nature portfolio

Peer Review File

Nonlinear dynamics of femtosecond laser interaction with the central nervous system in zebrafish

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The author describes a major issue about laser tissue interaction in nonlinear optical microscopy with a particular wavelength. the manuscript is written well. the comments are

what are the emission wavelengths and provide specifications of filters.

add figure number "e" inside the figure 2.

what kind of nonlinear signal authors are taking about ? is this study only for two photon fluorescence or can it be used for other types of nonlinear signals?

what is the excitation wavelength and emission wavelength used for fig 3?

author should mention the wavelengths used for frequency trippling in section - irradiation and live imaging.

what is the magnification of objective lens used to focus light to zebrafish in line 6, of section irradiation and live imaging.

what are the wavelengths used in line 8, of section irradiation and live imaging.

what are the average power of focused three beams ?

provide more information of the detector used ?

It will be useful if author include image of imaging setup.

It is also better to perform comparison study about the CNS damage threshold.

Is there any effect by the cell culture media ?

Reviewer #2 (Remarks to the Author):

As a set of results which helps to elucidate issues of relevance to the imaging community, I think the work could have been a useful contribution to the field. I think there is a mismatch between how comprehensively the results elucidate various aspects of laser-induced damage, and how the authors describe the work. In the introduction there are several very big open-ended questions, e.g. “What is the most accurate definition of biological damage caused by our observation?” and these are reasonable because they give the importance of the work presented. However, later in the introduction the authors then describe their work with several very big questions such as “How does the repetition rate of laser pulses impact the photo damage threshold?” These have been covered many times over decades (some but not all of which is referenced by the authors). One could argue it has not been comprehensively evaluated under a wide set of parameters by any one study, but I don’t think this presented work answers them comprehensively either. I would be more comfortable if the authors describe what questions can be answered, in what scope, and in what limitations. Otherwise it sounds like the authors have created the final word on these issues, which is absolutely not the case.

It’s stated that the Pockel’s cell was used to change repetition rate and therefore separate out thermal effects, but there are several other mechanisms that are also diffusive, such as molecular changes, ROS, etc. These must also be considered especially in the context of interpretation of the results.

In the labeled experiments, it is stated “Damage was defined as the absence of fluorescence in the region of interest”, but this seems to ignore the fact that absorption in the fluophores is many times higher than in the tissue, and cell damage is therefore less simple to evaluate in this way. The results may be vastly different when interpreted on unstained sample targets.

It also makes it harder to separate bleaching from “damage”. Any findings in the paper that are based on changes in fluorescence need to be independently assessed for how well that can be used as a metric for “damage”. The TUNEL assay is an accepted metric for cell death but not for damage.

“Short-term damage was evaluated by monitoring the immediate physiological response, that is loss of tissue integrity, at the whole tissue level as well as of specific fluorescently labeled cell types”. Loss of integrity is not the immediate physiological response for most living systems. There are many more subtle responses which occur immediately, but may not have been seen by the authors because they didn’t look for it. Action potential changes, modulation of calcium or other cell signaling pathways. The authors are evaluating essentially “explosive” levels of irradiation in this section. If this is the regime they wish to study, that’s fine, but as-written is misleading. I also wouldn’t call obvious loss of tissue integrity a “physiological” response.

The final conclusions seem to be surprised that the damage threshold at 1030 is much higher than at lower wavelengths. This is not at all surprising, and the authors seem to find that this is due to nonlinear photodamage. I don't think anyone reading this manuscript would be surprised that it's due to nonlinear damage. These studies (some of which are cited by the authors) go back decades with the same conclusions. Therefore the authors need to clarify what exactly is elucidated here.

They use words like "absence of gradual damage at this wavelength" but we need a better definition of damage, and it would be better if the authors could phrase this in terms of single or multiphoton effects, and although it may be complex, many of these issues have been treated in many different works, going back for decades. As one of *many* examples, and I'm not going to do the literature review for the authors, the following paper clearly defines two different modes of damage in a similar context:

Hopt A, Neher E. Highly nonlinear photodamage in two-photon fluorescence microscopy. Biophys J. 2001 Apr;80(4):2029-36. doi: 10.1016/S0006-3495(01)76173-5.

As a result of all this, I think they authors need to redefine what questions they are asking, what data they have, what has already been done in the field, and specifically how this work adds, and support all claims with data. I therefore cannot recommend publication.

Reviewer #3 (Remarks to the Author):

The paper submitted by Soyeon Jun and colleagues present experiments aiming at characterizing the induced phototoxicity in the spinal cord of zebrafish larvae induced by focused femtosecond laser pulses, centered at wavelengths 1030nm, 515nm and 343nm. To this end, several experiments are performed 1) to determine whether the phototoxicity is induced by linear or nonlinear light matter interaction, 2) to determine the influence of various experimental parameters such as: the peak intensity, the average power, repetition rate and the exposure time.

The subject is interesting and seems to be a hot topic in the community as I found a preprint when I was writing my review: https://www.biorxiv.org/content/10.1101/2023.10.09.561579v3.article-info which will probably have to be cited by the authors. I would like to say that I have no connection whatsoever with the authors of this study but the preprint seems to explore this subject in a more detailed and honest way than the paper submitted by Soyeon Jun and collegues. The fact that the wavelength at 1030nm can be used to generate nonlinear photo-perturbation and ablation contrary to wavelengths in the visible range namely 515nm and 343nm due to linear absorption is not new and not surprising. What

would be more pertinent here is to explore the influence of wavelength in the near infrared wavelength range, such as in the 700 to 1700nm wavelength range.

I would recommend the authors to temper some of their claims especially in the introduction and conclusion sections, which seems to be written in a different style than the Results section... I'm not sure that the preliminary observations made in this article on a specific case, namely the response to photoperturbation in the spinal cord of a zebrafish larvae can be generalized to other systems as it would require to explore in more details the various degree of freedom for different systems:

- Excitation wavelength in the near infrared regions: from 700 to 1700nm
- Peak power
- Pulse duration
- Pixel dwell time from a single pulse to a few µs
- Repetition rate, most people use 80MHz for two-photon imaging and 1-2MHz for three-photon imaging. The study here is done at 2MHz and 127kHz so another point with higher repetition rate is definitely important as the results could be different for 2MHz or 80MHz.

For example, they are various relaxation time in the system that can play a critical role even though the signal is created by a nonlinear process.

I would recommend the authors to change Figure 1 which looks nice but contains actually no useful information and insert graphics resuming the different effects that can induce directly or indirectly phototoxicity to the sample when using laser focused femtosecond laser pulses.

Other remarks for the authors:

The presence of markers can influence in a positive or negative manners phototoxicity so the authors may need to also perform experiments on wild type zebrafish larvae.

Please avoid using the word significantly, and use instead objective comparison ie 10 times larger, stronger. They are also several overstatements in the Introduction and Conclusion sections of the manuscript.

The following sentence in the abstract section is fairly surprising: “At this irradiation wavelength, photo damage becomes detectable only after exceeding a specifc peak intensity threshold, which is independent of the photon flux and irradiation time,”

What do you mean? To make such a claim you need to send only one impulsion to the sample and then observe visible damage. Was it the case? It also seems to contradict previously published work on Hela cells which reports increase of peak intensity damage threshold with decreasing integration time at wavelength 1040nm with the repetition rate of 80MHz: https://doi.org/10.1364/BOE.441225

Response to Reviewers' comments.

The reviewers' comments are in black. The authors' responses are shown in blue.

- Reviewer #1 (Remarks to the Author):

The author describes a major issue about laser tissue interaction in nonlinear optical microscopy with a particular wavelength. the manuscript is written well. the comments are what are the emission wavelengths and provide specifications of filters. add figure number "e" inside the figure 2.

RESPONSE: We are glad the reviewer appreciates that our work addresses an important topic and evaluates our manuscript as well-written. We thank the reviewer for their suggestions, which allowed us to improve the manuscript.

The fluorescent labels used for visualization of different cell types in transgenic zebrafish were GFP, mKate2, and mCherry, with the central emission wavelength at 507 nm and 633 nm and 610 nm. For imaging before and after irradiation, we used Plan-Apochromat 10x/0.45 M27 objective or Plan-Apochromat 20x/0.8 objective on a Zeiss LSM 980 confocal microscope. The detector ranges used were 490-588 nm for EGFP and 597-695 nm for mKate2 and mCherry. We used a main beam splitter (MBS) 488/594 for detection of EGFP, mKate2, and EGFP. We have added this information to the methods section of the revised manuscript and updated Figure 2.

what kind of nonlinear signal authors are taking about? is this study only for two photon fluorescence or can it be used for other types of nonlinear signals?

RESPONSE: We apologize for any lack of clarity that might have caused confusion. In this work, we study the damage mechanism in deep tissue at three different wavelengths 343 nm, 515 nm, 1030 nm with a particular focus on irradiation at 1030 nm. The irradiation peak intensity on the sample is increased until the damage or ablation is observed. To monitor tissue damage, specific cell types were labeled with fluorescence proteins using genetically modified zebrafish lines.

We observed that the damage at 343 nm scales linearly with the peak intensity of femtosecond pulses and the irradiation time. The damage threshold at 515 nm drifts from linear scaling and at 1030 nm the behavior becomes highly nonlinear. At this wavelength, the damage occurs at the ablation threshold and is highly dependent on the peak intensity of laser pulses and not the irradiation time. We identify that in this nonlinear regime, the repetition rate of the laser pulses can lead to different damage mechanisms. While a precise cavitation regime can be reached at 127 kHz, the tissue damage becomes collateral at 2 MHz repetition rates. We identify that the damage is not due to accumulated heat but is caused by low-density plasma generation, which could trigger plasma-mediated chemical effects and nonlinear photochemistry. Our findings agree well with the predictions of a theoretical model developed by Liang et al. (Optics Express 2019), which studies tissue damage due to excessive free electron generation. We have added this reference to the revised manuscript and refer to it in the discussion section.

The ROS generation from labels could also influence the damage threshold. Therefore, for irradiation at 1030 nm, we performed a second series of measurements, where neurons were labeled by either GFP with a nonlinear absorption pathway at 1030 nm, or mKate2 with no direct or nonlinear absorption pathway. We demonstrate that the influence of the labels on the peak intensity damage threshold at 1030 nm is minor at the near-infrared spectral range.

We went through the manuscript and edited the pertinent part to point this out more clearly.

what is the excitation wavelength and emission wavelength used for fig 3?

RESPONSE: We thank the reviewer for the question. The samples were irradiated at 1030 nm. For imaging before and after radiation, excitation, and emission wavelengths were chosen to be specific for the respective fluorophores. We have added this information to the materials section of the revised manuscript.

author should mention the wavelengths used for frequency tripling in section - irradiation and live imaging.

RESPONSE: We thank the reviewer for pointing this out. The triple frequency was generated by the sum frequency generation of the 1030 nm and 515 nm pulses. We added this information in the method section highlighted in red. Furthermore, we have expanded on the details of live imaging in the methods sections.

what is the magnification of objective lens used to focus light to zebrafish in line 6, of section irradiation and live imaging.

RESPONSE: The magnification factor of the objective lens used for irradiation was 40x. We added this missing information in the main text highlighted in red. Furthermore, we have expanded on the details of live imaging in the methods sections.

what are the wavelengths used in line 8, of section irradiation and live imaging.

RESPONSE: We thank the reviewer for the question. The samples were irradiated at 343 nm, 515 nm, and 1030 nm. We added this information in the main text.

what are the average power of focused three beams?

RESPONSE: Thank the reviewer for the comment. We made a new sub-figure that shows the dependence of the damage to the average power for the three irradiation wavelengths at different repetition rates (Figure 2.c)

provide more information of the detector used?

RESPONSE: We thank the reviewer for the question. The monitoring of the sample during the irradiation was done with a CCD camera mounted on top of the microscope (Allied Vision, Alvium 1800U-2050). Viewing modules on top of the microscope was used as another method to fix the position of the zebrafish (Thorlabs, LAURE2). Live examination and imaging before and after irradiation were done using the commercial microscope setups. We added the details to the methods section of the revised manuscript.

It will be useful if author include image of imaging setup.

RESPONSE: We thank the reviewer for the helpful suggestion. In the revised manuscript, we added a sketch of the setup that had been used to irradiate the zebrafish as Figure 1b. For live imaging of zebrafish, we used commercially available stereo fluorescence and confocal microscopes as detailed in the methods section.

It is also better to perform comparison study about the CNS damage threshold.

RESPONSE: We appreciate the reviewer's comment, Unfortunately, from the comment, it is not exactly clear to us what the reviewer refers to. From our interpretation, the reviewer suggests performing a comparative study of the damage threshold in CNS tissue. However,

we would like to emphasize that our measurements already report on the CNS damage threshold.

Is there any effect by the cell culture media?

RESPONSE: We thank the reviewer for the question. In all of our measurements, the conditions – including the embryo medium (E3 medium) which consists of a defined salt solution (5 mM NaCl, 0.17 mM KCl, 0.33 $CaCl_2$, 0.33 mM $MgSO_4$) and pH (7.2) – were the same except for the laser settings. Hence, we can exclude that the observed differences in tissue damage outcome were a consequence or effect of the embryo medium.

- Reviewer #2 (Remarks to the Author):

As a set of results which helps to elucidate issues of relevance to the imaging community, I think the work could have been a useful contribution to the field. I think there is a mismatch between how comprehensively the results elucidate various aspects of laser-induced damage, and how the authors describe the work. In the introduction there are several very big open-ended questions, e.g. “What is the most accurate definition of biological damage caused by our observation?” and these are reasonable because they give the importance of the work presented. However, later in the introduction the authors then describe their work with several very big questions such as “How does the repetition rate of laser pulses impact the photo damage threshold?” These have been covered many times over decades (some but not all of which is referenced by the authors). One could argue it has not been comprehensively evaluated under a wide set of parameters by any one study, but I don’t think this presented work answers them comprehensively either. I would be more comfortable if the authors describe what questions can be answered, in what scope, and in what limitations. Otherwise it sounds like the authors have created the final word on these issues, which is absolutely not the case. It’s stated that the Pockel’s cell was used to change repetition rate and therefore separate out thermal effects, but there are several other mechanisms that are also diffusive, such as molecular changes, ROS, etc. These must also be considered especially in the context of interpretation of the results.

RESPONSE: We are glad the reviewer appreciates that our work will help to elucidate issues of relevance to the imaging community and we thank the reviewer for their suggestions, which allowed us to improve the manuscript.

We apologize for any lack of clarity that might have caused confusion. The main questions that our manuscript addresses are:

1. How do the dynamics of different photodamage mechanisms unfold across a spectrum of femtosecond pulses, particularly at near-infrared (NIR) in deep tissue?
2. What are the dynamics behind the photodamage at high peak intensities at NIR, and does the repetition rate of the laser pulses influence the driving mechanism?
3. Is it feasible to reach plasma formation without inducing gradual damage to the sample in deep tissue?
4. What is the role of thermal effects in photodamage at high peak intensities and high repetition rates?
5. To what extent does the ROS generation due to fluorescence proteins cause acceleration in photodamage?
6. How does the damage threshold scale relative to the numerical aperture of focusing optics?

These questions have not been investigated in the interaction of femtosecond pulses at near-infrared with deep tissue, as only in the recent decade turn-key lasers delivering such pulses become available.

As correctly pointed out by the reviewer there are always several damage mechanisms occurring at the same time. Our study aims to address multi-dimensional aspects of the damage in deep tissue upon irradiation with 1030 nm femtosecond pulses.

We study the damage based on long working range focusing optics which are crucial for deep tissue imaging. At this focusing geometry, the generated free electron affects the beam propagation and peak intensity, before the beam reaches the waist. This causes a different damage threshold and mechanism compared to the damage caused by high numerical aperture, and short working distance focusing geometry.

Damage caused by the increased repetition rate of the laser has been associated with thermal damage in many previous works for samples irradiated at visible or the first window optical of tissue. In this work, we show that by increasing the repetition rate of laser pulses at 1030 nm, damage occurs at lower peak intensity not due to accumulated heat but due to the generation of low plasma density which results in triggering plasma-mediated chemical effects and nonlinear photochemistry. We identify two regimes of damage for irradiation at 1030 nm at two different repetition rates:

i) The peak intensity threshold at 127 kHz which is above the required threshold for bubble formation by a single laser pulse. Here, no collateral damage was observed, and the damage was confined to the region around the laser-induced plasma due to mechanical forces arising from bubble formation and shockwave emission.

ii) For samples irradiated at 2 MHz repetition rates, collateral damage is observed due to plasma-mediated accumulative chemical disintegration of biomolecules. Free electrons have ps lifetime and nanometer traveling range and cannot have a seminal contribution to the size of the observed damage size. However, the generated damaging "agents" at the focal spot, like ROS, have a high diffusivity and can travel over 25 μm within less than a second. These parameters match very well with the observed damage size. Moreover, for samples irradiated at 2 MHz repetition rate, the generated free electron density reaches the low plasma density. The inter-pulse interval at this repetition rate is 500 ns, which is considerably longer than the heat dissipation time. Therefore, the observed damage is not caused by thermal accumulation.

Our measurement agrees well with the theoretical modeling of Liang et al (Optics Express 2019).

ROS generation from the fluorescence labels plays a role in damage and cell death. To isolate the effect of ROS generation from labels and other damage mechanisms, we performed three series of measurements as described in the manuscript. The damage caused by irradiation at 343 nm and 515 nm, includes the ROS generation due to linear absorption in fluorescence labels and the tissue. In the measurements performed at 1030 nm, the samples were labeled either by i) GFP with a nonlinear absorption pathway at 1030 nm, or ii) mKate2 with no direct or nonlinear absorption pathway. We demonstrate that the influence of ROS generation from labels on the peak intensity damage threshold at 1030 nm is minor at the near-infrared spectral range compared to the effect of ROS generation from tissue.

We appreciate the reviewer's comment which encouraged us to edit the introduction and substantially change the discussion section to make the objectives and the results of the study more accessible to the reader.

In the labeled experiments, it is stated "Damage was defined as the absence of fluorescence in the region of interest", but this seems to ignore the fact that absorption in the fluophores is many times higher than in the tissue, and cell damage is therefore less simple to evaluate in this way. The results may be vastly different when interpreted on unstained sample targets.

RESPONSE: We appreciate the reviewer's comment. As it is correctly addressed by the reviewer the ROS generation from labels can influence the damage threshold due to the excessive ROS generation. As explained above, the damage caused by irradiation at 343 nm and 515 nm, includes the ROS generation due to linear absorption in fluorescence labels and the tissue. In the measurements performed at 1030 nm, the samples were labeled either by i) GFP with a nonlinear absorption pathway at 1030 nm, or ii) mKate2 with no direct or nonlinear absorption pathway. To address the extent of the influence of labels on damage threshold we

added Fig 2f to the manuscript. The figure shows that for samples irradiated at 1030 nm, the peak intensity damage threshold for ablation doesn't vary extensively in the presence (GFP labels) or absence (mKate2) of nonlinear absorption pathways in labels

We would like to point out that we assessed tissue damage by several complementary means, including loss of tissue integrity, loss of fluorescence, regeneration dynamics, TUNEL assay, and injury-associated recruitment of reactive fibroblasts and immune cells. Importantly, we observed tissue damage above the damage threshold also in animals in which macrophages were fluorescently labeled. As only very few resident macrophages are present in larval zebrafish trunk tissue but recruited in response to injury from distant sites, we can exclude that the absorption of laser light by fluorophore labeling neurons in the spinal cord solely accounts for tissue damage. We edited the manuscript in the Results and Discussion sections to point this out more clearly.

It also makes it harder to separate bleaching from "damage". Any findings in the paper that are based on changes in fluorescence need to be independently assessed for how well that can be used as a metric for "damage".

RESPONSE: We appreciate the reviewer's comment. We would like to point out, that we assessed tissue damage by evaluating both loss of fluorescence and loss of tissue integrity (as observed using transmitted light microscopy). Furthermore, we reassessed *elavl3*:rasmKate2 transgenic animals in which neurons were labeled (with mKate2) at different time points after irradiation (see Fig. 4a). This showed an imperfect fluorescence pattern of the white matter tracts at 48 hpi, supporting the regrowth of irradiation-damaged axonal fibers rather than recovery of fluorescence. We edited the manuscript to point this out more clearly.

The TUNEL assay is an accepted metric for cell death but not for damage.

RESPONSE: We appreciate the reviewer's comment. However, we respectfully disagree with the reviewer that TUNEL is not an appropriate assay for (cell) damage. While different types of cell death exist, it is well-accepted that cell death is a consequence of irreversible cell damage (e.g., https://www.nature.com/articles/s41418-017-0012-4). The TUNEL assay detects DNA breaks, which represents DNA damage. Hence, the TUNEL assay is an appropriate read-out for (tissue) damage.

"Short-term damage was evaluated by monitoring the immediate physiological response, that is loss of tissue integrity, at the whole tissue level as well as of specific fluorescently labeled cell types". Loss of integrity is not the immediate physiological response for most living systems. There are many more subtle responses which occur immediately, but may not have been seen by the authors because they didn't look for it. Action potential changes, modulation of calcium or other cell signaling pathways. The authors are evaluating essentially "explosive" levels of irradiation in this section. If this is the regime they wish to study, that's fine, but as-written is misleading. I also wouldn't call obvious loss of tissue integrity a "physiological" response.

RESPONSE: We thank the reviewer for the comment. We have edited the pertinent part in the revised manuscript to avoid any potential confusion. It now reads: "We employed two complementary criteria to assess photodamage at the level of the spinal cord and surrounding trunk tissue. Short-term damage was evaluated by monitoring loss of tissue integrity at the whole tissue level and by assessing specific cell types (neurons, glia cells, keratinocytes), which were monitored through fluorescence labels in specific transgenic zebrafish lines. Long-term damage was assessed by observing axon regeneration over a period of 2 days, histological stains to detect cell death, and injury-associated recruitment of reactive fibroblasts and immune cells (macrophages) within 24 hours post-irradiation (hpi)".

The final conclusions seem to be surprised that the damage threshold at 1030 is much higher than at lower wavelengths. This is not at all surprising, and the authors seem to find that this is due to nonlinear photodamage. I don't think anyone reading this manuscript would be surprised that it's due to nonlinear damage. These studies (some of which are cited by the authors) go back decades with the same conclusions. Therefore the authors need to clarify what exactly is elucidated here.

RESPONSE: We appreciate the reviewer's comment. However, we respectfully disagree with the reviewer's statement that our findings merely confirm previous reports. To the best of our knowledge, there is no measurement in label-free deep tissue in a live vertebrate species with long working range focusing optics. We demonstrate ablation is the major component for deep tissue damage at 1030 nm at 127 kHz. In addition, we report on a minor influence of the ROS generations from labels in changing the damage threshold when irradiated at 1030 nm. The measurements demonstrate that the damage at this wavelength and at irradiation at 2 MHz repetition rates becomes collateral. We identify, that the catastrophic damage is not due to accumulated heat but is caused by low-density plasma generation, which could trigger plasma-mediated chemical effects and nonlinear photochemistry. Our findings agree well with the predictions of a theoretical model developed by Liang et al. (Optics Express 2019) that studies damage at high nonlinearity and due to excessive free electron generation.

Based on the reviewer's comments and to make the findings of the manuscript clearer, we substantially changed the discussion section.

They use words like "absence of gradual damage at this wavelength" but we need a better definition of damage, and it would be better if the authors could phrase this in terms of single or multiphoton effects, and although it may be complex, many of these issues have been treated in many different works, going back for decades. As one of *many* examples, and I'm not going to do the literature review for the authors, the following paper clearly defines two different modes of damage in a similar context: Hopt A, Neher E. Highly nonlinear photodamage in two-photon fluorescence microscopy. Biophys J. 2001 Apr;80(4):2029-36. doi: 10.1016/S0006-3495(01)76173-5.

As a result of all this, I think they authors need to redefine what questions they are asking, what data they have, what has already been done in the field, and specifically how this work adds, and support all claims with data. I therefore cannot recommend publication.

RESPONSE: We apologise if the lack of clarity in our manuscript which led the reviewer to the conclusion that no new result is reported in this work. To summarize our data shows the following results:

1. the damage at 1030 nm is abrupt and highly nonlinear, reaching an ablation regime unlike the damage observed at the harmonics of the laser. While the severity of the damage scales gradually with the flux of the irradiation at 515 nm and 343 nm, the damage becomes independent of the flux for irradiation at 1030 nm and peak intensities below the ablation threshold.
2. For 1030 nm pulses at 127 kHz, the ablation is observed at 8 TW/cm^2 peak intensity, where the observed size of the damaged spot confirms the regime of bubble formation. No collateral damage was observed and the damage was confined to the region around the laser-induced plasma due to mechanical forces arising from bubble formation and shockwave emission. The reported value is in great agreement with the recent theoretical modelling shown by Liang et al. (optics Express 2019)
3. the required peak intensity for observing ablation for samples irradiated at 2 MHz repetition rates, is lower compared to irradiation at 127 kHz, while collateral damage is observed. No thermal-based damage at this regime was observed due to the 500 ns

intra-pulse time which is considerably longer than the heat dissipation time. We found that the damage occurs due to the generated low plasma density in tissue which leads to plasma-mediated accumulative chemical disintegration of biomolecules. The observed size of the damage agrees well with the anticipated size.

4. the sharpness of the damage nonlinearity at 2 MHz repetition rates is increased relative to the 127 kHz, although laser pulses have a lower peak intensity. We associate this sharpness with the collateral damage caused by the plasma-mediated accumulative chemical disintegration of biomolecules.
5. the sharpness of nonlinearity of the damage at the harmonics of the laser is smaller than the irradiation at 1030 nm, emphasizing the observation of the gradual damage at the lower wavelengths.
6. we demonstrate that the damage peak intensity threshold is decreased in the presence of the labels with the nonlinear absorption pathway at 1030 nm. However, the effect of absorption and reactive oxygen species generation in labels is marginal at this regime and the damage is dominated by low-density plasma generation (at 2 MHz) and ablation (at 127 kHz).
7. we demonstrate that only at irradiation at 1030 nm, the damage process depends on the peak intensity and not the photon flux. This means that if the laser pulses are adjusted to below the laser peak intensity threshold for ablation, the dwell time can be increased to photon flux beyond the associated photon flux to the peak intensity damage threshold. This is not the case for irradiation at 515 nm and 343 nm where the damage process is dominated by the photon flux threshold rather than the peak intensity threshold.

The reviewer cited paper by Hopt and Neher. 2001, which studies how cell damage occurs due to the two-photon fluorescence absorption of the fluorescent label. This effect has been studied extensively over the last decade. However, as mentioned above, our study not only addresses the damage due to the nonlinear absorption of fluorescence labels, but also addresses the damage mechanism in the presence and absence of absorption in labels. We study the case that the excitation wavelength is not within the multiphoton absorption regime of the used fluorescence labels (we irradiated the fish at 1030 nm for two scenarios i) labeled with mKate 2 with the absorption wavelength at 600 nm, and ii) GFP with the absorption wavelength at 500 nm and potential for two-photon absorption). Therefore, we cover the damage that is caused by ROS generation due to the absorption of fluorescent labels and the damage caused by direct nonlinear interaction of the CNS and 1030 nm pulses (which as described in the manuscript resulted in ablation). We presented the data for scenario II, where the fish were labled by GFP and mKate2), in newly added Figure 2-f in the manuscript for direct comparison.

- Reviewer #3 (Remarks to the Author):

The paper submitted by Soyeon Jun and colleagues present experiments aiming at characterizing the induced phototoxicity in the spinal cord of zebrafish larvae induced by focused femtosecond laser pulses, centered at wavelengths 1030nm, 515nm and 343nm. To this end, several experiments are performed 1) to determine whether the phototoxicity is induced by linear or nonlinear light matter interaction, 2) to determine the influence of various experimental parameters such as: the peak intensity, the average power, repetition rate and the exposure time.

The subject is interesting and seems to be a hot topic in the community as I found a preprint when I was writing my review:
https://www.biorxiv.org/content/10.1101/2023.10.09.561579v3.article-info which will probably have to be cited by the authors. I would like to say that I have no connection whatsoever with the authors of this study but the preprint seems to explore this subject in a more detailed and honest way than the paper submitted by Soyeon Jun and collegues. The fact that the wavelength at 1030nm can be used to generate nonlinear photo-perturbation and ablation contrary to wavelengths in the visible range namely 515nm and 343nm due to linear absorption is not new and not surprising.

RESPONSE: We appreciate the link to the pre-print by Wang et al. 2023. We would like to point out that the cited pre-print appeared online after the submission of our manuscript, and that it reports on an *ex vivo* study on chicken breast at 80 MHz repetition rates. In this regime, the damage mechanism is very well known and has been studied in many works already

The main topic of our manuscript is on the damage mechanism upon interaction of femtosecond pulses with deep tissue which we addressed via the following questions:

1. How do the dynamics of different photodamage mechanisms unfold across a spectrum of femtosecond pulses, particularly at near-infrared (NIR) in deep tissue?
2. How does the damage threshold scale relative to the numerical aperture of focusing optics, which are necessary means for deep-tissue interaction?
3. What are the dynamics behind the photodamage at high peak intensities at NIR, and does the repetition rate of the laser pulses influence the driving mechanism, and to what extent?
4. Is it feasible to reach plasma formation without inducing gradual damage to the sample in deep tissue?
5. What is the role of thermal effects in photodamage at high peak intensities and high repetition rates?
6. To what extent does the ROS generation due to fluorescence proteins cause acceleration in photodamage? And to what extent?

These questions have not been investigated in the interaction of femtosecond pulses at near-infrared with deep tissue. There are always several damage mechanisms occurring simultaneously. Our study aims to address multi-dimensional aspects of the damage in deep tissue upon irradiation with 1030 nm femtosecond pulses.

We study the damage based on long working range focusing optics which are crucial for deep tissue imaging. At the focusing geometry, the generated free electron affects the beam propagation and peak intensity before the beam reaches the waist. This causes a different damage threshold and mechanism compared to the damage caused by high numerical aperture, and short working distance focusing geometry.

Damage caused by the increased repetition rate of the laser has been associated with thermal damage in many previous works for samples irradiated at visible or the first window of tissue. In this work, we show that by increasing the repetition rate of laser pulses at 1030 nm, damage

occurs at lower peak intensity not due to accumulated heat but due to the generation of low plasma density which results in triggering plasma-mediated chemical effects and nonlinear photochemistry. We identify two regimes of damage for irradiation at 1030 nm at two different repetition rates:

iii) The peak intensity threshold at 127 kHz which is above the required threshold for bubble formation by a single laser pulse. Here, no collateral damage was observed, and the damage was confined to the region around the laser-induced plasma due to mechanical forces arising from bubble formation and shockwave emission.

iv) For samples irradiated at 2 MHz repetition rates, collateral damage is observed due to bubble formation based on plasma-mediated accumulative chemical disintegration of biomolecules. Free electrons have ps lifetime and nanometer traveling range and cannot have a seminal contribution to the size of the observed damage size. However, the generated damaging "agents" at the focal spot, like ROS, have a high diffusivity and can travel over 25 μm within less than a second. These parameters match very well with the observed damage size. Moreover, for samples irradiated at 2 MHz repetition rate, the generated free electron density reaches the low plasma density. The inter-pulse interval at this repetition rate is 500 ns, which is considerably longer than the heat dissipation time. Therefore, the observed damage is not caused by thermal accumulation.

Our measurement agrees well with the theoretical modeling of Liang et al (Optics Express 2019).

ROS generation from the fluorescence labels plays a role in damage and cell death. To isolate the effect of ROS generation from labels from other damage mechanisms, we performed three series of measurements as described in the manuscript. The damage caused by irradiation at 343 nm and 515 nm, includes the ROS generation due to linear absorption in fluorescence labels and the tissue. In the measurements performed at 1030 nm, the samples were labeled either by i) GFP with a nonlinear absorption pathway at 1030 nm, or ii) mKate2 with no direct or nonlinear absorption pathway. We demonstrate that the influence of ROS generation from labels on the peak intensity damage threshold at 1030 nm is minor at the near-infrared spectral range compared to the effect of ROS generation from tissue.

We appreciate the reviewer's comment which encouraged us to edit the introduction and substantially change the discussion section to make the objectives and the results of the study clearer.

What would be more pertinent here is to explore the influence of wavelength in the near infrared wavelength range, such as in the 700 to 1700nm wavelength range.

RESPONSE: We agree with the reviewer that it would be of interest to examine the tissue response to an excitation wavelength in the near-infrared regions. However, this is beyond the scope of our study. In our manuscript, we study the interaction of deep tissue and laser wavelength at 343 nm, 515 nm, and 1030 nm. The reason that we chose these three wavelengths is that the response of the tissue (scattering and transmission) varies substantially between these three wavelengths. However, the range from 700 nm- 1700 nm covers the first and second transparent windows of tissue where the variations in scattering and transmission in deep tissue are small, and the same response over this spectral range is expected.

I would recommend the authors to temper some of their claims especially in the introduction and conclusion sections, which seems to be written in a different style than the Results section... I'm not sure that the preliminary observations made in this article on a specific case,

namely the response to photoperturbation in the spinal cord of a zebrafish larvae can be generalized to other systems

RESPONSE: We appreciate the reviewer's comment. We would like to point out that the animal model used in our study, the zebrafish, presents a vertebrate species, which shares morphological and physiological similarities with mammals; 71% of human proteins and 82% of disease-causing human proteins have a corresponding equivalent in zebrafish (https://www.nature.com/articles/nature12111) . Hence, our findings are of potential relevance to other vertebrate species, including humans and other mammals. To our knowledge, our study is the first to address the deep-tissue damage in vivo in a vertebrate species in the case that the effect of the fluorescent labels in the damage is minimized. However, we agree that our statements can be toned down to better reflect the findings of our study. We edited the manuscript accordingly to point this out more clearly.

as it would require to explore in more details the various degree of freedom for different systems:

• Excitation wavelength in the near-infrared regions: from 700 to 1700nm

As stated above, we agree with the reviewer that it would be of interest to examine the tissue response to an excitation wavelength in the near-infrared regions. However, this is beyond the scope of our study. In our manuscript, we study the interaction of deep tissue and laser wavelength at 343 nm, 515 nm, and 1030 nm. The reason that we chose these three wavelengths is that the response of the tissue (scattering and transmission) varies substantially between these three wavelengths. On the contrary, the range from 700 nm- 1700 nm covers the first and second transparent windows of tissue where the changes in scattering and transmission in deep tissue are small.

• Peak power

The reviewer might have missed Fig. 2a and Fig. 2b and a newly added data in Fig 2f, where the peak power variation is shown. We pointed this out more clearly in the revised manuscript.

• Pulse duration

This is an interesting study, however beyond the scope of our work. We have already performed measurements with 2 times longer pulse duration and a similar effect was observed. We expect observation of similar behavior by making the pulses shorter and to sub 100 fs regime. Here, an increase in the kinetic energy of the free electrons at high intensity is expected. It is also predicted that the plasma generation differs for 1030 nm, sub-100 fs pulses at 1 TW/cm^2 compared to 250 fs pulses used in this study (please see the recent theoretical investigation by Liang et al, Optics Express 2019). Reducing the pulse duration to below 10 fs initiates a completely different regime as the spectral bandwidth of the pulse might contribute to the damage caused by the linear absorption.

• Pixel dwell time from a single pulse to a few µs

The reviewer might have missed Fig. 2d and Fig. 2e where the pixel dwell time is varied by changing the irradiation time and repetition rates

• Repetition rate, most people use 80MHz for two-photon imaging and 1-2MHz for three-photon imaging. The study here is done at 2MHz and 127kHz so another point with higher repetition rate is definitely important as the results could be different for 2MHz or 80MHz. For example, they are various relaxation time in the system that can play a critical role even though the signal is created by a nonlinear process.

The damage behavior at 80 MHz is very well studied. For example, see: https://doi.org/10.1364/OPTICA.454469. What is crucial for label-free microscopy is the possibility of increasing the laser peak power while minimizing the collateral damage. Sub 10 MHz repetition rates, which is studied in our manuscript, allow for operation in this regime where a high nonlinear signal can be achieved from the sample while the mechanisms for collateral damage are minimized. The presented data in our manuscript forms a base for comparison with the many available studies which are performed at 80 MHz repetition rates.

I would recommend the authors to change Figure 1 which looks nice but contains actually no useful information and insert graphics resuming the different effects that can induce directly or indirectly phototoxicity to the sample when using laser focused femtosecond laser pulses.

RESPONSE: We appreciate the reviewer's comments. Figure 1 carries important information relevant to the manuscript, like the position of the focus of the femtosecond pulses in zebrafish, and the types of cells that are used for monitoring the status of the tissue. Showing the role-play of several damage mechanisms is out of the scope of the graphical abstract of our manuscript. However, we added the scheme of the illumination setup in Fig 1 to provide more detailed information about the experiment performed in this manuscript.

Other remarks for the authors: The presence of markers can influence in a positive or negative manners phototoxicity so the authors may need to also perform experiments on wild type zebrafish larvae.

RESPONSE: As it is correctly addressed by the reviewer the ROS generation from labels can influence the damage threshold due to the excessive ROS generation. As explained above, the damage caused by irradiation at 343 nm and 515 nm, includes the ROS generation due to linear absorption in fluorescence labels and the tissue. In the measurements performed at 1030 nm, the samples were labeled either by i) GFP with a nonlinear absorption pathway at 1030 nm, or ii) mKate2 with no direct or nonlinear absorption pathway. To address the extent of the influence of labels on damage threshold we added Fig 2f to the manuscript. The figure shows that for samples irradiated at 1030 nm, the peak intensity damage threshold for ablation doesn't vary extensively in the presence (GFP labels) or absence (mKate2) of nonlinear absorption pathways in labels.

Please avoid using the word significantly, and use instead objective comparison ie 10 times larger, stronger. They are also several overstatements in the Introduction and Conclusion sections of the manuscript.

RESPONSE: We thank the reviewer for the comment. We went through the manuscript and revised the pertinent parts.

The following sentence in the abstract section is fairly surprising: "At this irradiation wavelength, photo damage becomes detectable only after exceeding a specifc peak intensity threshold, which is independent of the photon flux and irradiation time," What do you mean? To make such a claim you need to send only one impulsion to the sample and then observe visible damage. Was it the case? It also seems to contradict previously published work on Hela cells which reports increase of peak intensity damage threshold with decreasing integration time at wavelength 1040nm with the repetition rate of 80MHz: https://doi.org/10.1364/BOE.441225

RESPONSE: We appreciate the reviewer's comment. The reviewer refers to a previous work that was performed at 80 MHz by Talone et al (Biomedical Optics Express, 2021). This regime has been very well studied. Our work studies the behavior of deep tissue with long working range focusing geometry at 127 kHz and 2 MHz repetition rates. As explained above:

At this focusing geometry, the generated free electron affects the beam propagation and peak intensity before the beam reaches the waist. This causes a different damage threshold and mechanism compared to the damage caused by high numerical aperture, and short working distance focusing geometry.

Damage caused by the increased repetition rate of the laser has been associated with thermal damage in many previous works for samples irradiated at visible or the first window of tissue. In this work, we show that by increasing the repetition rate of laser pulses at 1030 nm, damage occurs at lower peak intensity not due to accumulated heat but due to the generation of low plasma density which results in triggering plasma-mediated chemical effects and nonlinear photochemistry. We identify two regimes of damage for irradiation at 1030 nm at two different repetition rates:

i) The peak intensity threshold at 127 kHz which is above the required threshold for bubble formation by a single laser pulse. Here, no collateral damage was observed, and the damage was confined to the region around the laser-induced plasma due to mechanical forces arising from bubble formation and shockwave emission.

ii) For samples irradiated at 2 MHz repetition rates, collateral damage is observed due to bubble formation based on plasma-mediated accumulative chemical disintegration of biomolecules. Free electrons have ps lifetime and nanometer traveling range and cannot have a seminal contribution to the size of the observed damage size. However, the generated damaging “agents” at the focal spot, like ROS, have a high diffusivity and can travel over 25 μm within less than a second. These parameters match very well with the observed damage size. Moreover, for samples irradiated at 2 MHz repetition rate, the generated free electron density reaches the low plasma density. The inter-pulse interval at this repetition rate is 500 ns, which is considerably longer than the heat dissipation time. Therefore, the observed damage is not caused by thermal accumulation.

Our measurement agrees well with the theoretical modeling of Liang et al (Optics Express 2019).

We also demonstrate that only at irradiation at 1030 nm, the damage process depends on the peak intensity and not the photon flux. This means that if the laser pulses are adjusted to below the laser peak intensity threshold for ablation, the dwell time can be increased to photon flux beyond the associated photon flux to the peak intensity damage threshold. This is not the case for irradiation at 515 nm and 343 nm where the damage process is dominated by the photon flux threshold rather than the peak intensity threshold. We edited the abstract, Discussion, and Conclusion to make the claims clearer.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The author addressed all the concerns raised.

Reviewer #2 (Remarks to the Author):

I'm not entirely thrilled with the way that the authors first wrote their manuscript with wide claims, then when challenged on those, seemed to feel it was a miscommunication or lack of understanding on the reviewers part (reviewers 2 and 3 in particular).

Nevertheless as the work stands, I don't think it would benefit from any specific further request from my side. It can be accepted for publication.

Reviewer #3 (Remarks to the Author):

I have now finished to read the revised paper from Soyeon Jun and colleagues. I am pleased that the authors have substantially modified the abstract, results, discussion and conclusion of their article, so that their conclusions and assertions now reflect their data. However, I still have some problems with the scope of the study chosen by the authors for several reasons listed below, that could be corrected by explaining more accurately what this article is about:

• As I explain below, the authors do not study the influence of phototoxicity in the case of deep tissue imaging, but rather in the case of a long interaction length, as this would require considering the effect of scattering and optical aberrations, which is not the case with the transparent samples used here.

• Given the differences in term of repetition rate (40 to 80MHz for two-photon imaging and 100kHz to 10MHz for three photon imaging) or numerical aperture (0.8 to 1.0) I wonder if the conclusion found here could be directly applied to the field of multiphoton microscopy? The authors need to better explain in which aspects this work could be interesting for this field as some of the parameters used here are different to what is usually used

• As for novelty, a previous study has already explored some of these aspects in zebrafish embryos at 1030nm for similar repetition rates and pulse durations, published in 2020 (Maioli et al Biomedical Optics Express, 11(10) 6012-6026, 2020, https://doi.org/10.1364/BOE.400113).

Please find below some aspects that needs clarifications:

Rebuttal letter: “As it is correctly addressed by the reviewer the ROS generation from labels can influence the damage threshold due to the excessive ROS generation. As explained above, the damage caused by irradiation at 343 nm and 515 nm, includes the ROS generation due to linear absorption in fluorescence labels and the tissue. In the measurements performed at 1030 nm, the samples were labeled either by i) GFP with a nonlinear absorption pathway at 1030 nm, or ii) mKate2 with no direct or nonlinear absorption pathway. To address the extent of the influence of labels on damage threshold we added Fig 2f to the manuscript. The figure shows that for samples irradiated at 1030 nm, the peak intensity damage threshold for ablation doesn’t vary extensively in the presence (GFP labels) or absence (mKate2) of nonlinear absorption pathways in labels.”

Concerning the evaluation of photodamage and phototoxicity with and without labelling, by looking at the two-photon excitation spectra available on the database fpbase (Lambert, Nature Methods 16, 277-278 2019, https://doi.org/10.1038/s41592-019-0352-8 & https://www.fpbase.org/):

• for mKate2, the two-photon absorption cross-section at 1030nm is about twice as small as at 1130nm, which corresponds to the peak of the two-photon cross-section, so in this case a non-linear absorption pathway is surely involved?

• For eGFP, the two-photon absorption cross-section at 1030 nm is around 25 times smaller than at 930 nm, corresponding to the peak of the two-photon cross-section. In this case, the non-linear absorption pathway is significantly smaller, but could still have an effect on phototoxicity ?

Could the authors clarify this aspect, as it seems to me that they state the opposite? However, the fact that there is a difference between the two cases (eGFP and mKate2, labeling) is already an indication in itself, but to conclude that the fluorophore has no influence is an extrapolation. To make such a claim, one would have to perform the experiment without fluorophores, for example with wild-type samples, and even then there may be endogenous fluorophores that have a non-negligible two- or three-photon absorption cross-section around 1030 nm, such as NADH.

Rebuttal letter: “We study the damage based on long working range focusing optics which are crucial for deep tissue imaging. At the focusing geometry, the generated free electron affects the beam propagation and peak intensity before the beam reaches the waist. This causes a different damage threshold and mechanism compared to the damage caused by high numerical aperture, and short working distance focusing geometry.”

The term "deep tissue imaging" regularly used in the manuscript and rebuttal letter generally refers to the imaging of scattering samples, such as brain tissue, at a depth of several mean free paths, i.e. around 500 to 1000 µm for two-photon and three-photon imaging. Zebrafish embryos are fairly transparent, scattering light only slightly for illumination and detection in the near infrared and visible range. The main source of disturbance here are optical aberrations caused by slow spatial variations in refractive index. The fact that the authors are able to provide very well contrasted Bright Field images is a good confirmation of this hypothesis. I think the use of the term "deep tissue imaging" is irrelevant here because the authors are not studying the influence of excitation beam scattering, which is another study in itself. However, I agree with the authors that it is interesting to quantify the effect of a "long" interaction length. Secondly, as far as the working distance of the microscope objectives usually used in multiphoton microscopy is concerned, it's around 2 to 3 mm, which never exceeds the penetration depth of most biological samples, unless they are totally transparent, but then there's no point in using multiphoton microscopy, so I don't understand why it would be necessary to study the effect of a longer working distance here? Finally, with regard to the numerical aperture (around 0.5 for this study), if it is too different from what people usually use in multiphoton microscopy, would the results of this study be transposable to the field of multiphoton microscopy? Does this mean that the authors need to refine their initial questions?

Main Text: “For the successful implementation of cutting-edge nonlinear microscopy techniques for deep-tissue, in vivo imaging and designing their use for medical applications, a comprehensive understanding of the non-invasive, optimal operational parameters and constraints of the imaging system is essential”. & Rebuttal letter: "We appreciate the reviewer’s comment. The reviewer refers to a previous work that was performed at 80 MHz by Talone et al (Biomedical Optics Express, 2021). This regime has been very well studied. Our work studies the behavior of deep tissue with long working range focusing geometry at 127 kHz and 2 MHz repetition rates. As explained above: At this focusing geometry, the generated free electron affects the beam propagation and peak intensity before the beam reaches the waist. This causes a different damage threshold and mechanism compared to the damage caused by high numerical aperture, and short working distance focusing geometry.”

Again, I have the same comments about these two sentences. Multiphoton microscopy uses high numerical aperture focusing optics with a repetition rate of 20 to 80 MHz for two-photon microscopy, and a few hundred kHz to a few MHz for three-photon microscopy. If this study does not concern the high repetition rate regime and high numerical aperture focusing optics, does this mean that the authors' conclusions would not be useful for the field of nonlinear microscopy? Could you clarify these aspects, which are currently unclear to me?

Rebuttal letter: “To the best of our knowledge, there is no measurement in label-free deep tissue in a live vertebrate species with long working range focusing optics” & “These questions have not been investigated in the interaction of femtosecond pulses at near-infrared with deep tissue. There are always several damage mechanisms occurring simultaneously. Our study aims to address multi-dimensional aspects of the damage in deep tissue upon irradiation with 1030 nm femtosecond pulses.”

The author should probably have a look to the following paper which has already investigated the effect of linear and nonlinear photodamage induced by excitation pulses centered at 1030nm and for repetition rate ranging from 0.1kHz to 4MHz with long working range focusing optics: Maioli et al Biomedical Optics Express, 11(10) 6012-6026, 2020. This has been done in live zebrafish embryos similarly to the authors but not in the same location, in the beating heart instead of the central nervous system. However, this study in itself is still interesting as it explore this issue in a more general manner and in a different sample location, the central nervous system which is common to many species.

Main text: “Moreover, we demonstrate that it is possible to extend the dwell time; therefore, photon flux, extensively and noninvasively for irradiation at 1030 nm, as long as the peak intensity is below the low-plasma density threshold. In this regime, the associated photon flux to the peak intensity damage threshold can be exceeded by increasing the dwell time without observation of the damage in the sample”

This would probably need to be tested over longer imaging duration than 30s. As mentioned by reviewer 2 the production of ROS can affect the cells viability over extended illumination period longer than 30s as they could accumulate gradually during the illumination with the laser beam.

Reviewer #1 (Remarks to the Author):

The author addressed all the concerns raised.

We thank this reviewer for their previously helpful suggestions and insightful comments, which have improved the manuscript. No further changes are necessary.

Reviewer #2 (Remarks to the Author):

I'm not entirely thrilled with the way that the authors first wrote their manuscript with wide claims, then when challenged on those, seemed to feel it was a miscommunication or lack of understanding on the reviewers part (reviewers 2 and 3 in particular). Nevertheless as the work stands, I don't think it would benefit from any specific further request from my side. It can be accepted for publication.

We thank this reviewer for their previously helpful suggestions and insightful comments, which have improved the manuscript. No further changes are necessary.

Reviewer #3 (Remarks to the Author):

I have now finished to read the revised paper from Soyeon Jun and colleagues. I am pleased that the authors have substantially modified the abstract, results, discussion and conclusion of their article, so that their conclusions and assertions now reflect their data.

We thank reviewer 3 for their previously helpful suggestions and insightful comments, which have improved the manuscript.

However, I still have some problems with the scope of the study chosen by the authors for several reasons listed below, that could be corrected by explaining more accurately what this article is about:

• As I explain below, the authors do not study the influence of phototoxicity in the case of deep tissue imaging, but rather in the case of a long interaction length, as this would require considering the effect of scattering and optical aberrations, which is not the case with the transparent samples used here.
• Given the differences in term of repetition rate (40 to 80MHz for two-photon imaging and 100kHz to 10MHz for three photon imaging) or numerical aperture (0.8 to 1.0) I wonder if the conclusion found here could be directly applied to the field of multiphoton microscopy? The authors need to better explain in which aspects this work could be interesting for this field as some of the parameters used here are different to what is usually used
• As for novelty, a previous study has already explored some of these aspects in zebrafish embryos at 1030nm for similar repetition rates and pulse durations, published in 2020 (Maioli et al Biomedical Optics Express, 11(10) 6012-6026, 2020, https://doi.org/10.1364/BOE.400113).

We provided answers to the reviewer in what follows as the above summary of the raised points by the reviewer are discussed in more detail in the following paragraphs.

Please find below some aspects that needs clarifications:

Rebuttal letter: “As it is correctly addressed by the reviewer the ROS generation from labels can influence the damage threshold due to the excessive ROS generation. As explained above, the damage caused by irradiation at 343 nm and 515 nm, includes the ROS generation due to linear absorption in fluorescence labels and the tissue. In the measurements performed at 1030 nm,

the samples were labeled either by i) GFP with a nonlinear absorption pathway at 1030 nm, or ii) mKate2 with no direct or nonlinear absorption pathway. To address the extent of the influence of labels on damage threshold we added Fig 2f to the manuscript. The figure shows that for samples irradiated at 1030 nm, the peak intensity damage threshold for ablation doesn't vary extensively in the presence (GFP labels) or absence (mKate2) of nonlinear absorption pathways in labels."

Concerning the evaluation of photodamage and phototoxicity with and without labelling, by looking at the two-photon excitation spectra available on the database fpbase (Lambert, Nature Methods 16, 277-278 2019, https://doi.org/10.1038/s41592-019-0352-8 & https://www.fpbase.org/):
• for mKate2, the two-photon absorption cross-section at 1030nm is about twice as small as at 1130nm, which corresponds to the peak of the two-photon cross-section, so in this case a non-linear absorption pathway is surely involved?

• For eGFP, the two-photon absorption cross-section at 1030 nm is around 25 times smaller than at 930 nm, corresponding to the peak of the two-photon cross-section. In this case, the non-linear absorption pathway is significantly smaller, but could still have an effect on phototoxicity ? Could the authors clarify this aspect, as it seems to me that they state the opposite? However, the fact that there is a difference between the two cases (eGFP and mKate2, labeling) is already an indication in itself, but to conclude that the fluorophore has no influence is an extrapolation. To make such a claim, one would have to perform the experiment without fluorophores, for example with wild-type samples, and even then there may be endogenous fluorophores that have a non-negligible two- or three-photon absorption cross-section around 1030 nm, such as NADH.

We thank the reviewer for this valuable comment and the important point raised. The reviewer has a valid argument for the mechanism behind the old Fig 2f. To understand the underlying mechanism of the damage in more detail, and following the suggestion of the reviewer we have repeated the damage threshold measurements on 40 (unlabeled) wild-type zebrafish and labeld zebrafish. The samples were illuminated at 127 kHz or 2 MHz under three conditions: 1. Wild-type zebrafish, 2. *elavl3*:rasmKate2 transgenic zebrafish in which neurons were labeled with mKate2, and 3. *elavl3*:GFP-F transgenic zebrafish in which neurons were labeled with GFP. Our results indicate a higher damage threshold for GFP-labeled samples compared to the mKate2 samples indicating the influence of the nonlinear absorption path in mKate2 for irraditation with laser at 127 kHz repetition rates. However, at higher repetition rates, the difference becomes negligible which we associate with the effect of nonlinear photochemistry which takes over in the presence of a higher number of photons and at lower laser peak intensity. Based on the request of the reviewer we also studied the case of wild zebrafish, showing a higher damage threshold than the labled cases. The new measurements are visualized in the new Fig 2f and the text was edited accordingly.

Rebuttal letter: "We study the damage based on long working range focusing optics which are crucial for deep tissue imaging. At the focusing geometry, the generated free electron affects the beam propagation and peak intensity before the beam reaches the waist. This causes a different damage threshold and mechanism compared to the damage caused by high numerical aperture, and short working distance focusing geometry. The term "deep tissue imaging" regularly used in the manuscript and rebuttal letter generally refers to the imaging of scattering samples, such as brain tissue, at a depth of several mean free paths, i.e. around 500 to 1000 μm for two-photon and three-photon imaging. Zebrafish embryos are fairly transparent, scattering light only slightly for illumination and detection in the near infrared and visible range. The main source of disturbance here are optical aberrations caused by slow spatial variations in refractive index. The fact that the authors are able to provide very well contrasted Bright Field images is a good confirmation of this hypothesis. I think the use of the term "deep tissue imaging" is irrelevant here because the authors are not studying the influence of excitation beam scattering, which is another study in itself.

However, I agree with the authors that it is interesting to quantify the effect of a "long" interaction length.

We thank the reviewer for this helpful comment. We would like to emphasize that the only parameter that our study doesn't include compared to the deep-tissue measurement of the brain is the effect of the scattering of the photons. The scattering results in lower intensity at the focal area, which requires higher pulse energy to be used to observe the same effect. However, to avoid any confusion by the readers and following the recommendation of the reviewer, we removed the emphasis on the deep-tissue and replaced the word deep-tissue with long-range imaging through the text when seemed proper (highlighted in red in the manuscript).

Secondly, as far as the working distance of the microscope objectives usually used in multiphoton microscopy is concerned, it's around 2 to 3 mm, which never exceeds the penetration depth of most biological samples, unless they are totally transparent, but then there's no point in using multiphoton microscopy, so I don't understand why it would be necessary to study the effect of a longer working distance here? Finally, with regard to the numerical aperture (around 0.5 for this study), if it is too different from what people usually use in multiphoton microscopy, would the results of this study be transposable to the field of multiphoton microscopy? Does this mean that the authors need to refine their initial questions?
Main Text: "For the successful implementation of cutting-edge nonlinear microscopy techniques for deep-tissue, in vivo imaging and designing their use for medical applications, a comprehensive understanding of the non-invasive, optimal operational parameters and constraints of the imaging system is essential". & Rebuttal letter: "We appreciate the reviewer's comment. The reviewer refers to a previous work that was performed at 80 MHz by Talone et al (Biomedical Optics Express, 2021). This regime has been very well studied. Our work studies the behavior of deep tissue with long working range focusing geometry at 127 kHz and 2 MHz repetition rates. As explained above: At this focusing geometry, the generated free electron affects the beam propagation and peak intensity before the beam reaches the waist. This causes a different damage threshold and mechanism compared to the damage caused by high numerical aperture, and short working distance focusing geometry." Again, I have the same comments about these two sentences. Multiphoton microscopy uses high numerical aperture focusing optics with a repetition rate of 20 to 80 MHz for two-photon microscopy, and a few hundred kHz to a few MHz for three-photon microscopy. If this study does not concern the high repetition rate regime and high numerical aperture focusing optics, does this mean that the authors' conclusions would not be useful for the field of nonlinear microscopy? Could you clarify these aspects, which are currently unclear to me?

We appreciate the reviewer's comment. The scope of the manuscript covers the illumination requirement beyond multi-photon or fluorescence microscopy as discussed in the conclusion. This study is particularly crucial for label-free imaging and novel multi-dimensional microscopy techniques especially due to the current availability of broadband laser sources at 1030 nm. Moreover, novel types of field microscopy are emerging where broadband femtosecond pulses are used for capturing label-free images based on the time-resolved detection of their molecular response. These novel detection techniques (please see a review paper by Herbst et al, Journal Of Physics B, 55, 172001 (2022)) allow for i) the detection of the molecular response with attosecond temporal resolution (praised by a Nobel prize in Physics in 2023), ii) high detection dynamic range and sensitivity, and iii) high spatial resolution. The laser sources required for these measurements are operating at below 5 MHz, they require a new type of focusing technique as lens objectives are highly dispersive. The condition studied in this work is beneficial for any type of broadband femtosecond label-free microscopy technique. Moreover, understanding of these types of focusing geometries is crucial for neuro-regeneration studies, where precise and reproducible damage areas, deep in the sample are required for research purposes.

Rebuttal letter: "To the best of our knowledge, there is no measurement in label-free deep tissue in a live vertebrate species with long working range focusing optics" & "These questions have not

been investigated in the interaction of femtosecond pulses at near-infrared with deep tissue. There are always several damage mechanisms occurring simultaneously. Our study aims to address multi-dimensional aspects of the damage in deep tissue upon irradiation with 1030 nm femtosecond pulses."
The author should probably have a look to the following paper which has already investigated the effect of linear and nonlinear photodamage induced by excitation pulses centered at 1030nm and for repetition rate ranging from 0.1kHz to 4MHz with long working range focusing optics: Maioli et al Biomedical Optics Express, 11(10) 6012-6026, 2020. This has been done in live zebrafish embryos similarly to the authors but not in the same location, in the beating heart instead of the central nervous system. However, this study in itself is still interesting as it explore this issue in a more general manner and in a different sample location, the central nervous system which is common to many species.

We thank the reviewer for the comment and for drawing our attention to this publication. As per the reviewer's suggestion, we have cited the paper by Maioli et al. in the main text. We would like to point out that the authors used a much lower NA (0.1) compared to us at 0.5 and they are studying the regime of wide-field microscopy. Their study includes repetition rates of 1-40 MHz, where they observe thermal damage of the sample. This effect is absent in our regime of high-intensity operation. However, they report damage at 30 mW for 1MHz pulses which is in good agreement with our 10 mW damage threshold report due to our higher NA. Inspired by the work of Maioli et al, we also performed an additional measurement on various repetition rates for the new 40 samples which are added to the SI as new Fig. 3.

Main text: "Moreover, we demonstrate that it is possible to extend the dwell time; therefore, photon flux, extensively and noninvasively for irradiation at 1030 nm, as long as the peak intensity is below the low-plasma density threshold. In this regime, the associated photon flux to the peak intensity damage threshold can be exceeded by increasing the dwell time without observation of the damage in the sample"

This would probably need to be tested over longer imaging duration than 30s. As mentioned by reviewer 2 the production of ROS can affect the cells viability over extended illumination period longer than 30s as they could accumulate gradually during the illumination with the laser beam.

We appreciate the reviewer's comment. As mentioned in the text, for this series of measurements we irradiated the samples for up to 900 s and did not observe any visible damage as plotted in Figure 2e)

REVIEWERS' COMMENTS:

Reviewer #3 (Remarks to the Author):

They are several problems with the bibliography, some articles are cited incorrectly and their contributions mentioned inaccurately. Below you will find the errors I spotted among the 116 references. For the rest, I am satisfied with the new version submitted by the authors, which can now be published after correction of the references mentioned.

“Moreover, due to its large penetration depth and image contrast, label-free microscopy has become a crucial tool for deep-tissue and long-range imaging [11, 45–50]” Ref 11 and 45 to 50 are about three-photon microscopy using fluorescent probes such as dextran, RFP, EGFP, GCAMP, Aggregation-induced emission luminogens (AIEgens), although some of the papers mentioned that third harmonic generation could be detected at the same time as three photon excited fluorescence. But the main scope of all these papers is not label free imaging, as is incorrectly stated in the manuscript.

“The complementary information provided by both categories of microscopy has led to the development of more advanced techniques, such as multimodal nonlinear microscopy [51–55]” Ref 54 is about the use of light-sheet microscopy with linear excitation ie CW lasers at 488 and 637nm for fast in-situ volumetric histological imaging. So, this ref is not about multimodal nonlinear microscopy.

“Over the last decades, a significant body of research has been conducted on the effects of short laser pulses on thin in vitro and ex vivo samples in nonlinear fluorescence microscopy [1, 19, 56–60]”. This is incorrect as Ref 60 explores the interplay between linear and nonlinear photoxicity in live zebrafish embryo in the beating heart. For this particular ref, it's neither in vitro nor ex vivo, but in vivo.

“These findings significantly contribute to advancements in innovative microscopy techniques, like multimodal microscopy [102, 103]”. In Ref 102 the authors convert fs pulses into ps pulses to get the proper spectral resolution in Stimulated Raman Scattering and use the same pulses to produce two-photon excited fluorescence. So, the results find here would not apply to these types of microscopy techniques.

Reviewer #3 (Remarks to the Author):

They are several problems with the bibliography, some articles are cited incorrectly and their contributions mentioned inaccurately. Below you will find the errors I spotted among the 116 references. For the rest, I am satisfied with the new version submitted by the authors, which can now be published after correction of the references mentioned.

“Moreover, due to its large penetration depth and image contrast, label-free microscopy has become a crucial tool for deep-tissue and long-range imaging [11, 45–50]” Ref 11 and 45 to 50 are about three-photon microscopy using fluorescent probes such as dextran, RFP, EGFP, GCAMP, Aggregation-induced emission luminogens (AIEgens), although some of the papers mentioned that third harmonic generation could be detected at the same time as three photon excited fluorescence. But the main scope of all these papers is not label free imaging, as is incorrectly stated in the manuscript.

“The complementary information provided by both categories of microscopy has led to the development of more advanced techniques, such as multimodal nonlinear microscopy [51–55]” Ref 54 is about the use of light-sheet microscopy with linear excitation ie CW lasers at 488 and 637nm for fast in-situ volumetric histological imaging. So, this ref is not about multimodal nonlinear microscopy.

“Over the last decades, a significant body of research has been conducted on the effects of short laser pulses on thin in vitro and ex vivo samples in nonlinear fluorescence microscopy [1, 19, 56–60]”. This is incorrect as Ref 60 explores the interplay between linear and nonlinear photoxicity in live zebrafish embryo in the beating heart. For this particular ref, it's neither in vitro nor ex vivo, but in vivo.

“These findings significantly contribute to advancements in innovative microscopy techniques, like multimodal microscopy [102, 103]”. In Ref 102 the authors convert fs pulses into ps pulses to get the proper spectral resolution in Stimulated Raman Scattering and use the same pulses to produce two-photon excited fluorescence. So, the results find here would not apply to these types of microscopy techniques.

RESPONSE: We thank the reviewer for their comments. We implemented the required changes by the reviewer in the bibliography of the manuscript.