

Three-photon population imaging of subcortical brain regions

Corresponding Author: Dr Hadas Frostig

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Frostig and colleagues present a large imaging field of view (FOV) three-photon microscope, denoted as LIFT. In combination with an optimized sampling strategy/custom scanning module and deep learning-based denoising, authors showcase calcium imaging in subcortical brain regions over a large FOV, while maintaining frame rate and signal-to-noise ratio (SNR). The proposed approach is well presented through both simulations and experimental validation. The inclusion of a detailed list of optical components is very helpful, as it facilitates reproducibility. However, the concept of sparse sampling itself is not novel and comes at a cost of reduced resolution.

1. The sparse sampling approach leads to a gain in FOV, frame rate and SNR, but the imminent loss in spatial resolution could be undesirable for certain applications, especially those requiring visualization of fine cellular structures, such as axons or fine cellular processes. On this note, could the authors show and discuss how their system performs apart from imaging neuronal somata. For example, by imaging astrocytic processes or capillary networks? Also, comment on this problem in the discussion.
2. Could authors discuss the advantages/disadvantages of their approach of increasing FOV, compared to already published work, f.e. in a table for ease of comparison?
3. It is unclear whether the comparison presented in Fig. 2c is based on equivalent spatial sampling rates, or if different sampling rates and corresponding spatial resolution were used in the referenced studies. This should be clarified. Since frame rate, sampling rate, FOV, resolution, SNR are interdependent parameters, discussing only one of these factors without accounting for the others results in a one-sided comparison.
4. In Fig.3 authors present a large FOV volumetric image of neurons in vivo, claiming to cover $2 \times 2 \times 1.23$ mm. However, the image shown (b,c) rather covers less area, as measured by the provided scale bar and considering the circular imaging FOV. Also provide a representative image from the stack corresponding to the CC. Please comment.
5. Please comment on the elliptical shape of neurons in Fig. 4.
6. Does the low sampling rate at $4 \mu\text{m}$ affect image co-registration between frames? This could be critical, particularly for awake imaging to reduce motion artifacts.
7. Authors mention the term “noninvasive”, which is an overstatement, considering the need for a cranial window. Please rephrase.
8. The sampling rate at the galvanometer’s turnaround is quite high (Ext.Fig.3), which could also lead to increased heat deposition. How would it influence the heating simulation in Fig. S4? And how is this issue mitigated?

Minor:

- Please adjust the order of figure/supplementaries and extended figure appearances in the manuscript, as they do not follow their numerical sequence causing some confusion.
- In Fig.1 change “um” to “ μm ”.

Reviewer #2

(Remarks to the Author)

Three-photon population imaging of subcortical brain regions

This manuscript describes the development of a Large Imaging Field-of-view Three-photon (LIFT) microscope, capable of imaging over fields-of-view $> 3 \text{ mm}^2$ using only off-the-shelf components, and its application to deep imaging in mouse and rat brains. The authors combine several key elements. First, they implement a simple and elegant approach to double the scan speed by adding a second galvanometer, while preserving flexibility in FOV size, frame rate, and focal spot spacing. Second, they perform optimized spatial and temporal sampling. This analysis is broadly relevant for anyone working with 3-photon imaging, independent of FOV size, and is therefore of clear value to the community. Although the specific sampling parameters are selected based on GCaMP6s kinetics and an assumed $\sim 10 \text{ }\mu\text{m}$ cell size, the underlying framework (e.g., Fig. 1i) is general and can be adapted to indicators with different kinetics or structures of differing spatial scale. Finally, the authors apply deep-learning-based denoising to calcium imaging data, enabling a substantial increase in the number of detected cells. Together, these advances allow high-SNR cellular-resolution functional imaging of neuronal populations in the mouse hippocampus and rat motor cortex, at depths up to 1.2 mm.

This is a technically strong and impactful contribution that advances the field of three-photon microscopy toward broader accessibility & practical large-scale neuronal population imaging. The manuscript is well organized and clearly written. The points below are intended to improve clarity and reproducibility.

- Imaging on turnaround and unwarping. In Extended Data Fig. 3, the authors show the beam position and corresponding spot spacing across the scan. This plot is presumably derived from the galvanometer position-feedback signal, combined with the known scan-relay geometry to convert angle to lateral position. Stating this explicitly (even briefly in the caption of Extended Data Fig. 3) would help clarify what was measured and how the plot was generated. Additionally, while the manuscript notes that imaging during the turnaround region is included, the implementation details are limited. It would be useful to expand on how pixel indices are remapped to their correct spatial coordinates (using the known position-versus-time waveform), the unwarping part. The Methods section (lines 310-315) specifies the turnaround time for Figs. 3,4,5, but explicitly stating how much the field of view increased in each example by including the turnaround region would help readers more clearly appreciate the improvement.
- Figure 3 illustrates the deep, large FOV that LIFT enables. However, the representative images in panels b and c show fluorescent structures over a smaller lateral extent than the full $2 \text{ mm} \times 2 \text{ mm}$ FOV. Figure 6 shows a similar effect. A brief clarification of why only part of the nominal FOV contains visible fluorescence (for example, due to expression boundaries or anatomical constraints) would be helpful.
- Fig S4: There appear to be some inconsistencies in the panel references in the caption.
- Extended Data Fig. 6 has panels a,c,d,e,g,h (missing b and f). The figure caption says that b and f should be the contours of the cells shown in a and e.
- Extended Data Fig. 11: The axial PSF reported here is $\sim 6 \text{ }\mu\text{m}$, which is somewhat larger than what might be expected under ideal diffraction-limited conditions for the stated NA and excitation wavelength. Because the axial extent can be influenced by several factors (e.g., degree of back-aperture filling), a brief clarification of the measurement conditions would be helpful for interpretation. Additionally, the fluorescein image appears spatially non-uniform and noisy. A brief note on whether this reflects raw (non-denoised and non-averaged) data would be useful.
- Supplementary Notes, page 7: The text refers to Fig. 1c at the end of the middle paragraph. It appears this should be Fig. 1d.
- Supplemental Table 2 provides ranges of excitation powers. It would be helpful to indicate the specific powers used in the representative frames shown in the main figures (for example, Fig. 3b,c). For Figs. 4 and 5, the reported range of 107–180 mW is quite large, even though the imaging depths are similar. Adding the actual excitation power used for the displayed data, either within the same table or in the corresponding figure captions, would improve clarity.
- Adding depth labels to Video 1 would be helpful.

Sulekh Fernando-Peiris and Cristina Rodríguez

Reviewer #3

(Remarks to the Author)

Intravital microscopy deep inside tissues was greatly improved using 3-photon (3P) microscopy, however this approach lacks the ability for large-scale cellular-resolution noninvasive imaging techniques with high scan speed. Frostic and co-workers developed a Large Imaging Field of view Three-photon (LIFT) microscope which delivers a field of view of $>3 \text{ mm}^2$ and reaches imaging depth in the brain beyond 1 mm. The approach contains a new strategy for scanning with galvanometers to maintain the strengths of 3-photon microscopy yet greatly improve scanning speed. The scanhead configuration seems to build upon the by-now discontinued Biorad confocal scanner, but here using 3 instead of 2 galvanometers. Parameters tested include effect of under sampling (large pixels, small psf) on image quality and linear phototoxicity burden. The strategy and application of AI-based denoising significantly improves the image quality. As outcome, live-scanning 3P microscopy of large brain areas with single-cell resolution over several minutes was achieved. This was showcased by fast (6 Hz) large-field scanning of calcium activity recorded at 1 mm depth in the mouse motor cortex during locomotion of the awake mouse, which allowed to detect a distinct signaling-active cell population during locomotion bouts.

The combined strategy is impressive and will advance the field of 3P microscopy in neuroscience. Potentially, with implementation of multi-color scanning, other fields depending on large-field scanning and fast acquisition rates may benefit, including, e.g., preclinical models in immunology/immunotherapy.

As main concerns, the extent of phototoxicity caused by the high average laser power and pulse energy used requires further clarification.

1. Very high average laser powers may lead to linear and nonlinear toxicity. The psf and nonlinear toxicity threshold have not been characterized and therefore, it is unclear if toxicity thresholds are reached upon repeated or prolonged scanning. The nonlinear effects were in part addressed for short-term imaging, by the lack of increased background GCamP6 signal during scanning. However, it remains unclear whether microanatomy and neuronal functionality after a prolonged scanning period (e.g., 15 or 30 min) remain preserved, which could be demonstrated, e.g., by re-recording the same region the following day. Why is the initial power at the brain surface 5x higher for rats? Does this not cause nonlinear toxicity? Could the larger psf of the 0.8 NA objective (as compared to 1.05 NA objectives used in other studies) cause higher toxicity thresholds in this study? This should be discussed.

2. In addition, the extent of linear toxicity in out-of-focus, upper tissue regions in between the objective and the focal plane, remain to be addressed.

Minor points

Pulse length: Was this also measured under the objective?

The optical resolution is not clear. What is the 3-photon excitation psf of the 0.8 NA objective @1300 nm in their setup?

In the figure captions it was mentioned 'below the dura' but in the methods it was mentioned that the dura has been removed in the rat model. Was the dura intact for the mouse model?

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have adequately addressed my previous concerns. The added experiments and clarifications significantly strengthen the work. Apart from minor corrections (below) I have no further comments and am happy with the manuscript in its current form.

Minor: - In Supplementary Table 1: Change Ref. 31, to Zhao et al. 2023.

- Source data link to googledrive was not functional at the time of review.

Reviewer #2

(Remarks to the Author)

The authors clearly addressed all of our questions, and we are satisfied with the corresponding changes made to the manuscript.

A couple typos:

- Supplementary Fig. 3: Panels f and g, red line legend reads 1 mW, 1 μm for both, but the captions say "1 mW excitation with 0.5 μm sampling". Is it 1 μm or 0.5 μm spacing?
- Supplementary Fig. 5: captions were corrected and refer to panels a,b,c,d,e,f (no more mention of contours) but the actual figure has labels a,c,d,e,g,h (instead of a,b,c,d,e,f)

Reviewer #3

(Remarks to the Author)

The authors have done a great job in revising this manuscript. I have no further concerns or comments.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

We sincerely thank the reviewers for their careful evaluation of our manuscript and for the constructive comments and suggestions. In response, we have performed new experiments, carried out additional numerical work, and conducted new analyses to address the reviewers' concerns. These additions are reflected in our revised manuscript, which includes new and revised figures and tables (Reviewer Table 1), as well as corresponding updates to the text. We have also revised the discussion section to clarify the interpretation and limitations of the results. We believe these changes have strengthened the manuscript and improved the quality and clarity of the work. We hope that the revised version satisfactorily addresses the reviewers' comments. We include a table summarizing all changes made to the manuscript's display items below.

Main text

Item	Panel	Changes
Fig. 2	c	Added ref. 31
Fig. 3	c	New
Fig. 4	c-n	Modified
Fig. 5	b-f	Modified

Supplementary

Item	Panel	Changes
Fig. S3	All	New (in Supplementary Note 1.3)
Fig. 1	h	New
Fig. 4	f,g	Modified
Fig. 6	All	Modified
Fig. 13	All	New
Fig. 14	All	New
Table 1	All	New
Table 3	N/A	Modified
Video 1	N/A	Modified

Reviewer Table 1: All changes made to display items of the manuscript on revision

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

We would like to thank the reviewer for the time and effort they have dedicated to this thorough review. We feel that the process of addressing the concerns they raised has served to improve our manuscript's presentation and clarity. We address these concerns point-by-point below.

Frostig and colleagues present a large imaging field of view (FOV) three-photon microscope, denoted as LIFT. In combination with an optimized sampling strategy/custom scanning module and deep learning-based denoising, authors showcase calcium imaging in subcortical brain regions over a large FOV, while maintaining frame rate and signal-to-noise ratio (SNR). The proposed approach is well presented through both simulations and experimental validation. The inclusion of a detailed list of optical components is very helpful, as it facilitates reproducibility. However, the concept of sparse sampling itself is not novel and comes at a cost of reduced resolution.

1. The sparse sampling approach leads to a gain in FOV, frame rate and SNR, but the imminent loss in spatial resolution could be undesirable for certain applications, especially those requiring visualization of fine cellular structures, such as axons or fine cellular processes. On this note, could the authors show and discuss how their system performs apart from imaging neuronal somata. For example, by imaging astrocytic processes or capillary networks? Also, comment on this problem in the discussion.

The reviewer raises an important point: multiphoton imaging encompasses many applications beyond calcium imaging of neuronal somata. To address this concern, we performed additional experiments and analyses and added a new paragraph to the discussion section.

As the reviewer notes, multiphoton imaging is used for a broad range of applications, each with different spatial and temporal sampling requirements. This versatility is precisely what motivated the flexible design of LIFT: sampling can be optimized for the specific biological structure and signal of interest. Unlike other sparser-sampling approaches in multiphoton imaging, which rely on scanners with fixed scan rates or fixed fields of view, the LIFT scanner enables the sampling and frame rates to be tailored to the size and dynamics of the imaged structure. The guiding principle is that, in 3P imaging, the optimal strategy is to acquire only the minimum information required to resolve the relevant spatial structures and temporal responses. This minimizes unnecessary sampling, improves SNR, and maximizes the achievable field of view and/or frame rate.

In this work we were interested in somas that are $\sim 10\ \mu\text{m}$ in size and therefore chose $4\ \mu\text{m}$ sampling. A general rule of thumb is to follow the Nyquist criterion and choose a focal spot spacing slightly smaller than half the diameter of the smallest structure that needs to be resolved. This allows the structure to be resolved without oversampling, which leads to excessive heating and an unnecessary reduction in FOV.

In order to demonstrate this in experimental data, we performed new experiments in which we imaged mice with GCaMP-labelled astrocytes and studied the effects of focal spot spacing on the retrieved width and contrast of their processes. Focal spot spacings that obeyed the Nyquist criterion generally correctly resolved the width and preserved contrast (Figure S3 in Supplementary Note 1.3). Since there is variability in the size of biological structures, it is best to run a check on acquired data, as detailed in Supplementary Note 1, to optimize the focal spot spacing and frame rate to the desired segmentation fidelity.

As the reviewer notes, spatial sampling, FOV size, and frame rate are coupled and therefore choosing smaller focal spot spacings will require a reduction in FOV or frame rate. We address this point in the revised discussion section and give an example in Supplementary Note 1.3.

To make this point clearer in the text, the following **changes were made to the manuscript**:

1. Added section 1.3 in Supplementary Note 1, which walks the reader through the rationale described above and illustrates it with the astrocyte example.
2. Added Fig. S3 in Supplementary Note 1, copied below.
3. Rearranged Supplementary Note 1 into sections to improve readability.
4. Added the following paragraph in the discussion section:
“While this work focuses on calcium imaging of neuronal somata, there are many other applications of in vivo multiphoton imaging. For imaging of finer cellular

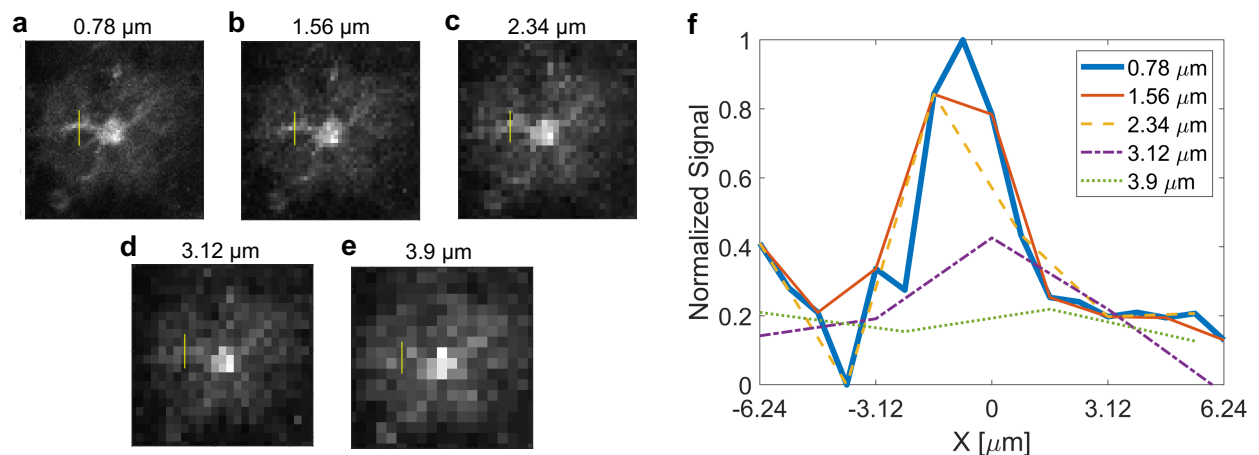


Figure S3 in Supplementary Note 1.3: The effect of focal spot spacing on the imaging of astrocytic processes. (a) An unprocessed three-photon image of a cortical astrocyte in mouse brain, acquired with $0.78\ \mu\text{m}$ focal spot spacing. (b)-(e) The same image as in a, with focal spot spacings of $1.56\ \mu\text{m}$, $2.34\ \mu\text{m}$, $3.12\ \mu\text{m}$, and $3.9\ \mu\text{m}$, respectively. (f) The intensity profiles along the yellow lines in a-e.

structures, focal spot distances smaller than 4 μm are required. A key advantage of LIFT is that it allows users to select the focal spot spacing, field of view, and frame rate best suited to their application (as in Supplementary Figs. 1a, 9, and 10). However, these three parameters are inherently coupled through a trade-off dictated by the optimal repetition rate for a given imaging depth. Thus, users can achieve finer spatial sampling by reducing either the FOV size or the frame rate. The optimal focal spot distance and frame rate for a particular application can be determined according to the procedures detailed in Supplementary Notes 1.1-1.3.”

2. Could authors discuss the advantages/disadvantages of their approach of increasing FOV, compared to already published work, f.e. in a table for ease of comparison?

LIFT can be compared to FOV expansion methods in other microscopy modalities (such as two-photon), to the one other work that expanded FOV in 3P imaging, or to other existing 3P systems. Please see all three comparisons below:

- i. For a comparison of the specifications of LIFT and all other 3P systems capable of performing calcium imaging around 1 mm depth, please see newly added Supplementary Table 1 (copied below), which was also added to address point 3 of this reviewer. The systems listed in this table are those compared to LIFT in Fig. 2c

	FOV size [mm²]	Lateral PSF size [μm]	Focal spot distance [μm]	Frame rate [Hz]	Animal mobility
LIFT large FOV mode	3.1	0.7	4	5.8	Head-fixed
LIFT video-rate mode	0.73	0.7	4	26.8	Head-fixed
LIFT high-resolution video-rate mode	0.02	0.7	0.78	27	Head-fixed
LIFT intermediate mode	0.28	0.7	1.95	9.65	Head-fixed
Ref. 25, Klioutchnikov et al. 2020	0.019	0.8	1.16	27.8	Head-mounted
Ref. 29, Ouzounov et al. 2017	0.04	N/A	0.78	8.5	Head-fixed
Ref. 30, Weisenburger et al. 2019	0.115	1.5	3.4	78	Head-fixed
Ref. 31, Zhou et al. 2023	0.0625	1	1	8.35	Head-mounted
Ref. 34, Mok et al. 2024	0.295	0.7	1.06	4.3	Head-fixed

Supplementary Table 1: A comparison of the specifications of LIFT and all other reported 3P systems capable of performing calcium imaging at around 1 mm depth.

of the manuscript.

- ii. LIFT is compared to the only other large FOV 3P system (Ref. 34) in Reviewer Table 2 below. Generally, the approach taken by Mok *et al.*, is to increase the number of focal spots scanned per frame in order to expand FOV (similar to Fig. 1c-d of main text). To do so, they both double the repetition rate and split the excitation light into two beamlets that scan the sample concurrently. However, because the heating limit constrains the total average excitation power delivered to the sample, it applies to the combined average power of the two beamlets. As a result, with twice the repetition rate and two simultaneous beamlets, the average power allocated to each beamlet must be reduced to one quarter of the original value. Since the 3P signal scales with the cube of the excitation power (Eq. 1 of the main text), this reduction in per-beamlet power leads to a substantial decrease in signal and, in turn, a reduction in maximum imaging depth. Consequently, the deepest calcium imaging demonstrated with this system is at 600 μm , a depth that can be imaged with many two-photon systems (please note that figure 4d of Ref. 34 was recorded with a different system that has a smaller FOV). Reviewer Table 2 compares all imaging parameters between the two systems. We did not compare to this system in Fig. 2c because they did not achieve hippocampal calcium imaging or imaging at hippocampus-equivalent depths ($\sim 1\text{ mm}$). We did compare LIFT to the smaller FOV system of Ref. 34 in Fig. 2c and Supplementary Table 1.

	LIFT large FOV mode	Ref. 34, Mok et al. 2024
Imaging depth [mm]	1.1	0.6
Imaging area	hippocampus	cortex
Frame rate [Hz]	5.8	2.6
FOV size [mm ²]	3.1	9.1
Focal spot distance [μm]	4	3.1
Advantages	• Large FOV functional imaging in the hippocampus • Flexible FOV and sampling rates • Resolves GCaMP6s calcium events	• Larger FOV • Adaptive excitation allows to shutter laser when scanning areas without cell bodies
Disadvantages	• Smaller FOV	• Cannot image layer 6b and below • Underresolves GCaMP6s events • Non-flexible resolution, FOV, and scan pattern

Reviewer Table 2: A comparison of LIFT and the one other published large FOV 3P imaging method.

- iii. LIFT is compared to FOV expansion methods used in two-photon microscopy and OCT in Fig. 1a-f of the manuscript and its accompanying text. FOVs in scanning microscopy can generally be increased either by expanding the focal spot size (Fig. 1a), or by increasing the number of focal spots scanned per image (Fig. 1c). The focal spot can be expanded by reducing the numerical aperture, which works relatively well in 2P, but causes a large drop in 3P signal (Fig. 1b). Alternatively, scanning more focal spots at the same frame rate requires either increasing the laser repetition rate directly or achieving an effective increase through the use of beamlets. In both cases, the 3P excitation power per focal spot must be reduced in proportion to the increase in the number of focal spots, leading once again to a substantial reduction in 3P signal (Fig. 1d). In contrast, high numerical aperture optimized sampling (Fig. 1e) preserves the signal magnitude (Fig. 1f).

Changes to manuscript: We added Supplementary Table 1, which compares the specifications of LIFT to those of all other reported 3P systems capable of performing calcium imaging around 1 mm depth.

3. It is unclear whether the comparison presented in Fig. 2c is based on equivalent spatial sampling rates, or if different sampling rates and corresponding spatial resolution were used in the referenced studies. This should be clarified. Since frame rate, sampling rate, FOV, resolution, SNR are interdependent parameters, discussing only one of these factors without accounting for the others results in a one-sided comparison.

We see this reviewer's point and have therefore added Supplementary Table 1, which lists all imaging parameters of the systems compared in Fig. 2c. There is, indeed, a trade-off between sampling rate, FOV and the frame rate. The purpose of this work was to design a system capable of performing large FOV, rapid imaging of somata, while maintaining the ability to choose any other set of acquisition parameters possible with a 1 MHz pixel rate.

Changes to manuscript:

1. Added Supplementary Table 1 and referenced it in the caption and text accompanying Fig. 2c.
2. Added a paragraph to the discussion section addressing the frame rate-FOV-spatial sampling rate trade-off.

4. In Fig.3 authors present a large FOV volumetric image of neurons in vivo, claiming to cover $2 \times 2 \times 1.23$ mm. However, the image shown (b,c) rather covers less area, as measured by the provided scale bar and considering the circular imaging FOV.

We thank the reviewer for this comment. We believe that the previous wording of the description of Fig. 3 might have been a bit confusing. We did not mean to claim to have a 2

x 2 mm FOV but rather a 2 mm-diameter FOV (Supplementary Fig. 12). While the scan performed to create Fig. 3 was in fact of a volume of 2 x 2 x 1.23 mm, the optical FOV is approximately a 2 mm-diameter circle (in fact it is a bit larger than 2 mm, as can be seen in Supplementary Fig. 7b). We therefore removed the parenthetical phrase: “(2 x 2 x 1.23 mm)” in the sentence introducing Fig. 3 (line 168) to avoid this confusion, and added wording that explicitly states that the optical FOV has a 2 mm diameter (see below).

In general, in all animals labeled with AAV injections, there is always nonuniformity in GCaMP labeling, which can reduce the apparent fullness of the FOV, as can shadows from blood vessels. This is the case also for the images in this work. To clarify this point, we added an explanation in the text in the paragraph describing Fig. 3. To assess LIFT's optical FOV independently of these two effects, we measured the FOV in a uniform fluorescein solution (see supplementary Fig. 7).

Changes to manuscript:

1. Removed the parenthetical phrase: “(2 x 2 x 1.23 mm)” in the sentence introducing Fig. 3, which caused confusion.
2. Added the sentence: “Although the diameter of the optical FOV is 2 mm (Supplementary Fig. 7b), spatial variability in GCaMP expression, as well as shadows from blood vessels, may result in dark regions, reducing the apparent FOV.” to the paragraph describing Fig. 3 in the main text.

Also provide a representative image from the stack corresponding to the CC. Please comment.

Great suggestion, we have added an image taken at a depth of 880 μm (newly added panel b in Fig. 3) which is representative of images taken in the corpus callosum.

Changes to manuscript: Added panel b in Fig. 3.

5. Please comment on the elliptical shape of neurons in Fig. 4.

We thank the reviewer for calling our attention to this matter. When looking at the structural stack (supplementary video 1), one can observe that some neurons, particularly those in the CC, are in fact a bit elongated (this corresponds to Fig. 4c). The hippocampal neurons, however, are mostly round but appeared a bit elongated in the previous version of Fig. 4. To correct this issue we implemented three changes to our processing/plotting pipeline:

1. We implemented background removal prior to the denoising of our time series. The background arises due to a slight asymmetry in the motion of the galvanometer, which creates an alternating line pattern in the direction of the fast axis (the vertical

direction of the images). In lower SNR data, the line pattern tends to 'smear' neurons along the fast axis.

2. We improved the accuracy of our unwarping algorithm to better correct the edges of our FOV. Inaccuracies in unwarping can elongate neurons along the fast axis as well.
3. We replotted all data so that the axis ratio is more accurate.

In Reviewer Fig. 1 below, we show a comparison of the shape of the neurons from the CA1 region in the previous version of Fig. 4f (panel a) and the revised version (panel b), showing improved neuron morphology. Pairs of arrows of the same color show examples of neurons that appeared elongated previously and no longer do.

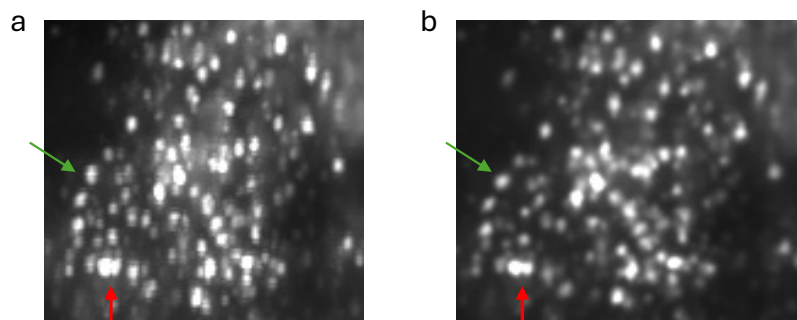
Changes to the manuscript: We reprocessed and replotted all our datasets with this issue.

6. Does the low sampling rate at 4 μm affect image co-registration between frames? This could be critical, particularly for awake imaging to reduce motion artifacts.

This is an important question. To test whether reduced spatial sampling degrades motion-correction performance, we performed new analyses of our data. We first denoised high-resolution time series and then removed data points to create additional time series with progressively larger focal-spot spacing. We then applied our motion-correction algorithm to these time series and compared the recovered motion along the x and y axes. The results for 0.8 μm and 4 μm spacing are compared in Supplementary Fig. 1h, where the x and y traces of the 4 μm spacing are vertically offset by 3 pixels for ease of comparison. We observed no difference between the x and y motion traces obtained from the two sampling conditions, suggesting that motion-correction performance is not measurably affected by optimized sampling.

Changes made to manuscript:

1. Added Supplementary Fig. 1h.
2. Added a paragraph in Supplementary Note 1.1 discussing the above analysis.



Reviewer Fig. 1: Data from Fig. 4f in the previous (panel a) and revised (panel b) versions of the manuscript. Arrows point to specific examples of neurons that appeared elongated in the previous version.

7. Authors mention the term “noninvasive”, which is an overstatement, considering the need for a cranial window. Please rephrase.

We see the reviewer’s point. All mentions of the term “noninvasive” were corrected to terms such as “in the intact brain”.

Changes made to manuscript: The term was replaced.

8. The sampling rate at the galvanometer’s turnaround is quite high (Ext.Fig.3), which could also lead to increased heat deposition. How would it influence the heating simulation in Fig. S4? And how is this issue mitigated?

We thank the reviewer for raising this concern and have addressed it in the manuscript with additional numerical work.

The distance between focal spots at the edge of LIFT’s FOV is indeed much smaller than 4 μm , which could lead to increased heating. However, the overall temperature rise is generally expected to be largest near the center of the FOV, where the greatest number of focal spots contribute to the accumulated heat load (as can be seen for the uniformly spaced 1 μm and 4 μm cases in Supplementary Fig. 3f, for example). Since the heat diffusion equation is linear, one can view the total heating as a sum of the heating from each focal spot. Each focal spot produces a temperature profile that is approximately Gaussian-like and centered on that spot. In the FOV center, long tails from many gaussians add up to cause a large increase in the temperature, whereas near the edges of the FOV, fewer neighboring focal spots contribute, resulting in a smaller cumulative temperature rise. In other words, at the edge of the FOV, heat can diffuse outward from the imaged region, without a corresponding inward heat flow from outside the FOV. Still, perhaps if the focal spots are close enough to each other at the FOV edge, the heating at the edges could be higher than the center despite this effect.

To test whether this is the case, we reran our in-plane heating simulation with nonuniformly spaced focal spots. We simulated a focal spot distribution that follows the distribution created by our x-galvo waveform. The resulting temperature profile was compared with those resulting from uniformly spaced 1 μm and 4 μm distributions, as shown in Supplementary Figs. 3f and 3g. The nonuniformly spaced distribution produced a temperature profile that was very similar to that of the uniformly spaced 4 μm distribution, with only a minor increase in heating near the FOV edges. The largest temperature increase remained at the center of the FOV, and the maximum tissue temperature was unchanged. This result is reassuring and shows that optimized sampling reduces tissue heating despite the increased sampling rate at the FOV edge.

Changes to the manuscript:

1. The results of the simulation with nonuniform focal spot spacing were added to Supplementary Figs. 3f and 3g.
2. A paragraph describing this simulation was added to Supplementary Note 2.

Minor:

- Please adjust the order of figure/supplementary and extended figure appearances in the manuscript, as they do not follow their numerical sequence causing some confusion.
- In Fig.1 change "um" to " μm ".

Thanks for calling our attention to these issues. **They have been fixed.**

Reviewer #2 (Remarks to the Author):

We would like to thank Sulekh Fernando-Peiris and Prof. Rodríguez for the time and effort they have dedicated to their thorough and helpful review. We feel that considering their comments has helped strengthen the clarity and overall presentation of the manuscript. Below we provide point-by-point responses to the issues they raised.

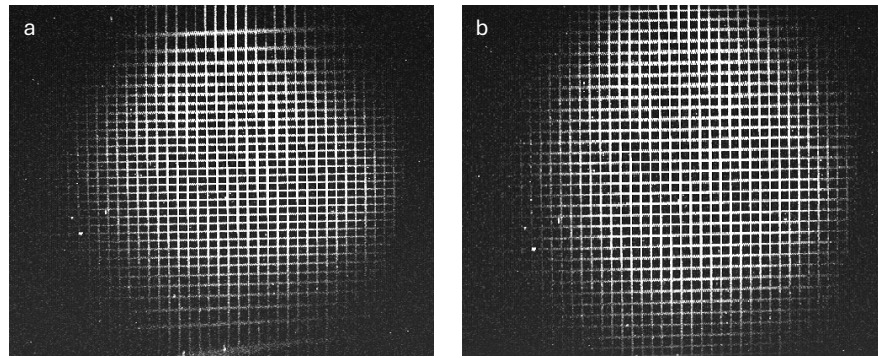
Three-photon population imaging of subcortical brain regions

This manuscript describes the development of a Large Imaging Field-of-view Three-photon (LIFT) microscope, capable of imaging over fields-of-view $> 3 \text{ mm}^2$ using only off-the-shelf components, and its application to deep imaging in mouse and rat brains. The authors combine several key elements. First, they implement a simple and elegant approach to double the scan speed by adding a second galvanometer, while preserving flexibility in FOV size, frame rate, and focal spot spacing. Second, they perform optimized spatial and temporal sampling. This analysis is broadly relevant for anyone working with 3-photon imaging, independent of FOV size, and is therefore of clear value to the community. Although the specific sampling parameters are selected based on GCaMP6s kinetics and an assumed $\sim 10 \text{ }\mu\text{m}$ cell size, the underlying framework (e.g., Fig. 1i) is general and can be adapted to indicators with different kinetics or structures of differing spatial scale. Finally, the authors apply deep-learning-based denoising to calcium imaging data, enabling a substantial increase in the number of detected cells. Together, these advances allow high-SNR cellular-resolution functional imaging of neuronal populations in the mouse hippocampus and rat motor cortex, at depths up to 1.2 mm.

This is a technically strong and impactful contribution that advances the field of three-photon microscopy toward broader accessibility & practical large-scale neuronal population imaging. The manuscript is well organized and clearly written. The points below are intended to improve clarity and reproducibility.

- Imaging on turnaround and unwarping. In Extended Data Fig. 3, the authors show the beam position and corresponding spot spacing across the scan. This plot is presumably derived from the galvanometer position-feedback signal, combined with the known scan-relay geometry to convert angle to lateral position. Stating this explicitly (even briefly in the caption of Extended Data Fig. 3) would help clarify what was measured and how the plot was generated.

The beam positions shown in Supplementary Fig. 11 (previously Extended Data Fig. 3) were derived by imaging a calibrated grid slide with our x-galvo waveform, measuring the



Reviewer Figure 2: (a) A fluorescent grid slide imaged with LIFT before unwarping. (b) Same image, after unwarping.

resulting FOV from the grid spacing, and scaling the waveform extrema to match the experimentally measured FOV. We then validated these estimated positions by using them to unwarp the grid-slide image, which restored the expected regular grid geometry and confirmed that the estimates captured both the scan extent and local spatial nonuniformity. Since unwarping grid-slide images with this beam position data produced satisfactory results (Reviewer Fig. 2), we assumed that the drive voltages and position-feedback voltages were sufficiently similar for this purpose and did not proceed to measure the position-feedback signal, which presented some technical difficulties.

Changes to manuscript: To clarify this in the text, we added the sentences “Beam positions were determined from the x-galvo drive voltages using an empirical FOV calibration from grid-slide images. The drive-voltage limits were mapped to the measured FOV boundaries. The computed beam positions were validated by unwarping distorted grid images and recovering the expected regular grid geometry.” to the caption of Supplementary Fig. 11.

Additionally, while the manuscript notes that imaging during the turnaround region is included, the implementation details are limited. It would be useful to expand on how pixel indices are remapped to their correct spatial coordinates (using the known position-versus-time waveform), the unwarping part.

This was indeed not fully explained in the text. To unwarp, we used the known pixel locations from the above calculation. However, instead of interpolating the image onto a regularly spaced grid, which can introduce artifacts due to the interpolation algorithm and due to the variability in SNR across the corrected pixels, we split the counts in each pixel between neighboring corrected pixels. We weighted the contribution to each corrected pixel according to how the position of the old pixel falls on the new grid. For example, if an old pixel was remapped to pixel 120.4 in the new grid, then 60% of the counts it contains would be assigned to pixel 120 and 40% to pixel 121.

Changes to manuscript: We added the sentence “The denoised time series was unwrapped to correct for the nonlinearity of the galvanometer motion, using the known pixel positions on the sample (Supplementary Fig. 11). To unwarp, pixel intensities were remapped onto a uniformly spaced grid by splitting each pixel’s contribution between neighboring corrected pixels with appropriate weights (see provided code).” to the methods section.

The Methods section (lines 310-315) specifies the turnaround time for Figs. 3,4,5, but explicitly stating how much the field of view increased in each example by including the turnaround region would help readers more clearly appreciate the improvement.

This is a great suggestion. For Figs. 4 and 5, which were acquired with LIFT’s rapid, large FOV mode (4 μm sampling, 6 Hz frame rate), we imaged on the turnaround portion of the galvanometer motion, which roughly doubles the FOV. This was illustrated in Fig. 2 but was not explicitly stated in the manuscript, so we have added “Acquiring data during the turnaround phase enables a 2.4-fold increase in FOV.” to the paragraph describing Fig. 2b in the main text.

Because Fig. 3 presents structural imaging, where rapid acquisition is not required, we used LIFT’s slower, high-resolution mode (1 μm sampling, 0.06 Hz frame rate) to resolve fine structural details. In this mode, imaging is not performed during the turnaround phase, as frame rate is not a limiting factor and image warping can be avoided. To clarify in the text, we added “Since frame rate is not a limiting factor for structural imaging, data points were not acquired during turnaround.” to the methods section.

Changes to the manuscript:

1. Added “Acquiring data during the turnaround phase enables a 2.4-fold increase in FOV.” to the paragraph describing Fig. 2b in the main text.

2. Added “Since frame rate is not a limiting factor for structural imaging, data points were not acquired during turnaround.” to the “Data acquisition and analysis” subsection of the methods section.

- Figure 3 illustrates the deep, large FOV that LIFT enables. However, the representative images in panels b and c show fluorescent structures over a smaller lateral extent than the full 2 mm $\times$ 2 mm FOV. Figure 6 shows a similar effect. A brief clarification of why only part of the nominal FOV contains visible fluorescence (for example, due to expression boundaries or anatomical constraints) would be helpful.

We thank the reviewers for raising this point. We believe that the previous wording of the description of Fig. 3 was a bit confusing. While the scan performed to create Fig. 3 was in fact of a volume of 2 x 2 x 1.23 mm, the optical FOV is approximately a 2 mm-diameter

circle (in fact it is a bit larger than 2 mm, as can be in Supplementary Fig. 7b). This was mentioned in the manuscript (and illustrated in Supplementary Fig. 12), though it may not have been conveyed as clearly as intended. We therefore removed the parenthetical phrase: “(2 x 2 x 1.23 mm)” in the sentence introducing Fig. 3 (line 168) to avoid this confusion and added wording that explicitly states that the optical FOV has a 2 mm diameter.

In addition, as the reviewers point out, animals labeled by AAV injection typically show some spatial nonuniformity in GCaMP expression, which can reduce the apparent fullness of the FOV, as can shadows from blood vessels. As a result, some regions within the optical FOV may appear dark in many images. To assess LIFT's true optical FOV independently of both labeling variability and vascular shadowing, we included an FOV measurement in a uniform fluorescein solution (Supplementary Fig. 7b).

Changes to manuscript:

1. We removed the parenthetical phrase: “(2 x 2 x 1.23 mm)” in the sentence introducing Fig. 3 to avoid this confusion.
2. We added the sentence: “Although the diameter of the optical FOV is 2 mm (Supplementary Fig. 7b), spatial variability in GCaMP expression, as well as shadows from blood vessels, may result in dark regions, reducing the apparent FOV.” to the paragraph describing Fig. 3 in the main text.

- Fig S4: There appear to be some inconsistencies in the panel references in the caption.

We thank the reviewers for calling our attention to this issue. **it has been fixed.**

- Extended Data Fig. 6 has panels a,c,d,e,g,h (missing b and f). The figure caption says that b and f should be the contours of the cells shown in a and e.

Thanks, **it has been fixed.**

- Extended Data Fig. 11: The axial PSF reported here is ~6 μm , which is somewhat larger than what might be expected under ideal diffraction-limited conditions for the stated NA and excitation wavelength. Because the axial extent can be influenced by several factors (e.g., degree of back-aperture filling), a brief clarification of the measurement conditions would be helpful for interpretation.

This is a good point. The excitation beam fills the objective back aperture. Under these conditions, we can use high-NA diffraction theory to estimate that the 1P transverse PSF should have a FWHM of ~0.9 μm , and the axial PSF should have a FWHM of ~4.5 μm . Due to the narrowing of the PSF in multiphoton microscopy, the corresponding 3P PSF is

expected to have a transverse FWHM of $\sim 0.5\ \mu\text{m}$ and an axial FWHM of $\sim 2.6\ \mu\text{m}$. Since we are measuring the PSF on $0.5\ \mu\text{m}$ beads, the lateral PSF should broaden to $0.7\ \mu\text{m}$. In our PSF measurement we report a transverse PSF of $0.7\ \mu\text{m}$ FWHM and an axial PSF of $6\ \mu\text{m}$. The elongation of the axial PSF is mostly due to spherical aberrations caused by the use of a coverslip together with our objective that is not corrected for one. The spherical aberrations elongate the axial PSF by a factor of 2-3 (depending on the exact coverslip thickness and spatial mode) while leaving the transverse width relatively unchanged. The use of a coverslip in this measurement follows our in vivo implementation, in which we use a #1 coverslip as our optical window and therefore reflects the PSF of the implemented imaging system.

Changes to manuscript: To address this issue, the following sentence was added in the description of Supplementary Fig. 7a (previously Extended Data Fig. 11):

“The axial width is larger than the diffraction-limited width due to spherical aberrations arising from the use of a coverslip with an objective that does not include a correction collar. This configuration is consistent with our in vivo imaging preparation, in which a coverslip of the same thickness is used as an optical window.”

Additionally, the fluorescein image appears spatially non-uniform and noisy. A brief note on whether this reflects raw (non-denoised and non-averaged) data would be useful.

The fluorescein image in Supplementary Fig. 7b is indeed a single raw image, which was not averaged or denoised. While the image is a bit noisy, we preferred to acquire the minimal number of images necessary to perform the FOV measurement in order to avoid heating the fluorescein solution, which could create artifacts.

Changes to the manuscript: We added “(raw image, no averaging or denoising used)” to the caption of Supplementary Fig. 7b.

- Supplementary Notes, page 7: The text refers to Fig. 1c at the end of the middle paragraph. It appears this should be Fig. 1d.

Thanks for catching that error, **it has been fixed.**

- Supplementary Table 2 provides ranges of excitation powers. It would be helpful to indicate the specific powers used in the representative frames shown in the main figures (for example, Fig. 3b,c). For Figs. 4 and 5, the reported range of 107–180 mW is quite large, even though the imaging depths are similar. Adding the actual excitation power used for the displayed data, either within the same table or in the corresponding figure captions, would improve clarity.

We see the reviewers' point. The powers for each panel of the figures in the main text and supplementary have been added to Supplementary Table 3. Due to the long working distance and the non-negligible absorption of 1300-nm light in the immersion water, both the post-objective power and the power on the brain surface have been included.

Changes to manuscript: The post-objective and on-brain-surface powers for each subfigure in the main text and supplementary have been added to Supplementary Table 3.

- Adding depth labels to Video 1 would be helpful.

Great suggestion, **it has been implemented.**

Sulekh Fernando-Peiris and Cristina Rodríguez

Reviewer #3 (Remarks to the Author):

We thank the reviewer for their careful and constructive review of our manuscript. In response to the reviewer's comments, we clarified several aspects of the study and performed additional experiments to further support our conclusions. We believe that these revisions and new data have enhanced the clarity and rigor of the manuscript.

Intravital microscopy deep inside tissues was greatly improved using 3-photon (3P) microscopy, however this approach lacks the ability for large-scale cellular-resolution noninvasive imaging techniques with high scan speed. Frostig and co-workers developed a Large Imaging Field of view Three-photon (LIFT) microscope which delivers a field of view of $>3 \text{ mm}^2$ and reaches imaging depth in the brain beyond 1 mm. The approach contains a new strategy for scanning with galvanometers to maintain the strengths of 3-photon microscopy yet greatly improve scanning speed. The scanhead configuration seems to build upon the by-now discontinued Biorad confocal scanner, but here using 3 instead of 2 galvanometers. Parameters tested include effect of under sampling (large pixels, small psf) on image quality and linear phototoxicity burden. The strategy and application of AI-based denoising significantly improves the image quality. As outcome, live-scanning 3P microscopy of large brain areas with single-cell resolution over several minutes was achieved. This was showcased by fast (6 Hz) large-field scanning of calcium activity recorded at 1 mm depth in the mouse motor cortex during locomotion of the awake mouse, which allowed to detect a distinct signaling-active cell population during locomotion bouts.

The combined strategy is impressive and will advance the field of 3P microscopy in neuroscience. Potentially, with implementation of multi-color scanning, other fields depending on large-field scanning and fast acquisition rates may benefit, including, e.g., preclinical models in immunology/immunotherapy.

As main concerns, the extent of phototoxicity caused by the high average laser power and pulse energy used requires further clarification.

1. Very high average laser powers may lead to linear and nonlinear toxicity. The psf and nonlinear toxicity threshold have not been characterized and therefore, it is unclear if toxicity thresholds are reached upon repeated or prolonged scanning. The nonlinear effects were in part addressed for short-term imaging, by the lack of increased background GCaMP6 signal during scanning. However, it remains unclear whether microanatomy and neuronal functionality after a prolonged scanning period (e.g., 15 or 30 min) remain preserved, which could be demonstrated, e.g., by re-recording the same region the following day.

This is a good point, and we thank the reviewer for suggesting a way to address this concern. Following their suggestion, we performed new experiments in which we imaged

the brain of awake mice for 15 minutes at a depth of 980 μm and then returned the next day to re-record from the same area. We performed this measurement with the same post-objective power as we have used for the data presented in Figs. 4f and 5 of the manuscript, which was also recorded at approximately 980 μm depth. We then compared the visual appearance of the imaging area, as well as the event rate, trace SNR, event magnitude and event width, between the two days. To avoid comparing neurons with different functional properties, we restricted our statistical analysis to the CA1 region. The results, presented in the newly added Supplementary Fig. 13, show no statistically significant difference in these parameters between the two days (panels g-j). The anatomy of the imaged region appears largely unchanged, with minor differences arising from variations in the neurons that happened to fire during image acquisition, as well as a small difference in the imaging location within the optical window, leading to differences in illumination of the FOV.

Changes to manuscript: Added Supplementary Fig. 13, which presents the results of newly performed experiments testing whether the excitation powers used in our previous results cause any damage to the scanned area.

Why is the initial power at the brain surface 5x higher for rats? Does this not cause nonlinear toxicity?

We thank the reviewer for calling our attention to this matter. The post-objective power at the shallowest imaging depth, 300 μm , was 28 mW (the power on the brain surface was 21 mW), and not 50 mW as previously stated. This power is roughly 3x the power used for the shallowest imaging depth for mice (Fig. 3); however, the shallowest imaging depth for mice was 0 μm . It has been shown that the effective attenuation length (EAL) for the rodent cortex is ~ 300 μm (Wang *et al.*, Elife 2020) and therefore 10 mW at 0 μm depth and 30 mW at 300 μm depth are roughly equivalent:

$$P_z = P_0 e^{-\left(\frac{z}{EAL}\right)}$$

$$P_{300} = P_0 e^{-\left(\frac{300}{300}\right)} = P_0 * 0.36 = 28 * 0.36 = 10$$

We think that the way the imaging powers were previously listed was confusing and hard to follow, and therefore we have updated Supplementary Table 3 to include the exact post-objective power for each subfigure in the main text and the supplementary. Due to the long working distance and the non-negligible absorption of 1300-nm light in the immersion water, both the post-objective power and the power on the brain surface have been included in the table. In addition, we corrected the statements about powers used both for mice and rats in the methods section.

Changes made to manuscript:

1. Added post-objective and on-brain-surface powers for each subfigure in the manuscript and supplementary in Supplementary Table 3.
2. Corrected the statement about the power on the brain surface for rat imaging to: “The excitation power on the brain surface was 21 - 104 mW.”
3. Added a separate statement about the power used in mouse structural imaging: “The excitation power on the brain surface was 10 - 141 mW.”
4. Changed the wording about powers used in mice imaging and added a separate statement about Fig. 4: “The excitation power on the brain surface was 64 - 141 mW.”

Could the larger psf of the 0.8 NA objective (as compared to 1.05 NA objectives used in other studies) cause higher toxicity thresholds in this study? This should be discussed.

Although 1.05 NA objectives are commonly used in 3P studies, many of those report underfilling the back aperture of the objective, reducing the excitation NA to 0.8-0.9. Others do not provide sufficient information to determine the effective NA. Only some studies report PSF measurements; among these, three show a PSF very similar to LIFT's (Mok *et al.* 2024, Wang *et al.* 2018, and Klioutchnikov *et al.* 2020), and two others show larger PSFs than LIFT's (Weisenburger *et al.* 2019, and Zhao *et al.* 2023). Therefore, it is hard to compare LIFT's excitation NA to a 'standard' effective 3P excitation NA.

In general, reducing the excitation NA, NA_{exc} , reduces excitation efficiency as NA_{exc}^2 . This could be concerning if the average power is then increased to compensate. In practice, however, scattering and aberrations in brain tissue preferentially degrade large-angle excitation rays, particularly for the imaging depths used in 3P microscopy (Theer and Denk, 2016, Akbari and Xu, 2021). Under these conditions, large-angle rays contribute to tissue heating while providing little or no additional signal, effectively reducing high excitation NAs to values similar to LIFT's NA. Thus, a 0.8 excitation NA can achieve a signal yield similar to that of higher nominal NAs, while reducing sample heating.

While this is an important point, we did not feel that there is enough detail in other 3P studies for a direct comparison of LIFT's NA and other systems' NA to benefit the reader and therefore did not include it in the revised manuscript.

2. In addition, the extent of linear toxicity in out-of-focus, upper tissue regions in between the objective and the focal plane, remain to be addressed.

We understand the reviewer's concern about damage in the brain regions dorsal to the scanned region. We have previously addressed this concern numerically (Supplementary Note 2 and Supplementary Fig. 3a-c) and showed that the extent of heating in the dorsal

regions is similar to that at the focal plane. These results agree with previous results by Wang *et al.*, Elife 2020. However, this concern has not been addressed experimentally.

To directly address this concern in the revised manuscript, we performed structural imaging at multiple depths in the regions dorsal to the plane scanned in Supplementary Fig. 13, on both day 1 and day 2 of imaging. Additionally, we performed calcium imaging in layer 6 of the cortex dorsal to CA1 on day 2, to examine neuronal health. Newly added Supplementary Fig. 14 compares the structural images of day 1 and day 2, and shows the activity recorded in the deep cortex on day 2. While minor differences are present in the structural images due to small differences in imaging location and animal head tilt between day 1 and day 2, as well as variability in neuronal activity during image acquisition, the imaged brain regions appear unchanged to the best of our assessment. The neuronal activity presented in panel I appears normal and provides reassurance regarding neuronal health.

Changes to manuscript: Added Supplementary Fig. 14, which presents the results of newly performed experiments testing whether the brain regions dorsal to the scanned brain area are affected by the excitation light traveling through them during the scan.

Minor points

Pulse length: Was this also measured under the objective?

We appreciate the reviewer pointing this out. We are aware that in some three-photon papers, particularly those establishing it as an *in vivo* and calcium imaging modality, pulse duration was measured under the objective lens. This indeed gives a more accurate result of the pulse duration. However, since this is not a major point of the present work and requires building an additional delay line, we opted to measure it with an autocorrelator after the compressor (before entering the microscope optics). We then found the optimal compression by careful optimization of the 3P signal from a uniform fluorescein solution *in-situ*. This procedure for determining the group delay dispersion (GDD) at the focal plane generally produces a pulse that is near transform limited at the focus, apart from residual higher-order dispersion terms that cannot be corrected with prism compressors.

Therefore, the main difference between the pulse duration measured by our autocorrelator and the duration under the objective is the broadening caused by the third-order dispersion (TOD) added to the pulse by the microscope optics. We know that the value of the GDD added to our pulse by the microscope optics is roughly $2,500 \text{ fs}^2$, from the difference in prism-to-roof mirror separations used to compensate dispersion with and without the microscope optics. From this, we can estimate that the TOD added by these optics to our pulse is roughly $20,000 - 40,000 \text{ fs}^3$. This range of TOD would broaden a 50 fs pulse to 53 – 62 fs, which is a relatively moderate change.

Changes to manuscript: To make this clearer in the text, we added the sentence “Since the pulse duration was not measured under the objective, a variation of 6 - 24% in duration is possible due to uncompensated third-order dispersion added by the microscope optics.” to the “excitation source” subsection of the methods section.

The optical resolution is not clear. What is the 3-photon excitation psf of the 0.8 NA objective @1300 nm in their setup?

A measurement of the three-photon excitation PSF of LIFT on 0.5 μm beads is presented in Supplementary Fig. 7a. The FWHM of the transverse and axial PSFs is 0.7 μm and 6 μm , respectively.

In the figure captions it was mentioned 'below the dura' but in the methods it was mentioned that the dura has been removed in the rat model. Was the dura intact for the mouse model?

The dura was indeed not removed in the mouse model, and this was not explicitly mentioned in the text. This has been fixed.

Changes made to manuscript: Added the sentence “A 3-mm diameter craniotomy was performed over the imaging region, leaving the dura intact.” to the “Animal imaging protocols and surgical procedures” subsection of the methods section.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed my previous concerns. The added experiments and clarifications significantly strengthen the work. Apart from minor corrections (below) I have no further comments and am happy with the manuscript in its current form.

Minor: - In Supplementary Table 1: Change Ref. 31, to Zhao et al. 2023.

Thanks, this has been fixed.

- Source data link to googledrive was not functional at the time of review.

We have now uploaded both data and code to public repositories. The data and code are available at: <https://doi.org/10.6084/m9.figshare.33109115>

Reviewer #2 (Remarks to the Author):

The authors clearly addressed all of our questions, and we are satisfied with the corresponding changes made to the manuscript.

A couple typos:

- Supplementary Fig. 3: Panels f and g, red line legend reads 1 mW, 1 μm for both, but the captions say “1 mW excitation with 0.5 μm sampling”. Is it 1 μm or 0.5 μm spacing?

0.5 μm spacing indeed. Thanks for calling out attention to this, it has been fixed.

- Supplementary Fig. 5: captions were corrected and refer to panels a,b,c,d,e,f (no more mention of contours) but the actual figure has labels a,c,d,e,g,h (instead of a,b,c,d,e,f)

Thanks, this has been fixed.

Reviewer #3 (Remarks to the Author):

The authors have done a great job in revising this manuscript. I have no further concerns or comments.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.