nature portfolio

Peer Review File

Photoinduced Chromophore Dissociation Resulting in Aggregation-induced Red Fluorescence

Corresponding Author: Professor Karthik Pushpavanam

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have submitted a thoroughly revised and substantially improved version of their manuscript. This third version benefits from a clear focus on the aggregation-induced fluorescence of CTPF and the complete removal of the MjRibK-related work. I remain supportive of this work, and it is my opinion that this manuscript could be potentially suitable for publication once the following minor issues are addressed.

1. I suggest that the authors should reconsider language such as “redefine the photochemistry of PhoCl1” in the conclusion and “reconsider the previously proposed model” in the abstract and on page 15. In my opinion, the fate of the CTPF was not part of the “previously proposed” model of PhoCl1. The key features of the previously proposed model were simply that photocleavage and dissociation result in the loss of red fluorescence from PhoCl itself. The aggregation of CTPF is a newly discovered phenomenon that occurs subsequently to CTPF dissociation, but does not redefine or change anything about the originally proposed model for PhoCl itself.

2. Figure S1 will need to be revised to correctly represent the photoconversion process. There are three issues with the current representation. The first issue is that the photoconversion proceeds from the protonated state of the chromophore (not the anionic form as represented in the figure). The clearest evidence for this is that it is the protonated state that absorbs at 405 nm, which is consistent with the wavelengths of light that most effectively induce the photocleavage. The second issue is that the two anionic forms (II and III) are resonance forms. Accordingly, it is not appropriate to describe them as in equilibrium with each other, and also not appropriate to use equilibrium arrows between them. A double-headed resonance arrow should be used. The third issue is that just one resonance form of the red chromophore is represented and, if I had to guess, I would say this is the lesser contributor to the resonance hybrid. The resonance form with the negative charge on the phenolate oxygen is more likely to be the major contributor to the resonance hybrid because it restores the aromaticity.

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4. In the abstract and conclusion, the authors mention “unexpected red fluorescence”, and “generates red fluorescence”, respectively. I suggest it would be appropriate in both cases to change these two phrases to “unexpected dim red fluorescence”, and “generates dim red fluorescence”, respectively.

5. I suggest that the first sentences of the figure legends in the main text and supporting information should use consistent capitalization, ideally following regular sentence capitalization rules.

Reviewer #2

(Remarks to the Author)
Kumar et al. have largely changed the manuscript in response to reviewer 1’s recommendation by removing the description of MjRibK. In this revised manuscript, the authors almost addressed my concern and they mostly focused on the mechanism of CTPF fluorogenesis induced by violet light irradiation of PhoCl1. They purified CTPF from violet-irradiated PhoCl1, characterized it by MALDI-TOF, SDSPAGE, dynamic light scattering, and absorption and fluorescence spectroscopy, and then, concluded that the violet light-induced generation of red fluorescence from PhoCl1 was attributed to CTPF aggregates.

Their MM and QM/MM studies also supported this conclusion. The authors took most effort to describe the mechanism of CTPF fluorogenesis and its characterization results. However, about the significance of this study, they only mentioned "These findings redefine the photochemistry of PhoCl1, highlighting its potential for developing fluorescence-based tools and expanding the applications of photocleavable proteins in optogenetics, bioimaging and molecular engineering". Although the criteria for publication in Communications Biology include an item of "The manuscript is important to scientists in the specific sub-field of biology", I do not think that the present manuscript meets the expectation of this journal's readers and I think that they need to claim the significance of their findings with more scientific insights and potential applications. Although in bioimaging field a labeling tag that forms aggregates of submicrons to microns to become fluorescent is difficult to use in general, I wonder if the authors have already come up with good ideas of CTPF use. I think that Scientific Reports, PLOS One, or a journal of a more specific field is likely to be more suitable for this manuscript unless it is revised.

Minor comments:
Figures 1E and 3A, and Figures 1B and 3B.
Figure 1E is no more than the subset of Figure 3A. Therefore, I think that Figure 3A can be moved to Figure 1A, the dashed red curve (Exposed) can be cited in the main text first, and the solid green curve (Unexposed) can be cited later in the text. Likewise, Figure 1B is no more than the subset of Figure 3B.

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The text writes "In this manuscript". According to Longman Dictionary of Contemporary English, the definition of "manuscript" is "a book or piece of writing before it is printed". I wonder if the authors hope that this manuscript continues to be in a "before it is printed" condition.

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Although the text writes "peak around 480 nm (Figure S3A)", such peak is not seen in Figure S3A.

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The authors investigated the effect of guanidium hydrochloride on CTPF fluorescence up to 2 M. Guanidium hydrochloride is very well-known as a typical denaturant in biology, and many biologists are likely to wonder what if guanidium hydrochloride is increased up to 6 M.

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The text writes "the previously proposed model". Here, a suitable paper should be cited.

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Reviewer #3

(Remarks to the Author)
This further revised manuscript has addressed this reviewer's prior concerns, with adequate clarifications and useful edits to

stay focused on an interesting new finding about the AIE-induced fluorescence from the photodissociated product (a C-terminal peptide) of PhoCl1. The response also pointed out future directions for a more detailed study of the aggregated CTPF chromophore in various environments, which will be a nice extension of this work. This reviewer can support the publication of this current version in Commun. Biol.

Version 1:

Reviewer comments:

Reviewer #1

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The authors have revised the manuscript to my satisfaction. It is my opinion that this work meets the standards of Communications Biology, and can be accepted for publication in its current form.

Reviewer #2

(Remarks to the Author)
The manuscript by Kumar et al. has been substantially improved. In this revised version, the authors have addressed nearly all of my previous concerns, and I acknowledge that they have convincingly demonstrated that the red fluorescence observed from PhoCl1 after photocleavage is attributable to the aggregation of CTPF.

However, in my previous review, I specifically pointed out that the authors had not sufficiently discussed the broader significance of their findings. Unfortunately, the current revision suggests that this point may not have been fully recognized. The authors mention the scientific relevance of their discovery only briefly, stating in the Conclusion: “These findings highlight the behavior of the photodissociated chromophore-containing peptide, highlighting its potential for developing fluorescence-based tools.”

This leaves me questioning how the authors themselves perceive the value of their results and what scientific message they intend to convey to the readers by publishing this work. While the first sentence of the Abstract refers to applications in “bioimaging and visualizing cellular localization, functionalities,” it should be noted that aggregating fluorescent dyes such as CTPF are generally not well-suited as probes for such purposes.

I strongly recommend that the authors provide a more thoughtful and critical discussion on the scientific importance of their discovery, its limitations, and potential future directions. Therefore, I recommend that the manuscript be revised accordingly.

Minor comment:
Page 6, Lines 3-4:
The sentences “We recorded the emission spectra…” and “We also recorded the corresponding excitation spectra…” both use the plural term “spectra.” However, Figure 2E presents only a single emission spectrum and a single excitation spectrum. The figure caption also incorrectly uses “spectra” in this context. This should be corrected.

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This minor-revised manuscript has largely addressed the other two reviewers’ remaining concerns, and this reviewer supports this further clarified/improved version for publication in Commun. Biol.

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Response: We agree with your interpretation and have revised the language accordingly to better reflect the scope of our findings. Specifically, we have removed the phrase “redefine the photochemistry of PhoCl1” from the conclusion and replaced “reconsider the previously proposed model” with more precise language that acknowledges the new observations regarding the aggregation behavior of the chromophore-containing peptide fragment (CTPF) without implying a revision of the original model of PhoCl1 photochemistry. The rephrased sentences are as follows

“These findings reveal a previously unrecognized role of the photodissociated chromophore-peptide complex in driving aggregation-induced emission.”

“These findings highlight the behavior of the photodissociated chromophore containing peptide”

2. Figure S1 will need to be revised to correctly represent the photoconversion process. There are three issues with the current representation. The first issue is that the photoconversion proceeds from the protonated state of the chromophore (not the anionic form as represented in the figure). The clearest evidence for this is that it is the protonated state that absorbs at 405 nm, which is consistent with the wavelengths of light that most effectively induce the photocleavage. The second issue is that the two anionic forms (II and III) are resonance forms. Accordingly, it is not appropriate to describe them as in equilibrium with each other, and also not appropriate to use

equilibrium arrows between them. A double-headed resonance arrow should be used. The third issue is that just one resonance form of the red chromophore is represented and, if I had to guess, I would say this is the lesser contributor to the resonance hybrid. The resonance form with the negative charge on the phenolate oxygen is more likely to be the major contributor to the resonance hybrid because it restores the aromaticity.

Response: We thank the reviewer for the helpful comments.

The figure has been revised to show the protonated chromophore as the light-absorbing species at 405 nm.

The anionic forms II and III are now connected with a resonance arrow, not an equilibrium arrow.

An additional resonance form of the red chromophore has been added. It shows the negative charge on the phenolate oxygen.

The updated figure is now included as Revised Figure S1.

3. I suggest that the title be revised, perhaps just to “Photoinduced chromophore dissociation resulting in aggregation-induced red fluorescence”.

Response: Thank you for your suggestion. The title has been revised to: “Photoinduced chromophore dissociation resulting in aggregation-induced red fluorescence” as recommended.

4. In the abstract and conclusion, the authors mention “unexpected red fluorescence”, and “generates red fluorescence”, respectively. I suggest it would be appropriate in both cases to change these two phrases to “unexpected dim red fluorescence”, and “generates dim red fluorescence”, respectively.

Response: Thank you for the suggestion. We have updated both the instances in the abstract and conclusion to “unexpected dim red fluorescence” and “generates dim red fluorescence,” respectively.

5. I suggest that the first sentences of the figure legends in the main text and supporting information should use consistent capitalization, ideally following regular sentence capitalization rules.

Response: Thank you for the suggestion. We have updated the first sentence of the figure legends to follow regular sentence capitalization rules.

Reviewer #2 (Remarks to the Author):

Kumar et al. have largely changed the manuscript in response to reviewer 1's recommendation by removing the description of MjRibK. In this revised manuscript, the authors almost addressed my concern and they mostly focused on the mechanism of CTPF fluorogenesis induced by violet light irradiation of PhoCl1. They purified CTPF from violet-irradiated PhoCl1, characterized it by MALDI-TOF, SDSPAGE, dynamic light scattering, and absorption and fluorescence spectroscopy, and then, concluded that the violet light-induced generation of red fluorescence from PhoCl1 was attributed to CTPF aggregates. Their MM and QM/MM studies also supported this conclusion. The authors took most effort to describe the mechanism of CTPF fluorogenesis and its characterization results. However, about the significance of this study, they only mentioned "These findings redefine the photochemistry of PhoCl1, highlighting its potential for developing fluorescence-based tools and expanding the applications of photocleavable proteins in optogenetics, bioimaging and molecular engineering". Although the criteria for publication in Communications Biology include an item of "The manuscript is important to scientists in the specific sub-field of biology", I do not think that the present manuscript meets the expectation of this journal's readers and I think that they need to claim the significance of their findings with more scientific insights and potential applications. Although in bioimaging field a labeling tag that forms aggregates of submicrons to microns to become fluorescent is difficult to use in general, I wonder if the authors have already come up with good ideas of CTPF use. I think that Scientific Reports, PLOS One, or a journal of a more specific field is likely to be more suitable for this manuscript unless it is revised.

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Figures 1E and 3A, and Figures 1B and 3B.

Figure 1E is no more than the subset of Figure 3A. Therefore, I think that Figure 3A can be moved to Figure 1A, the dashed red curve (Exposed) can be cited in the main text first, and the solid green curve (Unexposed) can be cited later in the text. Likewise, Figure 1B is no more than the subset of Figure 3B.

Response: Thank you for the suggestion. We agree that Figures 1B and 1E are subsets of Figures 3B and 3A, respectively. Accordingly, we have removed Figures 1B and 1E and replaced them with the original Figures 3B and 3A and renamed them appropriately.

L74

The text writes "In this manuscript". According to Longman Dictionary of Contemporary English, the definition of "manuscript" is "a book or piece of writing before it is printed". I wonder if the authors hope that this manuscript continues to be in a "before it is printed" condition.

Response: Thank you for bringing this to our attention. We have changed “In this manuscript” to “In this work”.

L112

Although the text writes “peak around 480 nm (Figure S3A)”, such peak is not seen in Figure S3A.

Response: We thank the reviewer for the observation. The manuscript has been revised to clarify that the excitation scan of unexposed PhoCl1 showed an increasing trend in the intensity towards larger wavelengths. The maximum could not be resolved due to spectral bleed-through of the excitation light into the emission channel fixed at 510 nm. The rephrased sentence reads as follows

“Concurrently, the excitation scan (Fixed emission of 510 nm) of unexposed PhoCl1 showed an increasing trend in the intensity towards larger wavelengths”

L165

The text writes “The quantum yield of the CTPF was determined to be 0.23 ± 0.08 % which is also corroborated by its dim visual appearance under the UV-transilluminator (λ = 365 nm)”. The phrase of “its dim visual appearance” is no more than subjective opinion, although the brightness of CTPF can be evaluated quantitatively. This phrase is little meaningful scientifically.

Response: Thank you for this suggestion, we have further calculated the brightness of the CTPF and rewritten the sentence in the manuscript.

“The quantum yield of the CTPF was determined to be 0.23 ± 0.08%. A low brightness of 25.85 $M^{-1}cm^{-1}$ for CTPF (38,000 $M^{-1}cm^{-1}$) of EGFP closely aligned with its dim visual appearance under the UV-transilluminator (λ = 365 nm).”

L230

In the sentence of “Additionally, we observed a r0 (fundamental anisotropy) value greater than 0.4 and non-zero residual anisotropy (r_∞ ~ 0.1) in the case of CTPF which also suggests the presence of a heterogeneous aggregates corroborating the DLS data”, I would like to point out two things. Firstly, they found that the zero-time anisotropy was >0.4. However, they need to consider Equation (10.22) in the 3rd Edition of Lakowicz’s textbook, because Equation (10.22) means that r0 is <= 0.4. I wonder what the authors suggest from the value of r0 > 0.4. Secondly, I hope that the authors explain in some more detail the logic flow for the claim “non-zero residual anisotropy (r_∞ ~ 0.1) in the case of CTPF which also suggests the presence of a heterogeneous aggregates corroborating the DLS data”. The relationship between rotational correlation time and rotating hydrated unit size (Equation (10.42) in

Lakowicz's textbook, which is also known as the Stokes-Einstein-Debye equation) may be helpful. In relation to this, for the time-resolved anisotropy decay like Figure S15A, some models can be used to decompose several anisotropy decay components for analysis. For example, Dutt, J. Phys. Chem. B 108, 3651–3657 (2004) as well as Lakowicz's book may be helpful. As another possibility, the fast anisotropy decay component in Figure S15A may reflect homo FRET among p-HBI chromophores contained in a CTPF aggregate.

Response: We thank the reviewer for their suggestion. We have now expanded further on the observed values and their significance to our study.

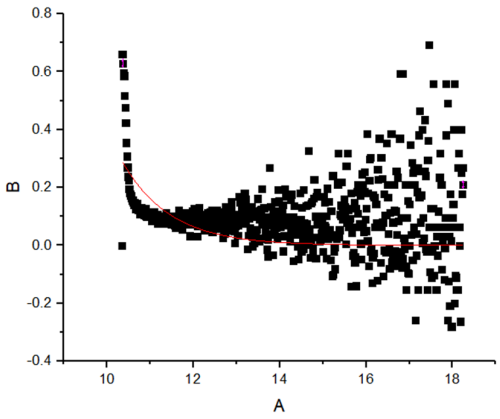
"

The rotating hydrated unit size of CTPF was determined to be ~0.5 nm, aligning with established observation of small molecules[1,2]. We observed a r_0 (fundamental anisotropy) value greater than 0.4 likely indicating light scattering contributions from the aggregates in addition to fluorescence.[3] Additionally, a non-zero residual anisotropy (r_∞ ~ 0.1) in the case of CTPF also suggests potential restricted rotational motion of the CTPF molecules in the aggregate.[3,4] Together, the results suggest that CTPF aggregates with restricted chromophore rotation within the larger framework.

"

We thank the reviewer for the suggestion regarding the use of models from Dutt et al. (J. Phys. Chem. B 2004) to decompose the time-resolved anisotropy decay data. As suggested, we attempted to fit the anisotropy decay using the biexponential model described in the reference ($r_{(t)}=r_0\beta\exp(-t/\tau_{slow})+(1-\beta)\exp(-t/\tau_{fast})$.

However, the fit quality was poor. As such, we chose to present the experimental decay profile based on the monoexponential decay.



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The authors investigated the effect of guanidium hydrochloride on CTPF fluorescence up to 2 M. Guanidium hydrochloride is very well-known as a typical denaturant in biology, and many biologists are likely to wonder what if guanidium hydrochloride is increased up to 6 M.

Response: Thank you for the comment. We have conducted additional experiments by increasing the guanidinium hydrochloride concentration up to 6 M. No significant change in CTPF fluorescence was observed, indicating that CTPF fluorescence is largely insensitive guanidinium hydrochloride.

L307

The text writes “the previously proposed model”. Here, a suitable paper should be cited.

Response: Suitable citations have been updated.

Supplementary information, P 6 L12

The text mentions CTPF concentration. Although PhoCl1 concentration can be measured by absorbance using its extinction coefficient, I wonder how the authors measured CTPF concentration. Its quantization method is preferred to be explained.

Response: Thank you for pointing this out. We have now added a description of the method used to quantify CTPF concentration in the Methods section of the revised manuscript.

“The purified PhoCl1 and CTPF concentration was measured using the Bicinchoninic Acid (BCA) assay.“

Supplementary information, P 6 L 20

I do not think that the phrase of “first order rate kinetics equation” is very common. It is preferrable to follow a textbook of kinetics.

Response: Thank you for the suggestion. This has now been changed to “first order rate kinetics model”

Supplementary information, P 8 L 11

About “slit widths of 1-1.5 nm”, the authors need to specify which slit width they meant, excitation, emission, or both. In most fluorescence spectrophotometers, slit widths of excitation and emission can independently be adjusted.

Response: Thank you for your suggestion. We have updated this in the Materials and Methods Section. Please see the revised text below

“Fluorescence spectra were recorded in quartz cuvettes with a 1 mm path length and slit widths of 1-1.5 nm (same for both, excitation and emission), using a peptide concentration of ~40 µM. “

Reviewer #3 (Remarks to the Author):

This further revised manuscript has addressed this reviewer's prior concerns, with adequate clarifications and useful edits to stay focused on an interesting new finding about the AIE-induced fluorescence from the photodissociated product (a C-terminal peptide) of PhoCl1. The response also pointed out future directions for a more detailed study of the aggregated CTPF chromophore in various environments, which will be a nice extension of this work. This reviewer can support the publication of this current version in Commun. Biol.

Response: We thank the reviewer for their positive comments about our study.

References:

1. Lakowicz, J. R., Maliwal, B., Cherek, H. & Balter, A. Rotational freedom of tryptophan residues in proteins and peptides. *Biochemistry* 22, 1741–1752 (1983).
2. Hay, C. E., Marken, F. & Blanchard, G. J. Solvent-Dependent Changes in Molecular Reorientation Dynamics: The Role of Solvent−Solvent Interactions. *J Phys Chem A* 114, 4957–4962 (2010).
3. *Principles of Fluorescence Spectroscopy*. (Springer US, Boston, MA, 2006). doi:10.1007/978-0-387-46312-4.
4. Akimoto, S. & Mimuro, M. Application of time-resolved polarization fluorescence spectroscopy in the femtosecond range to photosynthetic systems. *Photochem Photobiol* 83, 163–70 (2007).

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Answer: We thank the reviewer for their acceptance.

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I strongly recommend that the authors provide a more thoughtful and critical discussion on the scientific importance of their discovery, its limitations, and potential future directions. Therefore, I recommend that the manuscript be revised accordingly.

Answer: We have added the discussion on scientific importance of their discovery, its limitations, and potential future directions as follows:

“In summary, our experimental and computational analysis validate the aggregation of CTPF which in turn plays a crucial role in the emergence of red fluorescence following PhoCl1 photoexposure. These findings highlight the behavior of the photodissociated chromophore-containing peptide (Figure 5).6 The observation that photocleavage of PhoCl1 results in red fluorescence due to aggregation of CTPF

provides critical insights into the fate of photodissociated fragments in engineered fluorescent proteins. This is particularly important because many optogenetic tools rely on predictable post-cleavage behavior, often assuming the photofragments to be inert. Our findings reveal that the chromophore fragment can persist and undergo aggregation, leading to a new spectral output. While we do not propose CTPF as a direct tool for high-resolution bioimaging due to its aggregation-prone nature, the generation of red-emitting aggregates from a photocleaved product opens up avenues for designing triggered fluorescence turn-on systems. Future efforts may focus on (1) engineering the chromophore-containing peptide to control its aggregation, (2) leveraging this behavior for designing sensors based on aggregation-induced emission (AIE), and (3) using the aggregation of photocleaved peptides as a tunable parameter for material assembly and/or fluorescence tagging."

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Page 6, Lines 3-4:

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Answer: We have included the term spectrum instead of spectra wherever applicable.

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Answer: We thank the reviewer for their acceptance.