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Peer Review File

Measuring pH in insulin secretory granules by phasor-based fluorescence lifetime imaging of a genetically encoded sensor

Corresponding Author: Professor Francesco Cardarelli

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)
1. Brief summary of the manuscript
In the manuscript entitled “Measuring pH in insulin secretory granules using phasor fluorescence lifetime imaging of a genetically encoded sensor”, De Lorenzi et al. showcase the use of the green fluorescent protein (GFP) variant E1GFP inserted into the c-peptide of insulin as a pH sensor using FLIM microscopy with phasor analysis. The authors first characterize the purified E1GFP protein in solution, then measure the luminal pH of insulin secretory granules in INS1E cells. The authors identify a difference in the luminal pH between granules close to the plasma membrane and such further away.

2. Overall impression of the work
The manuscript can be split into two distinct parts. (A) The authors review and characterize the photo physics of the previously reported E1GFP reporter and highlight its pH sensitivity as measured by FLIM. (B) The authors use an Ins-E1GFP fusion construct to measure the luminal pH of insulin secretory granule in INS1E insulinoma cells.

The report has a focus on methodological aspects (E1GFP and FLIM) and less on the biological implications of the measured pH. Many reports have shown that FLIM-based reporters can be useful tools to measure organellar pH (for example: “Fluorescence Lifetime Imaging of pH along the Secretory Pathway – Linders et al. 2022”, or for secretory granules “The enhanced cyan fluorescent protein: a sensitive pH sensor for fluorescence lifetime imaging - Poëa-Guyon et al. 2013”, as well as “Orai-STIM-mediated Ca2+ release from secretory granules revealed by a targeted Ca2+ and pH probe – Dickson et al. 2012”). Using their reporter, the authors confirm numerous prior reports about the acidity of insulin secretory granules in insulinoma and/or beta cells.

To this reviewer, the manuscript lacks novelty and a clear methodological or biological hypothesis. The pH dependency of E1GFP has been characterized in vitro and in cells by the authors and colleagues before (“Real-time measurement of endosomal acidification by a novel genetically encoded biosensor – Serresi et al. 2008“), as well as the use of phasor analysis for pH measurements (“Intracellular pH measurements made simple by fluorescent protein probes and the phasor approach to fluorescence lifetime imaging – Battisti et al. 2012”). Similar reports have been published for the closely related E2GFP variant for pH measurements (“Development of a novel GFP-based ratiometric excitation and emission pH indicator for intracellular studies – Bizzarri et al. 2006”), thereby limiting the impact/novelty shown here dramatically.

This manuscript reads as a copy of the logic applied to endosomes (“Real-time measurement of endosomal acidification by a novel genetically encoded biosensor – Serresi et al. 2008“), but here to the exocytotic pathway (using a previously developed cloning strategy), making this report essentially a replication of prior observations in a slightly different setting. Considering that the main findings (ISG pH at steady state and upon glucose challenge) replicate data obtained by other groups and/methods (as indicated in the manuscript text), the manuscript does not provide relevant new data for beta cell biology and/or insulin secretion.

It is not clear whether this manuscript wishes to characterize and understand the photo physics of E1GFP or if the focus is on insulin secretory granule pH. Finally, the manuscript lacks a clear discussion of the findings in the context of prior work.

3. Specific comments

1) The introduction summarizes well prior work done to measure insulin secretory granule pH and highlights the shortcomings of using fluorescent dyes. However, pH sensitive fluorescent proteins have been employed to measure organelles (including secretory granules) using FLIM (see for example references above). This should be acknowledged either in the introduction or in the discussion part. How is the approach used here different and/or better?

2) Minor point: the authors cite a preprint as unpublished contribution. This seems contradictory, as it could not be cited if it were unpublished.

3) The authors show proficiency in using FLIM and subsequent analysis by phasor plot. Could the authors elaborate on the choice of analyzing the FLIM data using phasor plot over (multi)exponential fitting (as in Fig 1C)? How is this superior to other studies using pH measurements by FLIM? Due to using INS1E cells as a model, this manuscript may be read by readers not familiar with FLIM microscopy and phasor plot analysis. It may therefore be advisable to shortly introduce the approach and how to read phasor plots.

4) Minor point: it appears to this reviewer that the reference in line 214 should say Fig 1b

5) The first section of the manuscript “Characterization of E1GFP as a lifetime intracellular pH probe” constitutes a literature review, referring to previous reports and relating only little to data generated in this work. This section requires re-structuring to highlight the specific contributions of the authors. Since the manuscript title implies a focus on measuring insulin granule pH, the authors should consider omitting Fig 1 & 2 (or the entire section) or at least moving them into a supplementary figure. This is because the pH sensitivity; spectra of E1GFP have been described before (in essence same data/experiments as in Fig 1 of “Real-time measurement of endosomal acidification by a novel genetically encoded biosensor – Serresi et al. 2008“) and purified E1GFP is not used for pH measurements in INS1E cells. The reporter (Ins-E1GFP) used for experiments in living cells is established and characterized in the following section and is therefore more relevant.

6) Minor point: Fig 3A, the accepted nomenclature for insulin is B-chain, C-peptide and A-chain.

7) The authors refer to the reporter as proinsulin-E1GFP. Does this mean that insulin is not properly processed into mature insulin and a C-peptide-E1GFP hybrid? Immunoblots could help to address this question. If there is evidence for full insulin maturation, the reporter should be renamed to Ins-E1GFP.

8) The authors use the Zn2+ indicator ZIGIR to measure co-localization with Ins-E1GFP and show relatively poor co-colocalization as measured by Pearsons’ and Manders’ coefficients. The authors state that this is due to a lack of ZIGIR in immature insulin granules, without providing evidence. It would be helpful to repeat co-localization studies using fixed specimen and specific antibodies for different organellar compartments. This could include for example PTPRN, PTPRN2 for insulin granules, LAMP1, LAMP2 for lysosomes, Rab5, Rab7 for endosomes or LC3 for autophagosomes. ICA69 or proinsulin could be a useful marker for immature ISGs. Alternatively, co-overexpression of these markers (tagged with fluorescent proteins) could be used if co-localization in living cells is preferred.

9) Minor point: in line 304-305 the authors mention “crowding effects and self-quenching”. This should be explained better. To this reviewers’ knowledge, quenched fluorophores will not contribute to the fluorescence signal and therefore the measured lifetime. If by crowding effect the authors mean homo-FRET, then the effect would also not contribute to a change in the lifetime

10) In Fig 4, the authors measure the pH of insulin secretory granules in INS1E cells and state that ISGS in the cytoplasm have a higher pH (5.83) than those at the membrane (5.73). There are several questions: a) ISGs in the cytoplasm appear much larger than those close to the plasma membrane, which needs to be addressed by the authors. Given the low co-localization with ZIGIR, it cannot be excluded that these are non-ISG organelles. Attributing this to immature ISGs without evidence is speculation and needs to be named as such. b) the graph in Fig 4b shows a distribution of individual measurements for whole cells (this reviewer assumes this means total ISG pH), as well as for cytoplasmic and plasma membrane ISGs. Why do some ISGs in the cytoplasmic fraction have pH around 6, whereas this is not represented in the whole cell measurements which should include all ISGs? Are these data not from the same cells? C) a difference in pH of 0.1 seems very little and equates to a difference of ~380 nM protons. How is this difference biologically relevant, and, with a SD as shown in Fig 4b, can such small differences with a very low n be accurately assessed (given it appears the data is from a single experiment)? The authors need to contextualize their findings and explain the lack of effect of IAA-94 on ISG pH. How would a difference in pH of 0.1 be regulated by the cell?

11) The authors then measure the pH upon stimulation with glucose and speculate that the reduced amount of ISGs at the plasma membrane are due to secretion. It is noteworthy though, that the measured pH is not mentioned in the text, whereas Fig 5c suggests that ‘membrane’ ISGs have a lower pH (about 5.4 at timepoint 0) than in the prior results. This needs to be addressed. Where does this difference come from? Similar to Fig 4, large organelles can be seen. Are these still ISGs or potentially degradative organelles? Are these data from a single experiment? Repetitions should be performed to reduce potential effects during cell culture.

12) The manuscript lacks a clear discussion of the results obtained. The authors should more extensively discuss their findings in the context of prior research and highlight similarities and differences. In the case of the latter, the authors should discuss their view and opinion of potentially differing results.

Reviewer #2

(Remarks to the Author)
In this study, the authors develop a new tool to measure the pH of insulin granules of pancreatic beta cells, based on the targeting of the pH sensitive GFP variant E1GFP with favourable pKA of about 6 to insulin granules. The study has merit for two reasons: First, the approach can be likely easily adjusted to measure pH in other organelles and other systems by simply replacing the proinsulin in their DNA construct. Second, the pH in insulin granules has never been accurately determined, and this study fills this knowledge gap. This is important, given the well understood importance of luminal pH for loading of vesicles with cargo molecules and the secretion itself. The study is well designed, clearly presented and overall of high quality. I have no suggestions for further experiments, but have a couple of minor comments that can all be addressed by textual changes.

Figure 5 shows pH measurements upon glucose stimulation. From these measurements, it is concluded that the difference in pH between cell center and periphery is preserved during the early phase of glucose-induced secretion. However, I do not completely agree with this statement. First, the pH of the time points is not directly compared but visual inspection shows a clear increase in time in figure 5c. I understand that this difference is small, and it will be difficult to reach statistical significance due to the variation in the data, but the current conclusion seems to be too strong. Thus, the trend towards an higher pH needs to be acknowledged and briefly discussed.

The manuscript currently does not have a Discussion section. However, I miss some discussion on how the method can be adjusted for other systems. In addition, how does the pH compare with other secretory processes? The pH is determined Phasor, what is benefit over exponential fitting? Or compared to simple ratiometric acquisition with two different excitation wavelengths? A brief discussion and conclusion section would strengthen the paper.

On page 12 (lines 302-304), it is found that the absolute values of the fluorescence lifetimes measured in living cells were lower than those obtained in bulk solutions. This is a known phenomenon and we have also observed this for different constructs. However, I am not convinced by the authors' explanation that it is due to crowding and self-quenching. In this case, would you not expect large variation in figure 3, as self-quenching depends on expression levels which differ between experimental repeats and cells? In addition, shouldn't this be visible in the Phasor plot, as self-quenching likely will depend on the protonation state? This caveat should be discussed more. One likely alternative explanation might be the presence of other ions and biomacromolecules.

It should be mentioned in the results section and figure legend that the characteristics of A and A' in figure 1b and 1c were determined with purified protein at different pH values. I agree that the presence of A and A' is very likely under those conditions, but this is still inferred and not directly proven. This is no major issue, but is currently only explained in the Methods section and should be reiterated in the Results and figure legend as well.

I recommend separating the text in shorter paragraphs, especially for the Introduction section. Although the Introduction section is very good, is currently seems a single large paragraph which makes it difficult to read.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed all my comments, and I recommend publication.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

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The report has a focus on methodological aspects (E1GFP and FLIM) and less on the biological implications of the measured pH. Many reports have shown that FLIM-based reporters can be useful tools to measure organellar pH (for example: “Fluorescence Lifetime Imaging of pH along the Secretory Pathway – Linders et al. 2022”, or for secretory granules “The enhanced cyan fluorescent protein: a sensitive pH sensor for fluorescence lifetime imaging - Poëa-Guyon et al. 2013”, as well as “Orai-STIM-mediated Ca2+ release from secretory granules revealed by a targeted Ca2+ and pH probe – Dickson et al. 2012”). Using their reporter, the authors confirm numerous prior reports about the acidity of insulin secretory granules in insulinoma and/or beta cells.

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It is not clear whether this manuscript wishes to characterize and understand the photo physics of E1GFP or if the focus is on insulin secretory granule pH. Finally, the manuscript lacks a clear discussion of the findings in the context of prior work.

We thank the Reviewer for his/her critical reading of our manuscript and for highlighting lines of improvement aimed at increasing intelligibility, clearness and overall impact of the work. We are convinced that the revised version of the manuscript now tackles all the Reviewer's specific comments.

Concerning the criticism on novelty that the Reviewer anticipates here above, we do accept Reviewer's opinion. At the same time we are convinced, as now better stated in the revised manuscript, that the pH of insulin granules has never been accurately determined, combining the use of calibrated sensors and organelle-specific targeting: this study fills this knowledge gap.

3. Specific comments
1) The introduction summarizes well prior work done to measure insulin secretory granule pH and highlights the shortcomings of using fluorescent dyes. However, pH sensitive fluorescent proteins have been employed to measure organelles (including secretory granules) using FLIM (see for example references above). This should be acknowledged either in the introduction or in the discussion part. How is the approach used here different and/or better?

We thank the Reviewer for suggesting to integrate some additional previous contributions in the field of pH-sensitive fluorescent proteins applied to biological systems, including secretory granules. The revised introduction and the newly added discussion now satisfactorily tackle this request.

2) Minor point: the authors cite a preprint as unpublished contribution. This seems contradictory, as it could not be cited if it were unpublished.

We thank the reviewer for highlighting this minor but still relevant point. We now rephrased this citation into "a preprint and has not yet undergone peer review".

3) The authors show proficiency in using FLIM and subsequent analysis by phasor plot. Could the authors elaborate on the choice of analyzing the FLIM data using phasor plot over (multi)exponential fitting (as in Fig 1C)? How is this superior to other studies using pH measurements by FLIM? Due to using INS1E cells as a model, this manuscript may be read by readers not familiar with FLIM microscopy and phasor plot analysis. It may therefore be advisable to shortly introduce the approach and how to read phasor plots.

We thank the reviewer for this comment, as it gave us the opportunity to integrate the manuscript to better explain phasor conversion and its specific advantages over common fit-dependent procedures to analyze lifetime data.

4) Minor point: it appears to this reviewer that the reference in line 214 should say Fig 1b

We thank you for noting this typo, the text has been revised accordingly.

5) The first section of the manuscript "Characterization of E1GFP as a lifetime intracellular pH probe" constitutes a literature review, referring to previous reports and relating only little to data generated in this work. This section requires re-structuring to highlight the specific contributions of the authors. Since the manuscript title implies a focus on measuring insulin granule pH, the authors should consider omitting Fig 1 & 2 (or the entire section) or at least moving them into a supplementary figure. This is because the pH sensitivity; spectra of E1GFP have been described before (in essence same data/experiments as in Fig 1 of "Real-time measurement of endosomal acidification by a novel genetically encoded biosensor – Serresi et al. 2008") and purified E1GFP is not used for pH measurements in INS1E cells. The reporter (Ins-E1GFP) used for experiments in living cells is established and characterized in the following section and is therefore more relevant.

We thank the Reviewer for the suggestion of restructuring the section on E1GFP characterization. We agree that the introductory part of the Results section may distract the reader's attention from the biological implications of the measurements. To tackle this issue while preserving manuscript's overall intelligibility, we moved part of the content of the original Figure 1 to supplementary materials and revised and condensed the section. The new, restructured, Figure 1 now contains only previously unpublished contributions.

6) Minor point: Fig 3A, the accepted nomenclature for insulin is B-chain, C-peptide and A-chain.

The text has been corrected as noted, thank you.

7) The authors refer to the reporter as proinsulin-E1GFP. Does this mean that insulin is not properly processed into mature insulin and a C-peptide-E1GFP hybrid? Immunoblots could help to address this question. If there is evidence for full insulin maturation, the reporter should be renamed to Ins-E1GFP.

Thank you for this comment and for highlighting a potential source of confusion. In Watkins et al Traffic 2002 (doi: 10.1034/j.1600-0854.2002.30703.x), the authors performed western blot experiments on islets infected with C-pep-EGFP construct (named Ins-C-GFP in their work) and showed the presence of two electrophoretic bands corresponding to the proinsulin-GFP precursor and the C-pep-GFP product. They report that over two-thirds of the precursor was processed by the convertases. We have therefore changed proinsulin-E^{1}GFP to C-pep-E^{1}GFP in the text and figures.

8) The authors use the Zn2+ indicator ZIGIR to measure co-localization with Ins-E1GFP and show relatively poor co-colocalization as measured by Pearsons' and Manders' coefficients. The authors state that this is due to a lack of ZIGIR in immature insulin granules, without providing evidence. It would be helpful to repeat co-localization studies using fixed specimen and specific antibodies for different organellar compartments. This could include for example PTPRN, PTPRN2 for insulin granules, LAMP1, LAMP2 for lysosomes, Rab5, Rab7 for endosomes or LC3 for autophagosomes. ICA69 or proinsulin could be a useful marker for immature ISGs. Alternatively, co-overexpression of these markers (tagged with fluorescent proteins) could be used if co-localization in living cells is preferred.

This is a very important point and we thank once again the Reviewer for his/her careful reading of the manuscript and for having suggested this line of improvement. It gave us the possibility to perform new experiments using co-overexpression of the Cpep-EGFP and other markers either of the granule and of different organellar compartments, in living cells. In detail, we first performed a co-localization experiment among Cpep-EGFP and phogrin-mCherry as marker of the granule (which yielded robust co-localizatin scores); then we performed a similar co-localization assay among Cpep-EGFP and either mRFP-LC3b or Lysotracker Deep Red to evaluate possible mis-localization of our granule marker within autophagosomes or within lysosomes, respectively: these assay yielded poor co-localization. These results are now included in Figure S4 of the revised manuscript.

9) Minor point: in line 304-305 the authors mention "crowding effects and self-quenching". This should be explained better. To this reviewers' knowledge, quenched fluorophores will not contribute to the fluorescence signal and therefore the measured lifetime. If by crowding effect the authors mean homo-FRET, then the effect would also not contribute to a change in the lifetime

Thanks for the comment. Here we refer to the conventional reduction of lifetime that is observed in conditions of high fluorophore concentration that, in some literature, is referred to as self-quenching (see for instance: 10.1371/journal.pone.0247326). Yet, we do agree that this expression is somewhat

improper and may lead to some confusion. Thus, we removed this term and replaced it with "increase of the non-radiative rate from the excited state".

10) In Fig 4, the authors measure the pH of insulin secretory granules in INS1E cells and state that ISGS in the cytoplasm have a higher pH (5.83) than those at the membrane (5.73). There are several questions: a) ISGs in the cytoplasm appear much larger than those close to the plasma membrane, which needs to be addressed by the authors. Given the low co-localization with ZIGIR, it cannot be excluded that these are non-ISG organelles. Attributing this to immature ISGs without evidence is speculation and needs to be named as such. b) the graph in Fig 4b shows a distribution of individual measurements for whole cells (this reviewer assumes this means total ISG pH), as well as for cytoplasmic and plasma membrane ISGs. Why do some ISGs in the cytoplasmic fraction have pH around 6, whereas this is not represented in the whole cell measurements which should include all ISGs? Are these data not from the same cells? C) a difference in pH of 0.1 seems very little and equates to a difference of ~380 nM protons. How is this difference biologically relevant, and, with a SD as shown in Fig 4b, can such small differences with a very low n be accurately assessed (given it appears the data is from a single experiment)? The authors need to contextualize their findings and explain the lack of effect of IAA-94 on ISG pH. How would a difference in pH of 0.1 be regulated by the cell?

We would like to thank the reviewer for this comment which helped us to clarify, through new experiments and manuscript revision, a few putative critical points.

First, concerning point (a) on the size of granules in the cytoplasm with respect to those on the membrane: provided that the co-localization experiments already described in a previous point should have mitigated the reviewer's concerns on the exact nature of the vesicles observed in the cytoplasm; we have now explained in the text that the reason of the bigger apparent size of granules dynamically moving in the cytoplasm compared to granules that are docked at the plasma membrane is deeply linked to the FLIM technique that requires integration of the signal over time. We have performed an exemplary experiment in which we compare a single-frame image (a) with signal integration over time achieved by summing the indicated number of frames (b–e). The data included in the new Figure S5 show how the integration of the signal leads to the apparent enlargement and deformation of the cytoplasmic granules.

Second, concerning point (b) on the graph in Fig. 4b: we agree with the reviewer that the content of the graph may not be clear to the reader and took this opportunity to revise the figure description. In more detail, the values displayed come from the same cells, in which we have calculated the average pH of different ROIs corresponding to the cell membrane, the cytoplasm or the whole cell; the pH value of the whole cell-ROI is therefore a weighted average between membrane and cytoplasmic pH. The calculated pH values are not single granule measurements.

It was not a single experiment but N=15 cells from n=2 independent experiments. To exclude any possible concern regarding the robustness of the presented data, we expanded the analysis by adding 10 cells from 2 additional independent experiments.

Finally, concerning point (c) on the differences in pH observed: 0.1 is the difference between the average membrane pH and the average cytoplasmic pH. However, when examining individual cells, heterogeneity is observed: some cells show almost no difference, while others exhibit differences of up to approximately 0.3. This differing behavior may arise from genuine heterogeneity within the cell population or could be attributed to technical aspects of the measurements, such as the lowest resolution along the z-axis. This could result in plasma membrane granules being inadvertently included within the designated cytoplasmic ROI, thereby reducing the observed pH difference. Regarding the lack of effect of IAA-94 on ISG pH, we have better commented on the observed result and cited previous

work by Solimena group in which authors show the same lack of effect (even though the compound was used at a tenfold lower concentration compared to our experiments).

11) The authors then measure the pH upon stimulation with glucose and speculate that the reduced amount of ISGs at the plasma membrane are due to secretion. It is noteworthy though, that the measured pH is not mentioned in the text, whereas Fig 5c suggests that 'membrane' ISGs have a lower pH (about 5.4 at timepoint 0) than in the prior results. This needs to be addressed. Where does this difference come from? Similar to Fig 4, large organelles can be seen. Are these still ISGs or potentially degradative organelles? Are these data from a single experiment? Repetitions should be performed to reduce potential effects during cell culture.

We appreciate the reviewer's observations and provide the following clarifications:

The pH values observed in membrane-associated ISGs at timepoint 0 (approximately 5.4, new Fig. 4c) are indeed lower compared to the prior results (new Fig. 3b). INS-1E cells at timepoint 0 are incubated in serum-free SAB buffer containing 2.2 mM glucose while cells corresponding to Fig 3b are maintained in complete culture medium which contains 10% FBS and 11 mM glucose. To investigate whether the difference in pH arises from serum starvation or from reduction in glucose concentration, we performed a set of experiments comparing maintenance condition with SAB 2.2 mM glucose and SAB 11 mM (Fig. S6). Interestingly, we observed that the pH of membrane-associated ISGs also decreases in SAB 11 mM. This indicates that the decrease in pH of the membrane granules that is observed in time zero of the glucose experiment is likely due to the use of a "lean" buffer without serum rather than to the decrease in glucose concentration.

The data do not originate from a single experiment. Each analyzed cell was obtained from a dedicated dish, and the 11 cells were acquired over the course of three separate experimental sessions conducted on different days.

12) The manuscript lacks a clear discussion of the results obtained. The authors should more extensively discuss their findings in the context of prior research and highlight similarities and differences. In the case of the latter, the authors should discuss their view and opinion of potentially differing results.
We thank the reviewer for this comment. The revised manuscript contains an entirely new discussion section.

Reviewer #2 (Remarks to the Author):

In this study, the authors develop a new tool to measure the pH of insulin granules of pancreatic beta cells, based on the targeting of the pH sensitive GFP variant E1GFP with favourable pKA of about 6 to insulin granules. The study has merit for two reasons: First, the approach can be likely easily adjusted to measure pH in other organelles and other systems by simply replacing the proinsulin in their DNA construct. Second, the pH in insulin granules has never been accurately determined, and this study fills this knowledge gap. This is important, given the well understood importance of luminal pH for loading of vesicles with cargo molecules and the secretion itself. The study is well designed, clearly presented and overall of high quality. I have no suggestions for further experiments, but have a couple of minor comments that can all be addressed by textual changes.

First of all, we would like to thank the Reviewer for his/her critical reading of our manuscript and, especially, for having highlighted the aspects of novelty in the field and its overall high quality. Said this, we particularly appreciated the raised comments which helped us to improve manuscript's intelligibility and overall impact. In the following, for each raised point, the action taken.

Figure 5 shows pH measurements upon glucose stimulation. From these measurements, it is concluded that the difference in pH between cell center and periphery is preserved during the early phase of glucose-induced secretion. However, I do not completely agree with this statement. First, the pH of the time points is not directly compared but visual inspection shows a clear increase in time in figure 5c. I understand that this difference is small, and it will be difficult to reach statistical significance due to the variation in the data, but the current conclusion seems to be too strong. Thus, the trend towards an higher pH needs to be acknowledged and briefly discussed.

We appreciate the valuable feedback from the reviewer. To address this point , we have updated the Results section to include a statement recognizing this trend, despite the lack of statistical significance due to data variability.

The manuscript currently does not have a Discussion section. However, I miss some discussion on how the method can be adjusted for other systems. In addition, how does the pH compare with other secretory processes? The pH is determined Phasor, what is benefit over exponential fitting? Or compared to simple ratiometric acquisition with two different excitation wavelengths? A brief discussion and conclusion section would strengthen the paper.

We thank the reviewer for this comment as it gave us the opportunity to implement an entirely new "Discussion" section in the revised manuscript to present in more detail the methodological approach in the context of available literature and the biological implications of the obtained results.

On page 12 (lines 302-304), it is found that the absolute values of the fluorescence lifetimes measured in living cells were lower than those obtained in bulk solutions. This is a known phenomenon and we have also observed this for different constructs. However, I am not convinced by the authors' explanation that it is due to crowding and self-quenching. In this case, would you not expect large variation in figure 3, as self-quenching depends on expression levels which differ between experimental repeats and cells? In addition, shouldn't this be visible in the Phasor plot, as self-quenching likely will depend on the protonation state? This caveat should be discussed more. One likely alternative explanation might be the presence of other ions and biomacromolecules.

We thank the Reviewer for this comment. At first, we should state that we replaced the term "self quenching" with increase of the non-radiative rate from the excited state, following a suggestion of the

other reviewer. We do agree that in some cases crowding and concentration-effects may produce large variations in lifetime. Yet, to our knowledge, the lifetime reduction is often related to energy transfer to nonfluorescent dimers (or multimers). This in turn may imply a complex relationship between lifetime, fluorophore concentrations and the mutual distance among fluorophores, which could account for the observed trends. As for the phasor plot, we do observe the reduction of lifetimes. Yet, we agree with the reviewer that complex energy transfer processes among fluorophores could be different for the protonated and the anionic chromophore, i.e. depend on pH and possibly distort the titration curve. In this sense, changes in the protein environment constitute -also in our opinion- a better explanation of the observed photophysical phenotype. We added this consideration in the text.

It should be mentioned in the results section and figure legend that the characteristics of A and A' in figure 1b and 1c were determined with purified protein at different pH values. I agree that the presence of A and A' is very likely under those conditions, but this is still inferred and not directly proven. This is no major issue, but is currently only explained in the Methods section and should be reiterated in the Results and figure legend as well.

We thank the Reviewer for highlighting this putative source of confusion. We agree that reiterating the explanation on A and A' states in the Results section can help the reader. The text has been modified accordingly.

I recommend separating the text in shorter paragraphs, especially for the Introduction section. Although the Introduction section is very good, is currently seems a single large paragraph which makes it difficult to read.

Done, thanks.