:

Reviewer Comments & Decisions:

Decision Letter, initial version:

Dear Drew,

Your Brief Communication, "Engineering of bidirectional, cyanobacteriochrome-based light inducible dimers (BICYCL)s", has now been seen by three reviewers. As you will see from their comments below, although the reviewers find your work of considerable potential interest, they have raised a number of concerns. We are interested in the possibility of publishing your paper in Nature Methods, but would like to consider your response to these concerns before we reach a final decision on publication.

We therefore invite you to revise your manuscript to address these concerns. We think the technical concerns raised by reviewers 1 and 3 are reasonable and will improve the paper. We think the suggestion for additional demonstration by referee 2 would improve the paper, but is not strictly necessary to show that these tools enable new applications. We leave it to you whether you want to add a demonstration that highlights the increased spatial control possible with these tools. If you do add one, your paper would be promoted to an Article.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your paper:

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We hope to receive your revised paper within three months. If you cannot send it within this time, please let us know. In this event, we will still be happy to reconsider your paper at a later date so long as nothing similar has been accepted for publication at Nature Methods or published elsewhere.

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SUPPLEMENTARY PROTOCOL

To help facilitate reproducibility and uptake of your method, we ask you to prepare a step-by-step Supplementary Protocol for the method described in this paper (in this case it can be for one of the applications of the BICYCLs, not the engineering). We encourage authors to share

their step-by-step experimental protocols on a protocol sharing platform of their choice and report the protocol DOI in the reference list. Nature Research's Protocol Exchange is a free-to-use and open resource for protocols; protocols deposited in Protocol Exchange are citable and can be linked from the published article. More details can found at www.nature.com/protocolexchange/about.

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Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further. We look forward to seeing the revised manuscript and thank you for the opportunity to consider your work.

Sincerely,
Rita

Rita Strack, Ph.D.
Senior Editor
Nature Methods

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Optogenetic tools that take advantage of light-induced changes in protein-protein interactions are growing in number, but presently are limited by their large size, i.e. phyB-PIF6 systems, and their limited

spectra range, i.e. they are mostly confined to blue-regulated systems that require prolonged irradiation and the potential for off-target photooxidative side reactions. Here the authors develop a cyanobacteriochrome (CBCR)-based optogenetic tool by evolving binding partners that are based on the small albumin-binding domain of protein G, which can discriminate between the red and green photostates of their red/green Amg2 photoswitch. The authors demonstrate the binding specificity biochemically in vitro and optimize conditions for their use in vivo for photoreversible light-regulated target protein subcellular localization, e.g. mitochondrial tethering, nuclear localization or exclusion, or light-regulated gene expression in mammalian (HEK293 and CHO-K1) and yeast cells. Moreover, this work also assuages a critical concern - that of apoprotein signaling activity, by identifying binding partners that do not or poorly recognize the pool of photo-inactive apoprotein. Finally, the authors further combine their new red-ON/green-OFF and green-on/red-OFF Bidirectional, Cyanobacteriochrome-based Light inducible dimerization modules or BICYCLs with well-established blue light optogenetic systems to demonstrate the orthogonality of the newly developed reagents. This is quite nice and he specificity of the engineered BAmGreen systems is especially impressive. Overall - this work is novel, the methodology and data are well described, of high quality, and rigorously controlled, and the significance of the investigators' achievements shines through. Owing to the small size, the broad wavelength tunability and the bistable behavior of CBCRs, this work provides compelling support for the conclusion that that BICYCL technology will be rapidly adopted and further improved by the greater biomedical and cell biology communities. This work is likely to be recognized as a pioneering development and a classic paper in this field.

That said, some other issues remain to be addressed before BICYCLs will become a household name. BICYCLs as described require administration of the exogenous pigment phycocyanobilin, which is expensive and may be hard to administer into thick tissues or whole animals. Although phycocyanobilin production in situ is a potentially viable alternative, this may be difficult to genetically engineer in all experimental systems. A number of CBCRs, Amg2 included, can also bind biliverdin - a much more abundant pigment present in most eukaryote cells, including mammals. The parental CBCR from which Amg2 is derived, i.e., AM1_C0023g2, can form bistable adducts with both phycocyanobilin and biliverdin (see citation 23, Fushimi et al 2016), so this reviewer wonders why biliverdin was not examined in this study. A preliminary study using biliverdin begs to be performed to address this issue, perhaps via a companion study to Figs S9 and S10. Of course, the red-shifted spectra of the biliverdin adduct of Amg2 should shift the spectral response into the infrared which could be an added benefit. As a potential template for future studies, the authors might cite recent work on CBCRs that prefer verdins as chromophores (Moreno et al, 2020 A far-red cyanobacteriochrome lineage specific for verdins. PNAS 117(45): 27962-27970).

Other issues that the authors should consider are listed below.
1. On page 3, citation 13 appears incorrect. Should this be replaced by citation 23 (Fushimi et al, 2016)?

2. Re: Fig 1c. BAmRed 1.0 binding to Pr does not look 7-fold greater than that of Pg. Is this based on subtracting the fluorescence of phage binding to apo-Amg2 from those of Pr and Pg? The method for calculating MCR (and NCR later on) is buried in citation 14 in the supplement (Woloschuk et al, 2020) and perhaps could be articulated somewhat in the Supplemental Methods (to help the reader understand the difference between specificity and signal strength). In that paper, these investigators state that the mitochondrial to cytoplasmic tgRFP fluorescence ratio (MCR) is the ratio of the average 'corrected' mitochondria fluorescence divided by the average cytoplasmic fluorescence. This analysis assumes that the measured mitochondrial fluorescence is the sum of the mitochondrial-specific fluorescence and the average cytoplasmic fluorescence corresponding to the same cell area (or pixel number). This assumption needs to be stated herein. It is unclear that this assumption is strictly true since the mitochondrial matrix volume would lack the cytosolic fluorescence.
3. CItations to Fig. S6 and Table S1 (and brief discussion of results) appear missing from the main text on page 6. Also missing is citation to Fig. S14a,b on page 9 which show control green light 'orthogonality' measurements.
4. Regarding FR direct imaging of the Pg form of Amg2 in Figs. 7c, S8b, S9a&d, S10a&d, S11, S12, S13c and S14b&d, shouldn't this fluorescence be much lower than that of the Pr form? According to Fushimi et al, 2016, only the Pr form would exhibit fluorescence in the region, not the Pg form. What is being detected here or are the samples irradiated with green light to detect this fluorescence? In this regard, Fig. S12 presents the effect of the microscope's 640 nm laser on changes in the FR emission. There should be no changes for the Pg sample, while the FR emission should decrease for the Pr sample. This reviewer observes no changes in the latter.
5. For the LEXY-Amg2 and LINus-Amg2 fusion systems in Fig. 2c, why is a mCherry fusion needed? Seems like its presence might complicate imaging deconvolution.
6. Fig. S13 Panel C imaging wavelengths used are missing.
7. Fig. S16 For the red-BICYCL constructs analyzed for SEAP gene expression in Fig. S15 (panel d), why does G irradiation inhibit expression of SEAP with increasing fluence. Shouldn't G, like dark, sustain the Pr form of Amg2? This was not seen in panels f and h however, so why are these experiment different? As for other systems, the green-BICYCL constructs are clearly much more robustly ON/OFF (panel e). What time after PCB addition (+/- light) was gene expression determined for panels d and e?
8. Fig. S17 is not properly cited on page 9. Fig. S17 shows the loss of transcript abundances of the BICYCL Amg2-VP16 and E-BAm components in the cells during incubation/assembly of holoprotein with PCB.
9. For multiplex experiments in Fig. 2e&f, how can you control relative expression of the three systems, or can you, by transfection? By using different ratios of DNA?
10. Supplementary Methods
a. Page 5. What volume phage was added to each well of the MaxiSorp 96 plate?
b. Page 8. What is the mechanism of fluorescence quenching of Amg2 by BAmRed?
c. Page 32. Fig. S19, please define abbreviations or reference the different DNA binding proteins.

Reviewer #2:
Remarks to the Author:
The manuscript by Jang et al. presents the development and characterization of novel bidirectional photo-inducible dimerization systems based on cyanobacteriochrome. The authors chose a GAF domain from cyanobacteriochrome as a light-sensitive domain that can act as a conformational switches in response to irradiation in the green and red regions of the spectrum. They used this domain to systematically isolate state-specific binders (red or green state) from a library of GA domain variants by phage display and yeast two-hybrid methods. The best binders were characterized by ITC, size-exclusion chromatography and fluorescence quenching revealing low micromolar to sub-micromolar binding affinities and clear (>50 to >800-fold) preference for the state they were selected against. From this first part of the study, they identified BAmGreen2.4 and BAmRed1.4 as the most efficient green and red state binders of Amg2 (GAF domain) respectively. Next, they evaluated the properties of the binders in live cells. Control over the red and green binder capacity to recognize the Amg2 BAF domain was evaluated by respective localization of the binder and its target under green and red illuminations. Combination with LOV domain-based systems (irradiation with blue light) with LEXY and LINus was also evaluated. Finally, using three orthogonal reporter systems (SEAP, FLuc, GLuc) linked to the two binders and the LOV-domain based TULIP system, the authors demonstrated that they could selectively control each of the reporters expression with blue, green or red light. Altogether, these experiments confirmed the functionality of the tools in cells and their capacity to be used in combination with blue light controlled systems such as the ones derived from LOV domains.

This study is very well written and designed. It addresses a clear gap in the optogenetic field with so far an absence of tools that can be used in the green to red wavelengths thereby limiting multiplexing possibilities. With these two novel binders together with the Amg2 BAF domain, the authors elegantly demonstrate that, while these tools can still be improved, they importantly open new perspectives for setting up optogenetic-based studies with the possibility to combine up to three optogenetic modules with selective blue, green and red irradiation. The originality of the study is reinforced by a clear presentation of the results with numerous control experiments and a transparent reporting of the limitations. Statistics are treated adequately. References are appropriate but more may be added in the first paragraph (see minor comments). Overall, the manuscript is convincing and propose original tools that will with no doubt effectively complement the set of existing optogenetic tools by offering new possibilities.

My main comment would be that while the authors have focused on the multiplexing aspect, which is already an impressive achievement, I believe that the full photocontrol of the Amg2 state is also an aspect that opens up plenty of possibilities especially with respect to spatial control. Indeed, blue-light tools do not offer (or only partially and with poor control) this possibility as they rely on (fast) thermal relaxation to return to their basal state. Here, Amg2 combines a long half-life with the possibility to

control by light its return to basal state. An experiment that would illustrate the benefit of this fully photoreversible system would increase the impact and scope of the manuscript. For instance, showing that by irradiating two halves of a cell with red and green light respectively to locally and intracellularly impose two states (bound and unbound optodimers) without being affected by diffusion would constitute a major and impactfull addition to the current manuscript.

Minor comments:
-In the first paragraph of the main text, it would useful to clearly define the blue, green, red and far-red regions of the spectrum with their respective wavelength range.
-Reference should be included when CBCRs (1st main text paragraph) and blue-light tools (end of 2nd paragraph) are first mentioned.
-When reading fast, the red/green nomenclature turns out to be a bit confusing, a clear scheme in figure 1 summarizing which binder binds which state would be a plus for insuring that misunderstandings are avoided.

Reviewer #3:
Remarks to the Author:
The authors identify new binding partners (affibody) for Amg2 (15ZPr/15EPg) using a phage-display approach established by the group in 2018. Of the binding partners described, BAmGreen seems superior to BAmRed. Based on the BAmGreen/Red-Amg2 binding modules, the authors develop bidirectional, cyanobacteriochrome-based light inducible dimerization modules (BICYCL) systems for optogenetic control of protein-protein interactions and gene expression in cells.
This reviewer finds it a bit strange that all of the work done validating the BICYCL system in cells (mitochondrial recruitment data) was in supplemental, and not really referred to in the main text. This is probably due to figure restrictions of the journal, but if the manuscript is a method paper describing the BICYCL system, this reviewer would rather see more attention given the system on its own (vs. its compatibility with other previously established systems). Multiplexing system is interesting, but this paper focuses on the development of BICYCL, not on multiplexing optogenetic systems. To this extent, figures S7-S11 are important, but there is no description of them in the text. It is good to show in the main text an imaging panel showing cells cycled through multiple rounds of red-green switching. The authors show a long half-life of the thermal relaxation and claim BICYCLs act as bistable tools (Fig. S2c). However, a constant illumination for 24h by green/red light is required in many applications in cells. Would such a long illumination lead to phototoxicity or other cellular effects? This is a major drawback of the system, which is not discussed in the text.
Overall, the BICYCL system could be a valuable expansion of the current optogenetic toolbox. But the manuscript is not well written. There is a lot of supplemental data not referred to or discussed in the text.

Specific comments:
Fig. 1h, S5b, how to explain the binding at the initial titration? ITC measurements of BAmRed1.0, 1.4 binding to Pg-Amg2 are missing.
Page 6: “red-BICYCL1.4 (BAmRed1.4) afforded the tightest affinity.” Didn’t BAmGreen2.4 display the tightest affinity (Kd = 0.12 uM)?
Fig. S9-10: The authors did not discuss the effect of PCB concentration. No quantification was shown for no PCB and 10 uM PCB.
Fig. S11: Determination of the half-life of thermal relaxation in live cells is required.
Fig. S14: Quantification and statistical analysis are needed.
Fig. S15: d and e, please indicate the illumination time; (h, i) legend: “Relative expression of Amg2 and BAm transcript level.” Or you mean SEAP expression level? Shouldn’t the expression level of SEAP correlate with the activity shown in f and g, respectively?
Fig. S16: were b and d swapped? No switch was observed in d with the illumination time < 3h, suggesting that red-BICYCL1.4 requires a long illumination time (at least 24h).

Author Rebuttal to Initial comments

Responses to Reviewers' Comments:

Reviewer #1:
Remarks to the Author:

Optogenetic tools that take advantage of light-induced changes in protein-protein interactions are growing in number, but presently are limited by their large size, i.e. phyB-PIF6 systems, and their limited spectra range, i.e. they are mostly confined to blue-regulated systems that require prolonged irradiation and the potential for off-target photooxidative side reactions. Here the authors develop a cyanobacteriochrome (CBCR)-based optogenetic tool by evolving binding partners that are based on the small albumin-binding domain of protein G, which can discriminate between the red and green photostates of their red/green Amg2 photoswitch. The authors demonstrate the binding specificity biochemically in vitro and optimize conditions for their use in vivo for photoreversible light-regulated target protein subcellular localization, e.g. mitochondrial tethering, nuclear localization or exclusion, or light-regulated gene expression in mammalian (HEK293 and CHO-K1) and yeast cells.
Moreover, this work also assuages a critical concern - that of apoprotein signaling activity, by identifying binding partners that do not or poorly recognize the pool of photo-inactive apoprotein. Finally, the authors further combine their new red-ON/green-OFF and green-on/red-OFF Bidirectional, Cyanobacteriochrome-based Light inducible dimerization modules or BICYCLs with well-established blue light optogenetic systems to demonstrate the orthogonality of the newly developed reagents. This is quite nice and he specificity of the engineered BAmGreen systems is especially impressive. Overall - this work is novel, the methodology and data are well described, of high quality, and rigorously controlled, and the significance of the investigators' achievements shines through. Owing to the small size, the broad wavelength tunability and the bistable behavior

of CBCRs, this work provides compelling support for the conclusion that that BICYCL technology will be rapidly adopted and further improved by the greater biomedical and cell biology communities. This work is likely to be recognized as a pioneering development and a classic paper in this field.

We are delighted about the positive assessment of our study and would like to thank referee #1 for her/his very constructive comments for improving the manuscript. We have addressed all concerns as indicated below.

That said, some other issues remain to be addressed before BICYCLs will become a household name. BICYCLs as described require administration of the exogenous pigment phycocyanobilin, which is expensive and may be hard to administer into thick tissues or whole animals. Although phycocyanobilin production in situ is a potentially viable alternative, this may be difficult to genetically engineer in all experimental systems. A number of CBCRs, Amg2 included, can also bind biliverdin - a much more abundant pigment present in most eukaryote cells, including mammals. The parental CBCR from which Amg2 is derived, i.e., AM1_C0023g2, can form bistable adducts with both phycocyanobilin and biliverdin (see citation 23, Fushimi et al 2016), so this reviewer wonders why biliverdin was not examined in this study. A preliminary study using biliverdin begs to be performed to address this issue, perhaps via a companion study to Figs S9 and S10. Of course, the red-shifted spectra of the biliverdin adduct of Amg2 should shift the spectral response into the infrared which could be an added benefit. As a potential template for future studies, the authors might cite recent work on CBCRs that prefer verdins as chromophores (Moreno et al, 2020 A far-red cyanobacteriochrome lineage specific for verdins. PNAS 117(45): 27962-27970).

We entirely agree that a CBCR GAF domain that binds biliverdin would make application of these tools in thick tissues or whole animals more straightforward. As described by Fushimi et al, Amg2 does indeed bind biliverdin and the spectra are red-shifted compared to the PCB-bound version. As suggested, we have now added data to examine whether the Amg2-biliverdin adducts function in cells (See new Extended Data Fig. 1). When Amg2 is expressed and exogenous biliverdin is added BamRed1.0 and BamRed1.4 are observed to co-localize with the Pr state (actually Pfr since it is red-shifted). Some dissociation is observed upon exposure to 735 nm light. Likewise, in a transcriptional reporter setting (Extended Data Fig. 1) enhanced reporter gene expression is seen with the Pfr state vs. the Po state. The dynamic range of switching of the BV versions is smaller, however, than that seen with PCB. Importantly, when no exogenous BV was added, no colocalization (or switching) was seen. This indicates that the affinity of Amg2 for BV (or the rate of BV binding) is insufficient for functional chromophorylation with endogenous levels of BV, at least in HEK and CHO cells. Since adding exogenous BV provides no significant advantage over adding exogenous PCB, and the dynamic range was found to be better with PCB loaded proteins, we opted to focus on these. We agree that other templates may be useful for the development of BV versions of BICYCLs in future work and have added text and the suggested reference to make this point (pg. 9). We note also that Zhou et al (Zhou, Y., Kong, D., Wang, X. et al. A small and highly sensitive red/far-red optogenetic switch for applications in mammals. Nat Biotechnol 40, 262–272 (2022). https://doi.org/10.1038/s41587-021-01036-w) have successfully used PCB in whole animals, so this is also possible.

Other issues that the authors should consider are listed below.
1. On page 3, citation 13 appears incorrect. Should this be replaced by citation 23 (Fushimi et al, 2016)?

We thank the referee for catching this error. Indeed, this should be Fushimi et al (now corrected).

2. Re: Fig 1c. BAmRed 1.0 binding to Pr does not look 7-fold greater than that of Pg. Is this based on subtracting the fluorescence of phage binding to apo-Amg2 from those of Pr and Pg?

Yes, 7-fold is based on subtracting the background (equal to the apo signal). This has been added to the methods (SI pg. 5).

The method for calculating MCR (and NCR later on) is buried in citation 14 in the supplement (Woloschuk et al, 2020) and perhaps could be articulated somewhat in the Supplemental Methods (to help the reader understand the difference between specificity and signal strength). In that paper, these investigators state that the mitochondrial to cytoplasmic tgRFP fluorescence ratio (MCR) is the ratio of the average 'corrected' mitochondria fluorescence divided by the average cytoplasmic fluorescence. This analysis assumes that the measured mitochondrial fluorescence is the sum of the mitochondrial-specific fluorescence and the average cytoplasmic fluorescence corresponding to the same cell area (or pixel number). This assumption needs to be stated herein. It is unclear that this assumption is strictly true since the mitochondrial matrix volume would lack the cytosolic fluorescence.

We have now added the procedure we have used for calculating MCR to the SI methods (pg. 13). We agree that the assumption that the measured mitochondrial fluorescence is the sum of the mitochondrial-specific fluorescence and the average cytoplasmic fluorescence corresponding to the same cell area is not strictly correct but we have used this method to be consistent with published reports for other systems (Hallett, R. A., Zimmerman, S. P., Yumerefendi, H., Bear, J. E. & Kuhlman, B. Correlating in vitro and in vivo activities of light-inducible dimers: A cellular optogenetics guide. ACS Synth Biol 5, 53-64, doi:10.1021/acssynbio.5b00119 (2016).)

3. Citations to Fig. S6 and Table S1 (and brief discussion of results) appear missing from the main text on page 6.

Text referring to Fig. S6 and Table S1 has now been added (pg. 6)

Also missing is citation to Fig. S14a,b on page 9 which show control green light 'orthogonality' measurements.

Fig. S14 is now Fig. S24. These data have been quantified and text referring to this figure has now been added (pg. 15).

4. Regarding FR direct imaging of the Pg form of Amg2 in Figs. 7c, S8b, S9a&d, S10a&d, S11, S12, S13c and S14b&d, shouldn't this fluorescence be much lower than that of the Pr form? According to Fushimi et al, 2016, only the Pr form would exhibit fluorescence in the region, not the Pg form. What is being detected here or are the samples irradiated with green light to detect this fluorescence? In this regard, Fig. S12 presents the effect of the microscope's 640 nm laser on changes in the FR emission. There should be no changes for the Pg sample, while the FR emission should decrease for the Pr sample. This reviewer observes no changes in the latter.

Although Fushimi et al 2016 showed that the Pr form fluoresces, they did not show emission from the Pg state. Fluorescence emission measured with purified Amg2 protein upon excitation at 640 nm shows that the Pg form also fluoresces, and the peak emission is about 4-fold lower than the Pr form (these data are now added to Fig. S6a). When emission is measured on the microscope, it is collected over the range of the bandpass emission filter (700 ± 25 nm) so that this difference is only ~ 2.5-fold. A change of this magnitude is difficult to detect reliably using fluorescence imaging without careful calibration since absolute intensities can vary, for example, if cells move slightly, or if regions are slightly out of focus (incidentally, this is another reason for variability in the MCR data from cell to cell). Thus, the far-red fluorescence detected using the microscope reports primarily on the location of the Amg2 protein rather than directly which state (Pr/Pg) it is in.

Fig. S12 (now S14) shows the effects of the 640 nm laser on the system. This wavelength produces a mixture of Pg and Pr states, i.e. some Pr state is switched to Pg and *vice versa*. For the reasons just given, we do not expect to see a substantial change in overall fluorescence intensity since both Pg and Pr states fluoresce. Instead, the change in MCR (evident in the figure) is a more robust measure of the degree of Pg/Pr conversion.

5. For the LEXY-Amg2 and LINus-Amg2 fusion systems in Fig. 2c, why is a mCherry fusion needed? Seems like its presence might complicate imaging deconvolution.

We agree that the fusion is not essential, however it is convenient for monitoring relative expression levels of the construct in different cells as well as the apo/holo ratio.

6. Fig. S13 Panel C imaging wavelengths used are missing.

Fig. S13 is now Fig. S23. The imaging wavelengths have now been added (excited at 640 nm, detected at 700 ± 25 nm)

7. Fig. S16 For the red-BICYCL constructs analyzed for SEAP gene expression in Fig. S15 (panel d), why does G irradiation inhibit expression of SEAP with increasing fluence. Shouldn't G, like dark, sustain the Pr form of Amg2? This was not seen in panels f and h however, so why are these experiment different?

We thank the reviewer for drawing our attention to this. We have now repeated these experiments three times, with the same batch of cells for reproducibility as well as to ensure statistical

significance. These data are now part of the main text (Fig. 3f). As expected, dark-adapted and green-irradiated expressions show no significant difference.

As for other systems, the green-BICYCL constructs are clearly much more robustly ON/OFF (panel e). What time after PCB addition (+/- light) was gene expression determined for panels d and e?

Gene expression was measured 24h after adding PCB. This information has now been added to the figure legend. In response to reviewer 3, the BICYCL-Green data has now moved to main text (Fig. 2).

8. Fig. S17 is not properly cited on page 9. Fig. S17 shows the loss of transcript abundances of the BICYCL Amg2-VP16 and E-BAm components in the cells during incubation/assembly of holoprotein with PCB.

Due to transient transfection mRNA levels are decreasing over time (plasmid dilution). We have now added data with stably transfected cells. Fig. S20 shows that Amg2 and Bam levels are stable over time in this case. Both these figures are now referred to in the main text. (pg. 8 for Fig. S17, pg 10 for Fig. S20)

9. For multiplex experiments in Fig. 2e&f, how can you control relative expression of the three systems, or can you, by transfection? By using different ratios of DNA?

It is possible to control the expression of the individual components by adjusting the absolute and relative concentration of the respective plasmids encoding for the different systems. This is a standard practice and can be useful for titrating different levels of each component for optimal response. In contrast, with stable cell lines, where the components are stably inserted into the genome, the culture is homogeneous. It is possible during the generation process of the cell lines, to select for clones (individual insertion events) expressing the different levels of the components and showing the optimal activity.

10. Supplementary Methods.
a. Page 5. What volume phage was added to each well of the MaxiSorp 96 plate?

This information has now been added (SI page 5).

b. Page 8. What is the mechanism of fluorescence quenching of Amg2 by BAmRed?

The mechanism of quenching is unknown but, based on other examples, we expect that binding of BAmRed near the chromophore of Amg2 alters solvation or the local protein conformation in a manner that enhances non-radiative decay processes. (van de Weert, M. J Fluoresc 20, 625–629 (2010).)

c. Page 32. Fig. S19, please define abbreviations or reference the different DNA binding proteins.

Fig. S19 is now Fig. S26. Full names have been added to abbreviations for each of the DNA binding proteins.

Reviewer #2:
Remarks to the Author:

The manuscript by Jang et al. presents the development and characterization of novel bidirectional photo-inducible dimerization systems based on cyanobacteriochrome. The authors chose a GAF domain from cyanobacteriochrome as a light-sensitive domain that can act as a conformational switches in response to irradiation in the green and red regions of the spectrum. They used this domain to systematically isolate state-specific binders (red or green state) from a library of GA domain variants by phage display and yeast two-hybrid methods. The best binders were characterized by ITC, size-exclusion chromatography and fluorescence quenching revealing low micromolar to sub-micromolar binding affinities and clear (>50 to >800-fold) preference for the state they were selected against. From this first part of the study, they identified BAmGreen2.4 and BAmRed1.4 as the most efficient green and red state binders of Amg2 (GAF domain) respectively. Next, they evaluated the properties of the binders in live cells. Control over the red and green binder capacity to recognize the Amg2 BAF domain was evaluated by respective localization of the binder and its target under green and red illuminations. Combination with LOV domain-based systems (irradiation with blue light) with LEXY and LINus was also evaluated. Finally, using three orthogonal reporter systems (SEAP, FLuc, GLuc) linked to the two binders and the LOV-domain based TULIP system, the authors demonstrated that they could selectively control each of the reporters expression with blue, green or red light. Altogether, these experiments confirmed the functionality of the tools in cells and their capacity to be used in combination with blue light controlled systems such as the ones derived from LOV domains.
This study is very well written and designed. It addresses a clear gap in the optogenetic field with so far an absence of tools that can be used in the green to red wavelengths thereby limiting multiplexing possibilities. With these two novel binders together with the Amg2 BAF domain, the authors elegantly demonstrate that, while these tools can still be improved, they importantly open new perspectives for setting up optogenetic-based studies with the possibility to combine up to three optogenetic modules with selective blue, green and red irradiation. The originality of the study is reinforced by a clear presentation of the results with numerous control experiments and a transparent reporting of the limitations. Statistics are treated adequately. References are appropriate but more may be added in the first paragraph (see minor comments). Overall, the manuscript is convincing and propose original tools that will with no doubt effectively complement the set of existing optogenetic tools by offering new possibilities.

We are delighted with this very positive assessment of our study and the comments on the importance of BICYCLs to fill the void of optogenetic tools in the green-red area of the spectrum. We would like to thank the reviewer for the suggestions made to improve the manuscript.

My main comment would be that while the authors have focused on the multiplexing aspect, which is already an impressive achievement, I believe that the full photocontrol of the Amg2 state is also

an aspect that opens up plenty of possibilities especially with respect to spatial control. Indeed, blue-light tools do not offer (or only partially and with poor control) this possibility as they rely on (fast) thermal relaxation to return to their basal state. Here, Amg2 combines a long half-life with the possibility to control by light its return to basal state. An experiment that would illustrate the benefit of this fully photoreversible system would increase the impact and scope of the manuscript. For instance, showing that by irradiating two halves of a cell with red and green light respectively to locally and intracellularly impose two states (bound and unbound optodimers) without being affected by diffusion would constitute a major and impactfull addition to the current manuscript.

We entirely agree that the bistable nature of BICYCLs offers the possibility of improved green/red spatial control as has been demonstrated for the bistable far-red/red PhyB/PIF6 system. An experiment like the one proposed requires specialized optical hardware (e.g. digital micromirrors). This type of experiment, itself, is not new (e.g. Nature. 2009 Oct 15; 461(7266): 997–1001), but an important question is whether the switching efficiency of BICYCLs in both directions is compatible with such experiments. If switching in one direction (Pr-to-Pg or Pg-to-Pr) was very inefficient (e.g. required much higher light intensities than the other), then such experiments would be problematic. We show that BICYCLs are in fact compatible with two-color experiments for enhanced spatial control with several additional proof of principle experiments. First, we show with purified protein (where the state (Pg or Pr) can be ascertained from the color alone) that one state can be confined to a localized zone by switching off protein that diffuses out of the zone (Fig. 4d). This is, in effect, an in vitro, macroscopic version of the experiment that the reviewer proposes. Second, we show that one can profit from the bistability to restrict subcellular protein recruitment to mitochondria in individual cells without affecting adjacent cells by irradiating adjacent cells with the opposing wavelength (Fig. 4e,f). Third, we show that gene expression can be spatially regulated using a photo-mask (Fig. 4g). The bistability means that tighter localization is obtained with the masked zone is exposed to opposing wavelength (Fig. 4g, S22)

Minor comments:
-In the first paragraph of the main text, it would useful to clearly define the blue, green, red and far-red regions of the spectrum with their respective wavelength range.

We have now added this information.

-Reference should be included when CBCRs (1st main text paragraph) and blue-light tools (end of 2nd paragraph) are first mentioned.

Further references to prior work on CBCRs have been included (pg. 1) as well a comprehensive reference to blue light tools.

-When reading fast, the red/green nomenclature turns out to be a bit confusing, a clear scheme in figure 1 summarizing which binder binds which state would be a plus for insuring that misunderstandings are avoided.

As suggested, we have now added a panel to Fig. 1 summarizing which binder binds which state. Additionally, we have streamlined the schemes showing molecular design and mechanism of function of the optoswitches throughout (Figs. 1-5).

Reviewer #3:
Remarks to the Author:

The authors identify new binding partners (affibody) for Amg2 (15ZPr/15EPg) using a phage-display approach established by the group in 2018. Of the binding partners described, BAmGreen seems superior to BAmRed. Based on the BAmGreen/Red-Amg2 binding modules, the authors develop bidirectional, cyanobacteriochrome-based light inducible dimerization modules (BICYCL) systems for optogenetic control of protein-protein interactions and gene expression in cells.

This reviewer finds it a bit strange that all of the work done validating the BICYCL system in cells (mitochondrial recruitment data) was in supplemental, and not really referred to in the main text. This is probably due to figure restrictions of the journal, but if the manuscript is a method paper describing the BICYCL system, this reviewer would rather see more attention given the system on its own (vs. its compatibility with other previously established systems). Multiplexing system is interesting, but this paper focuses on the development of BICYCL, not on multiplexing optogenetic systems. To this extent, figures S7-S11 are important, but there is no description of them in the text. It is good to show in the main text an imaging panel showing cells cycled through multiple rounds of red-green switching.

We thank the reviewer for the comments and suggestions to improve the manuscript. Indeed, space constraints led us to put much of the data showing characterization of the BICYCL system alone (not multiplexed) in the SI. We have now moved some key data related to the development and implementation of the systems to the main text. This includes the molecular and functional characterization of the various optogenetic tools and imaging panels showing cells cycled through multiple rounds of red/green switching (new Figs. 2,3)

The authors show a long half-life of the thermal relaxation and claim BICYCLs act as bistable tools (Fig. S2c). However, a constant illumination for 24h by green/red light is required in many applications in cells.
Would such a long illumination lead to phototoxicity or other cellular effects? This is a major drawback of the system, which is not discussed in the text.

We show that the purified Amg2 protein acts as a bistable switch with very slow thermal relaxation ($\tau_{1/2}$: 25 h, 37°C; Fig. S2c). Cellular imaging data shows no relaxation over 1h (Fig. S13). Incubation in the dark for longer periods with live cells leads to cell movement and cell division so that images taken after many hours look very different from initial images. The stability of the switches *in vivo* is therefore best tested via gene expression studies. Our description of these experiments was not very clear in the previous version of the manuscript. While we did test effects of constant illumination of cells for 24h (and did not see toxicity), this is not required for stable gene expression. Indeed, as shown (in new Fig. 2) illumination times as short as 30 seconds are sufficient to achieve sustained levels of expression. This experiment (Fig. 2f) demonstrates that

when Amg2 is switched to the Pg state, it remains in this state over many hours in living cells. If considerable thermal relaxation occurred, gene expression would not be seen at this level (as demonstrated by deliberately switching the protein back to the Pr state with green light (Fig. 2f).

Overall, the BICYCL system could be a valuable expansion of the current optogenetic toolbox. But the manuscript is not well written. There is a lot of supplemental data not referred to or discussed in the text.

We appreciate the candid assessment and have now tried to clarify the manuscript by moving some supplemental data to the main text and ensuring that all SI figures are now mentioned in the main text as suggested by the reviewer.

Specific comments:
Fig. 1h, S5b, how to explain the binding at the initial titration?

As the reviewer points out, the first 1-2 injections in Fig 1h and the first injection in S5b indicate some binding is occurring. We attribute this to a fraction of the Pg-state being present. The commercial ITC instrument has a small white light at the loading site to guide syringe injection that cannot be turned off so that some Pr-to-Pg photoconversion is unavoidable. Fitting the data to this model indicates ~10% of the Pg state is present. This is noted in the figure legends. These curves cannot be used to extract accurate K_d values for binding but clearly indicate that binding to the dominant state (Pr) is much weaker upon photoconversion.

ITC measurements of BAmRed1.0, 1.4 binding to Pg-Amg2 are missing.

When BAmRed1.0 and BAmRed1.4 are added to Pg-Am2 thermal reversion rates are enhanced. We attribute this effect to weak binding of BAmRed1.0/BAmRed1.4 to a transition state structure on the pathway from Amg2-Pg state to Amg2-Pr state. Plotting the rate constant for thermal reversion vs. concentration of binder gives an apparent Kd for this effect >200 μM. This means the effect is negligible at concentrations of Amg2/BAmRed1.0/BAmRed1.4 in cells but at the high protein concentrations required for ITC the effect means that Pg reverts to Pr during the ITC run preventing the measurement of a Pg-state Kd.

Page 6: "red-BICYCL1.4 (BAmRed1.4) afforded the tightest affinity." Didn't BAmGreen2.4 display the tightest affinity (Kd = 0.12 uM)?

The reviewer is correct. We have now clarified this section of the text.

Fig. S9-10: The authors did not discuss the effect of PCB concentration. No quantification was shown for no PCB and 10 uM PCB.

These data are now shown in Figs S9-12. The effect of PCB concentration is now discussed in the main text (pg. 7). Quantification of images is now added.

Fig. S11: Determination of the half-life of thermal relaxation in live cells is required.

(See reply to point #2 above) The stability of the switch *in vivo* is best tested via gene expression studies, and we have now moved the gene expression time course data to the main text. The half-life of purified protein is 25 h at 37°C, and the gene expression data confirm that the Amg2 state is stable in the dark for ~24h (Figs. 2,3).

Fig. S14: Quantification and statistical analysis are needed.

Figure S14 is now Fig. S24. Quantification and statistical analysis have now been added to this figure.

Fig. S15: d and e, please indicate the illumination time;

The illumination time (24h) has now been added to the figure legend

(h, i) legend: "Relative expression of Amg2 and BAm transcript level." Or you mean SEAP expression level?

Data showing transcript (mRNA) levels (Fig. S17) have now been separated from protein expression data (Fig. S15). This wording has now been changed to: RT-qPCR were performed to measure the relative expression of (a,b) SEAP, (c,d) Amg2, (e) BAmRed1.4, and (f) BAmGreen2.4 transcripts. The expression was normalized by GAPDH

Shouldn't the expression level of SEAP correlate with the activity shown in f and g, respectively?

The SEAP protein expression (measured by protein activity) lags behind the transcription of its gene (measured by detecting the RNA transcript using RT-qPCR). This has been seen with other systems; e.g. Müller et al. (2013) Nucleic Acids Research doi:10.1093/nar/gkt340)

Fig. S16: were b and d swapped?

We thank the reviewer for noting this error. It has now been corrected and some of this data has been moved to the main text.

No switch was observed in d with the illumination time < 3h, suggesting that red-BICYCL1.4 requires a long illumination time (at least 24h).

Data showing the response of the red-BICYCL system is now shown in Figs 3e,f. Our description of these experiments was unclear in the previous version. Turn-on is seen after 1h.

Decision Letter, first revision:

Dear Drew,

Thank you for your letter detailing how you would respond to the remaining reviewer concerns regarding your Article, "Engineering of bidirectional, cyanobacteriochrome-based light inducible dimers (BICYCL)s". We have decided to invite you to revise your manuscript as you have outlined, before we reach a final decision on publication.

With regards to point four, we do not think a fluorescence titration is necessary for the revision. In your updated rebuttal that the referee(s) will see, please make note of the binding/chromatography data you already show in the SI.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further.

When revising your paper:

* include a point-by-point response to the reviewers and to any editorial suggestions

* please underline/highlight any additions to the text or areas with other significant changes to facilitate review of the revised manuscript

* address the points listed described below to conform to our open science requirements

* ensure it complies with our general format requirements as set out in our guide to authors at www.nature.com/naturemethods

* resubmit all the necessary files electronically by using the link below to access your home page

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We hope to receive your revised paper within four weeks. If you cannot send it within this time, please let us know. In this event, we will still be happy to reconsider your paper at a later date so long as nothing similar has been accepted for publication at Nature Methods or published elsewhere.

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Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further. We look forward to seeing the revised manuscript and thank you for the opportunity to consider your work.

Sincerely,
Rita

Rita Strack, Ph.D.
Senior Editor
Nature Methods

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
I am happy with the revised paper.

Reviewer #2:
Remarks to the Author:
I am satisfied with the revised manuscript by Pr Woolley and co-authors. My points were properly addressed and so were the points raised by the other reviewers.
The reporting summary is adequately filled.

Reviewer #3:
Remarks to the Author:
The manuscript has been improved. However, some key issues related to “bistable switch” remain to be solved.
1. “When BAmRed1.0 and BAmRed1.4 are added to Pg-Am2 thermal reversion rates are enhanced. We attribute this effect to weak binding of BAmRed1.0/BAmRed1.4 to a transition state structure on the pathway from Amg2-Pg state to Amg2-Pr state. Plotting the rate constant for thermal reversion vs. concentration of binder gives an apparent Kd for this effect >200 µM. This means the effect is negligible at concentrations of Amg2/BAmRed1.0/BAmRed1.4 in cells but at the high protein concentrations required for ITC the effect means that Pg reverts to Pr during the ITC run preventing the measurement of a Pg-state Kd.”
This issue is critical and should be discussed in the text. The results should be shown. The authors only measured the Pg to-Pr thermal reversion half-life of Amg2 alone (Fig. S2g). However, the half-life could be enhanced in the presence of binding partners (see also comments below). The authors should measure the half-lives of Amg2-BAmGreen/Red complexes under different illumination conditions in vitro and in cells. These are the key results, which are missing, to demonstrate the novelty of the system, i.e. bistabilty.
2. “While we did test effects of constant illumination of cells for 24h (and did not see toxicity), this is not required for stable gene expression. Indeed, as shown (in new Fig. 2) illumination times as short as 30 seconds are sufficient to achieve sustained levels of expression. This experiment (Fig. 2f) demonstrates that when Amg2 is switched to the Pg state, it remains in this state over many hours in living cells. If considerable thermal relaxation occurred, gene expression would not be seen at this level (as demonstrated by deliberately switching the protein back to the Pr state with green light (Fig. 2f).”
This result demonstrates that Amg2-BAmGreen binding may be stable in the dark for some time (1h as shown in Fig. S13). But to enhance gene expression required longer illumination time and constant illumination of 24h was required for maximal expression (Fig. 2f). Particularly, consistent with the authors’ finding in ITC (accelerated Pg-to-Pr conversion), the dissociation of Amg2-BAmRed was not stable, i.e. long-term illumination was required (Fig. 3f).
3. In the section of “Bi-stability and spatial control capabilities of the BICYCL optogenetic systems”, the authors attributed the spatial control to bistability. However, this reviewer is not convinced. The authors used constant (24h) illumination of activating light in combination with inhibitory light. The constant illumination conditions were not shown in the figure legend or text, but was hided somewhere in the legend of Figure S22. The results achieved here probably have nothing to do with bistability (stable in on or off state), unless they could do these by a short pulse of activating and inhibitory light illumination. Moreover, one could also make it with short-lifetime on/off tools using these conditions, i.e. constant illumination. “Bidirectional” would be a more appropriate description.

4. To avoid the contaminating light from the ITC cell, the authors could use an independent approach to measure the binding such as fluorescence titration as shown in Fig. S6. The authors should rule out the possibility that BAmGreen may also accelerate the Pg-to-Pr conversion of Amg2.

Author Rebuttal, first revision:

Response to Referees.

We are very pleased that referees 1 & 2 are satisfied with our revisions. Thank you for the opportunity to respond to reviewer 3. We are indebted to the reviewer for their careful reading of our work and their thoughtful comments. Please see our response and proposed revisions below (reviewer comments are in green).

Reviewer #3:
Remarks to the Author:
The manuscript has been improved. However, some key issues related to "bistable switch" remain to be solved.

1. "When BAmRed1.0 and BAmRed1.4 are added to Pg-Am2 thermal reversion rates are enhanced. We attribute this effect to weak binding of BAmRed1.0/BAmRed1.4 to a transition state structure on the pathway from Amg2-Pg state to Amg2-Pr state. Plotting the rate constant for thermal reversion vs. concentration of binder gives an apparent Kd for this effect >200 μM. This means the effect is negligible at concentrations of Amg2/BAmRed1.0/BAmRed1.4 in cells but at the high protein concentrations required for ITC the effect means that Pg reverts to Pr during the ITC run preventing the measurement of a Pg-state Kd."

This issue is critical and should be discussed in the text. The results should be shown. The authors only measured the Pg to-Pr thermal reversion half-life of Amg2 alone (Fig. S2g). However, the half-life could be enhanced in the presence of binding partners (see also comments below). The authors should measure the half-lives of Amg2-BAmGreen/Red complexes under different illumination conditions in vitro and in cells. These are the key results, which are missing, to demonstrate the novelty of the system, i.e. bistabilty.

The reviewer raises a very interesting topic that relates to the mechanism of thermal reversion and how these binding partners may influence conformational dynamics of the Amg2 domain. In fact, we characterized the effects of binding partners on relaxation rates of Amg2 but did not include this data previously because we considered it beyond the scope of the manuscript. We are happy to add these data to the SI. Using purified proteins *in vitro*, we measured thermal relaxation rates using UV-Vis

spectroscopy at 37ºC to match the temperature used for measurements in live cells. These data are now shown (Fig. S18)(shown below here as Fig. 1):

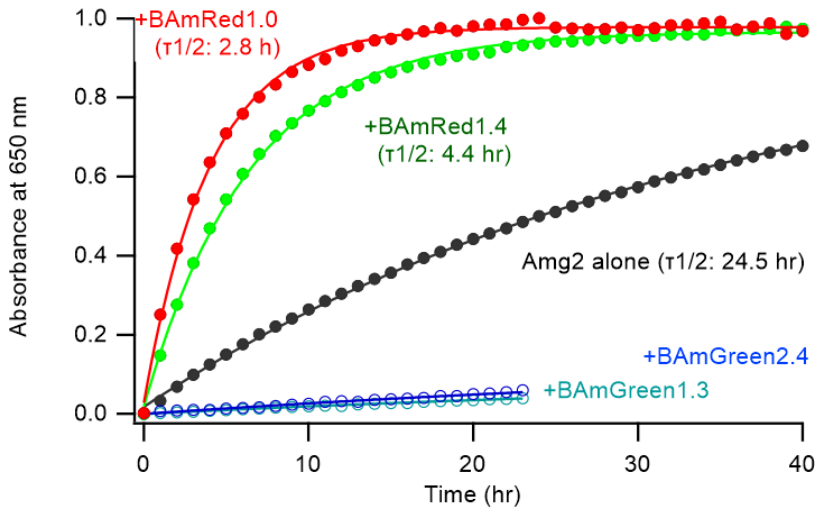

Figure 1. Thermal relaxation of Amg2 (10 μM) from the Pg state to the Pr state measured via absorbance at 650 nm in the absence (black) or presence of 20 μM BAmGreen1.3 (cyan), BAmGreen2.4 (blue), BAmRed1.0 (red), or BAmRed1.4 (green). Half-lives were calculated by fitting the curves to single exponential decay processes. BAmGreen1.3 and BAmGreen2.4 inhibit thermal reversion of Amg2. BAmRed1.0 and BAmRed1.4 accelerate thermal reversion Amg2.

While thermal relaxation becomes very slow (<5% relaxation in 24 h) in the presence of BAmGreen1.3 and 2.4, relaxation is accelerated in the presence of BAmRed1.0 and 1.4. Figure 2a shows Amg2 relaxation traces with increasing concentrations of BAmRed1.0 and Figure2b shows the calculated (observed) relaxation rates plotted vs. BAmRed1.0 concentration. This plot is almost linear indicating that the effective K_d for the interaction of BAmRed1.0 with Amg2 in the Pg state (or perhaps in some intermediate state/transition state *en route* from Pg to Pr) is >200 μM.

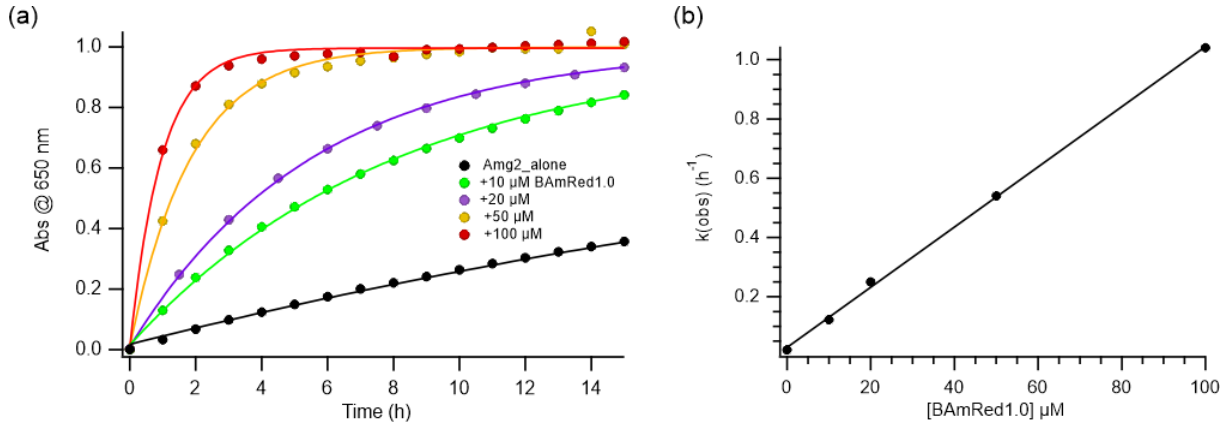

Figure 2. (a) Thermal relaxation of Amg2 (10 µM) from the Pg state to the Pr state measured via absorbance at 650 nm in the absence (black) or presence of increasing concentrations of BAmRed1.0 (see legend). **(b)** Rate constants for relaxation observed in (a) plotted versus concentration of BAmRed1.0 (an accurate Kd cannot be determined from these data but must be > 200 µM)

The practical implications of these data are that BAmRed proteins accelerate relaxation to the 2-4 h half-life range under conditions where both BAmRed and Amg2 are present at high concentrations (as during ITC measurements). In cells, however, even with proteins expressed under CMV promoters protein concentrations are more typically ~ 0.3 to 3 µM (refs. 1-4 below). Based on Fig. 2b this means relaxation rates <0.05 h^{-1} would be expected, or half-lives >12h.

Direct measurements of half-lives for thermal relaxation of Amg2 in living cells are not possible because proteins are continuously synthesized and degraded, and cells grow and divide on the time frames involved. Given the intrinsic experimental limitations, it is only possible to make a rough, indirect, estimate of the thermal relaxation half-life of Amg2 in cells via the quantification of gene expression data as shown in Figures 2f and 3f. These data show that for BAmRed/Amg2 (BICYCL-Red)(Fig. 3f), irradiation for 1h with red light, followed by 23 hours of dark incubation leads to gene expression levels that are significantly lower than those seen without irradiation (Fig. 3f) (*i.e.* 24h darkness), meaning that a significant fraction of Amg2 must remain in the Pg state for 23 h in darkness, even in the presence of BAmRed. For BAmGreen, persistence of gene expression is also seen 23 h after irradiation (Fig. 2f). While Amg2 undergoes Pg to Pr relaxation, which may be enhanced somewhat if BamRed is present (see above), these data confirm that the Pg state persists, at least for hours, under intracellular conditions.

2. “While we did test effects of constant illumination of cells for 24h (and did not see toxicity), this is not required for stable gene expression. Indeed, as shown (in new Fig. 2) illumination times as short as 30 seconds are sufficient to achieve sustained levels of expression. This experiment (Fig. 2f) demonstrates

that when Amg2 is switched to the Pg state, it remains in this state over many hours in living cells. If considerable thermal relaxation occurred, gene expression would not be seen at this level (as demonstrated by deliberately switching the protein back to the Pr state with green light (Fig. 2f)."

This result demonstrates that Amg2-BAmGreen binding may be stable in the dark for some time (1h as shown in Fig. S13). But to enhance gene expression required longer illumination time and constant illumination of 24h was required for maximal expression (Fig. 2f). Particularly, consistent with the authors' finding in ITC (accelerated Pg-to-Pr conversion), the dissociation of Amg2-BAmRed was not stable, i.e. long-term illumination was required (Fig. 3f).

The reviewer is correct that maximal expression is seen with longer illumination times. However, as noted above, measurements in living cells, in contrast to *in vitro* experiments, are affected by continuous synthesis and degradation of proteins and cell growth. Newly synthesized Amg2 will be in the Pr state if it is synthesized during a dark incubation period and newly synthesized Amg2 interacts with BAmRed, but not with BAmGreen. This can be observed in Fig. 3f vs. 2f (and Fig. 3e vs. 2e) where 24h dark incubation produces strong gene expression with BAmRed, but no signal with BAmGreen. As a result, gene expression changes that require dissociation of Amg2(Pr)-BAmRed (Fig. 3f) will appear less stable than those that require association Am2(Pg)-BAmGreen (*i.e.* Fig. 2f) because longer red light illumination times are required to dissociate *newly synthesized* Amg2 protein from BAmRed. We agree with the reviewer that some enhancement of Amg2-Pg to Amg-Pr relaxation by BAmRed could contribute to this observation but, based on measured half-lives with purified proteins *in vitro*, this effect is expected to be small, as argued above.

3. In the section of "Bi-stability and spatial control capabilities of the BICYCL optogenetic systems", the authors attributed the spatial control to bistability. However, this reviewer is not convinced. The authors used constant (24h) illumination of activating light in combination with inhibitory light. The constant illumination conditions were not shown in the figure legend or text, but was hided somewhere in the legend of Figure S22. The results achieved here probably have nothing to do with bistability (stable in on or off state), unless they could do these by a short pulse of activating and inhibitory light illumination. Moreover, one could also make it with short-lifetime on/off tools using these conditions, i.e. constant illumination. "Bidirectional" would be a more appropriate description.

We agree with the reviewer that the term "bi-directional" would better describe what is occurring in this experiment. Amg2 is distinct from proteins such as LOV that undergo thermal relaxation but cannot be actively "switched off" with light. The sharp spatial demarcation between "on" and "off" states seen in Figures 4d and 4g could, in principle, be achieved using a very fast relaxing version of a blue LOV photoswitch, but the downside of such an approach is that it requires intense irradiation (which is toxic) to achieve a large fraction of the on state.

However, as the reviewer points out, it is not the bi-stability *per se* that enables the spatial control in this experiment, but the fact that the protein can be *actively* switched in either direction by different colours of

light. Thus, we have reworded this section (Pg. 11 in main text) to reflect this, using the term "bi-directional" as suggested. In addition, we have now added the illumination conditions to the figure legend as requested.

4. To avoid the contaminating light from the ITC cell, the authors could use an independent approach to measure the binding such as fluorescence titration as shown in Fig. S6. The authors should rule out the possibility that BAmGreen may also accelerate the Pg-to-Pr conversion of Amg2.

We have shown (see Fig. 1 above, included as Fig. S18 in the revised manuscript) that BAmGreen inhibits, rather than accelerates, the Pg-to-Pr conversion of Amg2.

As the reviewer suggests, it might be possible to detect interaction of BAmGreen with the Pr state of Amg2 using fluorescence. However, we argue that the complete absence of a shift in the elution volumes seen using size exclusion chromatography (Fig. S4a) is good evidence for the absence of an interaction between Amg2-Pr and BAmGreen. No change in fluorescence would thus be expected if BAmGreen were added to Amg2-Pr.

References:

1. Nalaskowski MM, Ehm P, Giehler S, Mayr GW. A toolkit for graded expression of green fluorescent protein fusion proteins in mammalian cells. Anal. Biochem. (2012) 428:24-7.
2. Legewie S, Herzel H, Westerhoff H, Bluthgen N. Recurrent design patterns in the feedback regulation of the mammalian signaling network. Mol.Syst. Biol. (2008) 4:190 *supplementary material section II pp.6-7 table*
3. Li et al. System wide analyses have underestimated protein abundances and the importance of transcription in mammals. Peer J. (2014) 2:e270 *p.14 2nd paragraph*
4. Woloschuk et al. Yeast Two-Hybrid Screening of Photoswitchable Protein-Protein Interaction Libraries. J. Mol. Biol. (2020) 432:3113-3126.

Decision Letter, second revision:

Dear Drew,

Thank you for submitting your revised manuscript "Engineering of bidirectional, cyanobacteriochrome-based light inducible dimers (BICYCL)s" (NMETH-A48745B). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Methods, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

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Thank you again for your interest in Nature Methods Please do not hesitate to contact me if you have any questions.

Sincerely,
Rita

Rita Strack, Ph.D.
Senior Editor
Nature Methods

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Reviewer #3 (Remarks to the Author):

The authors have now addressed this reviewer's questions. The discussions on why constant illuminations are required in experiments (e.g. mainly to address newly synthesized Amg2 but the effect from enhancement of Amg2-Pg to Amg-Pr relaxation by BAmRed could be small) should be included in the main text. After this minor revision, this reviewer highly recommends publishing this nice piece of work in Nature Method.

Author Rebuttal, second revision:

Response to referee:

Reviewer #3 (Remarks to the Author):

The authors have now addressed this reviewer's questions. The discussions on why constant illuminations are required in experiments (e.g. mainly to address newly synthesized Amg2 but the effect from enhancement of Amg2-Pg to Amg-Pr relaxation by BAmRed could be small) should be included in the main text. After this minor revision, this reviewer highly recommends publishing this nice piece of work in Nature Method

Response:

We have added a sentence to the main text (Pg. 7) as follows:

Note that higher expression levels are observed with longer illumination times since measurements are affected by continuous synthesis and degradation of proteins and cell growth. Newly synthesized Amg2 will be in the Pr state if it is synthesized during a dark incubation period and newly synthesized Amg2 interacts with BAmRed, but not with BAmGreen.

Final Decision Letter:

Dear Drew,

I am pleased to inform you that your Article, "Engineering of bidirectional, cyanobacteriochrome-based light inducible dimers (BICYCL)s", has now been accepted for publication in Nature Methods. Your paper is tentatively scheduled for publication in our March print issue, and will be published online prior to that. The received and accepted dates will be March 22, 2022 and Dec 21, 2022. This note is intended to let you know what to expect from us over the next month or so, and to let you know where to address any further questions.

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Best regards,
Rita