nature portfolio

Peer Review File

Genetically encoded biosensor for fluorescence lifetime imaging of PTEN dynamics in the intact brain

Corresponding Author: Dr Tal Laviv

This file contains all editorial decision letters in order by version, followed by all author rebuttals in order by version.

Version 0:

Decision Letter:

15th May 2024

Dear Tal,

My apologies for our delay in reviewing your article, we were waiting on the third reviewer for some time for balanced reviews.

Your Article, "Genetically encoded biosensor for fluorescence lifetime imaging of PTEN dynamics in the intact brain", has now been seen by three reviewers. As you will see from their comments below, although the reviewers find your work of considerable potential interest, they have raised a number of concerns. We are interested in the possibility of publishing your paper in Nature Methods, but would like to consider your response to these concerns before we reach a final decision on publication.

We therefore invite you to revise your manuscript to address these concerns. We thought the comments were largely constructive and addressing them should improve the paper. We were particularly concerned about reviewer 2's comments regarding the probe having low signal and the comments about potential dominant negative effects of expression of the probe. We will pay close attention to how these are addressed upon resubmission.

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

When revising your paper:

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

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

publication at Nature Methods or published elsewhere.

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

Sincerely,
Rita

Rita Strack, Ph.D.
Senior Editor
Nature Methods

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In the manuscript by Kagan et al, the authors describe the development and validation of a novel biosensor that has the ability to measure the activity of PTEN protein. For this the authors cleverly take advantage of the degree of PTEN "openness" and "closedness" and have developed a FRET assay that is optimized for 2pFLIM measurements. Although novel, the biosensor developed in this study was inspired by a previous BRET version of PTEN (Lima-Fernandes, 2014). The FRET biosensor offers very important advantages that are tested in this study. There are obvious and important utilities for this tool, however there are some questions that need to be addressed prior to considering this as a gold standard. In particular, the biosensor validation could be performed using more optimal methodology. Furthermore, analyses are somewhat superficial and not based on solid rationales.

The following are some issues that need to be addressed:

Introduction
- Regarding the comment "there is currently no method to dynamically monitor its activity in intact biological systems", there PH domain sensors that can be recruited to PIP3 and recently an AKT FRET sensor was published which is an indirect measure of PTEN (Conway et al. Sci Adv, 2023). These tools could be mentioned and compared and contrasted with PTEN FRET.

- Expand intro to include what is known about PTEN activity states and the consequences of PTEN "openness" and "closedness". For instance, what processes control intramolecular interactions of C-terminal and core PTEN domains that are the basis of the biosensor design. Further, PTEN has been reported to function as dimer PMID: 24766807. How would the biosensor function in this context?

- PTEN is phosphorylated by the protein kinase CK2 at its C terminus, which supports the use of TBB. However, Torres and Pulido (Ref43) imply that CK2 phosphorylation leads to destabilization of PTEN by proteasome-mediated degradation. Please discuss this implication and how this could affectr the biosensor.

- Ref 44 is incorrectly interpreted. This paper does not show that EGF negatively regulates PTEN activity. Thus, the rationale for using EGF to suppress PTEN function is unclear.

- Experiments in Fig 1 should include controls showing that PTEN activity is affected as in ref 4, where pAKT output was used. This comment also applies to further experiments where pS6 is used as a proxy for PTEN function. The most proximal outputs for PTEN function are PIP3 levels and pAKT. These marker s should be considered and possibly discussed if they present limitations.

- How high is the conservation between human and worm PTEN sequence?

- Lysine 13 is a crucial Ub conjugation site for PTEN that may have roles in PTEN stability and localization PMID 26216063. Does R14G alter Ub status of PTEN? Localization? Could this limit the efficacy of the biosensor?

Reviewer #2:
Remarks to the Author:
Kagan et al. developed novel fluorescence lifetime sensors for PTEN, both in green and red channels. The design was to tag a pair of fluorophores for FRET to the two ends of PTEN, and the conformation change of PTEN between the active and inactive states can be read out using lifetime measurement of FRET. These sensors exhibit a dynamic range of ~0.2 ns. A point mutation was added to remove the endogenous function. The authors demonstrated the sensor usability in several ways: 1) to analyze disease related mutations on PTEN in vitro; 2) to quantify in vivo PTEN activity in C. elegans in the context of development and a gene mutation; 3 to monitor in vivo PTEN activity in mouse cortical neurons and how they were affected by CRISPR/Cas9-based knock-out of genes known to interact with PTEN signaling; 4) to demonstrate multiplexed imaging of PTEN (red version) with GCaMP8s was demonstrated, and 5) to multiplexed image PTEN dynamic in different neuronal types in response to sensory deprivation.

PTEN dephosphorylates PIP3 and in turn inhibits AKT, thereby controlling many aspects of cellular function. A robust PTEN sensor would be of interest to the field. However, with the presented data, the reviewer has questions regarding how robust the sensor is. This study could also be strengthened in several aspects.

Major.
1. The sensor dynamic range seems small. As a result, the data generally seem noisy, and the statistics rely on averaging hundreds of cells throughout the manuscript. This would potentially make the analysis within individual cells and to detect cell heterogeneity difficult. Can the sensor be further optimized?
2. Related to above, the sensor optimization effort seems limited at present. Has the author attempted different circular permutation forms of the donor and acceptor? Mutagenesis optimization of the linker?
3. Timelapse and longitudinal imaging are powerful for understanding cell biology, such as to reduce noise, to appreciate cell heterogeneity, and to reveal signaling kinetics. Other than one panel of in vitro experiment, longitudinal imaging of the same cells was not used. Wonder what the reason is, and whether longitudinal data can be presented.
4. PTEN overexpression alters cell physiology. Although the R14G mutation is introduced to eliminate the endogenous function, the R14G mutation still retain certain PTEN activities, as based on the cited reference #17 in one of the assays. The reviewer wonders whether the cell size is a sufficiently sensitive assay. Additional characterizations, such as electrophysiological properties of neurons, would greatly strengthen the study. Also, how many fold is the sensor overexpressed compared to endogenous PTEN?
5. Additionally, the R14G mutation is in the PIP3 binding domain, so the phosphatase domain and C2 domain are still intact. Could there be gain of functon(via phosphatase) or dominant negative (via C2) effects? Discussions along these lines together with additional characterizations, as discussed above, will enhance the study.
6. Based on the in vitro data in Fig. 1e-f, kinetics of this sensor seem slow. How are the kinetics in vivo? Is it consistent with the literature?
7. Is the PTEN sensor signal reversible within the same cells or animals?
8. With the modifications (FP tagging, R14G, overexpression), does the sensor activity still correlate with other assays of PTEN function, especially at an intermediate activation level?
9. Figure 5h. PTEN activities were different between spines and the adjacent dendritic shafts under a steady state condition when IGF1R or Tsc2 were knocked out. How might this happen? Particularly, the sensor appears to be a cytosolic protein, which would go in and out of a spine within less than a second. At the same time, the sensor kinetics seems to be slow (Fig. 1f). How would an activity gradient be possible (versus equilibration between the two compartments)? A discussion is needed. Measurement of the sensor mobility in and out of spines may help.
10. Fig. 7. Can the experiment be repeated by switching G- and R-PTEN in the two neuronal types? This controls for whether the effect is due to differential performance between the two sensors.
11. Fig. 5i. The drastic difference in spine density is not well represented in the example images (Fig. 5g).

Other.
1. Lines 341-342: "… we constructed an AAV with a double floxed Cre recombinase dependent expression of G-PTEN". Please consider rewriting to make it clearer.
2. Line 356: "…a distinct reduction of E/I levels of PTEN signaling". Probably will be more precise and easier to understand if change to "… differential regulation of PTEN signaling in excitatory versus inhibitory neurons".

Reviewer #3:
Remarks to the Author:
This study describes the development of a new fluorescence biosensor to monitor PTEN activity in living systems. In addition to its characterizations, the authors show several applications, from culture cells, to in vivo imaging of mouse brain and to complete organisms (C. elegans). As indicated by the authors, PTEN is a central regulator of multiple cellular processes with important physiological implications, from cell growth and differentiation to cellular metabolism, intracellular trafficking, etc. There have been attempts to develop sensors for phosphatydilinositol-3,4,5-trisphosphate (the substrate for PTEN activity and the key effector in the PI3K/PTEN pathway), but these have had limited success, probably because of the low abundance of

this phosphoinositide and the limited sensitivity of the sensor. Therefore, having a reliable sensor that directly reports PTEN activity would be extremely useful for the cell biology research community across multiple fields (neuroscience, cancer, physiology, development, etc).

This sensor is based on FRET changes (monitored by fluorescence lifetime imaging –FLIM) that correspond to conformational differences between inactive and active PTEN. They also develop a red fluorescence variant of this sensor, to use it in combination with other GFP-tagged proteins or even to simultaneously image two PTEN sensors (green and red) that could be expressed in different cell-types. The rationale for these sensors is that, under basal conditions, constitutive phosphorylation of several residues at the C-terminus of PTEN keeps it in a closed, inactive conformation, which is mostly cytosolic. Dephosphorylation opens up this conformation, leading to PTEN association with plasma membrane and/or with PDZ proteins and enhancing its catalytic activity.

The experiments described in this manuscript are sound and the results are well presented. Nevertheless, I do have a couple of technical questions that I think the authors should address.

1. The authors invest a significant effort in developing a PTEN sensor with reduced (or abolished) catalytic activity, to avoid undesired signaling effects of PTEN overexpression in the cell. This led them to use the R14G mutant as their best candidate for detecting PTEN activity changes without altering PTEN signaling. However, I am surprised that the authors do not seem consider that this inactive mutant might have a dominant negative effect, by interfering with the activity of the endogenous PTEN. In fact, this is what has been reported by several groups using the catalytically dead mutant C124S. In this sense, when the authors claim that the R14G sensor “has a minimal impact on endogenous PTEN signaling” (Fig. 3h-l), they are actually showing that this mutant is essentially inactive. But they do not show whether R14G would inhibit (dominant negative activity) the endogenous PTEN activity. To test this point, it would be necessary to express the R14G PTEN mutant in the presence of endogenous PTEN activity.
To the authors’ credit, this control appears to be equivalent to the experiment shown in Fig. 5. That is, if we look at soma size and spine density from control (CyRFP-labeled) neurons in Fig. 3j,l (scrambled gRNA, no recombinant PTEN), these values are quite similar to those reported for R14G PTEN-expressing cells in Fig. 5f,i (with scrambled gRNA). Therefore, at least with respect to soma size and dendritic spine density in pyramidal neurons from somatosensory cortex, the R14G PTEN sensor does not appear to have a dominant negative effect on endogenous PTEN activity. I am surprised that this point is not particularly emphasized by the authors, since I believe it is an important one. In fact, the authors could speculate why this inactive R14G mutant does not appear to have a dominant negative effect, while the catalytically dead mutant C124S does.

2. Another important methodological advance of this work is the development of a red-fluorescence variant of the PTEN activity sensor (R-PTEN versus G-PTEN). This allows for the co-expression with a green fluorescence calcium indicator (such as GCaMP8, Fig. 6) and potentially for the expression of G-PTEN and R-PTEN in different cell types to monitor their PTEN activity simultaneously. The authors describe this application for the expression of the PTEN sensors in excitatory versus PV inhibitory neurons in the mouse somatosensory cortex in vivo (Fig. 7). In this case, the strategy for cell-type specific expression is based on a PV-Cre mouse line co-injected with AAVs with R-PTEN under the synapsin promoter and G-PTEN as a floxed construct with a constitutive promoter. Under this configuration, the authors argue that R-PTEN will be expressed in excitatory cells (because of the Syn promoter) and G-PTEN in PV interneuros (because of Cre expression). However, the synapsin promoter is also active in GABA interneurons. Therefore, shouldn’t these neurons have co-expression of G- and R-PTEN? That is, excitatory neurons would have exclusive expression R-PTEN, but PV inhibitory neurons would have both green and red PTEN sensors. In principle, the fluorescence lifetime of G-PTEN and R-PTEN could be distinguished within the same cells from their different emission wavelengths. But this does not appear to be what the authors are reporting. Please clarify.
On the other hand, if R-PTEN is expressed in both excitatory and inhibitory neurons, one could compare the extent of PTEN activity under basal conditions in these two cell-types (which would be distinguished from the additional expression of G-PTEN). This would also be an interesting comparison (in addition to the effect of sensory deprivation shown in Fig. 7c-f). Again, the authors should clarify how they are carrying out these measurements.

Version 1:

Decision Letter:

Our ref: NMETH-A55616A

27th Nov 2024

Dear Tal,

Thank you for letting us know how you would respond to the remaining reviewer concerns regarding your revised manuscript "Genetically encoded biosensor for fluorescence lifetime imaging of PTEN dynamics in the intact brain" (NMETH-A55616A). Based on the remaining reviewer feedback and our editorial assessments, we'll be happy in principle to publish it in Nature Methods, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We ask that you provide a point-by-point rebuttal upon resubmission and that you modify the main text to clearly discuss issues regarding SNR and dynamic range and how these impact experimental observations.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements within two weeks or so. Please do not upload the final materials and make any revisions until you receive this

additional information from us.

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

Sincerely,
Rita

Rita Strack, Ph.D.
Senior Editor
Nature Methods

Reviewer #1 (Remarks to the Author):

The authors have satisfactorily addressed all of my comments. I have no further comments

Reviewer #2 (Remarks to the Author):

The authors address some of my questions, but not others. At this time, important concerns remain regarding the dynamic range, signal-to-noise ratio in vivo, and the ability to visualize single cell dynamics under physiological or behavioral manipulations. I feel that this study has not reached the bar of Nature Methods.

Major.
1. The sensor dynamic range is small. The authors cited several references in the rebuttal, arguing that the sensors dynamic range is on par. However, some of the papers did not achieve in vivo imaging. A paper from the same author did not report lifetime change (binding ratios were showed), and the most recent paper Massengill et al 2022 reported a lifetime dynamic range of ~0.65 ns, significantly larger than that of the PTEN sensors reported here (~0.25 ns).
2. Regardless of the dynamic range, for a user, the important parameter is the achievable signal-to-noise ratio. The data are noisy, and the statistics rely on averaging hundreds of cells throughout the manuscript. By eyeballing, the signal-to-noise ratio in most experiments are ~1 and sometimes even lower. This would potentially make the analysis within individual cells and to detect cell heterogeneity difficult. The authors seem to be unwilling to further optimize the sensor. However, the current sensor, as it is, is difficult to be adopted by the field.
3. Related to above, the authors state that "As for the suggestion for Circular permutations, it is an interesting point to consider. This manipulation is often used for intensity-based sensors …" Please take a look at the classic work by Nagai et al, PNAS 2004. There were also many subsequent works that followed.
4. The authors accredit some of the variability to biological reasons. However, even after TBB, significant variability remains. The 4A mutant also exhibits significant variability. This may be due to differential sensor maturation across cells. Regardless of the mechanism, this variability argues for the need of a sufficient sensor dynamic range that goes beyond this variability.
5. Timelapse and longitudinal imaging are powerful for understanding cell biology, such as to reduce noise, to appreciate cell heterogeneity, and to reveal signaling kinetics. In light of the variability discussed above, timelapse data normalized to each cell's respective baseline can effectively reduce noise and increase signal-to-noise ration. hte manuscript also emphasizes on "dynamics" at multiple places in the manuscript. The author now adds one HEK cell experiment for time-lapse imaging. However, no in vivo demonstration is included. This is important as
6. This sentence from my previous comments (part of point 4) has been removed in the rebuttal and is not addressed: "Also, how many fold is the sensor overexpressed compared to endogenous PTEN?".

7. The authors now provide a functional correlation in the newly added pAkt experiment. However, the request “especially at an intermediate activation level” has not been considered. The experiment used strong conditions, which may miss altered sensitivity (e.g., shifted EC50 of TBB, but the maximal dynamic range remaining the same).
8. In the newly added ED Fig. 8g, there is very little recovery in 100s. Is this consistent with what is known about PTEN or the sensor? Is it not a cytosolic protein?
9. ED Fig. 11 does not address former point 10 (“Fig. 7. Can the experiment be repeated by switching G- and R-PTEN in the two neuronal types? …”). The reviewer does not doubt the ability to separate color channels. The question is whether the two sensors may have different properties, such as sensitivity, etc. Also, related to point 5, can the authors provide a time course for the change, as this is the only physiological/behavioral manipulation that results in the sensor dynamics?
10. Fig. 4: Is Fig. 4g the duplicated data from Fig. 4e and 4f? Also, are they the same data as Figure 4d? It feels like unnecessary inflation of the figure panels.

Other.
1. Lines 397-398: “R-PTEN was expressed under the neuronal Synapsin promoter, which limited expression primarily to excitatory cells …” This is not true. It can probably be rephrased to: “R-PTEN was expressed under the neuronal Synapsin promoter, which limited expression to neurons, and the majority of neurons are excitatory cells…”.
2. Fig. 3g should be made larger to see cells.
3. Fig. 3l, please show example raw traces.

Reviewer #3 (Remarks to the Author):

The authors have been very responsive to all the reviewers’ comments. Particularly, they now included additional experiments to test the potential dominant negative effect of their PTEN biosensor, and they have ruled out interference with the physiological functions of endogenous PTEN. They have also carried out new imaging experiments to explore the potential of their G- and R-PTEN biosensors to monitor cell-type specific PTEN signaling.
Overall, this is now a thorough and rigorous study describing the development of a novel biosensor for PTEN activity, which is likely going to be of general interest for the scientific community.
I do recommend its publication.

Version 2:

Decision Letter:

21st Jan 2025

Dear Tal,

I am pleased to inform you that your Article, "Genetically encoded biosensor for fluorescence lifetime imaging of PTEN dynamics in the intact brain", has now been accepted for publication in Nature Methods. The received and accepted dates will be March 3, 2024 and Jan 21, 2025. This note is intended to let you know what to expect from us over the next month or so, and to let you know where to address any further questions.

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Please feel free to contact me if you have questions about any of these points.

Best regards,
Rita

Rita Strack, Ph.D.
Senior Editor
Nature Methods

From the editors:

Your article, "Genetically encoded biosensor for fluorescence lifetime imaging of PTEN dynamics in the intact brain", has now been seen by three reviewers. As you will see from their comments below, although the reviewers find your work of considerable potential interest, they have raised a number of concerns. We are interested in the possibility of publishing your paper in Nature Methods, but would like to consider your response to these concerns before we reach a final decision on publication.

We therefore invite you to revise your manuscript to address these concerns. We thought the comments were largely constructive and addressing them should improve the paper. We were particularly concerned about reviewer 2's comments regarding the probe having low signal and the comments about potential dominant negative effects of expression of the probe. We will pay close attention to how these are addressed upon resubmission.

We thank the editors for their potential consideration of our manuscript. Bellow we have addressed all the reviewers' comments with new experiments and analysis. We include the new figures bellow as supplements.

As mentioned by the editors, we have now addressed the reviewers' points regarding low signal to noise with new experiments, which we believe demonstrate the usefulness of the PTEN biosensor for in a variety of biological applications. We have carefully examined the potential dominant negative effect of the PTEN biosensor by performing new experiments assaying its biochemical activity, PTEN downstream signaling, and neuronal structure and function. Collectively, we show that the PTEN sensor does not perturb physiological function and is well suited to study PTEN activity in intact cells, tissues and organisms.

Reviewer #1:

Remarks to the Author:

In the manuscript by Kagan et al, the authors describe the development and validation of a novel biosensor that has the ability to measure the activity of PTEN protein. For this the authors cleverly take advantage of the degree of PTEN "openness" and

“closedness” and have developed a FRET assay that is optimized for 2pFLIM measurements. Although novel, the biosensor developed in this study was inspired by a previous BRET version of PTEN (Lima-Fernandes, 2014). The FRET biosensor offers very important advantages that are tested in this study.

We thank the reviewer for their positive evaluation of the PTEN biosensor.

There are obvious and important utilities for this tool, however there are some questions that need to be addressed prior to considering this as a gold standard. In particular, the biosensor validation could be performed using more optimal methodology. Furthermore, analyses are somewhat superficial and not based on solid rationales.

We have carefully addressed all the comments with new experiments and analysis, described for each comment.

The following are some issues that need to be addressed:

Introduction

- Regarding the comment “there is currently no method to dynamically monitor its activity in intact biological systems”, there PH domain sensors that can be recruited to PIP3 and recently an AKT FRET sensor was published which is an indirect measure of PTEN (Conway et al. Sci Adv, 2023). These tools could be mentioned and compared and contrasted with PTEN FRET.

Indeed, there are numerus sensors directed for the PTEN signaling pathway, but none to probe PTEN dynamics **directly**, let alone with cellular resolution in intact tissues. The importance of this fact and the rationale for designing this probe is discussed in the introduction:

“The PTEN signaling pathway is central to cell proliferation and survival, and includes growth factor activation, Akt, PIP3, and mTOR signaling. Numerus previous studies have engineered biosensors for live imaging of this signaling pathway, including Insulin growth Factor 1 Receptor (IGF1R) [1,2], Akt[3–6] , PIP3[7], and mTOR[8,9]. However, PTEN is uniquely positioned in this pathway as a major negative regulator, and is indispensable for cellular function and development [10–12]. Importantly, despite these collective efforts, none of the previous biosensors allowed monitoring protein signaling dynamics in vivo in with cell type specificity the intact brain”.

- Expand intro to include what is known about PTEN activity states and the consequences of PTEN “openness” and “closedness”. For instance, what processes control intramolecular interactions of C-terminal and core PTEN domains that are the basis of the biosensor design.

We thank the reviewer for this comment, we now include a description of the known consequences of PTEN conformational state in terms of catalytic activity, ubiquitination, self phosphorylation, and dimerization, and subcellular localization:

“PTEN conformation and activity is modulated by phosphorylation of serine residues in its C terminus by Casein Kinase 2 (CK2), a conserved kinase essential to cellular viability [13]. CK2 phosphorylation renders PTEN in inactive closed conformation[14,15]. Accordingly, PTEN phosphorylation and conformational state determines its catalytic activity[14], ubiquitination[16,17], dimerization[18] and subcellular localization [19].”

Further, PTEN has been reported to function as dimer PMID: 24766807. How would the biosensor function in this context?

We thank the reviewer for raising this important point. Indeed, PTEN has been reported to function as a dimer [18]. Furthermore, PTEN dimerization was shown to convey a LOF PTEN mutant with a dominant negative (DN) role, implicated with cancer progression [18]. To examine this, we performed experiments to directly test the dimerization of PTEN (**Extended Fig. 5e**). Using FRET analysis between PTEN tagged with donor and acceptor fluorescent proteins, we were able to confirm the induction of PTEN dimerization upon activation by TBB application. However, we found that the C124S mutation, which was previously shown to act as a DN, showed increased dimerization as compared to both WT and R14G. Critically, the R14G mutation used in the G-PTEN sensor, does not convey a DN effect, which is mirrored by lack of excess dimerization. Overall, the effect of dimerization is small in terms of FRET magnitude (0.02ns compared to 0.25ns for conformational change) and therefore we do not predict it significantly impacts our sensor design and practical utility.

- PTEN is phosphorylated by the protein kinase CK2 at its C terminus, which supports the use of TBB. However, Torres and Pulido (Ref43) imply that CK2 phosphorylation leads to destabilization of PTEN by proteasome-mediated degradation. Please discuss this implication and how this could affect the biosensor.

We have now tested the effect of proteosome inhibition on the PTEN sensor, these new results are shown in **extended fig. 6c-d**. We find that proteasomal inhibition leads to an increase in PTEN activity, as shown by increase in the PTEN sensor lifetime. Furthermore, we find that both TBB induced activation and the 4A mutation, both of which antagonize CK2 phosphorylation of PTEN, lead to similar increase in PTEN activity. We conclude that it is possible that CK2 induced phosphorylation is associated with proteasomal inhibition. However, proteasomal inhibition has very broad effects on multiple signaling pathways so it is difficult to conclude this is a casual relationship.

- Ref 44 is incorrectly interpreted. This paper does not show that EGF negatively regulates PTEN activity. Thus, the rationale for using EGF to suppress PTEN function is unclear.

We thank the reviewer for pointing out this error about Ref 44. However, other previous studies have demonstrated compelling evidence for EGF mediated PTEN inhibition, which is mediated by direct activation of CK2[20,21] . We now include these references in the revised MS.

Furthermore, we have repeated the EGF experiments and found that EGF robustly decrease PTEN activity, which can be readily reversed by TBB application. We believe that our data and the reference studies do support the idea that EGF induced inhibition of PTEN is via CK2 activation (**Fig. 3d-f**).

- Experiments in Fig 1 should include controls showing that PTEN activity is affected as in ref 4, where pAKT output was used. This comment also applies to further experiments where pS6 is used as a proxy for PTEN function. The most proximal outputs for PTEN function are PIP3 levels and pAKT. These marker s should be considered and possibly discussed if they present limitations.

We thank the reviewer for these comments. We have included in the revised MS, new experiments to address these points. First, we used an in vitro phosphatase assay using a PIP3 analogue (**Extended Fig. 3b**). We find that while the WT PTEN sensor retains phosphatase activity, the R14G is a LOF variant and does not show any detectable phosphatase activity. In addition, we have performed additional characterization of the sensor using immunofluorescence for pAkt (**Fig. 3g-h**). Our results further support the utility of the biosensor, since we show that unlike the catalytically active WT PTEN sensor which lower pAkt levels, the PTEN R14G does

not impact pAkt levels and its regulation by endogenous PTEN. We have also included western blot analysis and found that WT and R14G PTEN sensors show similar expression levels (**Extended Fig. 3g-h**).

Finally, we conducted in vivo experiments to test the effect of the PTEN sensor on neuronal morphology and function as well as staining for pS6 (**Fig. 3i-m, Extended Fig. 5, Extended Fig. 9, Fig. 6f-i**). We used pS6 staining for analysis of PTEN signaling in the brain since it was not possible to perform pAkt staining in brain tissue due to diffuse cytosolic signals. The somatic localization of S6 provides a useful estimate for downstream PTEN activity, and has been routinely used as such in previous studies [22,23].

- How high is the conservation between human and worm PTEN sequence? The worm PTEN ortholog, DAF-18, is more than double the length of PTEN (962 compared with 403). However, the PTEN domain is in the first half of DAF-18 and the second half of DAF-18 has no recognized domain. So, if we compare only the PTEN sequences, then the conservation is 34% identical, 50% similar. Importantly, a recent study [24] found that Daf18 KO can be rescued by the human PTEN sequence.

lysine 13 is a crucial Ub conjugation site for PTEN that may have roles in PTEN stability and localization PMID 26216063. Does R14G alter Ub status of PTEN? Localization? Could this limit the efficacy of the biosensor?

We thank the reviewer for raising this important point. As the reviewer points out, previous studies have found that K13 is important for PTEN's subcellular localization, which is likely mediated by ubiquitination [16]. To address this in the context of our biosensor and the R14G mutation, we have carried out additional experiments to examine the subcellular localization of the PTEN sensor (**Extended Fig. 6a-b**). We found that both the WT PTEN sensor and the R14G mutants showed similar enrichment in cytosol/nucleus expression under basal conditions. However, we found that the PTEN sensor containing a K13E significantly enhanced nuclear expression and impaired PTEN subcellular localization. These new experiments further support

the use of the R14G mutation, and its lack of effect on PTEN signaling and cellular physiology.

Reviewer #2:

Remarks to the Author:

Kagan et al. developed novel fluorescence lifetime sensors for PTEN, both in green and red channels. The design was to tag a pair of fluorophores for FRET to the two ends of PTEN, and the conformation change of PTEN between the active and inactive states can be read out using lifetime measurement of FRET. These sensors exhibit a dynamic range of ~0.2 ns. A point mutation was added to remove the endogenous function. The authors demonstrated the sensor usability in several ways: 1) to analyze disease related mutations on PTEN in vitro; 2) to quantify in vivo PTEN activity in C. elegans in the context of development and a gene mutation; 3 to monitor in vivo PTEN activity in mouse cortical neurons and how they were affected by CRISPR/Cas9-based knock-out of genes known to interact with PTEN signaling; 4) to demonstrate multiplexed imaging of PTEN (red version) with GCaMP8s was demonstrated, and 5) to multiplexed image PTEN dynamic in different neuronal types in response to sensory deprivation.

PTEN dephosphorylates PIP3 and in turn inhibits AKT, thereby controlling many aspects of cellular function. A robust PTEN sensor would be of interest to the field. However, with the presented data, the reviewer has questions regarding how robust the sensor is. This study could also be strengthened in several aspects.

We thank the reviewer for his/her enthusiasm regarding the potential use of the sensor. We have addressed all the specific issues below and have conducted a thorough validation of the robustness and validity of the PTEN sensor. We provide details for each comment below.

Major.

1. The sensor dynamic range seems small. As a result, the data generally seem noisy, and the statistics rely on averaging hundreds of cells throughout the manuscript. This

would potentially make the analysis within individual cells and to detect cell heterogeneity difficult. Can the sensor be further optimized?

While we can understand the reviewer's comment, in fact, the magnitude of response of our PTEN sensor is highly similar to other FRET based FLIM sensors used by the research community [25–29]. The dynamic range of the sensor, as determined by pharmacology, is 0.25ns, since it is the range between the PTEN inhibition and activation. The maximal difference in conformation can be read out by the 4A mutant, since it represents a maximal open state devoid of PTEN phosphorylation and is 0.35ns. We would like to emphasize that in the initial characterization of the PTEN biosensor, we do compare absolute fluorescence lifetime across different populations of cells in some of the analysis (**Fig. 1**). We do not perform this type of comparison to compensate for large noise of the biosensor, as its dynamics are well in line with longitudinal measurements of the same cells over time (**Fig. 1e-g and Fig. 3d-f**). In contrary, we consider this to be an advantage of FLIM based sensors. As opposed to intensity-based FRET analysis, it is possible to obtain lifetime data, which is insensitive to photon numbers and expression levels, and thus make statistical comparisons across different cell populations. This feature is what allowed us to compare PTEN mutants in **Fig. 2** and **Fig. 5** compare differences in PTEN lifetime across different point mutations, as well as examine the effect of genetic perturbations on PTEN activity levels.

We agree with the reviewer that in basal state, we can detect heterogenous levels of PTEN lifetime. This heterogeneity can be attributed to two main factors: experimental variability arising from system errors and photon noise, and biological variability reflecting differential PTEN activity across cells. To clearly dissociate between these two factors, we **performed additional experiments** to address and analyse single to noise analysis of the PTEN sensor, both in vitro in HEK cells (**Extended Fig. 4**) and in vivo in the mouse brain (**Extended Fig. 7**). In these experiments, we continually measured the fluorescence lifetime of the PTEN sensor in individual cells, while we increase the photon counts over long image acquisition. This allows us to experimentally assess how increasing the number of photons affect the standard deviation of PTEN sensor. We compared the standard deviation of lifetime measurements with the estimated deviation of shot noise, which is the theoretical system error due to noise at different photon numbers. As can be seen from our

analysis, indeed frame to frame variability of lifetime is expectedly cantered around the shot noise curve. However, when we analyse this relationship across single cells in the same field of view, there is a significantly larger range of lifetime variability. This variability likely represents biological variability of PTEN activity across cells. We confirm this, by repeating this analysis following application of TBB, which renders PTEN active by inhibiting CK2 activity which leads to de-phosphorylation of PTEN. Following TBB application, our signal to noise analysis shows greatly reduced variability of lifetime across cells. These results confirm that variable levels of lifetime are indeed reflecting biological variability rather than experimental variability. In addition, these experiments further exemplify the utility of the PTEN sensor for analysis of single cell activity.

Overall, we would like to emphasize that this is the first demonstration of a genetically encoded FLIM based sensor for PTEN. As the reviewer points out, we show that this approach is useful for 5 different biological applications:

1. Structural integrity of PTEN mutations in vitro.

2. developmental dynamics of PTEN in C. Elegans.

3. In vivo imaging of PTEN activity in the mouse brain.

4. Impact of genetic perturbations to PTEN activity in vivo in the brain.

5. Cell-type specific PTEN activity in vivo.

As any technique or biosensor has limitations, we believe the most important factor to consider here is: does this biosensor allows one to make biological discoveries? We believe that the answer to this question is undoubtedly yes for the PTEN sensor, since none of the 5 applications we demonstrate were known or even could be directly demonstrated with prior tools and methodologies in the field.

2. Related to above, the sensor optimization effort seems limited at present. Has the author attempted different circular permutation forms of the donor and acceptor? Mutagenesis optimization of the linker?

We have tested and optimized linker size between the donor and acceptor fluorescent proteins and PTEN, which are described in **extended Fig. 1**.

Our major effort in terms of sensor design was to find a mutation that would allow the sensor to minimize perturbation to PTEN signaling, while leaving cellular function intact. These efforts are described in **Fig. 3**, and extended **Figs. 3, 4, 5**. We believe this point is critical to address since protein biosensors with a similar design (Conformational based sensors) are inherently tricky since they utilize overexpression of the protein which they seek to monitor (for example CaMKIIa [30,31]). We describe an elegant solution to this, by inserting a loss of function mutation, R14G, which allows us to monitor PTEN signaling and conformation while minimizing the impact on endogenous PTEN signaling and maintaining cellular function. We indeed find that overexpression of WT PTEN leads to reduced cell size, alterations in synaptic morphology, and neuronal function (**Fig. 3, Extended Fig. 3, 4**). Of note, the previous study which designed a PTEN BRET based biosensors, used WT PTEN and therefore would ultimately lead to aberrant cellular function in all the assays one would use it. We believe this represent an important contribution to the field and showcases a potential solution for the important issue of the physiological impact of biosensor expression on their intrinsic signaling pathways.

As for the suggestion for Circular permutations, it is an interesting point to consider. This manipulation is often used for intensity-based sensors such as GPCR sensors [32,33] , in which conformational change would lead to fluorescence only upon protein folding, and would thus yield a higher signal to noise. As for FRET based sensors, changes in fluorescence of the FRET donor upon conformational change would be unwarranted, since donor fluorescence (and lifetime for FLIM) should be kept at the same levels at closed and open forms to yield a quantitative readout. For the acceptor FP, it could be an interesting modification to test, which we had not yet tested.

Ultimately, we think that every biosensor can be further optimized and improved over time, using linker optimization and modification of fluorescent proteins as suggested by the reviewer. Here, we describe the first version of the PTEN sensor, and it is possible that future studies might build upon it and further improve its dynamic range. Ultimately, similarly to the first point we addressed, we believe that the current version of the biosensor is useful and allow to dynamically monitor PTEN activity, which allows to make new biological discoveries.

3. Timelapse and longitudinal imaging are powerful for understanding cell biology, such as to reduce noise, to appreciate cell heterogeneity, and to reveal signaling kinetics. Other than one panel of in vitro experiment, longitudinal imaging of the same cells was not used. Wonder what the reason is, and whether longitudinal data can be presented.

We thank the reviewer for this important point. In the revised manuscript, we **conducted new experiments** of timelapse imaging of the same cells using the G-PTEN sensor (**Fig. 3d-f, Extended movie 2**). In these experiments, we monitored the same cells over time and show that EGF application robustly lowers lifetime and inhibits PTEN. Then, we showed that this effect is reveres and that PTEN lifetime and activity could be increased by TBB application of the same cells. We believe this data demonstrates the ability to conduct longitudinal measurements using the PTEN sensor in living cells. As for kinetics, it is difficult to speculate what is the kinetics of PTEN conformational changes, as well as the sensor's kinetics since there is no known direct pharmacological agent that would inhibit or activate PTEN, via changes to PTEN conformation. Both EGF and TBB act via modification of CK2 and therefore act in an indirect manner, which would explain the relatively slow time course we observe in these experiments.

4. PTEN overexpression alters cell physiology. Although the R14G mutation is introduced to eliminate the endogenous function, the R14G mutation still retain certain PTEN activities, as based on the cited reference #17 in one of the assays. The reviewer wonders whether the cell size is a sufficiently sensitive assay. Additional characterizations, such as electrophysiological properties of neurons, would greatly strengthen the study.

We thank the reviewer for raising this critical point.

We have spent considerable efforts to address this issue, by introducing a LOF point mutation, R14G, in the PTEN biosensor. We based our screen on the previous study by Post et al [24], where numerus PTEN mutations were mapped and tested for LOF or GOF phenotypes. The R14G mutation was unique since it showed LOF, while it kept the PTEN expression levels within normal levels, whereas the majority of LOF mutations showed greatly reduced protein stability. Originally, this mutation was tested in some of the assays in the previous study and was found to show almost complete

loss of PTEN function. We further confirmed these findings by performing a phosphatase in vitro assay on the PTEN R14G sensor variant. While the WT-PTEN sensor retained phosphatase activity upon addition of PIP3 ligand, the R14G showed no detectable enzymatic activity (**Extended Fig. 3b**). To further test the function and signaling of the R14G containing sensor, we previously performed in vivo imaging experiments in which we tested the effect of PTEN on cellular morphology and dendritic spine density (now in **Extended Figure 5**). We used CRISPR/Cas9 to KO PTEN in single L2/3 cortical cells, which led to hypertrophic soma and increased spine density. We find that while overexpression of WT PTEN completely rescues soma size and spine density, the R14G did not show any rescue and was indistinguishable from PTEN KO cells. We interpret these results as further evidence showing that the R14G PTEN lacks signaling activity in vivo.

We now show additional functional characterization of the R14G PTEN as suggested by the reviewer. We chose to use calcium imaging to analyze neuronal function since it is possible to combine this approach to simultaneously monitor G-PTEN and red-shifted calcium indicators, to test in vivo neuronal physiology within intact neuronal circuits. We show that dendritic spine number as well as spontaneous neuronal activity, which correlates with neuronal connectivity, are dramatically reduced in L2/3 neurons overexpressing the WT-PTEN sensor. However, the PTEN sensor with R14G mutations does not show any change in neuronal activity or synaptic density compared to GFP expressing control cells. These experiments are presented in **Fig. 3i-o** in the revised MS. These experiments were carried out and compared across different mice, since we used IUE which permits that both XCaMP-R a G-PTEN are co-expressed in the same cells. We also previously included a comparison of neuronal function across cells within the same mice, in the experiments presented in **Fig. 6f-i**. We used AAVs to express R-PTEN alongside GCaMP8s. This experimental approach allowed us to compare neuronal activity in neurons with and without PTEN R14G sensor, within the same animal. We find that neuronal activity is unaffected by R14G overexpression (**Fig. 6i**), further supporting the lack of physiological perturbation by overexpression of the G-PTEN biosensor.

5. Additionally, the R14G mutation is in the PIP3 binding domain, so the phosphatase domain and C2 domain are still intact. Could there be gain of functon (via phosphatase) or dominant negative (via C2) effects? Discussions along these lines

together with additional characterizations, as discussed above, will enhance the study.

As we previously discussed for point number 4 raised by the reviewer, both in vitro experiments of PIP3 phosphatase activity, and in vivo imaging in combination with endogenous PTEN KO, demonstrated lack of signaling, and therefore lack of gain of function for the R14G sensor.

As for the dominant negative effect, we examined this with a series of experiments to characterize the PTEN R14G biosensor:

- We measured pAkt levels, the downstream target of PTEN signaling, using Immunofluorescence.
  We found that while WT PTEN greatly diminished pAkt signaling, overexpression of R14G PTEN showed no DN effect, as compared to control cells (**Fig. 3g-h**).
- We performed immunofluorescence measurements in brain slices, following overexpression of the PTEN sensor with R14G and found no difference in terms of pS6 levels between control cells and cells expressing the sensor in the same field of view (**Extended Fig. 9)**.
- We performed in vivo imaging and compared neuronal morphology, namely the density of dendritic spines in apical dendrites of L2/3 neurons. While overexpression of WT PTEN reduced spine density in vivo, as was previously demonstrated in vitro, we find that overexpression of the R14G mutant sensor did not change spine density (**Fig. 3i-j**).
- We performed simultaneous imaging of spontaneous neuronal function with a red calcium indicator and the G-PTEN variant (**Fig. 3k-o**) and a green calcium indicator with the R-PTEN variant (**Fig. 6f-h**). In both cases, we find no changes in neuronal activity across animals (**Fig. 3**) and within individual cells within the same animal (**Fig. 6i**). Notably, WT-PTEN expression led to a dramatic reduction in neuronal activity, which can be explained by the reduction in synaptic density.
- Finally, we examined a possible mechanism to explain the lack of dominant negative effect of PTEN R14G mutation. A previous study suggested possible dimerization of PTEN mutants with WT PTEN, which reduce overall PTEN

activity [18]. We directly tested for dimerization induced FRET between tagged WT, R14G and C124S. We found that C124S, which was previously shown to function as a DN mutation, induced significant dimerization with WT PTEN, while R14G did not show aberrant dimerization over WT PTEN (**Extended fig. 6e**).

Overall, we conclude that based on the aforementioned experiments, the PTEN sensor containing the R14G mutation, servs as a reliable proxy of PTEN activity via conformational change but is devoid of PTEN signalling activity and does not impact cell physiology.

6. Based on the in vitro data in Fig. 1e-f, kinetics of this sensor seem slow. How are the kinetics in vivo? Is it consistent with the literature?

This is an important point raised by the reviewer. However, as previously mentioned, the pharmacological agents we used to inhibit and activate PTEN rely on modulation of CK2 activity, the main upstream regulator of PTEN [14,15]. There are currently no direct positive pharmacological modulators of PTEN that would allow to determine the kinetics of PTEN in a straightforward manner. This also explains the relatively slow kinetics the sensor shows in Fig. 1e-f, as well as in Fig. 3d-f. We are not aware of any previous measurements that allowed to monitor PTEN kinetics directly with existing biochemical or imaging-based tools.

In addition, TBB was not validated for in vivo studies extensively, so it remains unclear if it can be used and penetrate the BBB, to determine in vivo kinetics. On a more general note, we believe kinetic sensor measurements should be conducted in vitro, which allows more careful experimental control. Chronic application of TBB is predicated to induce neuronal damage and dysfunction and thus should be avoided for in vivo application.

7. Is the PTEN sensor signal reversible within the same cells or animals?

To address this point, we demonstrate the reversibility of the PTEN sensor by first increasing PTEN activity by application of TBB, which was followed by a reversible decrease in activity following TBB washout and EGF application (**Fig. 1j-l**). We also show that EGF induced inhibition is readily reversible and the sensor shows increase

activity once TBB is applied, as demonstrated by longitudinal imaging of the same cells overt time (**Fig. 3d-f**).

8. With the modifications (FP tagging, R14G, overexpression), does the sensor activity still correlate with other assays of PTEN function, especially at an intermediate activation level?

We address this point by preforming pAKT measurements using immunofluorescence, as shown in **Fig. 3g-h**. Indeed, the WT PTEN sensor is still functional and can lead to significant decrease in pAkt levels. This contrasts with overexpression of R14G, which does not alter basal pAkt levels. TBB induced activation of PTEN significantly reduced pAkt levels in cells overexpressing R14G PTEN sensor, and this effect is augmented with overexpression of the WT PTEN sensor.

In addition, the experiments in **Extended Fig. 5** demonstrate that despite all modification, the PTEN sensor can still reverse the morphological deficits following endogenous PTEN KO and restore neuronal morphology to normal levels.

9. Figure 5h. PTEN activities were different between spines and the adjacent dendritic shafts under a steady state condition when IGF1R or Tsc2 were knocked out. How might this happen? Particularly, the sensor appears to be a cytosolic protein, which would go in and out of a spine within less than a second. At the same time, the sensor kinetics seems to be slow (Fig. 1f). How would an activity gradient be possible (versus equilibration between the two compartments)? A discussion is needed. Measurement of the sensor mobility in and out of spines may help.

Indeed, the distinct compartmentalization detected following genetic perturbations to IGF1R and Tsc2 is surprising. We agree with the reviewer that the steady state concentration of the sensor should be equally distributed since it should freely diffuse in and out of dendritic spines. However, one previous study found evidence for preferential interaction of PTEN with PSD95 [34] which could suggest preferential synaptic PTEN localization. To directly test this, we performed measurements of sensor mobility as suggested by the reviewer. We performed in vivo Fluorescence Recovery After Photobleacing (FRAP) of the G-PTEN sensor (**Extended fig. 8g**). We found that PTEN fluorescence recovery occurs with the same kinetics in dendritic spines and shafts.

We consider other potential mechanisms which might induce PTEN compartmentalized signaling. Numerus previous studies have shown that synaptic plasticity can induce local protein signaling in the postsynaptic compartment, with extensive evidence for loclaized synaptic signaling activity for some proteins (For example CaMKIIa[30], BDNF[35]). However, other signaling protein show spillover of signaling activity to nearby spines and dendritic branch (RhoA[36], Erk[26], PKA[26] for example). Accordingly, we hypothesize that upstream local signaling mechanisms which modulate PTEN rather, then its expression levels, might account for the aberrant PTEN activity following genetic perturbations we performed. We discuss this as follows:

"To explore the potential mechanisms of PTEN compartmentalized activity, we examined if the mobility of PTEN is differentially regulated in dendritic spines and shaft. We performed Fluorescence Recovery after Photobleaching (FRAP) of the G-PTEN sensor during in vivo imaging of dendrites. We found that dendritic shaft and spine displayed similar fluorescence kinetics, which implies equal distribution of PTEN in these compartments **(Extended fig. 8g)**. We hypothesize that upstream local signaling mechanisms[37], which could be restricted in dendritic spines but also spread to the dendritic shaft, might differentially affect PTEN signaling following genetic perturbations".

10. Fig. 7. Can the experiment be repeated by switching G- and R-PTEN in the two neuronal types? This controls for whether the effect is due to differential performance between the two sensors.

We thank the reviewer for this comment. We tested the performance of the two sensor variants **in a new series of experiments** where we expressed both G and R-PTEN in the same cells (**Extended Fig. 11**). We found that the G and R-PTEN sensors perform irrespective of the expression of the other variant, with similar sensitivity and dynamic range. In addition, the changes we detect in **Fig. 7** are in opposite directions, where R-PTEN in excitatory neurons shows decreased activity and lifetime and G-PTEN in inhibitory neurons showed increased activity. Accordingly, these changes cannot be due to differential sensitivity of the sensor variants.

11. Fig. 5i. The drastic difference in spine density is not well represented in the example images (Fig. 5g).

Thank you for this comment, we have replaced the example images with ones that better represent the difference in spine density.

Other.

1. Lines 341-342: "… we constructed an AAV with a double floxed Cre recombinase dependent expression of G-PTEN". Please consider rewriting to make it clearer.

Thank you for this correction, we corrected the sentence to:

"To express G-PTEN in inhibitory cells, we first inserted the G-PTEN sensor into an AAV vector which allow restricted expression of the sensor to Cre expressing cells (FLEX G-PTEN, Extended fig. 12)".

2. Line 356: "…a distinct reduction of E/I levels of PTEN signaling". Probably will be more precise and easier to understand if change to "… differential regulation of PTEN signaling in excitatory versus inhibitory neurons".

We thank the reviewer for pointing this out, we corrected this sentence according to the suggestion.

Reviewer #3:

Remarks to the Author:

This study describes the development of a new fluorescence biosensor to monitor PTEN activity in living systems. In addition to its characterizations, the authors show several applications, from culture cells to in vivo imaging of mouse brain and to complete organisms (C. elegans). As indicated by the authors, PTEN is a central regulator of multiple cellular processes with important physiological implications, from cell growth and differentiation to cellular metabolism, intracellular trafficking, etc. There have been attempts to develop sensors for phosphatydilinositol-3,4,5-trisphosphate (the substrate for PTEN activity and the key effector in the PI3K/PTEN pathway), but these have had limited success, probably because of the low abundance of this phosphoinositide and the limited sensitivity of the sensor.

Therefore, having a reliable sensor that directly reports PTEN activity would be extremely useful for the cell biology research community across multiple fields (neuroscience, cancer, physiology, development, etc).

We thank the reviewer for their positive evaluation of the PTEN sensor.

This sensor is based on FRET changes (monitored by fluorescence lifetime imaging – FLIM) that correspond to conformational differences between inactive and active PTEN. They also develop a red fluorescence variant of this sensor, to use it in combination with other GFP-tagged proteins or even to simultaneously image two PTEN sensors (green and red) that could be expressed in different cell-types. The rationale for these sensors is that, under basal conditions, constitutive phosphorylation of several residues at the C-terminus of PTEN keeps it in a closed, inactive conformation, which is mostly cytosolic. Dephosphorylation opens up this conformation, leading to PTEN association with plasma membrane and/or with PDZ proteins and enhancing its catalytic activity.

The experiments described in this manuscript are sound and the results are well presented.

We thank the reviewer for the positive evaluation of the work.

Nevertheless, I do have a couple of technical questions that I think the authors should adderss.

1. The authors invest a significant effort in developing a PTEN sensor with reduced (or abolished) catalytic activity, to avoid undesired signaling effects of PTEN overexpression in the cell. This led them to use the R14G mutant as their best candidate for detecting PTEN activity changes without altering PTEN signaling. However, I am surprised that the authors do not seem consider that this inactive mutant might have a dominant negative effect, by interfering with the activity of the endogenous PTEN. In fact, this is what has been reported by several groups using the catalytically dead mutant C124S. In this sense, when the authors claim that the R14G sensor “has a minimal impact on endogenous PTEN signaling” (Fig. 3h-l), they are actually showing that this mutant is essentially inactive. But they do not show whether

R14G would inhibit (dominant negative activity) the endogenous PTEN activity. To test this point, it would be necessary to express the R14G PTEN mutant in the presence of endogenous PTEN activity.

To the authors' credit, this control appears to be equivalent to the experiment shown in Fig. 5. That is, if we look at soma size and spine density from control (CyRFP-labeled) neurons in Fig. 3j, l (scrambled gRNA, no recombinant PTEN), these values are quite similar to those reported for R14G PTEN-expressing cells in Fig. 5f, i (with scrambled gRNA). Therefore, at least with respect to soma size and dendritic spine density in pyramidal neurons from somatosensory cortex, the R14G PTEN sensor does not appear to have a dominant negative effect on endogenous PTEN activity. I am surprised that this point is not particularly emphasized by the authors, since I believe it is an important one. In fact, the authors could speculate why this inactive R14G mutant does not appear to have a dominant negative effect, while the catalytically dead mutant C124S does.

In the revies MS we conduct experiments in which we test the physiological impact of the PTEN sensor on neuronal structure and function, as well as on PTEN signaling:

- We performed in vitro experiments of PIP3 phosphatase activity (**Extended Fig. 3b)**, as well as in vivo imaging in combination with endogenous PTEN KO (**Extended Fig. 5**), and demonstrated lack of signaling, and therefore lack of gain of function for the R14G sensor.

To test for the dominant negative effect, we examined this with:

- Measurements of pAkt levels, the downstream target of PTEN signaling, using Immunofluorescence. We found that WT PTEN greatly diminished pAkt signaling, while overexpression of R14G PTEN showed no DN effect, as compared to control cells (**Fig. 3g-h**).
- We performed immunostaining of pS6, downstream to PTEN and pAkt signaling, following overexpression of the PTEN sensor with R14G. We found no difference in terms of pS6 levels between control cells and cells expressing the sensor in the same field of view (**Extended Fig. 9)**.
- We performed in vivo imaging and compared the density of dendritic spines in apical dendrites of L2/3 neurons in the somatosensory cortex. We found that overexpression of WT PTEN greatly reduced spine density in vivo, but

overexpression of the R14G mutant sensor did not change spine density (**Fig. 3i-j**).

- We performed simultaneous imaging of spontaneous neuronal function with a red calcium indicator and the G-PTEN variant (**Fig. 3k-o**) or a green calcium indicator with the R-PTEN variant (**Fig. 6f-h**). In both cases, we find no changes in neuronal activity across animals (**Fig. 3**) and within individual cells within the same animal (**Fig. 6**). Notably, WT-PTEN expression led to a dramatic reduction in neuronal activity, which can be explained by the reduction in synaptic density.
- We examined a possible mechanism to explain the lack of dominant negative effect of PTEN R14G mutation. As previously suggested, we tested for the dimerization of PTEN mutants with WT PTEN [18]. We tested for dimerization induced FRET between tagged WT, R14G and C124S PTEN variants. We found that C124, which was previously shown to function as a DN mutation, induced significant dimerization with WT PTEN, while R14G did not show aberrant dimerization over WT PTEN (**Extended fig. 6e**).

Altogether, we show that PTEN activity can be reliably monitored by the PTEN R14G sensor. We show that physiological regulation of PTEN signaling, and neuronal structure and function are not affected by overexpression of the PTEN sensor.

2. Another important methodological advance of this work is the development of a red-fluorescence variant of the PTEN activity sensor (R-PTEN versus G-PTEN). This allows for the co-expression with a green fluorescence calcium indicator (such as GCaMP8, Fig. 6) and potentially for the expression of G-PTEN and R-PTEN in different cell types to monitor their PTEN activity simultaneously. The authors describe this application for the expression of the PTEN sensors in excitatory versus PV inhibitory neurons in the mouse somatosensory cortex in vivo (Fig. 7). In this case, the strategy for cell-type specific expression is based on a PV-Cre mouse line co-injected with AAVs with R-PTEN under the synapsin promoter and G-PTEN as a floxed construct with a constitutive promoter. Under this configuration, the authors argue that R-PTEN will be expressed in excitatory cells (because of the Syn promoter) and G-PTEN in PV interneurons (because of Cre expression). However, the synapsin promoter is also active in GABA interneurons. Therefore, shouldn't these neurons have co-expression of G- and R-PTEN? That is, excitatory neurons would have exclusive expression R-

PTEN, but PV inhibitory neurons would have both green and red PTEN sensors. In principle, the fluorescence lifetime of G-PTEN and R-PTEN could be distinguished within the same cells from their different emission wavelengths. But this does not appear to be what the authors are reporting. Please clarify.

We thank you for this comment. We further tested our experimental approach and validate the expression of the AAV expressing R-PTEN under the Synapsin promoter. We find that in our experimental setting, this AAV is predominantly expressed in excitatory cortical cells, which we detect by lack of VGAT expression (**Extended Fig. 9a**, 91% of cells). This is likely due to the relatively young age of mice injection (p4). Indeed, we realize that in this experiment we might account for some level of "contamination" from inhibitory expressing cells, which we further discuss in the next point the reviewer raises.

On the other hand, if R-PTEN is expressed in both excitatory and inhibitory neurons, one could compare the extent of PTEN activity under basal conditions in these two cell-types (which would be distinguished from the additional expression of G-PTEN). This would also be an interesting comparison (in addition to the effect of sensory deprivation shown in Fig. 7c-f). Again, the authors should clarify how they are carrying out these measurements.

We thank the reviewer for this critical point. This is indeed a fascinating point to consider and would allow further exploration of cell-type specific PTEN signaling.

We performed additional characterization of the ability to conduct dual G and R-PTEN measurements in the same cells in vitro (**Extended Fig. 11**). We find that under these experimental conditions, it is possible to simultaneously monitor both variants in an independent manner.

As for the direct comparison of the two sensor variants in the same cells suggested by the reviewer, unfortunately it is not possible to analyze inhibitory cells expressing both G and R-PTEN. In our analysis, we detected a significant decrease in R-PTEN lifetime in the small population of PV cells co-expressing the G-PTEN sensor. This is likely the result of blead-thorough of GFP fluorescence into the red channel due to the inherent differences in expression of the sensors with different promoters (CAG Vs Synapsin). We therefore excluded from our analysis a minor population of inhibitory PV cells co-expressing both G and R-PTEN, which we have specifically now pointed

out in the MS. We are currently further optimizing plasmids and AAVs, to allow to conduct such measurements in the future, which would allow to further elucidate cell-type specific PTEN activity in the brain.

Figure 3

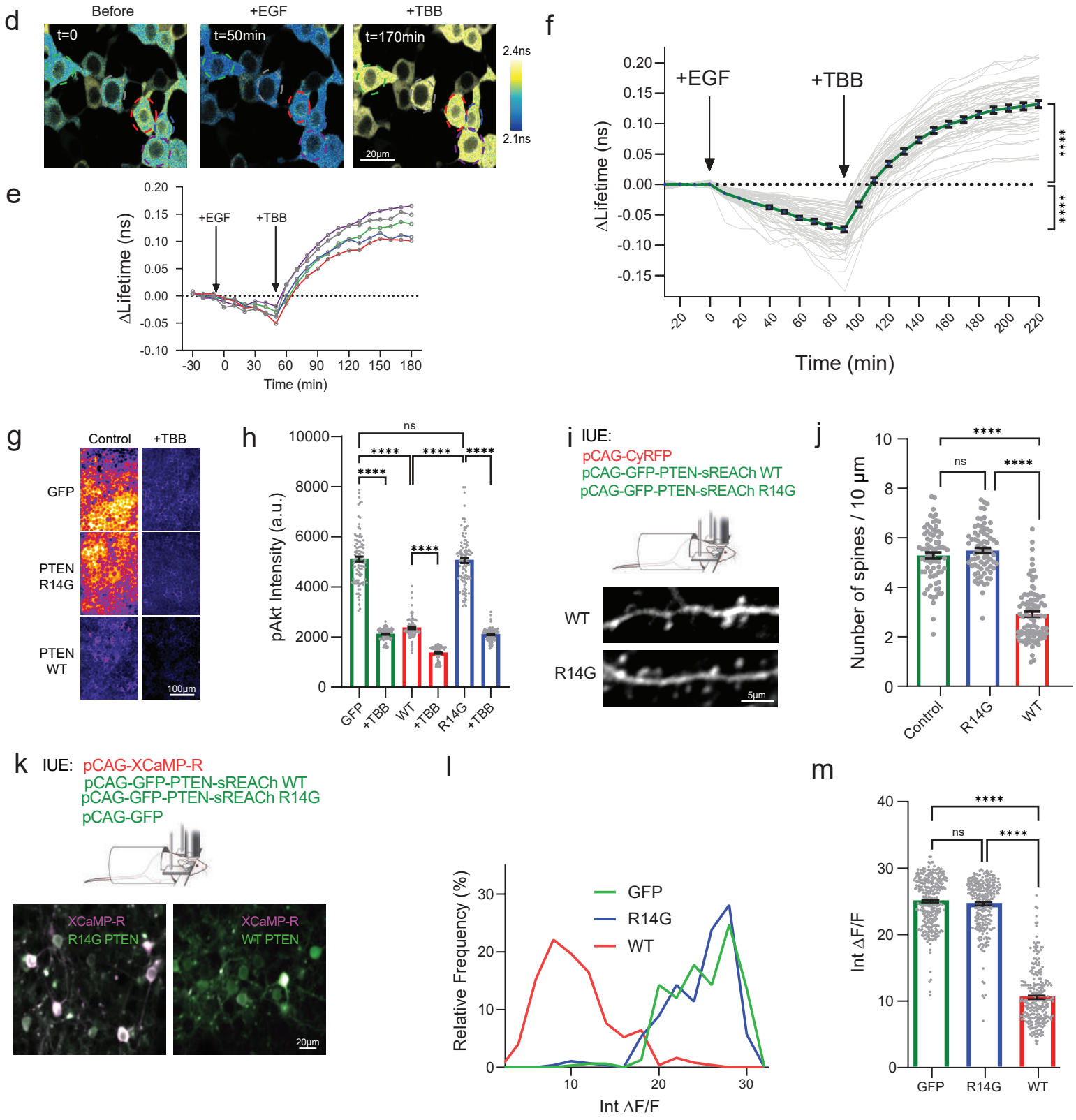

d
Before
+EGF
+TBB
t=0
t=50min
t=170min
2.4ns
2.1ns
20μm
e
ΔLifetime (ns)
+EGF
+TBB
Time (min)
f
ΔLifetime (ns)
+EGF
+TBB
****
****
Time (min)
g
Control
+TBB
GFP
PTEN R14G
PTEN WT
100μm
h
pAkt Intensity (a.u.)
ns
****
GFP
+TBB
WT
+TBB
R14G
+TBB
i
IUE:
pCAG-CyRFP
pCAG-GFP-PTEN-sREACh WT
pCAG-GFP-PTEN-sREACh R14G
WT
R14G
5μm
j
Number of spines / 10 μm
****
ns
****
Control
R14G
WT
k
IUE:
pCAG-XCaMP-R
pCAG-GFP-PTEN-sREACh WT
pCAG-GFP-PTEN-sREACh R14G
pCAG-GFP
XCaMP-R
R14G PTEN
XCaMP-R
WT PTEN
20μm
l
Relative Frequency (%)
GFP
R14G
WT
Int ΔF/F
m
Int ΔF/F
****
ns
****
GFP
R14G
WT

Figure 3

(d) - (e) Pseudo-colored FLIM images and continuous time-course of single cells expressing mEGFP-PTEN-sREACh containing the R14G mutation before, 50 minutes after 100ng/ml EGF application, and following 2 hours from 50 µM TBB application. Scale bar; 20 µm.

(f) Time course for changes in fluorescence lifetime of the HEK cells expressing the mEGFP-PTEN-sREACh containing the R14G mutation, following 90 minutes of 100ng/ml EGF application (****, -0.08±0.004 ns) and after adding 50 µM TBB for 130 minutes (****, 0.13±0.005 ns). Single cells are shown in grey lines, average changes in green line, n=55 cells.

(g) Representative images of pAkt staining before and after application of TBB, in cells expressing GFP, mEGFP-PTEN-sREACh WT, and mEGFP-PTEN-sREACh R14G. Scale bar; 100 µm

(h) Quantification of phospho-Akt (pAkt) staining in cells expressing GFP (5111±100.5 units, n = 100, and 2116±22.92 units, n = 100), mEGFP-PTEN-sREACh WT (2366±41.52 units, n = 100, and 1366± 27.97 units, n = 100) and mEGFP-PTEN-sREACh R14G (5059±101.7 units, n = 100, and 2103±25.47 units, n = 100), with and without 50 µM TBB application, respectively.

(i) Schematics of experimental design, and representative in vivo images of fluorescently labeled apical dendrites expressing CyRFP, co-expressing either mEGFP-PTEN-sREACh WT or mEGFP-PTEN-sREACh R14G. Scale bar; 5 µm.

(j) Quantification of spine density per 10 µm for dendrites expressing CyRFP alone (5.29±0.13, n = 76), CyRFP + mEGFP-PTEN-sREACh R14G (5.49±0.11, n = 75), or CyRFP + mEGFP-PTEN-sREACh WT (2.91±0.11, n = 91). p = 0.475 for CyRFP and R14G comparison.

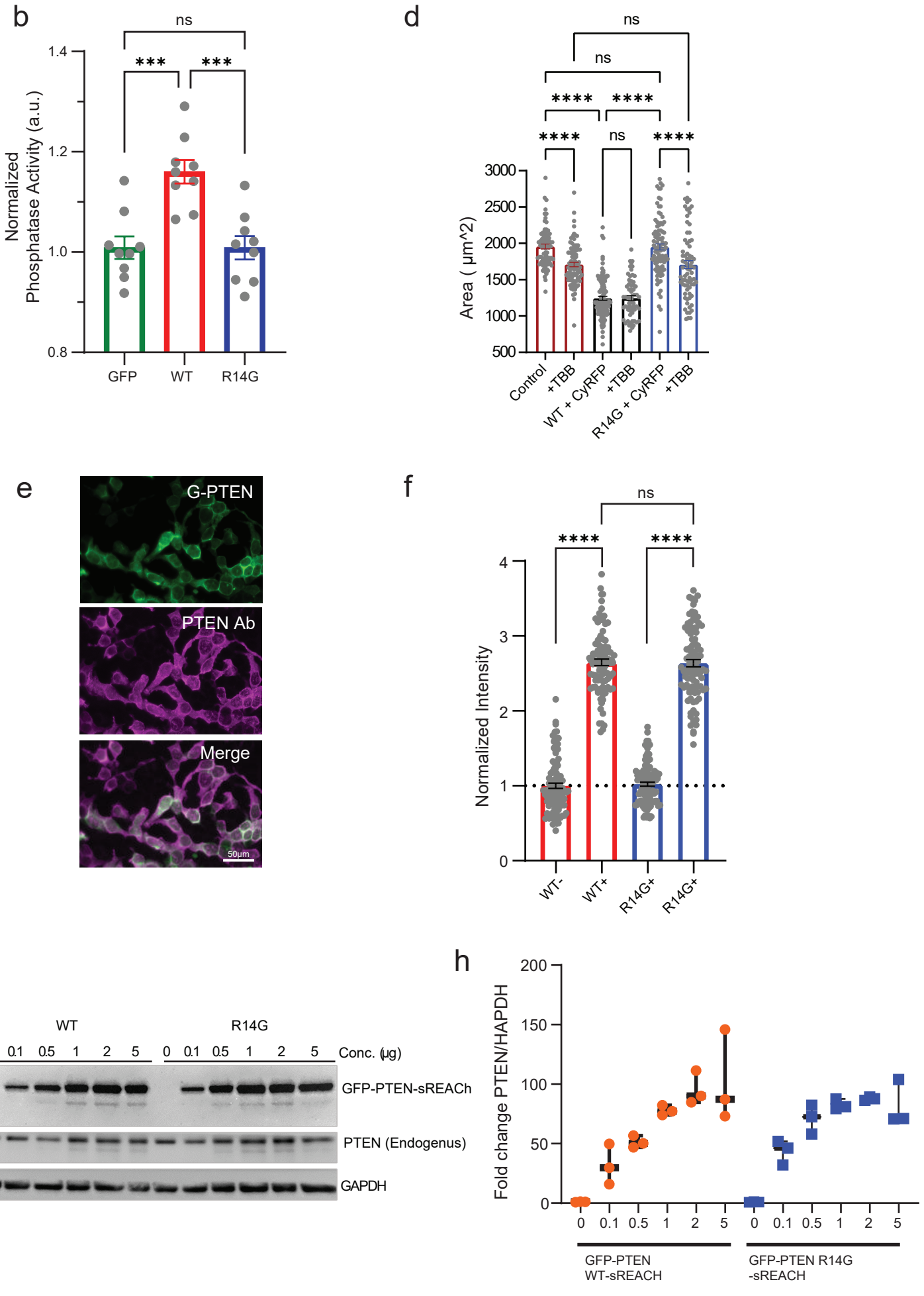
(k) Schematics of experimental design, and representative in vivo images of fluorescently labeled L2/3 neurons expressing XCaMP-R (magenta) and mEGFP-PTEN-sREACh WT or R14G sensors (green). Image intensity is summed for 400 frames at a frame rate of 4Hz. Scale bar; 20 µm.

(l) Relative frequency histogram of Integrated ΔF/F distribution for L2/3 neurons expressing XCaMP-R and co-expressing either GFP (n=4 mice, 316 cells), mEGFP-PTEN-sREACh R14G (ns p = 0.94, n=3 mice, 281 cells) or mEGFP-PTEN-sREACh WT (****, n=3 mice, 249 cells).

(m) Quantification of Integrated ΔF/F of L2/3 neurons expressing XCaMP-R and co-expressing either GFP (25.04±0.21, n=4 mice, 316 cells), mEGFP-PTEN-sREACh R14G (ns, 24.63±0.23, n=3 mice, 281 cells) or mEGFP-PTEN-sREACh WT (****,10.58±0.27, n=3 mice, 249). ns p = 0.403.

Error bars represent s.e.m; statistical differences for (c, f, h, j, m) were measured using one-way ANOVA followed by post-hoc Tukey's multiple comparison test. Statistical differences for (l) was measured using Kolmogorov-Smirnov. Statistical difference for (e) was measured using unpaired two-tailed student t-test. **** $p < 0.0001$.

Extended Figure 3



Extended Figure 3

(b) Quantification of normalized phosphatase activity of GFP (1±0.02, n = 9), mEGFP-PTEN-sREACh WT (1.16±0.02, n = 9) and mEGFP-PTEN-sREACh R14G (1±0.02, n = 9) on the substrate PI(3,4,5)P3 diC8. *** p = 0.0003, ns p >0.9999.

(d) Quantification of cell area of CyRFP alone, before (1958±33.40 μm^2, n = 82, 1709±30.15 μm^2, n = 88), + mEGFP-PTEN-sREACh WT (1245±26.78 μm^2, n = 109, 1247±34.35 μm^2, n = 62), or with mEGFP-PTEN-sREACh R14G (1951±42.25 μm^2, n = 92, 1707±53.80 μm^2, n = 75) before and after TBB application, respectively. ns p > 0.9999 for CyRFP Control compared to R14G + CyRFP Control, for CyRFP TBB compared to R14G + CyRFP TBB, and for WT + CyRFP Control compared to WT + CyRFP TBB.

(e) Representative images for staining with a PTEN Ab (magenta) in cells expressing the mEGFP-PTEN-sREACh R14G (green). Scale bar; 50 µm.

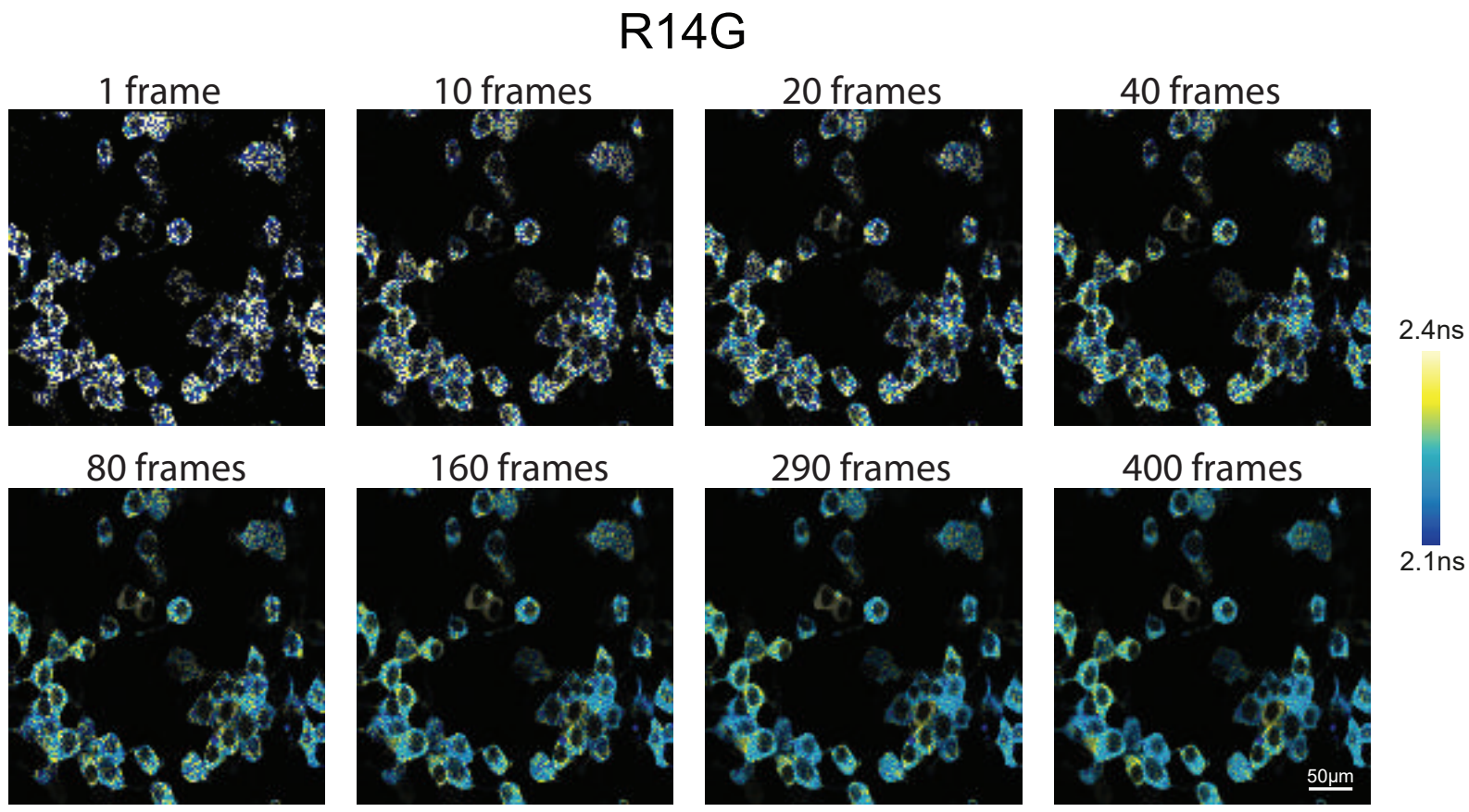
(f) Quantification of normalized intensity for PTEN staining in HEK cells transfected with either mEGFP-PTEN-sREACh WT or mEGFP-PTEN-sREACh R14G. Comparison of cells without (1±0.04, n = 122), and with WT sensor (2.24±0.07, n = 122), without (1±0.05, n = 99) and with R14G sensor (2.03±0.07, n = 99). ns p > 0.9999 and p = 0.0899 for WT- vs. R14G- and WT+ vs. R14G+, respectively.

(g) - (h) Representative western blot image and quantification of PTEN expression in HEK293 cells transfected with mEGFP-PTEN-sREACh WT or mEGFP-PTEN-sREACh R14G at indicated concentrations. Cells were lysed and analyzed by SDS-PAGE/western blotting using anti-PTEN and anti-GAPDH antibodies. GFP-PTEN (overexpressed) and PTEN (endogenous) bands were detected on the same blot but with different exposures. n = 3.

Error bars represent s.e.m; statistical differences for (a, c) were measured using one-way ANOVA followed by post-hoc Tukey's multiple comparison test. For (h) the one-way ANOVA was followed by Kruskal Wallis post-hoc test. Statistical difference for (e) was measured using unpaired two-tailed student t-test. **** p < 0.0001.

Extended Figure 4

a



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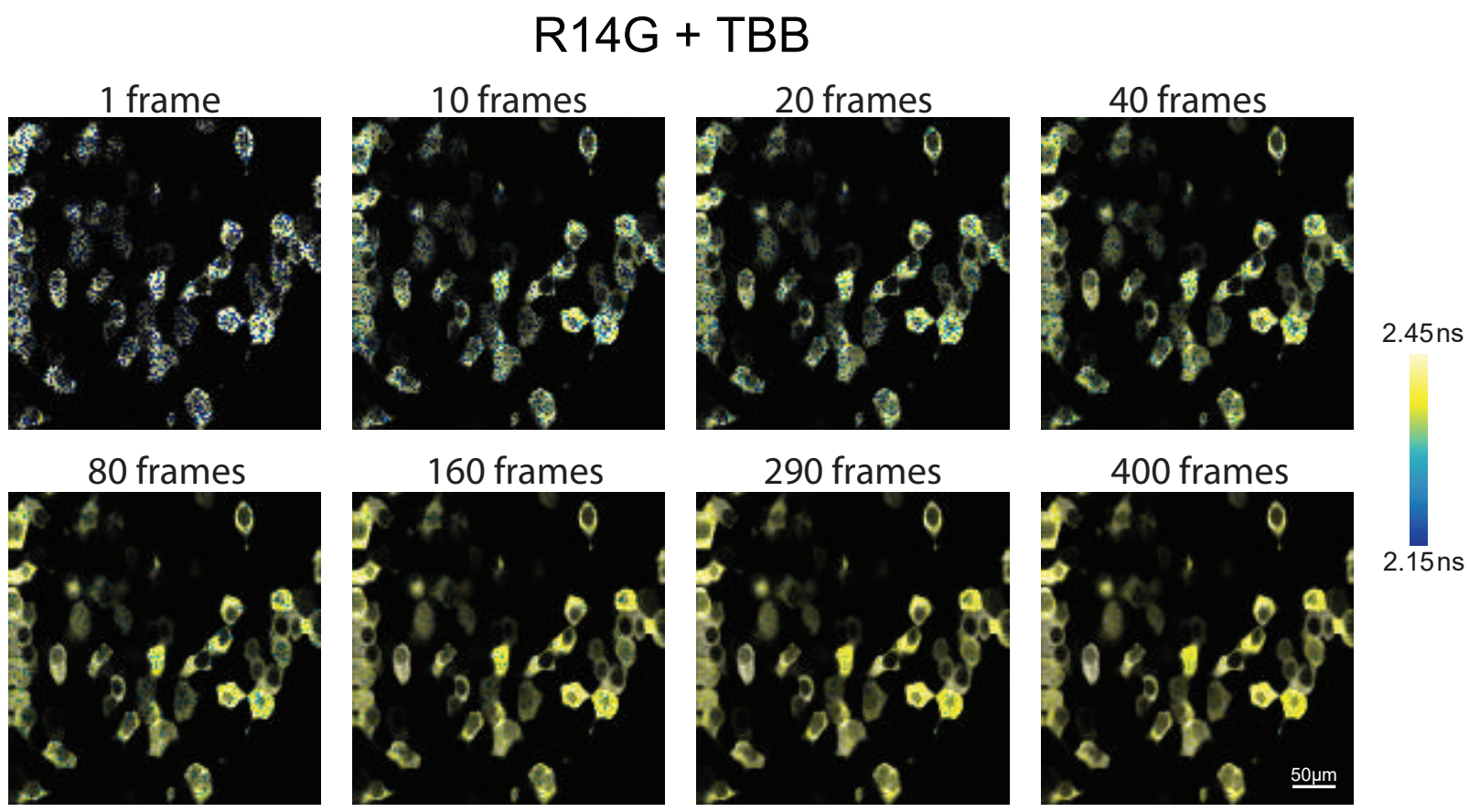

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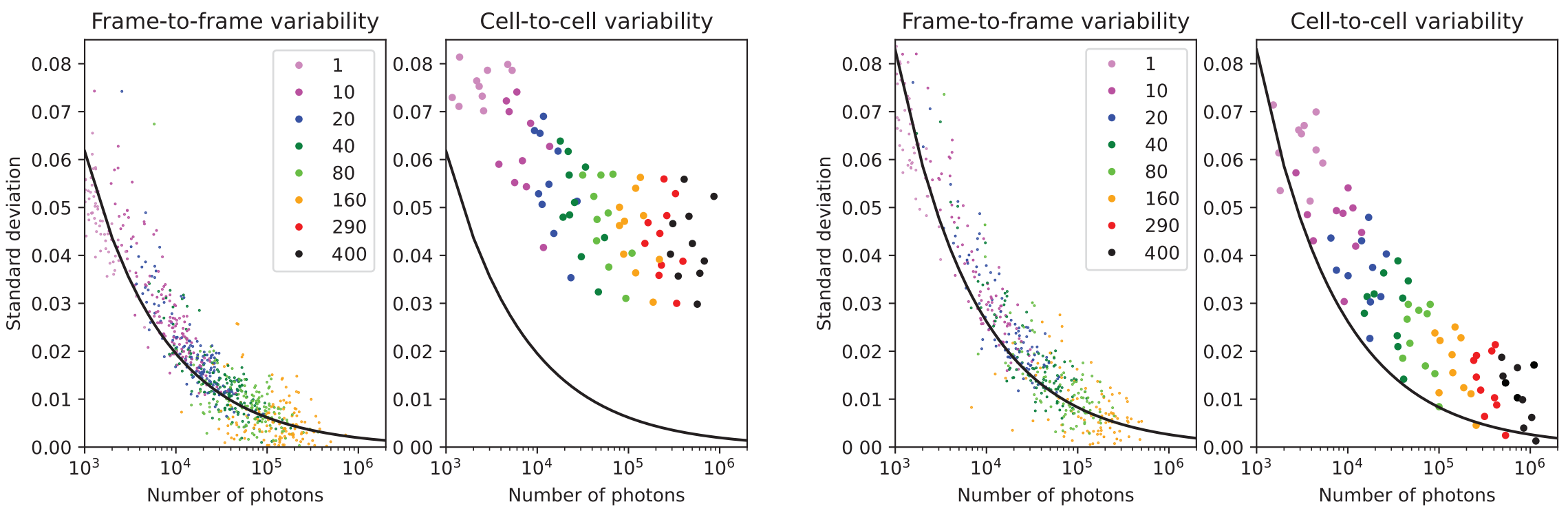

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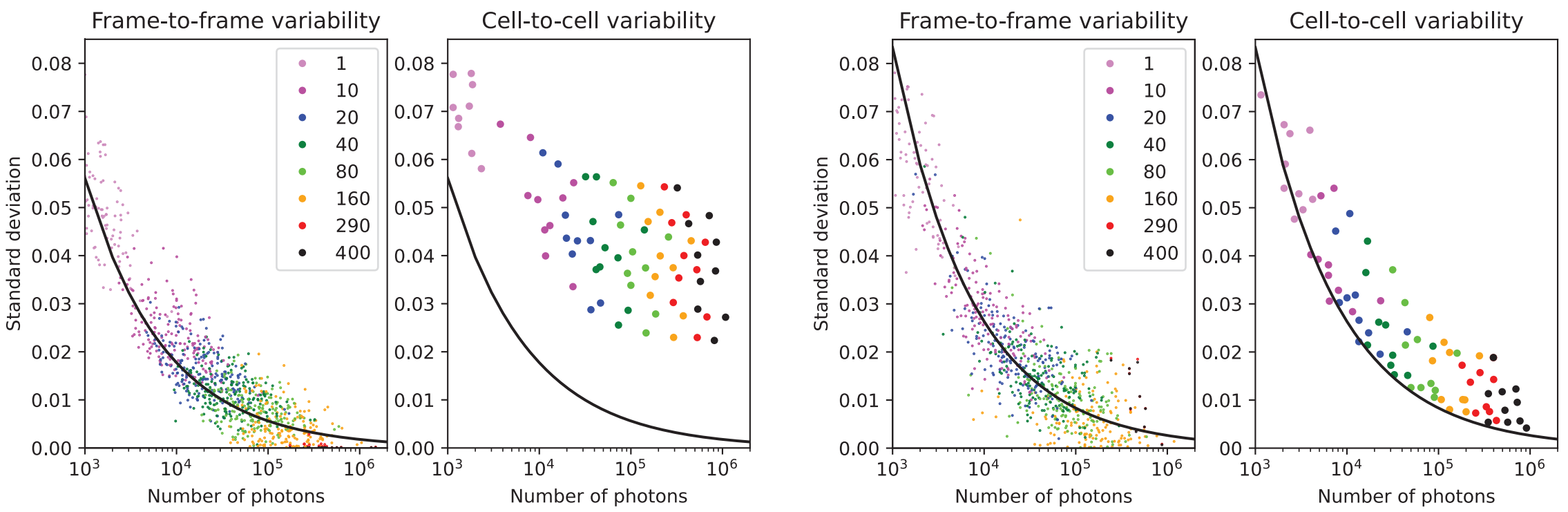


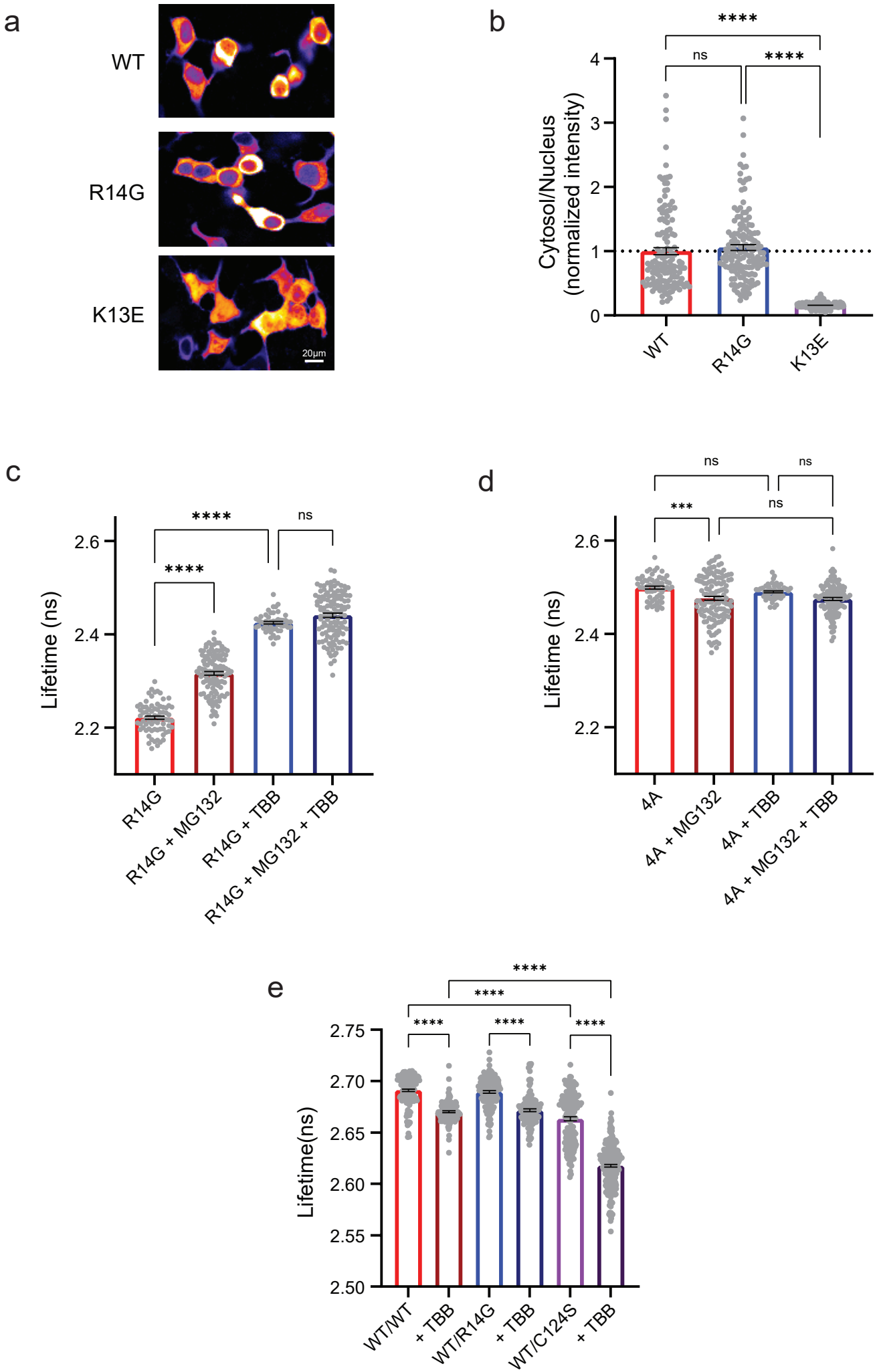
Extended Figure 4

(a) - (b) Representative pseudo-colored images of fluorescent lifetime summed for different frame duration, for G-PTEN before and after 3hr of 50 μM TBB application. Scale bar; 50 μm.

(c) Standard deviation of signal-to-noise of mEGFP-PTEN-sREACh before and after TBB, as a function of number of photons from each cell. Each point represents individual cells for frame-to-frame (left) or individual experiments over one field of view for cell-to-cell (right) variation. The binding fraction's theoretical change caused by shot-noise is represented by the black curve (see methods for equation).

(d) Standard deviation of signal-to-noise of G-PTEN before and after TBB, as a function of the amount of photons from each cell. Each point represents individual cells for frame-to-frame (left) or individual experiments over one field of view for cell-to-cell (right). The binding fraction's theoretical change caused by shot-noise is represented by the black curve.

Extended Figure 6



Extended Figure 6

(a) Representative images of fluorescent intensity in HEK293 cells expressing either mEGFP-PTEN-sREACh WT, and with the R14G, or the K13E mutation. Scale bar; 20 µm.

(b) Quantification of normalized intensity of Cytosol / Nucleus in HEK293 cells expressing either mEGFP-PTEN-sREACh WT (1±0.05, n = 137), with the R14G (ns, 1.05±0.04, n = 143) or K13E mutation (****,0.16±0.003, n = 209). ns p = 0.5206.

(c) Quantification of fluorescent lifetime in HEK293 expressing G-PTEN before pharmacology application (2.22±0.004 ns, n = 75), after overnight application of 10 µM MG132 (****, 2.32±0.004 ns, n = 117), after 3hr of 50 µM TBB application (****, 2.38±0.003 ns, n = 50), and after 3hr of 50 µM TBB application following overnight application of 10 µM MG132 (****, 2.44±0.004 ns, n = 120). ns p = 0.088 for R14G + TBB vs. R14G + MG132 + TBB.

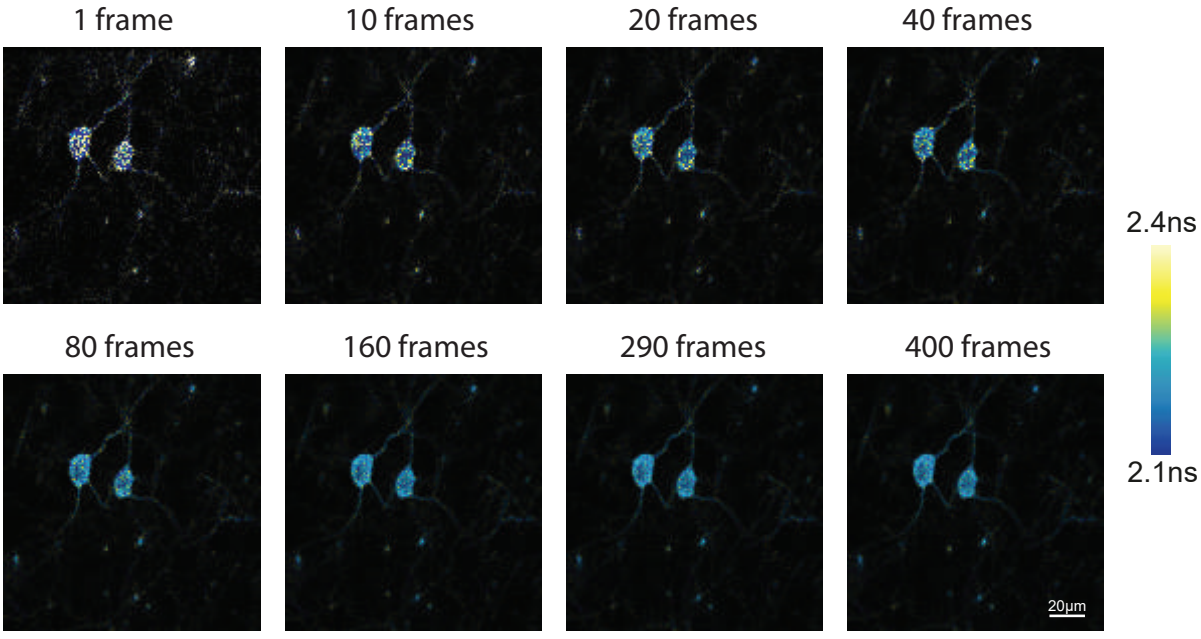
(d) Quantification of fluorescent lifetime in HEK293 expressing mEGFP-PTEN-sREACh 4A before pharmacology application (2.50±0.003 ns, n = 60), after overnight application of 10 µM MG132 (***, 2.48±0.004 ns, n = 126), after 3hr of 50 µM TBB application (ns, 2.49±0.002 ns, n = 50), and after 3hr of 50 µM TBB application following overnight application of 10 µM MG132 (ns, 2.47±0.003 ns, n = 108). *** p = 0.0004 for 4A vs. 4A + MG132. ns p = 0.5678, p = 0.0654 and p = 0.9890 for 4A vs. 4A + TBB, 4A + TBB vs. 4A + MG132 + TBB, and 4A + MG132 vs. 4A + MG132 + TBB respectively.

(e) Quantification of dimerization, measured by fluorescent lifetime in HEK293 expressing PTEN-sREACh WT and co-expressing either mEGFP-PTEN WT (****, 2.69±0.001 ns, n = 144 and 2.67±0.001 ns, n = 140), mEGFP-PTEN R14G (****, 2.69±0.001 ns, n = 140 and 2.67±0.001 ns, n = 130) or mEGFP-PTEN C124S (****, 2.66±0.001 ns, n = 161 and 2.62±0.001 ns, n = 244), before and after 3hr of 50 µM TBB application respectively.

Error bars represent s.e.m; statistical differences for (b, c, d, e) were measured using one-way ANOVA followed by post-hoc Tukey's multiple comparison. **** $p < 0.0001$.

Extended Figure 7

a



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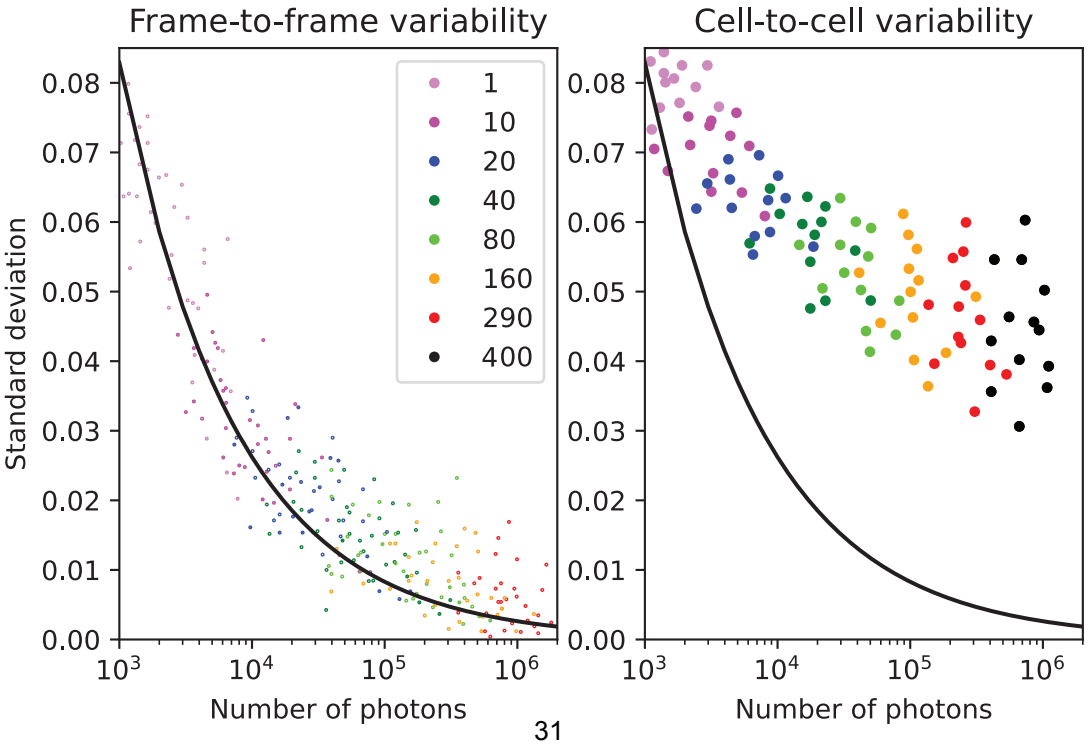
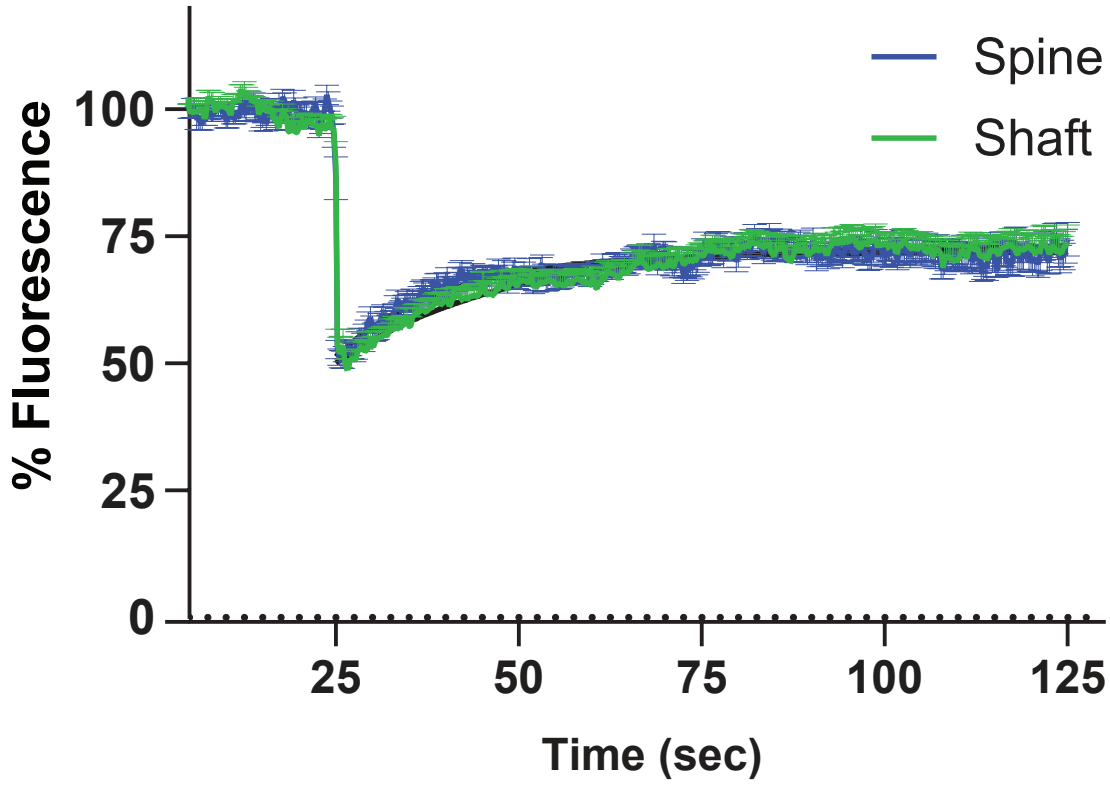


Extended Figure 7

(a) Representative images of fluorescent lifetime summed over different frames numbers, for G-PTEN in single L2/3 cells in vivo. Scale bar; 20 μm.

(b) Standard deviation of signal-to-noise of G-PTEN in vivo, as a function of the amount of photons from each cell. Each point represents individual cells for frame-to-frame (left) or individual experiments over one field of view for cell-to-cell (right). The binding fraction's theoretical change caused by shot-noise is represented by the black curve (see methods for equation).

g


Spine
Shaft
% Fluorescence
100
75
50
25
0
25
50
75
100
125
Time (sec)

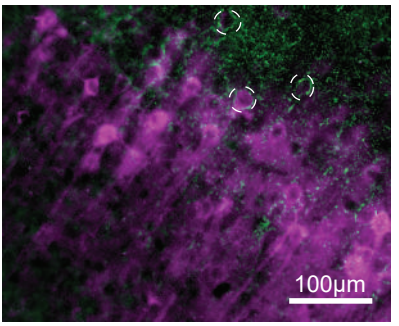
Extended Figure 8

(g) Analysis of in vivo FRAP time-course for dendritic shaft (green) and spines (blue), showing similar fluorescence recovery kinetics. n=36 spines and 20 dendrites, in 3 mice.

Extended Figure 9

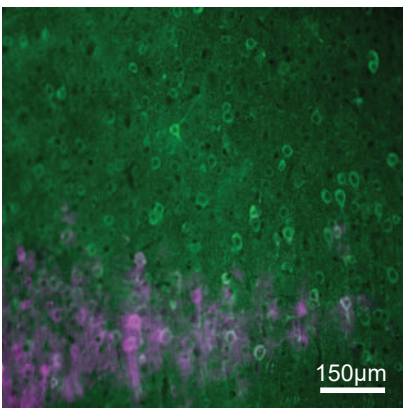
a

AAV-pSyn R-PTEN
IHC-PTEN Ab



b

AAV-pSyn R-PTEN
IHC-PTEN Ab



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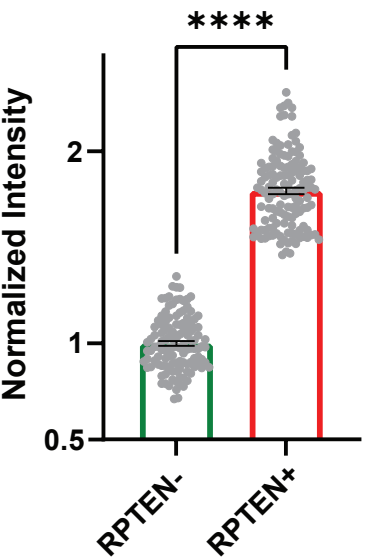
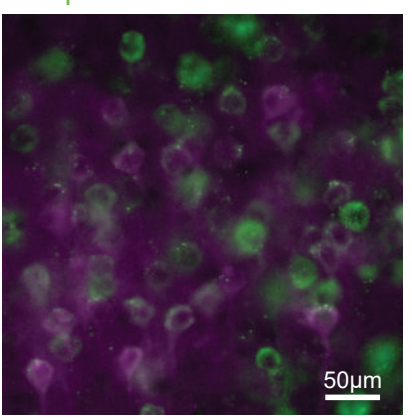


d

AAV-pSyn R-PTEN
IHC-pS6 Ab



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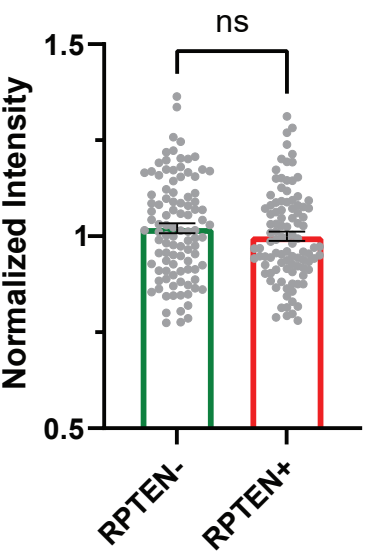


Extended Figure 9

(a) Representative image of VGAT staining in cortical brain slices, for cells co-expressing AAV pSyn-R-PTEN. Scale bar; 100 µm, VGAT positive and R-PTEN positive cells are marked. Overall, for n=7736 cells, 90.81% were negative for VGAT.

(b) Representative image of a cortical slice, stained for PTEN, for cells expressing AAV pSyn-R-PTEN. Scale bar; 150 µm.

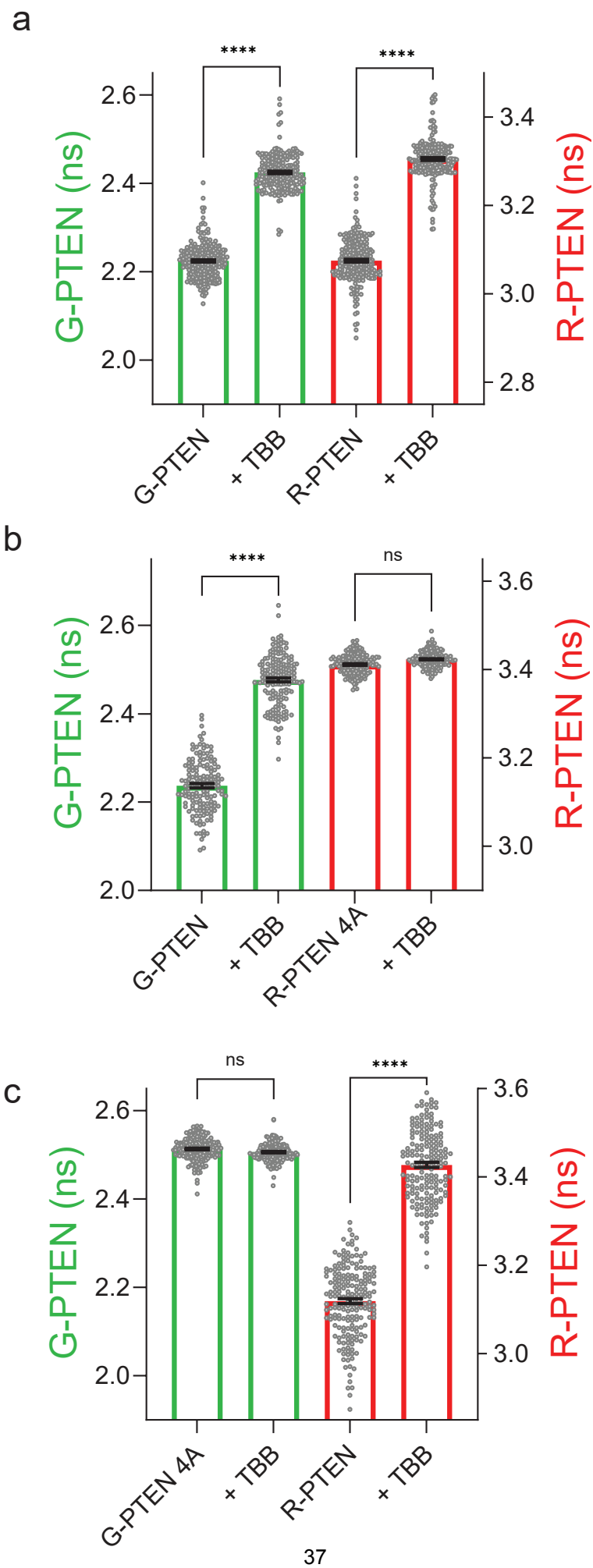
(c) Quantification of normalized intensity for total PTEN levels L2/3 cortical neurons without (1±0.01, n = 121) and with R-PTEN (****, 1.79±0.01, n = 134) in the same field of view.

(d) Representative image of a cortical slice stained for pS6, in a mouse expressing injected with AAV pSyn-R-PTEN. Scale bar; 50 µm.

(e) Quantification of normalized intensity for pS6 in cells without (1±0.01, n = 100) and with R-PTEN (1.02±0.01, n = 100) in the same field of view. ns p = 0.2358.

Error bars represent s.e.m; statistical difference for (c, e) were measured using unpaired two-tailed student t-test. **** $p < 0.0001$.

Extended Figure 11



Extended Figure 11

(a) Quantification of fluorescence lifetime in HEK293 co-expressing G-PTEN (****, 2.22±0.003 ns, n = 231 and 2.43±0.003 ns, n = 217) and R-PTEN (****, 3.08±0.003 ns, n = 234 and 3.31±0.003 ns, n = 207), before and after 3hr of 50 µM TBB application, respectively.

(b) Quantification of fluorescence lifetime in HEK293 co-expressing G-PTEN (****, 2.23±0.004 ns, n = 149 and 2.48±0.004 ns, n = 165) and mCyRFP2-PTEN-mMaroon 4A (ns, 3.41±0.002 ns, n = 169 and 3.42±0.001 ns, n = 158), both before and after 3hr of 50 µM TBB application, respectively. ns p = 0.0713.

(c) Quantification of fluorescence lifetime in HEK293 co-expressing mEGFP-PTEN-sREACh 4A (ns, 2.51±0.001 ns, n = 195 and 2.51±0.001 ns, n = 178) and R-PTEN (****, 3.12±0.005 ns, n = 195 and 3.43±0.006 ns, n = 178), both before and after 3hr of 50 µM TBB application, respectively. ns p = 0.6557.

Error bars represent s.e.m; statistical differences for (a, b, c) were measured using one-way ANOVA followed by post-hoc Tukey's multiple comparison test. **** $p < 0.0001$.

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Reviewer #1 (Remarks to the Author):

The authors have satisfactorily addressed all of my comments. I have no further comments

We thank the reviewer for their helpful comments.

Reviewer #2:
Remarks to the Author:
The authors address some of my questions, but not others. At this time, important concerns remain regarding the dynamic range, signal-to-noise ratio in vivo, and the ability to visualize single cell dynamics under physiological or behavioural manipulations. I feel that this study has not reached the bar of Nature Methods.

In our revised MS, we have addressed the reviewer's specific concerns by performing further experiments which precisely measure the signal to noise of the PTEN sensor, as well as its usability for monitoring PTEN activity in single cells during longitudinal imaging. We therefore demonstrated that the PTEN sensor is on par with other similar sensors which are used by the scientific community. In addition, we performed careful characterization to demonstrate that the PTEN sensor has no dominant negative function and does not alter neuronal physiology.

We demonstrate the utility of the PTEN sensor with 6 different biological applications:

1. The impact of PTEN pathogenic mutations on PTEN structural and conformational state (Extended Fig. 2).

2. PTEN activity during development and due to daf2 levels in C. elegans (Fig. 3).

3. In vivo imaging of PTEN activity in the mouse brain (Fig. 4).

4. Impact of genetic perturbations to in vivo PTEN compartmentalized activity in the mouse brain (Fig. 4).

5. Development of red-shifted PTEN sensor and its use for in vivo simultaneous cellular imaging of neurons alongside GFP based calcium indicator in the mouse brain (Fig. 5).

6. In vivo imaging of cell-type specific PTEN activity in excitatory and inhibitory neurons, following sensory deprivation in the mouse brain (Fig. 6).

Accordingly, we think that the most important factor to consider here is: does this biosensor allows one to make biological discoveries which were not previously possible with existing methods? The answer to this question is undoubtedly yes, since none of the 6 applications we demonstrate were known or could be directly demonstrated with pre-existing tools. Accordingly, the argument here for generating an improved version of the sensor is unsubstantiated by the data we present.

Major.
1. The sensor dynamic range is small. The authors cited several references in the rebuttal, arguing that the sensors dynamic range is on par. However, some of the papers did not achieve in vivo imaging. A paper from the same author did not report lifetime change (binding ratios were showed), and the most recent paper Massengill et al 2022 reported a lifetime dynamic range of ~0.65 ns, significantly larger than that of the PTEN sensors reported here (~0.25 ns).

In respect to this comment, the ability to use a first-generation biosensor in vivo is not trivial, and this is a point which argues in favour of the PTEN biosensor we developed. In the paper the reviewer is referring to (Laviv et al Neuron 2020), the reported binding fraction is numerically equivalent to absolute lifetime changes (binding fraction is often stated in respect to inter-molecular FRET). In this case, the sensor dynamic range is ~0.15ns, slightly lower than the dynamic range of our PTEN sensor, which allowed to perform extensive in vivo imaging.

As for the example the reviewer pointed out (Massengill et al 2022), while we did cite this reference, this work describes the fifth generation and optimization of a cAMP sensor, originally developed in 2004 (Ponsioen *et al* 2004, *Embo J*). Therefore, we do not think this is an equivalent comparison to our first-generation biosensor in terms of dynamic range.

We assembled a table summarizing several existing FLIM sensors used for in vivo imaging (Table 1). This is not a comprehensive list of all sensors, but rather providers an overview of existing sensors for in vivo FLIM and their dynamic range, as compared to the PTEN sensor.

Altogether, the signal to noise of the PTEN biosensor is on par to existing FLIM based biosensors developed so far and used for in vivo imaging.

2. Regardless of the dynamic range, for a user, the important parameter is the achievable signal-to-noise ratio. The data are noisy, and the statistics rely on averaging hundreds of cells throughout the manuscript. By eyeballing, the signal-to-noise ratio in most experiments are ~1 and sometimes even lower. This would potentially make the analysis within individual cells and to detect cell heterogeneity difficult. The authors seem to be unwilling to further optimize the sensor. However, the current sensor, as it is, is difficult to be adopted by the field.

Throughout the MS, we perform two different types of measurements:

1. Basal fluorescence lifetime of different cell populations.
2. Normalized fluorescent lifetime in the same cells upon pharmacological manipulations.

The reviewer is referring to the first type of comparison here. We disagree that the achievable signal-to-noise ratio is ~1. We do not understand what definition the reviewer is referring to in respect of noise here. In FLIM measurements, the signal-to-noise is proportionally related to the number of photons acquired. Therefore, one can optimize and adjust the size of the filed of view and acquisition time. Therefore, the achievable signal to noise greatly depends on and can be optimized by the user. For example, when imaging a single spine (~2um), it is possible to achieve sufficient signal to noise by zooming in on a small dendritic segment (For example, Chang *et al*, Neuron 2017). In the first set of experiments characterizing the sensor (Fig. 1, 2), we use a typical imaging setting in which we image a filed of view of 100x100µm, where we average 24 frames during a total acquisition time of 6 seconds. In these conditions, if we consider an empirical definition, the noise levels can be referred to as the deviation within each cell population. The noise, or variability could be a result of biological noise (different levels of PTEN activation across cells) and/or instrumental noise related to the sensor and measurement error. If we disregard the source of noise, we can compare the standard deviation of measurements within each group (SD of lifetime) with the change in lifetime within groups as the signal. This comparison yields a S/N ratio of ~6 in our measurements in Fig. 1g as an example, when comparing the SD of the sensor group to the mean change in lifetime. Importantly, it should be noted that the user can adjust the FOV and image size, as well as acquisition time, to increase

S/N if needed for a specific experiment. This is always contrasted by compromising with temporal resolution.

To substantiate this point and calculate the S/N of the sensor, we experimentally addressed this point in a straightforward manner in extended figure 4. In this experiment, we continually measured the fluorescence lifetime of the PTEN sensor in individual cells, while we increased the photon counts over long image acquisition periods. This manipulation allows us to experimentally assess how increasing the number of photons affect the standard deviation of PTEN sensor across cells. We compared the standard deviation of lifetime measurements with the estimated deviation of shot noise, which is the fluorescent lifetime system error due to noise at different photon numbers. This is the most accurate S/N measurements using FLIM.

As can be seen from our analysis, indeed frame to frame variability of lifetime is expectedly reflecting instrumental variability and is centered around the shot noise curve. However, when we analyse this relationship across single cells in the same field of view, there is a significantly larger range of lifetime variability. Based on our measurements, this variability is 8-10 fold larger than instrumental variability. We confirm the origin of this variability by repeating this analysis following application of TBB, which renders PTEN maximally active. Following TBB application, our signal to noise analysis shows greatly reduced variability of lifetime across cells, a 3-fold reduction then the variability we measured in control cells. These results confirm that variable levels of the PTEN sensor lifetime largely reflect biological variability rather than instrumental variability. We repeated this analysis for typical FOV for in vivo imaging, which is shown in extended figure 7, and show that during in vivo imaging of the PTEN sensor, we can monitor single cells with sufficient resolution, where the cell-to-cell variability is significantly larger by a factor of ~6, then the frame to frame variability representing system shot noise and error. Therefore, we show that this S/N ratio allows for high quality in vitro and in vivo imaging of FLIM based PTEN activity.
To address the general considerations and limitations for an end user in regard to S/N, we have added a supplementary note (Supplementary Note 2) which discusses this issue at length.

3. Related to above, the authors state that “As for the suggestion for Circular permutations, it is an interesting point to consider. This manipulation is often used for intensity-based sensors …” Please take a look at the classic work by Nagai et al, PNAS 2004. There were also many subsequent works that followed.

The work the reviewer is citing is for fluorescence intensity-based FRET reporters, which would benefit from increase of fluorescence intensity of the FRET acceptor upon conformational changes and FRET increase. For a FLIM based application, measurements are based on the lifetime of the donor and the changes are reflected in energy absorbance by the acceptor. In the PTEN biosensor, we use a dark version of YFP, which has much reduced emission. Empirically, it is possible that replacing the acceptor with a circular permutation version might be beneficial. Again, we understand that every biosensor can be further optimized and improved over time, using linker optimization and modification of fluorescent proteins as suggested by the reviewer. Here, we describe the first version of the PTEN sensor, and it is possible that future studies might build upon it and further improve its dynamic range. We show that the current version of the biosensor is useful to dynamically monitor PTEN activity and can be used to make new and impactful biological discoveries.

4. The authors accredit some of the variability to biological reasons. However, even after TBB, significant variability remains. The 4A mutant also exhibits significant variability. This may be due to differential sensor maturation across cells. Regardless of the mechanism, this variability argues for the need of a sufficient sensor dynamic range that goes beyond this variability.

With respect to the variability of the sensor, we address this point specifically in extended figure 4, as previously discussed in response to point number 2 which was raised by the reviewer. We show that the standard deviation of our measurements is significantly larger compared to the noise, which is monitored by frame-to-frame variability. For the WT and R14G sensors, this results in a 8-10 fold difference, when comparing cell to cell/frame to frame variability in control conditions. After TBB application, this variability is greatly diminished. This includes a ~3-fold reduction before and after TBB application, which likely reflects biological variability. Our results clearly show that the PTEN biosensor allows to monitor PTEN activity in single cells with large dynamic range (As discussed in point 1) and substantial S/N (discussed in point 2).

5. Timelapse and longitudinal imaging are powerful for understanding cell biology, such as to reduce noise, to appreciate cell heterogeneity, and to reveal signaling kinetics. In light of the variability discussed above, timelapse data normalized to each cell's respective baseline can effectively reduce noise and increase signal-to-noise ration. the manuscript also emphasizes on "dynamics" at multiple places in the manuscript. The author now adds one HEK cell experiment for time-lapse imaging. However, no in vivo demonstration is included. This is important as

It appears that the reviewer's comment wasn't finished, so it is difficult to know exactly what they are referring to. Based on what text remains, we specifically address the point the reviewer is referring to. In our revision, we performed an experiment in which we carried out longitudinal measurements of the same cells using the PTEN sensor, as presented in **Fig. 3d-f**. These experiments exemplify that the sensor can be efficiently used for single cell dynamic measurements over the time course of minutes to hours, with minimal variability. We show that application of EGF reduces PTEN activity, which can be reversed by application of TBB which blocks CK2 activity and therefore increase PTEN activity over time, in the same cells. These changes reflect "dynamic" PTEN activity which we show this sensor can accurately capture, in a controlled and careful set of experiments. We also address here the point which the reviewer previously raised regarding the reversibility of the biosensor.

As for the suggestion to demonstrate this in vivo- we performed measurements in whole organisms (*C. elegans*) (Fig. 3b), where we added TBB to the worm bacteria, and this led to an increase in PTEN lifetime. These experiments showed the PTEN sensor allows to capture changes of PTEN activity (dynamics) in a whole living organism. We also show in a different set of experiments, the ability to track PTEN activity throughout the worm developmental stage over days.

Unfortunately, we do not see any reasonable experiments using in vivo imaging in the mouse brain, which could be accomplished for purely demonstrative purposes. There is no clear evidence that we found in the literature that TBB, the main pharmacological agent we use to increase PTEN activity, can be effectively applied and through systemic administration and penetrate the BBB. On a practical and ethical note, this experiment would require gaining ethical approval since it involves animal experiments, and would be difficult to prove necessary. Moreover, based on the slow kinetics of TBB in cells and in worms, we predict that if this works,

it would take many hours to affect PTEN activity, which would be very difficult to implement for in vivo imaging of the same cells in the live brain. We are very excited to use or see our tool used to capture PTEN activity during development, or longitudinally over days, but the reviewer probably understands those types of experiments are incredibly challenging. Accordingly, we think these requests are well outside the scope of this MS.

6. This sentence from my previous comments (part of point 4) has been removed in the rebuttal and is not addressed: "Also, how many fold is the sensor overexpressed compared to endogenous PTEN?".

We greatly apologize for this error. This sentence was indeed accidently deleted, but we address this point, as we provide an estimate of PTEN overexpression in cells (2-3 fold, Extended Fig. 3e-f) and neurons in vivo (~2 fold, Extended Fig. 8i-j).

7. The authors now provide a functional correlation in the newly added pAkt experiment. However, the request "especially at an intermediate activation level" has not been considered. The experiment used strong conditions, which may miss altered sensitivity (e.g., shifted EC50 of TBB, but the maximal dynamic range remaining the same).

We thank the reviewer for this clarification. We performed these experiments to correlate between downstream signaling (pAkt) and sensor expression. We showed that overexpression of the PTEN sensor containing the R14G mutation does not impact downstream pAkt signaling, as appose to overexpression of the WT PTEN sensor, which greatly diminished it. TBB is not a direct modulator of PTEN and thus its kinetics are slow. In addition, assessment of pAkt levels using IHC does not provide a direct readout and is not amenable to quantitative comparison as suggested for this type of analysis. Importantly, other experiments in our MS provide ample evidence of PTEN activity at "intermediate" levels and due to various manipulation to RhoA activity (Fig. 1l), during worm development (Fig. 3), following in vivo genetic perturbations to Tsc2 and IGF1R (Fig 4) and due to sensory deprivation (Fig. 6).

8. In the newly added ED Fig. 8g, there is very little recovery in 100s. Is this consistent with what is known about PTEN or the sensor? Is it not a cytosolic protein?

We performed these experiments to address the reviewer comments regarding differential compartmentalization we observed between spines and dendrite. Accordingly, we performed photobleaching on an entire dendritic branch and spines, which probably led to a relative low level of fluorescence recovery. Nevertheless, we did not find any difference between spine and dendrite recovery rates which suggests the PTEN sensor is cytosolic.

9. ED Fig. 11 does not address former point 10 ("Fig. 7. Can the experiment be repeated by switching G- and R-PTEN in the two neuronal types? ..."). The reviewer does not doubt the ability to separate color channels. The question is whether the two sensors may have different properties, such as sensitivity, etc. Also, related to point 5, can the authors provide a time course for the change, as this is the only physiological/behavioural manipulation that results in the sensor dynamics?

In our revision, we demonstrated in a new set of experiments (Extended Fig. 9a-c), that when the two sensors are expressed in the same cells, the R and G sensors show similar sensitivity to pharmacological and genetic manipulations. This addresses the reviewer's previous remarks regarding differential sensitivity and provides direct evidence for lack of difference in terms of

performance of the two sensor variants. As we stated in our previous point by point rebuttal, we find no reason to perform this experiment in vivo, since our results indicate different response in cell types, meaning G PTEN sensor shows an increase and R-PTEN shows a decrease in lifetime. We therefore do not see the logic behind the claim that the results could be explained by differential sensitivity.

As for the reviewer's suggestion to perform new experiments which include an in vivo time-course, we do not think this is a plausible experiment to pursue. Sensory deprivation likely takes place over several days to weeks. As we previously discussed, these types of experiments are highly challenging and will take a significant amount of time to complete. Accordingly, we think this request is well outside the scope of this MS.

10. Fig. 4: Is Fig. 4g the duplicated data from Fig. 4e and 4f? Also, are they the same data as Figure 4d? It feels like unnecessary inflation of the figure panels.

This is indeed the same data presented. The reason we chose to show these graphs separately is for the ability to show statistical comparison within each group (4e and 4f), while 4g was used only for visual representation (The legends state that these are the same data). We believe this provides the reader easy visual comparison between the two experiment groups, after viewing the two separate graphs.

Other.
1. Lines 397-398: "R-PTEN was expressed under the neuronal Synapsin promoter, which limited expression primarily to excitatory cells …" This is not true. It can probably be rephrased to: "R-PTEN was expressed under the neuronal Synapsin promoter, which limited expression to neurons, and the majority of neurons are excitatory cells…".

We provide an estimate using IHC that indeed most cells expressing the sensor are excitatory (~90%), which is exactly what we stated.

3. Fig. 3g should be made larger to see cells.

This image shows a large field of view to show pAkt levels following response to TBB application.

3. Fig. 3l, please show example raw traces.

Our representative image shows the summed df/f for the duration of 100 seconds, in the revised MS we now provide the raw data for all figures.

Reviewer #3 (Remarks to the Author):

The authors have been very responsive to all the reviewers' comments. Particularly, they now included additional experiments to test the potential dominant negative effect of their PTEN biosensor, and they have ruled out interference with the physiological functions of endogenous PTEN. They have also carried out new imaging experiments to explore the potential of their G- and R-PTEN biosensors to monitor cell-type specific PTEN signaling.
Overall, this is now a thorough and rigorous study describing the development of a novel biosensor for PTEN activity, which is likely going to be of general interest for the scientific

community.
I do recommend its publication.

We thank the reviewer for their positive evaluation and helpful comments.

Table 1: existing 2pFLIM based biosensor used for in vivo imaging:

Paper	Target	Dynamic range	In vivo use
This work: "Genetically encoded biosensor for fluorescence lifetime imaging of PTEN dynamics in the intact brain"	PTEN	0.2-0.3ns	Yes
"A PKA activity sensor for quantitative analysis of endogenous GPCR signaling *via* 2-photon FRET-FLIM imaging" Chen et al 2014 PMID: 24765076	PKA	0.2ns	Used in subsequent studies for in vivo imaging: *Lee et al* 2020 PMID: 33361810
"In vivo imaging of the coupling between neuronal and CREB activity in the mouse brain" Laviv et al 2020 PMID: 31883788	CREB	0.15ns	Yes
"A highly sensitive A-Kinase activity reporter for imaging neuromodulatory events in awake mice" Ma *et al* 2018 PMID: 30100256	PKA (improved version)	0.25ns	Yes, also used in subsequent work for in vivo imaging in the awake mouse brain: Ma *et al* 2022 PMID: 36352228
"Quantitative in vivo imaging of neuronal glucose concentrations with a genetically encoded fluorescence lifetime sensor" Diaz-Garcia *et al* 2019 PMID: 31106909	Glucose	0.2-0.3ns	Yes
"Fast and slow: Recording neuromodulator dynamics across both transient and chronic time scales" Ma *et al* 2024 PMID: 38381826	Dopamine	0.15ns	Yes

“Sensitive genetically encoded sensors for population and subcellular imaging of cAMP in vivo” Massengill et al 2024 PMID: 36303019	cAMP (improved version, 5th generation of the original EPAC sensor).	0.5-0.6ns	Yes