We thank the Referees for their interest in our work and for helpful comments that will greatly improve the manuscript. We endeavored to respond to all the points raised. The Referees have brought up some good points and we appreciate the opportunity to improve our manuscript accordingly.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
Manko et al establish a clear protocol for the FRET-FLIM approach used in Gasser et al, although I found the protocol well developed, and the software used easy to install/use I have a few suggestions to help clarify and also not raise expectations too much for an unexperienced user.

Major Concerns:
One of the main points that are missed is that choosing how to tag PvdA and PvdL is an essential question. Indeed when PvdA is tagged with eGFP and PvdL with mCherry, the FRET-FLIM signal is really low compared to when PvdA is tagged with mCherry and PvdL with eGFP. Fig.8A and Fig.8C are almost undistinguishable. It would be necessary to make this a clear point and add not only the fitted curves, but all the data points to Fig.8, as in Fig.9. Clearly in the case of Fig.8A many of the pixels will fall in a place where the change in tau is not significant and this interaction is inconclusive.

Choosing how to tag the proteins of interest is indeed a key point for successful FLIM-FRET experiments. Most critically, tags should not modify or alter the interaction of proteins. Unfortunately, excepted in rare cases where the structure of the proteins are known or can be predicted, in most cases one is compelled to trial-and-error approaches. Interpretations of FLIM-FRET in the absence of energy transfer have therefore always to consider the possibility that labels can alter the interaction. For this reason, FLIM-FRET can be seen as a confirmatory technique in the sense that if an interaction is observed, it should exist in the absence of label. Disposing of an external functional readout - like checking that the production of pyoverdine by the mutated strains expressing doubly labelled proteins is similar to wt strains - is particularly useful to interpret FLIM-FRET results. (this paragraph has been added to the main manuscript in the discussion section)

If protein of interest are interacting in a 1-to-1 complex, labelling one or the other protein with eGFP - the donor - (and the other with the acceptor) should give concordant FRET signals. In our manuscript, we further demonstrate that non 1-to-1 interactions can also be characterized. In this case, changing the labelling from one protein to the other leads to very different FRET signals from almost no apparent FRET in the presence of excess of donor, to (high) FRET value with almost 100% of species interacting in the presence of an excess of acceptor. Empirical cumulative distribution functions of the average mean lifetime for one or the other labelling scheme illustrate these differences (significant changes of the distribution in the presence of an excess of acceptor - and tiny or hardly discernable changes in the presence of an excess of donor). Disposing of both labelling schemes is a key part of the exploration.

So for example I disagree with statement: line 589 "As a consequence, it is not dependent on local concentrations of fluorophores neither on the intensity of the light excitation." which contradicts the following paragraph.

There is likely a misunderstanding on this point. The whole context of the sentence was "Fluorescence lifetime is an intrinsic parameter of the fluorophore. As a consequence, it is not dependent on local concentrations of fluorophores neither on the intensity of the light excitation." The absence of dependence on concentration and light excitation was standing for fluorescence lifetime and not for FRET. If it is true that FRET depends on concentrations - and particularly in the context of complexes with unbalanced stoichiometries - the fluorescence lifetime (i.e. the mean by which to access to FRET values) does not.

Minor Concerns:

There are always newer versions of fluorescent proteins in use, they depend on each organism, are eGFP and mCherry the latest available in P.aeruginosa? Would be good to review.

Choosing the optimal FP FRET pairs is a complex and ever moving forward field and we thought it was out of the scope of this protocol. In the revised version of the manuscript, we propose the interested reader to refer to recent reviews about palette of available FP FRET pairs.

What is the green line in Figure 6?

The green line corresponds to the Instrumental Response Function. The IRF results mostly from the convolution of the response functions of the detector and of the laser pulse duration. As we are using two-photons with fs-pulses, the IRF is not measured but estimated from the data. (see *The bh TCSPC Handbook* cited in ref 25).

The sentence "The experimental decay curve of the pixel pointed in the above image, its fit (red curve) deconvoluting the decay from its calculated instrumental response function (green curve) can be seen in the green panel." has been added to the legend of figure 6

I would add in Figures 8 and 9 the names of the fusions PvdA, PvdJ and PvdL because they are referred to in the text and it would make the story easier to follow/

Done

What version of R is needed?

The package has been developed using R version 3.5.2 (December 2018). The package is compatible with any latest R versions (including the major update to the 4.0 version) and is also likely compatible with all versions ≥ 3.0.2 (although not tested) because it does not call or import any other package. A *Depends* section has been added to the package description file to indicate dependency on a particular version of R (>= 3.5)

Please on the HowTo document clarify that you are running all commands from R and no need to go/clone from github is needed.

The sentence “Note that there is no need to clone the repository from github to use the package. Instead the package can be installed from the github source repository directly in R. “ has been added.

line 119: "immobilized so that they do not move during the imaging acquisition time." please change to "immobilized during the imaging acquisition time."

This sentence has been removed following the remark of referee 2

line 581: "mCherry with which eGFP is transferring energy. " change to "mCherry proteins with which eGFP is transferring energy.

Done

line 630: "Similarly,machine learning applied to FLIM data in term of pixel classification or regression, denoising or signal restauration will make possible to achieve outstanding image reconstruction and analysis" replace with Similarly, machine learning applied to FLIM data regarding pixel classification or regression, denoising or signal restOration will allow outstanding image reconstruction and analysis"

The sentence has been changed for “Similarly, machine learning applied to FLIM data regarding pixel classification or regression, denoising or signal restoration will allow outstanding image reconstruction and analysis that will further increase the interest of FRET-FLIM methods”

Reviewer #2:

Manuscript Summary:

The manuscript of Manko et al. presents a very complete protocole of FLIM-FRET measurements in bacteria to reveal and caracterize PPI. All the protocol is well presented and the manuscript is wrtitten in a comprehensive manner. This protocol is definitely of high interest to be published in JOVE (and particularly with the video support). To improve this work I have few suggestions.

Major Concerns:

- Other FLIM methods than TCSPC are not cited in the introduction. there is no any information regarding the acquisition time, photon budget.

This point has been addressed by adding this paragraph in the introduction

« FLIM-FRET allows measuring the fluorescence lifetime decay of a FRET donor at every pixel of a FLIM image (Figure 1A). There are two major techniques to determine fluorescence lifetime which differ in acquisition and analysis: Frequency-domain (FD)[14] and Time-domain (TD). TD FLIM is more widespread and is performed using a pulsed illumination combined with different possible detection configurations including gating methods[15], streak camera[16] or Time-correlated single photon counting (TCSPC) techniques[8]. For both FD and TD techniques, fluorescence lifetime is not directly measured but requires an analysis of the measured data to estimate the lifetime(s) or the presence of interactions. For TCSPC techniques, the most widely used analysis relies on fitting the decays with single or multi exponential functions using least square iterative re-convolutions that minimize the weighted sum of the residuals. »

- In fact the introduction presents that FLIM is abble to study spatial location of PPI but there is no real use of image regarding the data analysis, particularly in fig8 and 9. This point has to be clarified. If yous sale spatial location in the introduction you have to show interesting results regarding this approach.

We do agree with this statement. Even if FLIM-FRET has the potential to extract spatial location of PPI, it can be exploited in situations where PPI are localized at fixed position in the cell. It is obviously not the case in our data where the interacting proteins are diffusing freely throughout the cytoplasm. To make the manuscript clearer, we removed the section about spatial characterization in the introduction part and added the following comment in the discussion part: " As FLIM measure fluorescence decays for every pixel of the image, it can additionally provide information about where the interactions take place in cells at the pixel resolution. But the space component of FLIM can be exploited only if the observed structures are sufficiently stable over the acquisition time."

- In term of data analysis, only fitting procedure with models seems to be used. I have two concerns:

(i) what means lifetime in fig8? do you use é expo model and mean lifetime calculaition? single expo model? this has to be clarified.

In figure 8, lifetime stands for the mean lifetime of a single exponential model. This value is calculated for every pixel with more photons that the defined threshold. The ecdf of the distribution of these values are then plotted. To make the legend of fig 8 more precise, we added the expression "(single exponential model)" at the end of the figure 8 title.

(ii) in the perspective, you mention new methods to analyze FLIM with AI. But you do not consider already available non-fiting approches with mean lifetme coming from the mean arrival time or phasor determination. this is missed in the introduction and in the perspective. Moreover, this is an important aspect when considering photon budget and acquisition time (see above).

We indeed missed to report the non-fitting approaches. In the revised version, we added the following paragraph in the discussion section "In this context, fitting procedures using least square methods that require high count statistics might be poorly adapted to estimate FRET. A variety of alternative methods have been developed [16,32], including non-fitting methods (reviewed in Padilla-Parra et al. 2011 [33]). These methods differ in calculation speed, minimal number of photons required for proper analysis, accuracy, complexity and type of data that can be efficiently processed. Techniques like the minimal fraction of interacting donor[34] or phasor approach[35,36,37] have the potential to perform high speed acquisitions in FRET–FLIM and still be quantitative to process large amount of data or even to reach video-rate speeds.

"

Minor Concerns:
Add more details regarding data analysis strategies and fitting methods in order to understand well the different models and the different values. Particularly you can include it in the figure legends (fig6, 8, 9)

Please see answer to the first question

Legends of figures 6,7, 8 and 9 have also been updated

Reviewer #3:

Manuscript Summary:

This protocol describes a method that combines the use of fluorescence lifetime imaging microscopy and Forster resonance energy transfer (FILM-FRET) to study protein-protein interactions. Within they include the steps to construct the bacterial strains, preparation of the culture slide and parameters for efficient FLIM-FRET cell imaging. They also go through the data analysis of images and describe batch analysis systems. The highlighted portions seem to match well to the goals of the protocol and all images include in the written portion are well constructed to compliment the protocol.

Minor Concerns:

A few grammatical issues but nothing major.

We read carefully the manuscript and corrected some grammatical errors.

Reviewer #4:

Manuscript Summary:

In the manuscript titled with "FLIM-FRET measurements of protein-protein interactions in live bacteria", the authors described a protocol for FLIM-FRET measurements to monitor Protein-Protein Interactions (PPIs) in live Pseudomonas aeruginosa. FRET has been used as a high spatial resolution assay to probe the protein-protein interactions in living cells. The authors demonstrated that FLIM-FRET measurements could be used to characterize the interactions between PvdA and PvdL. Overall, the manuscript is well written, and it fits the scope of the journal. The paper provides detailed protocol for FLIM-FRET measurements. The references also reflect the recent progress in the field.

Major Concerns:

1. Since this choice of FRET pair is critical for FRET measurement, the author should discuss the choice of FRET pair for FLIM measurement.

We do agree that the choice of the FRET pair is a critical point. However, the considerations when using FP pairs is a complex issue that would require a full reviewing - out of the scope of this protocol. To assist the interested reader, we have suggested in the revised version of the manuscript two dedicated references

2. In Fig.2 the authors should provide more details about the biological design of fusion. For example, the linker sequence and length between the fluorescent proteins and PvdA (or PvdL).

Additional information has been added in the plasmid construction section of the protocol

"For PvdA labelled at the C-terminus with eGFP, the 700 bp region upstream relative to the stop codon was amplified by the primers in blue, and the 700 bp downstream region containing the stop codon was amplified with the primers in green. For PvdL labelled at the N-terminus with mCherry, the 700 bp region upstream to the PvdL gene, including the start codon, was amplified by the primers in blue, and the 700 bp downstream region was amplified with the primers in green"

3. Provide brief biological background of PvdA and PvdL to make the report more readable. How stable is the interaction between PvdA and PvdL? What about the binding affinity?

A paragraph has been added in the introduction to provide the biological context of the interaction between PvdA and PvdL.

About the PvdA/PvdL interaction, unfortunately, little is known in term of stability or binding affinity between these two proteins. This is mainly explained by the relative large size of PvdL (480 kDa) - making this protein difficult to express and purify and to be characterized using classical biochemistry experiments.

Minor Concerns:
Give a brief summary about the advantages of FLIM vs other methods for FRET measurement (e.g sensitized emission).

Advantages of FLIM vs other intensity based FRET imaging techniques are discussed in paragraphs 1 and 2 of the discussion

Editorial comments:
You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.
NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

*• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:*
1) 2.12: unclear how gentamycin sensitivity is measured.

Done

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4) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
5) Notes cannot be filmed and should be excluded from highlighting.

Highlighting was removed for notes - all others points sounds OK

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• ***References:*** *Please spell out journal names.*

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