nature portfolio

# Peer Review File

## Two-photon all-optical neurophysiology for the dissection of larval zebrafish brain functional and effective connectivity

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This manuscript by Dr Turrini and colleaguesa presents a fully two-photon all-optical setup employs light-sheet microscopy for rapid whole-brain imaging and acousto-optic deflectors (AODs) for 3D optogenetic stimulation. This setup enable high spatio-temporal resolution volumetric imaging of the larval zebrafish brain accompanied by simultaneous three-dimensional optogenetic stimulation. Utilizing two-photon excitation and the inertia-free light targeting capabilities of AODs, the system functionality was validated through reconstructing the efferent functional connectivity of the left habenula, a cerebral nucleus mainly composed of excitatory neurons, linking forebrain and midbrain structures.

The research showcases meticulous execution, experimental design, and data analysis using pertinent statistical methods. The figures are presented in a clear and easily comprehensible manner and the supplementary data serves to address numerous concerns while incorporating essential control measures.

Concerns
- My main concern lies in the limitations of functional connectivity as a metric for establishing causal relationships between neuronal populations across the entire brain.
Functional connectivity (FC) and causal relationships, while both concerned with understanding the interactions between different brain regions, have distinct characteristics. FC describes the statistical associations between brain regions, often in terms of correlation or coherence of activity, without implying a direction or causality of the relationship. It is typically assessed using correlational methods and is often used to map the network structure of the brain. On the other hand, causal relationships, or effective connectivity, involve determining the influence one neural system exerts over another, including directionality and often the mechanism of interaction. They are investigated using methods that can infer directionality, such as Granger causality or dynamic causal modeling, and aim to establish a cause-and-effect relationship between brain activities. Therefore, while FC provides a map of the brain's network structure based on statistical relationships, causal relationships delve deeper into the dynamics of how one region may influence another, providing insights into the direction and potential mechanisms of these interactions.

Here are a few references for your consideration.
PMID: 37823962
PMID: 31611705
PMID: 36301683

- My second concern pertains to the thresholding of the functional connectivity matrix. I am interested in understanding how the optimal value is calculated and why it appears to be relatively low.

- My final concern is using inter-region analysis instead of cell-wise analysis in single-cell data. Cell-wise analysis offers a detailed understanding of heterogeneity, identification of rare cell populations, and detection of subtle differences between cells. This approach is especially relevant in single-cell genomics and spatial transcriptomics, capturing spatial and functional relationships at the individual cell level. In contrast, inter-region analysis may overlook these nuances by averaging variations across cells within a region.
Here are a few references for your consideration.
PMID: 32033589
PMID: 37002403
PMID: 33058349

Reviewer #2 (Remarks to the Author):

The manuscript reports about an all optical configuration leveraging 2P light sheet imaging in combination with targeted 2P optogenetic stimulation. As far as I know this is the first time where such a 2P+2PAOD configuration is presented. Said that, I have some major points that require the author's attention. In general, this all optical configuration should be capable of working at high spatial resolution, either in imaging and in photostimulation. I found it extremely disappointing that data are presented, quantified and processed with no or minimal spatial reference and no reference at all to the underlying cells. No information is shown about the effective quality of the imaging and precision of the photostimulation, even what is actually stimulated.
Because of this and the points below I consider this manuscript not acceptable in the current version for this journal. Of course, I'm available to evaluate a substantially revised version in case.

Reviewer #3 (Remarks to the Author):

This manuscript describes an application of a previously described system for two-photon, all-optical electrophysiological observation of the larval zebrafish brain. The platform studies transgenic zebrafish expressing genetically encoded calcium indicator GCaMP6s as a physiological reporter and opsin ReaChR as a voltage actuator in a crosstalk free manner. The transgenic zebrafish are stimulated using to reveal functional connectivity between the left habenula (LHb) and interpeduncular nucleus (IPN). The manuscript demonstrates an innovative, noninvasive way to study electrical activity in translucent samples, claiming high spatial and temporal resolution. However, it is lacking in explanations of biological significance and figure design hinders comprehension of the results.
Major comments:
1. The biological significance and background throughout the manuscript is lacking. In the introduction, a stated limitation is that current methods to assess human brain connectivity, EEG and fMRI, are lacking in spatial and temporal resolution. However, EEG is regarded to have high

temporal resolution. There is also no introduction to what time scale single-neuron events in humans and zebrafish occur on, and what temporal is required to observe them. Authors should justify their choice of volume rate and optical stimulation frequencies with proper biological basis.

2. There is no introduction to the advantages of zebrafish as a model, except for that they are tiny and translucent. Why is uncovering the functional connectivity important? Does it have significance for human disease? Is the purpose only to show the capabilities of the system? The authors should answer these questions to convince readers of the significance of their chosen model.

3. Many figures are presented in a manner that cannot be well understood.
• In Fig 1d, nothing is being compared in the bar plot. It would be better to only state a value and a standard deviation as is done in the text.
• In Fig 1e, the colors are hard to separate. Authors should consider for this and all figures of this style.
• In Fig 2c-i, 3c, 4d, it is unclear what the asterisks are indicating statistical significance between.
• Figures 4d-e are referenced as Figure 3d-e.
• Figure 3g and Supplementary Figure 4 are presented as n=1. It is unclear if other samples show the same cross power spectrum distribution.

Minor comments:
• One of the main advantages of the system is that it is crosstalk free, however, there is not much mention of how this is achieved until the discussion. More background on how the optogenetic actuators and sensors (ReaChR and gCaMP6s) were chosen and a figure showing that their activation spectra minimally overlap would allow readers to be better convinced of the crosstalk-free nature of the system.
• Limitations of the system are not well discussed.
• All sample sizes are n=6 or less. The reason for the small sample size is not mentioned.

**Point-by-point response to Reviewers' comments**

**Manuscript ID: COMMSBIO-24-0129**

**Two-photon all-optical electrophysiology**
**for the dissection of larval zebrafish brain functional and effective connectivity**

Dear Editor, we would like to thank you and the Reviewers for sending us detailed and constructive comments, which have greatly helped us to improve this manuscript. We have prepared a revised version of the manuscript following Reviewers' suggestions.
Below is our point-by-point response to each of the comments.

Reviewer #1 (Remarks to the Author):

This manuscript by Dr Turrini and colleaguesa presents a fully two-photon all-optical setup employs light-sheet microscopy for rapid whole-brain imaging and acousto-optic deflectors (AODs) for 3D optogenetic stimulation. This setup enable high spatio-temporal resolution volumetric imaging of the larval zebrafish brain accompanied by simultaneous three-dimensional optogenetic stimulation. Utilizing two-photon excitation and the inertia-free light targeting capabilities of AODs, the system functionality was validated through reconstructing the efferent functional connectivity of the left habenula, a cerebral nucleus mainly composed of excitatory neurons, linking forebrain and midbrain structures.

The research showcases meticulous execution, experimental design, and data analysis using pertinent statistical methods. The figures are presented in a clear and easily comprehensible manner and the supplementary data serves to address numerous concerns while incorporating essential control measures.

We thank the Reviewer for appreciating our work.

Concerns
- My main concern lies in the limitations of functional connectivity as a metric for establishing causal relationships between neuronal populations across the entire brain.
Functional connectivity (FC) and causal relationships, while both concerned with understanding the interactions between different brain regions, have distinct characteristics. FC describes the statistical associations between brain regions, often in terms of correlation or coherence of activity, without implying a direction or causality of the relationship. It is typically assessed using correlational methods and is often used to map the network structure of the brain. On the other hand, causal relationships, or effective connectivity, involve determining the influence one neural system exerts over another, including directionality and often the mechanism of interaction. They are investigated using methods that can infer directionality, such as Granger causality or dynamic causal modeling, and aim to establish a cause-and-effect relationship between brain activities.

Therefore, while FC provides a map of the brain's network structure based on statistical relationships, causal relationships delve deeper into the dynamics of how one region may influence another, providing insights into the direction and potential mechanisms of these interactions.

Here are a few references for your consideration.
PMID: 37823962
PMID: 31611705
PMID: 36301683

We thank the Reviewer for highlighting this aspect and for giving us helpful hints. Following the Reviewer's suggestions we set up the Granger causality analysis among the average activities of brain regions. The analysis confirmed a causality link between the triggered activity in the habenula and that in the IPN. Notably, the strength of the causal link LHb-IPN is similar to that of other causal links emerging from spontaneous activity.
We also performed partial correlation analysis which highlighted that the observed LHb-IPN causality link is of a direct type, thus not being intermediated by other regions.

Results of the analysis are presented in Figure 4 as novel panels c and d.

Novel text about these results was introduced in the Results, Discussion and Methods sections.

In accordance with the novel results added to the work, we modified the manuscript title as follows (additions in bold text):

"Two-photon all-optical electrophysiology for the dissection of larval zebrafish brain functional **and effective** connectivity"

- My second concern pertains to the thresholding of the functional connectivity matrix. I am interested in understanding how the optimal value is calculated and why it appears to be relatively low.

We set a threshold on the voxel-wise functional connectivity map (previously Figure 4c, now Figure 4e) based on the statistical comparison carried out in Figure 4d (now Figure 4f). From this comparison emerges that voxels of the LHb and IPN are the only ones having significantly higher correlation with the activity triggered in the LHb. The chosen 0.12 threshold on Pearson's seed-correlation coefficient represents the highest value separating significant from non-significant correlations.

This aspect was mentioned in the Methods section (at the end of Data analysis → Activation probability and correlation maps) and in Figure 4 legend.

The Reviewer's comment, however, highlighted a point of the manuscript that was not sufficiently clear. We added to the plot in Figure 4d (now Figure 4f) a gray horizontal dashed line at the value of 0.12 in order to graphically visualize the threshold in the context of brain regions correlations values. We modified the Figure legend accordingly. We also modified the description of Supplementary Movie 9 (previous Suppl. Movie 6) showing the 3D rotation of the thresholded functional connectivity map. Moreover, we specified in the Results section how the threshold value on correlation was chosen, as follows:

"In order to visually isolate the neuronal circuit underlying LHb stimulation, we set a threshold on the correlation coefficient. Based on the results shown in Figure 4f, we chose a threshold of 0.12 as the highest value separating regions showing significantly higher correlation with the seed activity."

Also the description of the thresholding in the Methods section (Data analysis → Activation probability and correlation maps) was modified (additions in bold text):

"The binarized functional connectivity map shown in Figure 4h was obtained after applying a threshold on the Pearson's correlation coefficient to the average correlation map shown in Figure 4e. The 0.12 value **adopted** represented the correlation coefficient threshold separating **significant from non-significant correlations among brain** regions **(see Figure 4f)** ~~showing significantly higher connectivity~~."

- My final concern is using inter-region analysis instead of cell-wise analysis in single-cell data. Cell-wise analysis offers a detailed understanding of heterogeneity, identification of rare cell populations, and detection of subtle differences between cells. This approach is especially relevant in single-cell genomics and spatial transcriptomics, capturing spatial and functional relationships at the individual cell level. In contrast, inter-region analysis may overlook these nuances by averaging variations across cells within a region.
Here are a few references for your consideration.
PMID: 32033589
PMID: 37002403
PMID: 33058349

As we discuss in the answer to a point raised by Reviewer #2, our custom 2P light-sheet microscope, despite producing images with a voxel size of 2.2×2.2×5 $\mu m^2$, representing approximately 30% of the neuron size laterally, it does not have sufficient signal-to-noise ratio (SNR) to consistently resolve single neurons throughout the entire brain. Zebrafish neurons are tightly packed (except for ventral-most regions) and to resolve individual cells it is necessary to have high contrast between the neuronal nuclei and the small space in between them. While 1P light-sheet microscopy can effortlessly achieve this goal, 2P LSFM (owing to the nonlinear nature of the excitation and the need to elongate the axial PSF of the illumination beam to produce the lightsheet) is typically prone to SNR issues. In this regard, our system performs in line with the

other few 2P LSF microscopes employed for zebrafish brain volumetric functional imaging [Ref_1; Ref_2; Ref_3], though achieving higher volumetric rate and a much more thorough sampling of the larval brain (volumetric rate 2.5 Hz, 200 µm depth/5 µm z-step Vs 1 Hz, 64 µm/8 µm in Ref_1; 1 Hz 90 µm/9 µm in Ref_2; 1 Hz 100 µm/ NA in Ref_3). To the best of our knowledge, only one 2P LSF microscope published achieves sufficient SNR to obtain single neuron resolution [Ref_4, volumetric rate 0.5 Hz, 250 µm depth/ 4.5 µm step], however needing 490 mW of pulsed laser impinging on the sample which is, in our experience, frankly too much for not producing photodamage. For this reason we performed voxel-wise (choosing quasi-isotropic voxels, having approximately half of the size of a neuronal body: 4.4×4.4×5 $\mu m^2$) and region-wise analyses.
We explained this limitation in the Discussion section.

References

1 Wolf S, Supatto W, Debrégeas G, Mahou P, Kruglik SG, Sintes JM, Beaurepaire E, Candelier R. Whole-brain functional imaging with two-photon light-sheet microscopy. Nat Methods. 2015 May;12(5):379-80. doi: 10.1038/nmeth.3371. PMID: 25924070.

2 Wolf S, Dubreuil AM, Bertoni T, Böhm UL, Bormuth V, Candelier R, Karpenko S, Hildebrand DGC, Bianco IH, Monasson R, Debrégeas G. Sensorimotor computation underlying phototaxis in zebrafish. Nat Commun. 2017 Sep 21;8(1):651. doi: 10.1038/s41467-017-00310-3. PMID: 28935857; PMCID: PMC5608914.

3 Truong TV, Holland DB, Madaan S, Andreev A, Keomanee-Dizon K, Troll JV, Koo DES, McFall-Ngai MJ, Fraser SE. High-contrast, synchronous volumetric imaging with selective volume illumination microscopy. Commun Biol. 2020 Feb 14;3(1):74. doi: 10.1038/s42003-020-0787-6. Erratum in: Commun Biol. 2022 Apr 11;5(1):363. Erratum in: Commun Biol. 2022 May 27;5(1):533. PMID: 32060411; PMCID: PMC7021898.

4 Keomanee-Dizon K, Fraser SE, Truong TV. A versatile, multi-laser twin-microscope system for light-sheet imaging. Rev Sci Instrum. 2020 May 1;91(5):053703. doi: 10.1063/1.5144487. PMID: 32486724; PMCID: PMC7255815.

Reviewer #2 (Remarks to the Author):

General aspects:
The manuscript reports about an all optical configuration leveraging 2P light sheet imaging in combination with targeted 2P optogenetic stimulation. As far as I know this is the first time where such a 2P+2PAOD configuration is presented. Said that, I have some major points that require the author's attention. In general, this all optical configuration should be capable of working at high spatial resolution, either in imaging and in photostimulation. I found it extremely disappointing that data are presented, quantified and processed with no or minimal spatial reference and no reference at all to the underlying cells. No information is shown about the effective quality of the imaging and precision of the photostimulation, even what is actually stimulated.

Because of this and the points below I consider this manuscript not acceptable in the current version for this journal. Of course, I'm available to evaluate a substantially revised version in case.

Main points:

LINE076: high spatial resolution of the AOM stimulation. This is in theory, here in the manuscript tha author used the system for a rather bulk stimulation of a relative large brain volume encompassing hundreds of cells.

Lines 74-77 report "In this work, we present an all-optical setup consisting of a light-sheet microscope and a light-targeting system equipped with AODs, both employing nonlinear excitation, which enable **high spatio-temporal resolution volumetric imaging** of the larval zebrafish brain along with concomitant three-dimensional optogenetic stimulation.", thus "high spatio-temporal resolution" actually referred to the volumetric imaging.

Considering the Reviewer's comment, we deemed that the sentence could be misinterpreted. We thus rephrased the concept as follows:

"In this work, we present an all-optical setup combining a light-sheet microscope and a light-targeting system equipped with AODs, both employing nonlinear excitation. The light-sheet microscope enables high spatio-temporal resolution volumetric imaging of the larval zebrafish brain, while the light-targeting system is employed to perform concurrent three-dimensional optogenetic stimulation."

In this kind of collective stimulation of cells and neuronal processes what is the advantage of the AOM based stimulation path?

The advantage of using AODs over galvo mirrors in the experiments presented in the manuscript is the possibility of targeting the excitation spot at different focal positions (defocusing) without the need to actually move the objective lens. On one hand, since we use the same 20x objective both for stimulation and for collecting calcium fluorescence, moving the objective to address light at different axial positions would have strongly hampered the temporal homogeneity of the volumetric photostimulation. On the other hand, renunciation to remote focusing would have irretrievably linked the optogenetic stimulation to the same *z* planes sampled with the light-sheet and to its timing. In addition, in both scenarios the periodic movements of the bulky objective inside the water-filled imaging chamber would have produced waves impinging on the sample that disrupt the volumetric reconstruction. A possible solution, specific for these very experiments, would have been to perform volumetric optogenetic stimulation using galvo mirrors for *x-y* displacement and an electrically tunable lens (ETL) for remote focusing of the excitation spot. This solution, however, would have severely hindered future applications of the system for multispot excitation experiments.
To better clarify these aspects, we modified a sentence in the Discussion section as follows (addition in bold text):

"Notably, an intriguing aspect of our approach is that, owing to the use of remote focusing of the detection objective **and of AODs for stimulation light defocusing**, the localization of the photostimulation volume remains entirely independent of the sequential acquisition of different brain planes, thus affording greater flexibility in our experimental investigations."

More importantly, because of the claims in the introduction could the author reference or provide data supporting the cellular activation and the response on the targeted cell? if the title is all-optical protocol this is an aspect that should be presented to support the idea of high resolution.

As explained before, in the Introduction no claims to the high resolution achievable by the optogenetic stimulation using AODs were made. In addition to this, we disagree with the Reviewer on all-optical protocols necessarily meaning single-cell stimulation/recording, since there is plenty of literature showing all-optical investigations using wide-field imaging and/or stimulation (e.g., Ref_1 to 5).

References

1 Lim DH, Mohajerani MH, Ledue J, Boyd J, Chen S, Murphy TH. In vivo Large-Scale Cortical Mapping Using Channelrhodopsin-2 Stimulation in Transgenic Mice Reveals Asymmetric and Reciprocal Relationships between Cortical Areas. Front Neural Circuits. 2012 Mar 15;6:11. doi: 10.3389/fncir.2012.00011. PMID: 22435052; PMCID: PMC3304170.

2 Lin JY, Knutsen PM, Muller A, Kleinfeld D, Tsien RY. ReaChR: a red-shifted variant of channelrhodopsin enables deep transcranial optogenetic excitation. Nat Neurosci. 2013 Oct;16(10):1499-508. doi: 10.1038/nn.3502. Epub 2013 Sep 1. PMID: 23995068; PMCID: PMC3793847.

3 Crocini C, Ferrantini C, Coppini R, Scardigli M, Yan P, Loew LM, Smith G, Cerbai E, Poggesi C, Pavone FS, Sacconi L. Optogenetics design of mechanistically-based stimulation patterns for cardiac defibrillation. Sci Rep. 2016 Oct 17;6:35628. doi: 10.1038/srep35628. PMID: 27748433; PMCID: PMC5066272.

4 Resta F, Montagni E, de Vito G, Scaglione A, Allegra Mascaro AL, Pavone FS. Large-scale all-optical dissection of motor cortex connectivity shows a segregated organization of mouse forelimb representations. Cell Rep. 2022 Nov 8;41(6):111627. doi: 10.1016/j.celrep.2022.111627. PMID: 36351410; PMCID: PMC10073205.

5 Chai Y, Qi K, Wu Y, Li D, Tan G, Guo Y, Chu J, Mu Y, Shen C, Wen Q. All-optical interrogation of brain-wide activity in freely swimming larval zebrafish. iScience. 2023 Nov 3;27(1):108385. doi: 10.1016/j.isci.2023.108385. PMID: 38205255; PMCID: PMC10776927.

What are the authors targeting for optogenetic stimulation? cells or neuropil regions? the video with the activity shows a corona of cells that become active with a dark spot at the center

The target of the stimulation is the entire left habenular volume which comprises both neuronal bodies and processes. Supplementary Movie 2 (now Suppl. Movie 5) shows the activity of 8 brain planes (out of the 41 sampled) in response to habenular stimulation. Reviewer's observation about a dark spot at the center is correct and it depends on two factors: the habenular structure and the specific transgenic line employed. On the one hand, the plane at 90 µm in Supplementary Movie 5 shows an optical section of the habenula at a depth where its structure is composed by outer neuronal bodies and inner processes. On the other hand, since the transgenic line employed expresses GCaMP in neuronal nuclei, only the outer layer shows fluorescence and thus activity.

Following the Reviewer's comment, we added to the Supplementary Movie 5's caption the following sentence:

"Due to the nuclear localization of the calcium indicator and the specific habenular structure (a dome of neuronal bodies surmounting neuronal processes), left habenula in the plane at 90 µm depth appears as an active fluorescent rim (neuronal nuclei) with dark inner (neuronal processes)."

LINE108: please do not introduce useless labeling, ReaChR+ and ReaChR- are clear and easy to associate

Following the Reviewer's comment, we removed from the text the "R" labeling. References to R+ and R- were left in the Figures for representation purpose.

LINE 108. Crosstalk-free configuration, is the Hellinger distance between the SDs (R- vs R-) a good proxy with respect to counting the relative number of spontaneous events? I mean, no direct evidence is shown that the brain is active during the baseline recordings. Is the increasing trend of the SD with the power somehow expected? I would strongly recommend adopting a more precise metric, actually closer to neuronal activity.

Following the Reviewer's comment we performed a voxel-wise automatic counting of calcium peaks on a whole-brain scale. The number of peaks per minute (and consequently the relative number of peaks) however, probably due to the slow kinetic of calcium indicators and to the fact of measuring a resting state brain, proved to be poor in discriminating between different conditions (i.e., different 920 nm laser powers tested). We thus wondered whether the amplitude of detected calcium peaks could be a good metric for activity discrimination. This seems to be the case. The results we obtained with peak amplitude reflect what we observed using SD as a metric. We thus decided to use SD as a metric for quantifying neuronal activity level since, contrary to peak amplitude, it does not need the setting of any threshold.

In order to convince the readers of the choice made, we prepared a novel Supplementary Figure 3 showing a comparison between the three metrics (SD, peak/min and peak amplitude). We modified the text in the Results and in the Methods section accordingly.

LINE 136. The authors are referring to normalized counts of voxels, but how many fish have been actually tested?

The number of larvae tested is 6 ReaChR+ and 6 ReaChR- and it is reported in the Figure 1f (now Figure 1j) legend, and in the Statistics and Reproducibility section. Normalized bin counts of the distributions were pooled (method: average), independently for the two groups, and presented as average normalized bin counts ± sem.

What is the typical size of the voxel for image processing? Could the authors provide. This information should be moved from the methods up in the main results. The reported voxel size corresponds to 60-70% of the typical cell size in the packed regions of the brain. Why did authors not consider any segmentation algorithms to identify the neurons? This is today freely available and straightforward to apply. Is the image quality sufficient to use this type of pipelines?

Following the Reviewer's suggestion we have reported the voxel size used for image processing in the Results section.
As we discuss in the answer to point 1 raised by Reviewer #1, 2P LSFM is typically prone to low SNR and our microscope performs in line with other similar systems yet with a higher volumetric rate and more thorough sampling of the larval brain. Our microscope has an imaging voxel size of 2.2×2.2×5 $\mu m^3$ representing approximately 30-35% of a typical zebrafish neuronal nuclei diameter (6-7 μm), laterally. Despite this voxel size satisfying Nyquist-Shannon sampling theorem in the x and y dimensions, due to the nonlinear excitation process and the elongated illumination PSF, the need to keep the laser power as low as possible for ethical (i.e., for the sake of larvae) and experimental reasons (i.e., the need to perform measurement on healthy animals, without perturbing their physiological homeostasis), and the cell density inside the larval brain, images do not have sufficient contrast to reliably allow the use of segmentation algorithms to automatically identify neurons. For this reason we decided to perform a 2×2 (x-y) image binning, resulting in quasi-isotropic voxels on which we performed the analysis.

Following the Reviewer's comment, we deemed it important to specify this aspect in the manuscript. We thus modified the Discussion section as follows (additions in bold text):

"On the imaging side, the use of NIR light to produce the sheet of light leads to a significant reduction of common striping artifacts that otherwise could severely hinder the interpretation of functional data. **Nevertheless, due to the nonlinear nature of its excitation and the need to elongate the axial point spread function (PSF) of the illumination beam to produce the light sheet (thus reducing photon density), 2P LSFM is also typically prone to low signal-to-noise ratio. As a result, despite a voxel size (2.2×2.2×5 $\mu m^3$) being 30-35% than the average**

**diameter of a neuronal nucleus (6-7 µm), we did not achieve consistent detection of single neurons throughout the entire brain.**
[...] **On the imaging side, technical implementations will be made, in order to improve image contrast while maintaining a low laser power on the sample. This advancement will enable the use of automated segmentation algorithms for single neuron detection. Cell-wise analyses will allow to refine the reconstruction of neuronal connectivity, capturing the nuanced differences between individual cells.**"

LINE162: The authors evaluated the calcium response elicited by the optogenetic stimulation as function of the stimulus duration and excitation power. Looking at the corresponding part in the methods (555) it is not clear what they actually measured. I would assume that they are referring to voxel time series averaged across the lHb Area.

Following Reviewer's comment we modified the description of the method as follows (additions in bold):

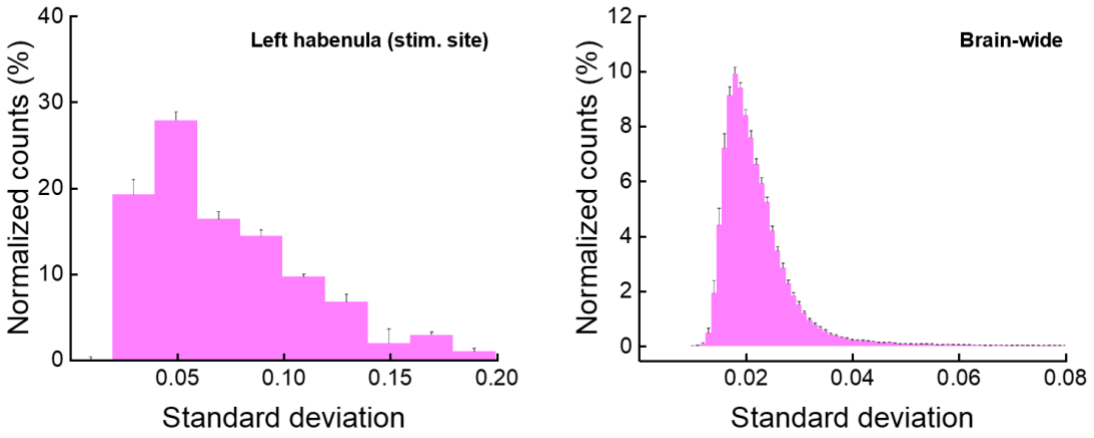
"To characterize the neuronal activation as a function of the stimulation parameters (scan time and laser power), we first extracted the ~~fluorescence~~ **voxel** time **series** ~~traces~~ averaged over the entire stimulation site (i.e., left habenula) from 4D *ΔF/F* hyperstacks."

Again there is no information on the number of cells present in the recording area, the number of cells that get actually activated, the number of non-responsive or non-recordable cells. This is information that I would consider fundamental to assess the applicability of the protocol.

As previously highlighted in response to other points raised by the Reviewer, despite a good spatial sampling for neuron detection, the SNR achieved (while maintaining a low laser power on the sample) is not enough to enable consistent cell-wise analyses. This aspect has been discussed in the manuscript.

Importantly, what is the impact of the stimulated volume in the activity elicited in the lHb and brain wide?

We assumed that the Reviewer is referring to the quantification of the effect of optogenetic excitation at the stimulation site and brain wide used in Figure 1, yet for the left habenular nucleus. We thus produced average SD distributions of the voxel time series contained in the left habenula and brain wide, following stimulation of the entire left habenula (error bar is sem; N = 6 ReaChR+ larvae).




The nice plot 2g what is actually telling us in terms of cell activity? We could get something very similar with LFP, without any spatial or cellular information.

Figure 2g shows the trend of calcium transients' amplitude obtained averaging over time the fluorescence of the habenular volume. The increase in amplitude which we observe as a function of stimulation power suggests that increasing laser power produces a proportional increment in the firing rate of the underlying cells. Thus, for what pertains to this very case, the Reviewer is, in principle, right when saying that a similar result could be obtained with an LFP recording. However, differently from what could be achieved through LFP recordings, our measurements enable simultaneous whole-brain investigation with a spatial parcelization of $2.2\times2.2\times5\ \mu m^3$.

Other points
- it is hard to believe, without any confirming evidence, that 60mW of a Gaussin beam entering the eye do not produce any alteration of the vision circuit functionality. It also surprises me the recurrent activation of the hindbrain circuits, shown in the raster plots. That is one of the typical signs indicating fish struggling. With tubocurarine you get no movement artifacts, but the pattern of the activity are enough to show that the fish is not well tolerating the light beam.

Following the Reviewer's comment we performed a set of experiments designed for evaluating the effect of laser exposure during imaging on behavior/brain activity, in order to assess whether larvae are well tolerating or not the light beam. We employed a sample mounting procedure in which larvae had agarose restrained head and free tail. This enabled simultaneous brain activity and behavioral (tail beats) recording in larvae not treated with the paralyzing agent. The protocol consisted of 200 s of behavioral recording. The first half in the dark (imaging OFF) and the second half with whole-brain imaging ON (same parameters as in the measurements performed throughout the manuscript: 60 mW laser power, 2.5 Hz volumetric rate, 200 µm z sampling). Measurements were carried out both on ReaChR- and ReaChR+ larvae. The results of this experimental set show a slight but not significant increase in tail beats upon laser exposure with respect to pre-exposure, equally affecting ReaChR- and ReaChR+. The pattern of hindbrain

activity observed by the Reviewer is indeed a sign of intended tail movements. However, those movements are not significantly due to the laser exposure during whole-brain light-sheet imaging, being present also during imaging OFF. Some signs of struggle can happen due to the restrained condition, which not acclimated larvae have to face.
Given the importance of this control experiment in demonstrating, with a different approach, the absence of imaging crosstalk, we added the results obtained to Figure 1 as novel panels g, h, i (figure legend was modified accordingly). Novel text about this new experiment was introduced in the Results, Discussion and Methods sections.

- what is the size of the lightsheet along the light propagation direction on both the two arms?

The Gaussian profile of each of the beams has a longitudinal FWHM of 327 µm. Information regarding the imaging setup resolution were skipped since part of a previous paper by our group (reference n°40, de Vito, G. *et al.* Fast whole-brain imaging of seizures in zebrafish larvae by two-photon light-sheet microscopy. *Biomed. Opt. Express, BOE* **13**, 1516-1536, doi:10.1364/BOE.434146 (2022).)
Following the Reviewers comment, however, we deemed it important to report those informations in the manuscript. We thus added to the "Methods → Optical characterization of the system" section the following text:

"Summarizing, each of the light sheets coming from the two excitation arms has a transversal FWHM at waist of 6 µm and a longitudinal FWHM of 327 µm. The lateral FWHM of the detection PSF is 5.2 µm. "

- if you put the hardware diagram, one would expect to find all the information about the components actually shown.

It is not clear what the Reviewer is referring to since all the components shown in Figure 1a are described in Methods → Optical Setup. In the revised version of the manuscript the complete hardware scheme is moved in Supplementary Materials as novel Supplementary Figure 1.

- In the complete manuscript there is no image demonstrating the actual resolution of the imaging configuration with respect to the sample imaged. It is not clear whether the system can resolve individual cells across the complete brain, also in the deeper regions of the brain.

Supplementary Movie 2 (now Suppl. Movie 5) shows the image quality of a raw volumetric functional acquisition. However, as the Reviewer correctly pointed out, we deemed it important to add a figure panel showing the actual image quality achievable with the system.

To this end, we added this information in Figure 1, modifying panel a. We thus changed the Figure legend accordingly.

Moreover, we added a novel Supplementary Figure (new Supp. Figure 2), showing image quality along 20-µm spaced planes of the larval brain.

As explained in response to a previous point from the Reviewer, we added in the Discussion section text regarding the limitations of the system in terms of image quality.

Reviewer #3 (Remarks to the Author):

This manuscript describes an application of a previously described system for two-photon, all-optical electrophysiological observation of the larval zebrafish brain. The platform studies transgenic zebrafish expressing genetically encoded calcium indicator GCaMP6s as a physiological reporter and opsin ReaChR as a voltage actuator in a crosstalk free manner. The transgenic zebrafish are stimulated using to reveal functional connectivity between the left habenula (LHb) and interpeduncular nucleus (IPN). The manuscript demonstrates an innovative, noninvasive way to study electrical activity in translucent samples, claiming high spatial and temporal resolution. However, it is lacking in explanations of biological significance and figure design hinders comprehension of the results.
Major comments:
1. The biological significance and background throughout the manuscript is lacking. In the introduction, a stated limitation is that current methods to assess human brain connectivity, EEG and fMRI, are lacking in spatial and temporal resolution. However, EEG is regarded to have high temporal resolution.

Since the sentence which the Reviewer is referring to could be misleading, we modified it as follows (addition in bold text):

"Critical insights into the complex interplay among large populations of neurons have been provided by electroencephalography and functional magnetic resonance imaging. Those gold standard methods, however, do provide a noninvasive means to detect neuronal activity, but with limited spatial **(the former) and** temporal resolution **(the latter),** and lack equally noninvasive possibilities to precisely control it."

There is also no introduction to what time scale single-neuron events in humans and zebrafish occur on, and what temporal is required to observe them. Authors should justify their choice of volume rate and optical stimulation frequencies with proper biological basis.

Single-neuron events occur on timescales which are consistent across *phyla* [REF_1]. Clearly, in order to temporally resolve individual action potentials (i.e. the electrical depolarization occurring in neurons) the sampling rate of the "detector" (in its broader sense, any instrument used for recording some events) should be at least in the kHz range. In our case, i.e. in the case of calcium

imaging, an indirect measurement of the underlying electrical activity is performed. Calcium indicators integrate high-frequency action potentials, thus exhibiting a slower kinetics. With some differences depending on the specific dynamic of the calcium indicator chosen, typical calcium transients happen in the second range. In the case of GCaMP6s (the slow and sensitive version of the GCaMP6 sensor) in its nuclear-localized form, the typical time constant of the exponential decay is in the range of 3.5 s [REF_2] or higher. This is the reason why a volumetric rate of 2.5 Hz is a more than optimal sampling for the calcium dynamics to be highlighted.

We thus added this information to the Methods → Simultaneous calcium imaging and optogenetic stimulation section as follows (additions in bold text):

"Whole-brain calcium imaging was performed at 2.5 Hz **(a more than optimal volumetric rate considering the typical time constant of the exponential decay for the nuclear localized version of the GCaMP6s sensor $\tau$: 3.5 s [REF_2])** with 41 stacked z-planes spanning a depth of 200 μm."

Regarding the stimulation frequency, after the experiments of characterization of the calcium response (Figure 2), we chose a frequency of 1/16 Hz in order to trigger activation events only when the previous calcium transient had come back to the baseline.

We better specified this aspect in the same Methods section as follows (additions in bold text):

"Each stimulation trial consisted of 100 s of whole-brain calcium imaging during which 5 optogenetic stimuli (interstimulus interval: 16 s**, based on the characterization experiments performed, in order to trigger activation events only after the end of the previous calcium transient**) were performed in the same volumetric site."

References

1 Buzsáki G, Logothetis N, Singer W. Scaling brain size, keeping timing: evolutionary preservation of brain rhythms. Neuron. 2013 Oct 30;80(3):751-64. doi: 10.1016/j.neuron.2013.10.002. PMID: 24183025; PMCID: PMC4009705.

2 Migault G, van der Plas TL, Trentesaux H, Panier T, Candelier R, Proville R, Englitz B, Debrégeas G, Bormuth V. Whole-Brain Calcium Imaging during Physiological Vestibular Stimulation in Larval Zebrafish. Curr Biol. 2018 Dec 3;28(23):3723-3735.e6. doi: 10.1016/j.cub.2018.10.017. Epub 2018 Nov 15. PMID: 30449666; PMCID: PMC6288061.

2. There is no introduction to the advantages of zebrafish as a model, except for that they are tiny and translucent.

Eversince George Streisinger pioneered its use as an animal model for developmental biology studies in the late seventies, zebrafish (*Danio rerio*) has been increasingly used in the most disparate fields of life science research. Since then, this animal model has been the topic of more than 55k publications, with an average of more than 3600 papers published per year in the last decade (source PubMed). With such numbers, we frankly deemed unnecessary to repeat the usual information about zebrafish advantages which can be straightforwardly found in the literature.

Nevertheless, we modified the sentence which the Reviewer is referring to as follows (addition in bold text):

"In this framework, the ever-increasing use of the tiny and translucent zebrafish larva as **a reliable** animal model **recapitulating manifold features of vertebrate species physiology**, has provided moment for the development [...]"

Why is uncovering the functional connectivity important? Does it have significance for human disease? Is the purpose only to show the capabilities of the system? The authors should answer these questions to convince readers of the significance of their chosen model.

Uncovering the functional connectivity (intended in its broader sense, not only as a mere statistical correlation) is fundamental for understanding how the brain works (as we state in the first sentence of the Introduction). With that said, it is implied that knowing brain physiological functional connectivity can have a long-range huge impact on disease understanding. Ours is a work of basic research aimed at validating an advanced all-optical methodology. We thus deem misleading to discuss in the manuscript the possible implications on human disease one could achieve by studying larval zebrafish brain functional connectivity.

3. Many figures are presented in a manner that cannot be well understood.
• In Fig 1d, nothing is being compared in the bar plot. It would be better to only state a value and a standard deviation as is done in the text.

Even though there are no other bars in the plot, Figure 1d (now Figure 1e) represents *per se* a comparison. It shows the imaging crosstalk index which comes out from the comparison of the distributions shown in Figure 1c (now Figure 1d). For this reason, we deem it correct and more impactful to represent it as a single bar plot.

• In Fig 1e, the colors are hard to separate. Authors should consider for this and all figures of this style.

Following the Reviewer's suggestion we modified the color palette of Figure 1e (now Figure 1f).

• In Fig 2c-i, 3c, 4d, it is unclear what the asterisks are indicating statistical significance between.

We thank the Reviewer for pointing out a part of the work that was not sufficiently clear.
Regarding Figure 2c-i, the horizontal bars below asterisks embrace conditions (i.e., specific scan time/power values) which are statistically significant. When the asterisk is black, it means that those significance apply with respect to all the conditions not embraced by the horizontal bar. When the asterisks are colored, color/s indicate/s with respect to which condition the significance exerts.

In order to clarify this aspect, we added the following text to Figure 2 legend:

"Black horizontal bars underlying asterisks embrace conditions which are statistically significant with respect to: all the other conditions in the plot (black asterisk), the condition(s) indicated by the color(s) of the asterisk(s)."

• Figures 4d-e are referenced as Figure 3d-e.

We thank the Reviewer for pointing out this error. Reference to the proper figure numbers was restored in the text.

• Figure 3g and Supplementary Figure 4 are presented as n=1. It is unclear if other samples show the same cross power spectrum distribution.

We thank the Reviewers for highlighting this point. Indeed, we realized that this aspect was not explained with sufficient clarity. Following Reviewer's comment, we specified both in the text and in the figure legend that the plot of the ΔF/F traces shown in Figure 3f (from which the cross power spectrum in Figure 3g was calculated) is a representative case.

Moreover, in order to highlight the consistency among samples of the result shown in Figure 3g, we added a new Supplementary Figure (new Suppl. Figure 7) adding cross power spectra of the other larvae. We also modified Supplementary Figure 4 (now Suppl. Fig. 8), adding cross power spectra between LHb and RHb activities for all the larvae. We modified the Figure legends accordingly.

Minor comments:
• One of the main advantages of the system is that it is crosstalk free, however, there is not much mention of how this is achieved until the discussion. More background on how the optogenetic actuators and sensors (ReaChR and gCaMP6s) were chosen and a figure showing that their activation spectra minimally overlap would allow readers to be better convinced of the crosstalk-free nature of the system.

Following the Reviewer's suggestion, we added the following text (in bold) to the Results section:

"After having demonstrated the absence of crosstalk activation of ReaChR channels upon 2P light-sheet scanning, we investigated the capability of our AOD-based photostimulation system to effectively induce optogenetic activation of targeted neurons. For this purpose, **we selected a stimulation wavelength (1064 nm) that is red-shifted relative to the opsin's 2P excitation peak (975 nm). By doing so, we increased the separation between the wavelength used for optogenetic stimulation and the 2P excitation peak of GCaMP6s (920 nm), thus further reducing the potential for stimulation-induced artifacts.**"

We did not add a figure showing the 2P excitation spectra of the two proteins since we do not have these data produced in the lab.

• Limitations of the system are not well discussed.

We added some text to the Discussion section regarding the limitations of the setup in terms of signal-to-noise ratio which prevents from applying automated segmentation algorithms for consistent single neuron detection throughout the brain.

• All sample sizes are n=6 or less. The reason for the small sample size is not mentioned.

Overall, we observed a high grade of consistency in the results obtained from different larvae. For this reason we deemed sufficient the sample size employed, also taking into account the 3Rs principle. According to Reviewer's comment, we added the following text in the "Statistics and Reproducibility" paragraph:

"No a priori sample size calculation was performed. The sample size employed was justified by the high grade of consistency in the results obtained from different larvae."

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

- I appreciate the authors for addressing this aspect. Following the suggestions, the authors conducted Granger causality analysis among the average activities of brain regions, which confirmed a causality link between the triggered activity in the habenula and that in the IPN. The strength of the causal link LHb-IPN is similar to that of other causal links arising from spontaneous activity. The authors also performed partial correlation analysis, which highlighted that the observed LHb-IPN causality link is of a direct type, not being intermediated by other regions. The results of the analysis are presented in Figure 4 as novel panels c and d. The authors have appropriately introduced new text discussing these results in the Results, Discussion, and Methods sections. In accordance with the novel results added to the work, the authors modified the manuscript title as follows: “Two-photon all-optical electrophysiology for the dissection of larval zebrafish brain functional and effective connectivity.”

- I also appreciate the authors for addressing the concerns that were previously raised regarding the thresholding process and its significance in the analysis. The revised explanation and modifications made to the figure legends, methods section, and results section have significantly clarified the thresholding process and its significance in the analysis. The addition of a gray dashed line in Figure 4f to visually represent the threshold value of 0.12 is a helpful addition. The revised description in the results section and methods section also provide a clear understanding of how the threshold was chosen based on the correlation coefficients. These changes greatly improve the clarity and interpretation of the results.

- Regarding the authors’ response to Reviewer #2, their custom 2P light-sheet microscope, despite producing images with a voxel size of $2.2\times2.2\times5\ \mu m^2$, does not have sufficient signal-to-noise ratio (SNR) to consistently resolve single neurons throughout the entire brain. The tight packing of zebrafish neurons requires high contrast between neuronal nuclei and the small space between them. While 1P light-sheet microscopy can achieve this, 2P LSFM is typically prone to SNR issues due to its nonlinear excitation and elongated axial point spread functon(PSF) of the illumination beam. In comparison to other 2P LSF microscopes used for zebrafish brain imaging, their system performs similarly, but with a higher volumetric rate and more thorough sampling of the larval brain. However, achieving single neuron resolution without excessive laser power remains a challenge. To address this limitation, the authors performed voxel-wise and region-wise analyses. They acknowledged this limitation in the Discussion section. I share the concern raised about using inter-region analysis instead of cell-wise analysis in single-cell data. Cell-wise analysis offers a detailed understanding of heterogeneity, identification of rare cell populations, and detection of subtle differences between cells, which is especially relevant in single-cell genomics and spatial transcriptomics. Inter-region analysis may overlook these nuances by averaging variations across cells within a region.

Reviewer #2 (Remarks to the Author):

In the revised manuscript from Turrini et al., authors addressed most of my points.
A few final remarks regarding the revised text
- “electrophysiology” on the title sounds, to me at least, a little weird if not wrong. Could the authors consider a proper rephrasing? Maybe calling it approach or paradigm as referred by the authors in their text?
- "is capable of recording the entire larval brain (5400×800×200 μm3) at volumetric rates up to 5 Hz" Is 500x800x200, correct?
- sd vs calcium events
I get the point from the authors and appreciate the evaluation of the calcium events. One point, though: can they exclude that the same outcome obtained in figure 1d-f could not be obtained with a normal fluorescent protein, but only with a calcium-based sensor?
-figure1J, the calcium transients shown has a decay time at least twice the size indicated and reported in the text, how the authors are explaining this? See figure 2b and e.
- in the discussion, authors are referring to the slow ReaChR kinetics, would be good to have numbers as well as for the normalized value for the action spectrum of the Reacher at the 920nm wavelength used for the imaging before the place where these are already cited
-in the discussion, "calcium amplitude", what does it means?
- in the discussion authors propose the idea that LSM optimized the imaging power density with respect to point scanning approach, so making the reduction of the imaging-due cross talk more effective. Without numbers and models supporting, the statements are quite debatable, as different factors should be considered, like detection efficiency, effective SNR achievable, effective resolution achievable, transversal size of the excitation beam, etc... This goes along with a more general consideration that is inherent to all the all-optical configuration published so far, i.e. that, independently from the technique and the particular pair of molecules, you have to find a trade-off either in the resolution or in other parameters. Maybe the authors would like to consider this aspect.

As I said, I think that the authors addressed most of my point.
I see that the configuration has strong potential for improvements, in particular on the stimulation side, but nevertheless the application scenario is interesting and promising. There is not so much improvement of the current state of the art in using AOD 3D stimulation for the bulk stimulation of an entire region, here the habenula. Stating the approach as high resolution and at the same confirming that the imaging does not allow cellular resolution in imaging is somehow a current limiting factor, that the authors in the last version acknowledged. I think that the contribution is suitable for publication without requiring further evaluation from my side.

Reviewer #3 (Remarks to the Author):

The authors have sufficiently addressed my earlier concerns.

**Point-by-point response to Reviewers' comments**

**Manuscript ID: COMMSBIO-24-0129A**

**Two-photon all-optical neurophysiology for the dissection of larval zebrafish brain functional and effective connectivity**

Reviewer #1 (Remarks to the Author):

- I appreciate the authors for addressing this aspect. Following the suggestions, the authors conducted Granger causality analysis among the average activities of brain regions, which confirmed a causality link between the triggered activity in the habenula and that in the IPN. The strength of the causal link LHb-IPN is similar to that of other causal links arising from spontaneous activity. The authors also performed partial correlation analysis, which highlighted that the observed LHb-IPN causality link is of a direct type, not being intermediated by other regions. The results of the analysis are presented in Figure 4 as novel panels c and d. The authors have appropriately introduced new text discussing these results in the Results, Discussion, and Methods sections. In accordance with the novel results added to the work, the authors modified the manuscript title as follows: "Two-photon all-optical electrophysiology for the dissection of larval zebrafish brain functional and effective connectivity."

- I also appreciate the authors for addressing the concerns that were previously raised regarding the thresholding process and its significance in the analysis. The revised explanation and modifications made to the figure legends, methods section, and results section have significantly clarified the thresholding process and its significance in the analysis. The addition of a gray dashed line in Figure 4f to visually represent the threshold value of 0.12 is a helpful addition. The revised description in the results section and methods section also provide a clear understanding of how the threshold was chosen based on the correlation coefficients. These changes greatly improve the clarity and interpretation of the results.

- Regarding the authors' response to Reviewer #2, their custom 2P light-sheet microscope, despite producing images with a voxel size of 2.2×2.2×5 μm², does not have sufficient signal-to-noise ratio (SNR) to consistently resolve single neurons throughout the entire brain. The tight packing of zebrafish neurons requires high contrast between neuronal nuclei and the small space between them. While 1P light-sheet microscopy can achieve this, 2P LSFM is typically prone to SNR issues due to its nonlinear excitation and elongated axial point spread functon(PSF) of the illumination beam. In comparison to other 2P LSF microscopes used for zebrafish brain imaging, their system performs similarly, but with a higher volumetric rate and more thorough sampling of the larval brain. However, achieving single neuron resolution without excessive laser power remains a challenge.
To address this limitation, the authors performed voxel-wise and region-wise analyses. They acknowledged this limitation in the Discussion section. I share the concern raised about using inter-region analysis instead of cell-wise analysis in single-cell data. Cell-wise analysis offers a

detailed understanding of heterogeneity, identification of rare cell populations, and detection of subtle differences between cells, which is especially relevant in single-cell genomics and spatial transcriptomics. Inter-region analysis may overlook these nuances by averaging variations across cells within a region.

We thank the Reviewer for appreciating the revision work done.

Reviewer #2 (Remarks to the Author):

In the revised manuscript from Turrini et al., authors addressed most of my points.
A few final remarks regarding the revised text

- “electrophysiology” on the title sounds, to me at least, a little weird if not wrong. Could the authors consider a proper rephrasing? Maybe calling it approach or paradigm as referred by the authors in their text?

We considered the Reviewer’s suggestion and changed the term “electrophysiology” with “neurophysiology”. The title of the revised manuscript is “Two-photon all-optical neurophysiology for the dissection of larval zebrafish brain functional and effective connectivity”.

- "is capable of recording the entire larval brain (5400×800×200 μm3) at volumetric rates up to 5 Hz"
Is 500x800x200, correct?

We thank the Reviewer for highlighting the typo. We corrected the text.

- sd vs calcium events
I get the point from the authors and appreciate the evaluation of the calcium events. One point, though: can they exclude that the same outcome obtained in figure 1d-f could not be obtained with a normal fluorescent protein, but only with a calcium-based sensor?

Unlike a fluorescent calcium indicator, a fluorescent protein exhibits a constant fluorescence emission (net of photobleaching) that in no way depends on neuronal activity. This would have made it impossible to assess the potential excitation of neurons due to the ReaChR activation by the wavelength used to generate the lightsheet.

-figure1J, the calcium transients shown has a decay time at least twice the size indicated and reported in the text, how the authors are explaining this? See figure 2b and e.

We verified that the average calcium transient in Figure 1j has a decay time of 10.2 s, while the decay time of the average transient in Figure 2b (blue trace, 500 ms scan time) is 12.3 s and the average decay time reported in Figure 2e (blue data, 500 ms scan time) is 12.4 s. The transient shown in Figure 1j has a decay time that is below the average yet within the observed variability range.

- in the discussion, authors are referring to the slow ReaChR kinetics, would be good to have numbers as well as for the normalized value for the action spectrum of the Reacher at the 920nm wavelength used for the imaging before the place where these are already cited.

Regarding ReaChR slow kinetic, the channel off-rate is already reported in the text (line 352, Discussion).
Following the Reviewer comment we added to the Discussion section numbers regarding ReaChR 2P cross-section at 920nm, as follows (addition in bold text):
"It is worth noting that, despite the negligible crosstalk, 2P light-sheet imaging may still lead to subthreshold activation of ReaChR$^{+}$ neurons **(at 920 nm the opsin retains approximately 25% of the peak action cross-section[67])**, potentially resulting in altered network excitability[68]."

-in the discussion, "calcium amplitude", what does it means?

We thank the Reviewer for highlighting this point. It was actually a typo. In the revised version of the manuscript we corrected in "calcium peak amplitude".

- in the discussion authors propose the idea that LSM optimized the imaging power density with respect to point scanning approach, so making the reduction of the imaging-due cross talk more effective. Without numbers and models supporting, the statements are quite debatable, as different factors should be considered, like detection efficiency, effective SNR achievable, effective resolution achievable, transversal size of the excitation beam, etc... This goes along with a more general consideration that is inherent to all the all-optical configuration published so far, i.e. that, independently from the technique and the particular pair of molecules, you have to find a trade-off either in the resolution or in other parameters. Maybe the authors would like to consider this aspect.

In the Discussion section we hypothesized that the discrepancy between our observations and those of Chen et al. (Ref. 42) regarding the power dependency of the crosstalk activation of ReaChR by the 920 nm wavelength, may be due to the different optical system employed to perform imaging. Generally speaking, at equal power, 2P point-scanning imaging presents increased photon density with respect to 2P light-sheet microscopy due to the different PSF dimensions which impacts crosstalk.
To better clarify this point we modified the Discussion as follows (additions in bold text):

"Previous research has demonstrated that the slow channel closing of ReaChR makes this opsin more susceptible to crosstalk activation when scanning the 920 nm imaging laser at power levels exceeding 60 mW[42]. However, in our work, we did not observe a significant increase in cross-activation even at power levels as high as 100 mW. This divergence can be attributed to ~~our use~~ **the peculiar excitation features** of 2P light-sheet **imaging** ~~instead of~~ **compared to** 2P point scanning imaging."

As I said, I think that the authors addressed most of my point.
I see that the configuration has strong potential for improvements, in particular on the stimulation side, but nevertheless the application scenario is interesting and promising. There is not so much improvement of the current state of the art in using AOD 3D stimulation for the bulk stimulation of an entire region, here the habenula. Stating the approach as high resolution and at the same confirming that the imaging does not allow cellular resolution in imaging is somehow a current limiting factor, that the authors in the last version acknowledged. I think that the contribution is suitable for publication without requiring further evaluation from my side.

We thank the Reviewer for appreciating the work done during revisions.

Reviewer #3 (Remarks to the Author):

The authors have sufficiently addressed my earlier concerns.

We thank the Reviewer.