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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In this work, Tittelmeier and co-worker report an original method to follow the a-synuclein aggregation directly in cell using FLIM. The most interesting point is that the analysis of the mean fluorescent lifetime distribution can inform on the polymorphism of aggregates. The manuscript is clear and the discussion is supported by convincing results. However, several points should be clarified.
The FLIM involved the label of the a-syn, here with YFP and ATTO647. The mean fluorescent lifetime of these dyes grafted and not on the a-syn should be given as well as different stage aggregation of aggregation (with THT control) without cell.
The impact of YFP and ATTO647 affect the dynamic and the morphology of a-syn should be evaluated and discussed.
It is possible to effect a fluorescent lifetime to an aggregate morphology ?
For the phasor image correspondence colour/ value of mean fluorescence lifetime should be given.

Reviewer #2 (Remarks to the Author):

Tittelmeier et al. used phasor approach of fluorescence lifetime imaging microscopy (FLIM) to study the aggregation properties of and seeding dynamics of different α-Syn fibers in HEK 297T cell lines. Aggregation was assessed as decreased fluorescence lifetime of α-SynA53T-YFP. Seeding capacity was assessed with different α-Syn-Atto647 polymorphs. Interestingly, cellular clearance pathways yielded fibrillar species with higher fluorescence lifetime (less processed) but increased seeding capacity. Previous studies used phasor approach of FLIM to study the aggregation of proteins (e.g. https://doi.org/10.1016/j.jcis.2020.03.107), and FLIM has been used to study α-Syn oligomerization (https://faseb.onlinelibrary.wiley.com/doi/10.1096/fj.05-5422com). Thus, as a method paper, the novelty of this study is limited. However, this study adds to the proof of principle application of these methods as well as the biology by 1) distinguish the seeding and aggregation property of different α-Syn polymorphs; and 2) showing how cellular clearance pathways regulate fibrillar species as well as aggregate formation. The study is thus of interest to the field of protein aggregation and neurodegeneration, if the major concerns can be addressed.

Major concerns

1. Quantification and statistical analysis are lacking, and hence the conclusions are not well supported. Below are a few examples.
a. The authors conclude that FLIM is a powerful tool to distinguish different amyloid structures. Without quantification and statistical analysis, it is unknown that whether the fluorescence lifetimes of different polymorph are distinguishable.
b. Figure 1c, the phasor plot of Ribbon treated α-Syn-A53T-YFP seems similar to control and different from other polymorph treated ones, inconsistent with the statement 'no robust difference in lifetimes of α-Syn-A53T-YFP upon seeding with distinct α-Syn-A53T-YFP polymorphs could be observed' (line 112-113). Please quantitate and perform statistical analysis.
c. Figure 2., quantification and statistical analysis are needed to compare the lifetime properties of α-Syn-Atto647 from all conditions. Is the lifetime distribution of Ribbon significantly different from α-Syn monomers?
d. Figure 4 and 5., please perform statistical analysis for lifetime distribution between control and treatment groups.
2. It is unclear what we learn in a cell line can generalize to neurons or disease conditions. Please include data from neurons, or at least discuss this limitation.

Specific comments:
1. The study uses a mixture of α-Syn-Atto647 fibrils and α-SynA53T-YFP in most experiments. Please discuss whether this mixture is clinically relevant, and why A53T is relevant to use here. Do wild type α-Syn-YFP display the same properties?
2. Figure 1., please include histograms of lifetime distribution.
3. Figure 2. Why would monomeric α-Syn display lifetimes between 1.5ns and 1.8ns. Should we expect it to be entirely 1.8ns?
4. Supplement Figure 1D, Ribbon fiber is not able to induce significantly more foci in cells, consistent with that the phasor plot of Ribbon treated α-Syn-A53T-YFP that looks like control in Fig. 1C. Please describe and discuss.
5. Supp. Figure 3C shows lifetime change of seeding fibers is caused purely by cellular processes without interactions with native α-Syn. This is important. Please include it in main figures.
6. The study should cite more previous papers, including but not limited to the 2 mentioned in the opening paragraph.

Reviewer #3 (Remarks to the Author):

This study describes the use of Fluorescence lifetime imaging microscopy (FLIM) combined with phasor analysis to monitor the properties of seeded a-synuclein aggregates in mammalian cells. The study shows convincingly that the phasor analysis is suited to identify differences in the in vivo properties of a-synuclein polymorphs, and provides evidence that amyloid seeds are remodeled in vivo over time. The authors attempt to include some mechanistic studies by evaluating the effects of lysosomal, proteasomal and chaperone inhibition on the properties of the a-synuclein seeds. However, the effects on the FLIM properties are generally quite subtle, and leave the reviewer wonder about what new information we learn form this analysis. Altogether, this study is very descriptive and provides little new insights into a-synuclein aggregation in vivo.

Major comments:
1) Although the authors conclude from their data that highly seeding competent degradation products are produced from some of their polymorphs, there is no direct evidence provided that there are more seeding competent species formed.
2) The effects of inhibiting the proteasome/DnaJ according to FLIM are quite subtle especially given that the controls themselves seem highly variable
3) What is the physiological consequence of cells having more or fewer foci? Does the size of the foci change?

Dear Reviewers,

Thank you for the thorough and insightful evaluation of our manuscript. We have carefully addressed your remarks and submitted a revised manuscript for reconsideration. The changes we made are labeled in red. Detailed responses to each of your concerns are listed under the individual comments.

We believe that the new data strengthen our initial findings and conclusions about FLIM being a powerful tool that can distinguish different amyloid structures and can be used to monitor the dynamic process of amyloid remodeling by the cellular environment. We therefore hope that you and the Editor will find our manuscript suitable for publication in Communications Biology.

Sincerely,

Carmen Nußbaum-Krammer

Reviewers' comments:

Reviewer #1

In this work, Tittelmeier and co-worker report an original method to follow the α-synuclein aggregation directly in cell using FLIM. The most interesting point is that the analysis of the mean fluorescent lifetime distribution can inform on the polymorphism of aggregates. The manuscript is clear and the discussion is supported by convincing results. However, several points should be clarified.

We thank the reviewer for the overall positive feedback on the manuscript. We have addressed your comments below.

1: The FLIM involved the label of the α-syn, here with YFP and ATTO647. The mean fluorescent lifetime of these dyes grafted and not on the α-syn should be given as well as different stage aggregation of aggregation (with THT control) without cell.

The fluorescence lifetime is an intrinsic characteristic of a fluorophore. According to the fluorophore database FBbase, the fluorescent lifetime of enhanced YFP is 3.1 ns (https://www.fpbase.org/protein/eyfp/). However, many different aspects can affect the lifetime of fluorophores, for example attaching a protein. When YFP is fused to α-Syn and in a cellular environment, its lifetime is around 2.9 ns (Schierle et al. 2011). The same is true for Atto674. The lifetime of the fluorophore alone is 2.4 ns (https://www.atto-tec.com/ATTO-647.html?language=en), while monomeric α-Syn-Atto647 has a lifetime of 1.7 ns (Figure 2E). Since both fluorophores do not aggregate, a reference value for the aggregated protein itself cannot be provided.

Therefore, we think it is more meaningful to measure the lifetimes of non-aggregated α-SynA53T-YFP and α-Syn-Atto647 to determine a reference value for the soluble proteins in our system and then compare it to the values obtained with the aggregated proteins.

2: The impact of YFP and ATTO647 affect the dynamic and the morphology of α-syn should be evaluated and discussed.

Attaching a fluorophore might impact the dynamic and morphology of the respective protein. We have added the following sentences to discuss that with respect to α-SynA53T-

YFP: “While seeded aggregation did lead to a shorter fluorescence lifetime signal of the endogenous α-SynA53T-YFP reporter, the characteristic fluorescence lifetimes of the respective seeds were not replicated (Fig. 1). This was unexpected as amyloid propagation is generally thought to involve templated incorporation of monomers at the ends of the filaments, thereby preserving the conformation of the seed. Two reasons could explain why endogenous α-SynA53T-YFP showed minimal variation when its aggregation is triggered by different α-Syn polymorphs in FLIM. First, α-SynA53T-YFP might not be able to adopt the exact conformation of the seed due to the specific conditions of the cellular environment, which substantially differ from the conditions the seeds were made in regarding salt concentration, pH, etc. (Bousset et al. 2013; Grozdanov et al. 2019). Second, the attached fluorophore could prevent α-SynA53T-YFP from adopting the exact conformation of the seed (Afitska et al. 2017; Caputo et al. 2020). In both cases, the observed seeded aggregation would result primarily from secondary nucleation events rather than from a templated addition of monomers at fibrils ends (de Oliveira and Silva 2019). Thus, the conformation of fibrils formed by secondary nucleation events would be predominantly determined by solution conditions and intrinsic structural preferences rather than by the seed conformation (Hadi Alijanvand, Peduzzo, and Buell 2021).” (page 10, line 281).

We want to point out that this is less likely for the ATTO647-labelled α-Syn polymorphs, since the fluorophore is attached to the fibers after and not during their assembly. We have added the following sentence to indicate this: “In this case, fluorophores are attached to lysine residues exposed at the surfaces of the fibrils after assembly and therefore do not affect the conformation of the distinct polymorphs.” (page 11, line 297). We have also described this in the materials and methods section.

3: It is possible to effect a fluorescent lifetime to an aggregate morphology?

We believe this is possible and we hope that the additional data we provided support this claim. We show in Figure 3A that different α-Syn polymorphs display different fluorescence lifetime signatures. To better highlight that the fluorescence lifetime of the Atto647 fluorophore is significantly affected by the aggregate morphology, we have expanded Figure 3 to show the weighted mean fluorescence lifetime with statistics (new Figure 3C).
The distinct structure of these polymorphs can be appreciated in the EM images displayed in Supplementary Figure S1A. Moreover, they have been extensively characterized previously (Rey et al. 2019; Shrivastava et al. 2020; Makky et al. 2016; Landureau et al. 2021). To clarify that the polymorphs have distinct morphologies we added the following statement with additional references (page 5, lines 113): “In addition to Fibrils, we seeded the biosensor cell line with other structurally well-characterized fibrillar α-Syn polymorphs, F65, F91, and Ribbons (Makky et al. 2016; Landureau et al. 2021; Rey et al. 2019; Shrivastava et al. 2020). These polymorphs differ in the amino acids located in their core and those exposed at their surface, resulting in a distinct fiber architecture (Supplementary Fig. 1A)(Makky et al. 2016; Landureau et al. 2021; Rey et al. 2019; Shrivastava et al. 2020).”

4: For the phasor image correspondence colour/ value of mean fluorescence lifetime should be given.

We thank the reviewer for this important suggestion. We have added the mean fluorescence lifetime to all of the figures. By comparing the means we can now show that the differences we claimed were indeed significant, which strengthens the study.

Reviewer #2 (Remarks to the Author):

Tittelmeier et al. used phasor approach of fluorescence lifetime imaging microscopy (FLIM) to study the aggregation properties of and seeding dynamics of different α-Syn fibers in HEK 297T cell lines. Aggregation was assessed as decreased fluorescence lifetime of α-SynA53T-YFP. Seeding capacity was assessed with different α-Syn-Atto647 polymorphs. Interestingly, cellular clearance pathways yielded fibrillar species with higher fluorescence lifetime (less processed) but increased seeding capacity. Previous studies used phasor approach of FLIM to study the aggregation of proteins (e.g. https://doi.org/10.1016/j.jcis.2020.03.107), and FLIM has been used to study α-Syn oligomerization (https://faseb.onlinelibrary.wiley.com/doi/10.1096/fj.05-5422com). Thus, as a method paper, the novelty of this study is limited. However, this study adds to the proof of principle application of these methods as well as the biology by 1) distinguish the seeding and aggregation property of different α-Syn polymorphs; and 2) showing how cellular clearance pathways regulate fibrillar species as well as aggregate formation. The study is thus of interest to the field of protein aggregation and neurodegeneration, if the major concerns can be addressed.

We thank the reviewer for the comments highlighting what our study adds to the fields of protein aggregation and neurodegeneration.

Major concerns

1. Quantification and statistical analysis are lacking, and hence the conclusions are not well supported. Below are a few examples.
a. The authors conclude that FLIM is a powerful tool to distinguish different amyloid structures. Without quantification and statistical analysis, it is unknown that whether the fluorescence lifetimes of different polymorph are distinguishable.
We agree with the reviewer that quantification and statistical analysis were missing. We have added the additional quantification of the weighted mean of the fluorescence lifetimes to every experiment and have performed statistical analysis. We believe that this supports our conclusions.

b. Figure 1c, the phasor plot of Ribbon treated α-Syn-A53T-YFP seems similar to control and different from other polymorph treated ones, inconsistent with the statement 'no robust difference in lifetimes of α-Syn-A53T-YFP upon seeding with distinct α-Syn-A53T-YFP polymorphs could be observed' (line 112-113). Please quantitate and perform statistical analysis.

We thank the reviewer for pointing this out. With the addition of the new analysis, we show that Ribbons are indeed similar to the control and significantly different from the other polymorphs. We have updated the manuscript (page 5 lines 117) to state:
"F65 and F91 polymorphs induced the formation of α-SynA53T-YFP foci (Supplementary Fig. 1C), leading to a decreased mean fluorescence lifetime of α-SynA53T-YFP in seeded compared to non-seeded cells similar to Fibrils (Fig. 1E-G). Ribbons had the lowest seeding capacities of the different polymorphs in our experimental model (Supplementary Fig. 1C), which was reflected by an unchanged fluorescence lifetime distribution and no significant difference in the mean lifetime of α-SynA53T-YFP compared to the control (Fig. 1E-G). Selection of short-lived α-SynA53T-YFP species on the phasor plot in Ribbon-seeded cells revealed that they localized to foci (Fig. 1E). However, these foci also contained α-SynA53T-YFP species with longer fluorescence lifetimes, resulting in a significantly higher mean than the mean fluorescence lifetime of α-SynA53T-YFP species in foci seeded with Fibrils, F65 or F91 polymorphs (Fig. 1E, Supplementary Fig. 1D). No robust difference in the mean fluorescence lifetimes of α-SynA53T-YFP in foci seeded with Fibrils, F65 or F91 polymorphs could be detected (Supplementary Fig. 1D). Hence, the seeded aggregation of endogenous α-SynA53T-YFP by the addition of exogenous α-Syn polymorphs Fibrils, F65 and F91 led to the accumulation of short-lived protein species, whereby the conformation of the added seeds did not cause a significant difference in the respective fluorescence lifetimes."

c. Figure 2., quantification and statistical analysis are needed to compare the lifetime properties of α-Syn-Atto647 from all conditions. Is the lifetime distribution of Ribbon significantly different from α-Syn monomers?
Analysis of the mean fluorescence lifetime shows there is no significant difference between monomeric α-Syn and Ribbon polymorphs (new Figure 2F).

d. Figure 4 and 5., please perform statistical analysis for lifetime distribution between control and treatment groups.
This analysis was added to the new Figure 5B, D, F and Figure 6B, E, H (previously figures 4 and 5).

2. It is unclear what we learn in a cell line can generalize to neurons or disease conditions. Please include data from neurons, or at least discuss this limitation.

We have added the following paragraph to discuss this limitation (page 12, lines 336):
"Having limited this study to a HEK biosensor cell line, it would be of interest to investigate seeded aggregation of α-Syn in other cell types, such as neurons or oligodendrocytes to assess whether the processing of fibrillar α-Syn is also differentially affected by the cellular milieu (Peng et al. 2018). Future studies using patient-derived α-Syn conformers in more disease-relevant cell types may reveal potential disease-specific members of the proteostasis network that influence the seeded aggregation of α-Syn, which could explain the heterogeneity of synucleinopathies and pave to way toward disease-specific therapeutics (Hoppe, Uzunoğlu, and Nussbaum-Krammer 2021).

However, regardless of the exact degradation machinery involved, processing of α-Syn fibers by cellular clearance pathways generally yielded species with high seeding capacity that enhanced aggregation of endogenous α-Syn (Fig. 5, 6, Supplementary Fig. 5, 6, 7)".
Of note, we did not investigate neuron-specific pathways or genes. Autophagy and the ubiquitin-proteasome system are the two major proteolytic systems in all eukaryotic cells, and the Hsp70 disaggregase in conserved in metazoan. While there might be some differences in the composition of the proteostasis network between neuronal and non-neuronal cells, the core machinery is highly conserved between all cell types, and therefore it is very likely that our findings can be generalized to neurons or disease conditions.

Specific comments:

1. The study uses a mixture of α-Syn-Atto647 fibrils and α-SynA53T-YFP in most experiments. Please discuss whether this mixture is clinically relevant, and why A53T is relevant to use here. Do wild type α-Syn-YFP display the same properties?

We used the biosensor cell line expressing A53T mutant α-Syn because it is more sensitive to the addition of exogenous seeds than cells expressing WT α-Syn. This is based on the description in the original study that established this biosensor cell line, which states: "In the HEK cells expressing α-syn140–YFP, we found 25–30% of the cells developed aggregates upon exposure to 30 nM α-syn140*A53T fibrils, whereas over 50% of the cells expressing α-syn140*A53T–YFP exhibited aggregates in the presence of the fibrils (Fig. 2). Based on these findings, we chose the α-syn140*A53T–YFP cells for further study (Woerman et al. 2015)."
Moreover, this cell line was successfully used to detect fibrillar material of different α-Syn mutants (Boyer et al. 2019; 2020; Woerman et al. 2015). We have added this information to the materials and methods section (page 13, line 400) "This biosensor cell line was shown to be highly sensitive in detecting a variety of different fibrillar α-Syn species". As to the fibrillar polymorphs we used, they have been shown to trigger different pathologies in vivo (Peelaerts et al. 2015; Rey et al. 2019) and to seed the aggregation of α-Syn to different extents in vitro (Shrivastava et al. 2020).

2. Figure 1., please include histograms of lifetime distribution.

We have moved the histograms from Supplementary Figure 1 to the main Figure 1C and F.

3. Figure 2. Why would monomeric α-Syn display lifetimes between 1.5ns and 1.8ns. Should we expect it to be entirely 1.8ns?

In an undisturbed environment in a well-defined buffer solution, we would expect it to be a single value of 1.8 ns. However, in our microscopy setup, we are not able to image monomeric α-Syn-Atto647 in a test tube. We have to transfect it into cells instead. Since the local concentration of a monomeric protein is significantly lower than that of an aggregate, it is much harder to detect than aggregated protein. Therefore, to detect monomeric α-Syn-Atto647, we transfected 10x more protein (1µM of the monomer compared to 100 nM used for the respective polymorphs), which might favor intermolecular interactions. Also, α-Syn is able to form tetrameric forms and interact with membranes(Bartels, Choi, and Selkoe 2011; Musteikytė et al. 2021). This could all affect the lifetime of the attached fluorophore. However, this is pure speculation. After statistical analysis, we now state the mean fluorescent lifetime of the monomer in the cellular environment (page 6 line 152).

4. Supplement Figure 1D, Ribbon fiber is not able to induce significantly more foci in cells, consistent with that the phasor plot of Ribbon treated α-Syn-A53T-YFP that looks like control in Fig. 1C. Please describe and discuss.

The reviewer raises an important issue that is also related to their point 1b. Ribbons show a similar fluorescence lifetime as the control, as they are not able to induce significantly more foci in cells compared to the control. We have updated the manuscript to state the following (page 5, line 120) "Ribbons had the lowest seeding capacities of the different polymorphs in our experimental model (Supplementary Fig. 1C), which was reflected by an unchanged fluorescence lifetime distribution and no significant difference in the mean lifetime of α-SynA53T-YFP compared to the control (Fig. 1E-G). Selection of short-lived α-SynA53T-YFP species on the phasor plot in Ribbon-seeded cells revealed that they localized to foci (Fig. 1E). However, these foci also contained α-SynA53T-YFP species with longer fluorescence lifetimes, resulting in a significantly higher mean than the mean fluorescence lifetime of α-SynA53T-YFP species in foci seeded with Fibrils, F65 or F91 polymorphs (Fig. 1E, Supplementary Fig. 1D)."

5. Supp. Figure 3C shows lifetime change of seeding fibers is caused purely by cellular processes without interactions with native α-Syn. This is important. Please include it in main figures.

We agree with the reviewer that this is an important control and have moved data from Supplementary Figure 3C to the main Figure 3C.

6. The study should cite more previous papers, including but not limited to the 2 mentioned in the opening paragraph.

We have added the following citations (bold) for the manuscript to cite more papers that apply FLIM to investigate aggregation (page 3, line 61).

Aggregation into an amyloid fiber leads to quenching of an attached fluorophore due to compaction and crowding, thus reducing its fluorescence lifetime (Schierle et al. 2011; Chen et al. 2016). This can be measured using fluorescence lifetime imaging microscopy (FLIM) (Schierle et al. 2011; Chen et al. 2016; **Gallrein et al. 2021**; **Hardenberg et al. 2021**; **De Luca et al. 2020)**. Therefore, FLIM is increasingly used to investigate aggregation processes in various models (Sandhof et al. 2020; Esbjörner et al. 2014; Laine et al. 2019; **Klucken et al. 2006; Pigazzini et al. 2020**).

Reviewer #3 (Remarks to the Author):

This study describes the use of Fluorescence lifetime imaging microscopy (FLIM) combined with phasor analysis to monitor the properties of seeded α-synuclein aggregates in mammalian cells. The study shows convincingly that the phasor analysis is suited to identify differences in the in vivo properties of α-synuclein polymorphs, and provides evidence that amyloid seeds are remodeled in vivo over time. The authors attempt to include some mechanistic studies by evaluating the effects of lysosomal, proteasomal and chaperone inhibition on the properties of the α-synuclein seeds. However, the effects on the FLIM properties are generally quite subtle, and leave the reviewer wonder about what new

information we learn from this analysis. Altogether, this study is very descriptive and provides little new insights into α-synuclein aggregation in vivo.

Major comments:

1) Although the authors conclude from their data that highly seeding competent degradation products are produced from some of their polymorphs, there is no direct evidence provided that there are more seeding competent species formed.

We have actually dedicated an entire study to the detailed characterization of the specific products generated by the Hsp70 disaggregation machinery. We have disaggregated the Fibril polymorph with the Hsp70 disaggregation machinery and separated the liberated products by centrifugation and tested their individual seeding capacity in our biosensor cell line (Figure for Reviewers). The data show that disaggregation of Fibrils generates more seeding competent species. Smaller fragments and oligomeric species isolated from the total disaggregation reaction have a higher seeding capacity. Monomeric species isolated from the total disaggregation reaction were not seeding competent.

This dataset is part of a comprehensive collaborative study with the laboratory of Bernd Bukau, which includes the use of well controlled *in vitro* experiments with purified components. The manuscript will be published separately at a later time.
In the study described here, our scope was to characterize the seeded aggregation in the cellular environment *in situ* by using FLIM, without additional manipulations by lysis and centrifugation. Our data show that the occurrence of fibrillar species with intermediate fluorescence lifetimes nicely/perfectly correlates with the seeding efficiency of the polymorphs. Whenever we significantly interfere with this processing by pharmacological or genetic inhibition of major degradation or disaggregation pathways, there is a significant reduction in the seeding capacity of the respective polymorphs. FLIM is able to directly visualize these intermediate fibrillar species and shows that they preferentially colocalize with endogenous α-SynA53T-YFP foci (Figure 4D). Moreover, we now provide quantifications and statistical tests that strengthen our results. Therefore, we think it is valid to conclude from these data that the products produced from the polymorphs by cellular processing exhibit increased seeding propensity.

2) The effects of inhibiting the proteasome/DnaJ according to FLIM are quite subtle especially given that the controls themselves seem highly variable

This is an important point that needed clarification. We performed additional quantifications and statistical analysis of the different treatments to show that the mean fluorescence lifetime of the seeds is significantly shorter in treated samples in comparison to the controls. This supports our claim that that the seeds are significantly less processed and that significantly less species with longer lifetimes are generated, which correlates with a lower seeding capacity. We have added these data to Figure 5D, F and 6E, H and Supplementary Figure 5H, 6H, and 7H (previously Figure 4 and 5).

3) What is the physiological consequence of cells having more or fewer foci? Does the size of the foci change?

The HEK biosensor cells used in this study are widely used to detect and quantify the presence of seeding-competent protein species (Boyer et al. 2019; 2020; Woerman et al. 2015). These seeding-competent protein species are believed to drive disease progression because they are able to self-propagate and correlate with cytotoxicity in patients and animal models (Boyer et al. 2019; 2020; Woerman et al. 2015). However, HEK cells are quite robust and there is only little physiological consequence for these cells having foci. This is likely due to the fact that this cell line is rapidly dividing, unlike neurons in the human brain or other cell types that are terminally differentiated. Therefore, toxic protein species do not accumulate but are diluted during continuous cell divisions. The question regarding physiological consequences needs to be addressed in more sophisticated model systems, such as animal models. However, we believe that this is beyond the scope of our study, which was to establish the use of FLIM to monitor α-Syn seeding and aggregation dynamics in the cellular environment and to show that FLIM allows the detection of different fibrillar species in cells *in situ*.

Regarding the size of the foci, we did observe an intriguing change in the size and shape of the foci after knockdown of DNAJB1. We have described this observation on page 9, line 229: "Of note, while DNAJB1 KD reduced foci formation in general, we noticed an increase in elongated foci as opposed to the typical spherical foci (Supplementary Fig. 5D zoom, 5E), suggesting that DNAJB1 may affect not only exogenously added seeds but also endogenous α-SynA53T-YFP aggregates."

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REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The authors have made an effort to take into account the referees comments correct the manuscript. I feel that the manuscript could be accepted in present form.

Reviewer #2 (Remarks to the Author):

Tittelmeier et al. significantly improved their manuscript by adding statistical analyses of their data, including measured interpretation that match their results, and discussing the limitation of the study. I have three minor comments.
1. Most statistical analyses used unpaired T-tests. Did the authors test for normality of the data? If not, or if the data do not follow normal distribution, please use non-parametric statistical tests.
2. It is still unclear to me whether α-SynA53T-YFP is clinically relevant? Does the mutation happen in human patients? If so, please discuss and cite relevant papers
3. The writing is often unclear, contains many grammar mistakes and typo, and does not do justice to the study. The manuscript can benefit from a good writing or editing service. Here are only a few examples.
a. page 12, lines 336): Incorrect grammar
"Having limited this study to a HEK biosensor cell line, it would be of interest to investigate seeded aggregation of α-Syn in other cell types, such as neurons or oligodendrocytes to assess whether the processing of fibrillar α-Syn is also differentially affected by the cellular milieu (Peng et al. 2018).
b. typo: "pave to way forward"
"Future studies using patient-derived α-Syn conformers in more disease relevant cell types may reveal potential disease-specific members of the proteostasis network
that influence the seeded aggregation of α-Syn, which could explain the heterogeneity of synucleinopathies and pave to way toward disease-specific therapeutics (Hoppe, Uzunoğlu, and Nussbaum-Krammer 2021).
c. page 5 lines 117: Unclear and long sentence, too many comparisons in a single sentence.
"F65 and F91 polymorphs induced the formation of a-SynA53T-YFP foci (Supplementary Fig. 1C), leading to a decreased mean fluorescence lifetime of a-SynA53T-YFP in seeded compared to non-seeded cells similar to Fibrils (Fig. 1E-G).
d. Unclear and long sentence.
However, these foci also contained a-SynA53T YFP species with longer fluorescence lifetimes, resulting in a significantly higher mean than the mean fluorescence lifetime of a-SynA53T-YFP species in foci seeded with Fibrils, F65 or F91 polymorphs (Fig. 1E, Supplementary Fig. 1D).

Reviewer #3 (Remarks to the Author):

The authors have addressed my concerns to a reasonable extent.

Dear Reviewer #2,

we have addressed your additional remarks and submitted a revised manuscript for reconsideration. Detailed responses to each of your concerns are listed under the individual comments.

Sincerely,
Carmen Nußbaum-Krammer

Reviewers' comments:

Reviewer #2

Tittelmeier et al. significantly improved their manuscript by adding statistical analyses of their data, including measured interpretation that match their results, and discussing the limitation of the study. I have three minor comments.

1. Most statistical analyses used unpaired T-tests. Did the authors test for normality of the data? If not, or if the data do not follow normal distribution, please use non-parametric statistical tests.
Data sets were tested for normality and results are provided in the supplementary data files. We have also added a statement to the methods to highlight the employed normality testing.

2. It is still unclear to me whether α-SynA53T-YFP is clinically relevant? Does the mutation happen in human patients? If so, please discuss and cite relevant papers
The A53T mutation is a clinically relevant missense point mutation that has been associated with autosomal dominant, early-onset PD. We have now highlight that A53T is a disease-related mutation in the description of the biosensor cell line (Line 93).

3. The writing is often unclear, contains many grammar mistakes and typo, and does not do justice to the study. The manuscript can benefit from a good writing or editing service. Here are only a few examples.
a. page 12, lines 336): Incorrect grammar
“Having limited this study to a HEK biosensor cell line, it would be of interest to investigate seeded aggregation of α-Syn in other cell types, such as neurons or oligodendrocytes to assess whether the processing of fibrillar α-Syn is also differentially affected by the cellular milieu (Peng et al. 2018).
The sentence was changed to: “It would be of interest to investigate seeded aggregation of α-Syn in other cell types, such as neurons or oligodendrocytes, to assess whether the processing of fibrillar α-Syn is also differentially affected by the cellular milieu[6].”

b. typo: “pave to way forward”
“Future studies using patient-derived α-Syn conformers in more disease relevant cell types may reveal potential disease-specific members of the proteostasis network
that influence the seeded aggregation of α-Syn, which could explain the heterogeneity of synucleinopathies and pave to way toward disease-specific therapeutics (Hoppe, Uzunoğlu, and Nussbaum-Krammer 2021).
Typo was changed to “pave the way forward”.

c. page 5 lines 117: Unclear and long sentence, too many comparisons in a single sentence.
“F65 and F91 polymorphs induced the formation of a-SynA53T-YFP foci (Supplementary Fig. 1C), leading to a decreased mean fluorescence lifetime of a-SynA53T-YFP in seeded compared to non-seeded cells similar to Fibrils (Fig. 1E-G).
The sentence was changed to “Similar to Fibrils, F65 and F91 polymorphs induced the formation of α-SynA53T-YFP foci. This led to a decreased mean fluorescence lifetime of α-SynA53T-YFP in seeded compared to non-seeded cells (Fig. 1E-G, Supplementary Fig. 1C).”

d. Unclear and long sentence.
However, these foci also contained a-SynA53T YFP species with longer fluorescence lifetimes, resulting in a significantly higher mean than the mean fluorescence lifetime of a-SynA53T-YFP species in foci seeded with Fibrils, F65 or F91 polymorphs (Fig. 1E, Supplementary Fig. 1D).
The sentence was changed to: “However, these foci also contained α-SynA53T-YFP species with longer fluorescence lifetimes. Therefore, α-SynA53T-YFP foci seeded by Ribbons have a significantly higher mean fluorescence lifetime than α-SynA53T-YFP foci seeded by Fibrils, F65, or F91 polymorphs (Fig. 1E, Supplementary Fig. 1D).”