nature portfolio

Peer Review File

A Multimodal Adaptive Optical Microscope For In Vivo Imaging from Molecules to Organisms

Corresponding Author: Professor Srigokul Upadhyayula

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

Version 0:

Decision Letter:

30th Jun 2025

Dear Gokul,

Happy summer!

Your Article, "A Multimodal Adaptive Optical Microscope For In Vivo Imaging from Molecules to Organisms", has now been seen by three reviewers. As you will see from their comments below, while they have no substantive technical criticisms, they have raised a number of concerns about presentation. We therefore invite you to revise your manuscript to address these concerns for what will hopefully be a quick sign off.

We ask that you provide the decision tree (ref 1), discuss rationale behind MOSAIC design, and better discuss complexity and other potential downsides, including big data handling. We ask that you do this in the spirit of making the paper read a bit less like an ad (ref 3).

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests you think are outside the scope of this work.

When revising your paper:

* include a point-by-point response to the reviewers and to any editorial suggestions

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

* address the points listed described below to conform to our open science requirements

* ensure it complies with our general format requirements as set out in our guide to authors at www.nature.com/naturemethods

* resubmit all the necessary files electronically by using the link below to access your home page

Link Redacted

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

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When revising your manuscript, please update your reporting summary.

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Please submit these with your revised manuscript. They will be available to reviewers to aid in their evaluation if the paper is re-reviewed. If you have any questions about the checklist, please see http://www.nature.com/authors/policies/availability.html or contact me.

Please note that these forms are dynamic 'smart pdfs' and must therefore be downloaded and completed in Adobe Reader. We will then flatten them for ease of use by the reviewers. If you would like to reference the guidance text as you complete the template, please access these flattened versions at http://www.nature.com/authors/policies/availability.html.

For any revision that includes light microscopy data, we ask our authors to please include a completed light microscopy reporting table [https://www.nature.com/documents/Light_microscopy_reporting_table.xlsx] to ensure the methods are described thoroughly. The table will be available to reviewers and ultimately published should the manuscript be accepted at the journal.

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When submitting the revised version of your manuscript, please pay close attention to our Digital Image Integrity Guidelines and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

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When re-submitting your manuscript, please ensure that any supplementary figures and tables that are crucial to the manuscript's conclusions are converted into Extended Data figures and tables to increase visibility of these data. Extended Data figures and tables are online-only (present in the online PDF and full-text HTML versions of the paper), peer-reviewed display items that provide essential background to the article but are not included in the main article due to space constraints. A maximum of ten Extended Data display items (figures and tables) is permitted.

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We strongly encourage you to deposit all new data associated with the paper in a persistent repository where they can be freely and enduringly accessed. We recommend submitting the data to discipline-specific and community-recognized repositories; a list of repositories is provided here: http://www.nature.com/sdata/policies/repositories

All novel DNA and RNA sequencing data, protein sequences, genetic polymorphisms, linked genotype and phenotype data, gene expression data, macromolecular structures, and proteomics data must be deposited in a publicly accessible database, and accession codes and associated hyperlinks must be provided in the "Data Availability" section.

For papers containing bioimaging data, we strongly recommend depositing the data to Bioimage Archive (https://www.ebi.ac.uk/bioimage-archive/). Associated accession codes and hyperlinks should be provided in the "Data Availability" section.

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please select the Source Data file type and use the Title field in the File Description tab to indicate which figure the source data pertains to.

Please include a “Data availability” subsection in the Online Methods. This section should inform readers about the availability of the data used to support the conclusions of your study, including accession codes to public repositories, references to source data that may be published alongside the paper, unique identifiers such as URLs to data repository entries, or data set DOIs, and any other statement about data availability. At a minimum, you should include the following statement: “The data that support the findings of this study are available from the corresponding author upon request”, describing which data is available upon request and mentioning any restrictions on availability. If DOIs are provided, please include these in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see: http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf

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More details about our materials availability policy can be found at https://www.nature.com/nature-portfolio/editorial-policies/reporting-standards#availability-of-materials

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

Sincerely,
Rita

Rita Strack, Ph.D.
Senior Editor
Nature Methods

Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

In this manuscript, TM Fu et. al. present MOSAIC (Multimodal Optical Scope with Adaptive Imaging Correction), a reconfigurable microscope that incorporates multiple imaging modalities
(widefield epifluorescence, label-free oblique illumination, lattice light-sheet (LLS), 3D Structured Illumination Microscopy (SIM), LLS-SIM, Image Scanning Microscopy (ISM), 2-photon), all of which are equipped to minimize sample-induced aberrations by applying adaptive optics correction. This work embodies a herculean effort to combine a broad array of microscopy techniques, while sharing key hardware and software modules wherever possible. To demonstrate the utility, the authors showcase MOSAIC across a wide array of imaging scenarios encompassing multiple time and length scales.

Suggested Improvements:
A. Although the examples presented in the manuscript give a flavor of what is attainable with MOSAIC, the readers would benefit greatly from a decision tree in navigating the myriad possibilities with this instrument. To that end, a flowchart or a table in the main text that highlights the following would help the readers greatly:
1. the nominal specifications (FOV, resolution, acquisition speed) of each modality
2. native imaging context as a starting point for a given modality
3. Modalities that extend the capabilities further (e.g., LLS-SIM for LLM)
4. Modalities for correlative imaging (e.g., Oblique Illumination interleaved with LLS)
5. Trade-offs (e.g., with Adaptive Optics ON vs OFF, Denoising for 3D-SIM, acquisition speed, light exposure and such)

B. I commend the authors' sincerity about the complexity of MOSAIC, not just to assemble one but even to operate across all available modalities. This alone makes MOSAIC challenging for a typical imaging core that needs to serve multiple research projects simultaneously. Further discussion on the challenges presented by a system such as MOSAIC, and the opportunity via an alternative model of an Observatory, as cited in ref 49 (Betzig E., Nature Methods 22, 2025), would help the readers appreciate the possibilities unlocked by this new ecosystem.

C. In the "Microscope design and characterization" section, please provide summary statistics of measurements from Figures S2, S3, S4, and S8

D. In the "Adaptive optical LLSM in living organisms" section, paragraph 2, please quantify the FOV size for which a single isoplanatic patch suffices

E. Supplementary Fig S2 (c), S3 (b), label deformable mirror. Are all components in magenta shared across modalities? If so, please mention in figure text

All in all, the authors present a single reconfigurable instrument that not only incorporates multiple microscopy modalities but also extends the utility of these modalities to image multicellular systems by incorporating adaptive optics correction. This work is a treasure trove for tool builders and the nearly 1000 pages of documentation mentioned in the manuscript will be invaluable for any research lab or imaging core interested in replicating MOSAIC in its entirety or as a subset.

-Raghav K. Chhetri

Reviewer #2 (Remarks to the Author):

A. Summary of Key Results
The authors present MOSAIC, a reconfigurable and multimodal adaptive optics microscope that integrates multiple advanced imaging modalities: LLSM, SIM (3D and LLS-SIM), SMLM/SPT, ISM, two-photon point scanning, and label-free oblique illumination, within a unified optical platform. The system allows for switching between modalities and incorporates adaptive optics (AO) for all imaging modes.
The manuscript demonstrates MOSAIC’s utility across a wide range of biological applications, including long-term live imaging of cell division, optogenetic perturbation, single-molecule tracking, and nanoscale imaging of expanded human tissue. These results span a large spatial and temporal range and convincingly demonstrate the versatility of the system. The final sentence in the “Main” section is especially important, as it emphasizes the platform's integrative power to bridge multiscale biological processes.

B. Originality and Significance
MOSAIC is a technically innovative and highly significant contribution to the field of optical microscopy, facilitating complex experiments that previously required multiple specialized microscopes. While modular microscopes capable of multiple imaging modes do exist, this platform is unique in its combination of:
• Multiple imaging modalities (SIM, LLSM, SMLM, ISM, two-photon),
• Full integration of adaptive optics,
• Unified control and sample registration,
• Live and fixed imaging across diverse model systems.
This integration is not matched by any existing published system to my knowledge. If comparable systems do exist, the authors should provide a brief comparison in the Discussion to help readers understand how MOSAIC is differentiated.

C. Data and Methodology
The optical design, hardware configuration, and control software are well described and technically sound. The Supplementary Information offers extensive detail on the optics, wavefront correction, and imaging paths. The sample preparation and biological protocols are appropriate for the applications shown.

Specific suggestions for improvement include:
• Add a citation to the original LLSM paper when referencing the earlier system using 0.65 NA excitation and 1.1 NA detection objectives.
• Quantify the “modest reduction in resolution” due to design trade-offs. Include lateral and axial resolution comparisons.
• When 3D FOVs are reported, clearly define axis order (e.g., X × Y × Z).
• In the “Long-term multimodal imaging” section, specify whether the reported FOV (1.218 × 0.975 mm^2) is a measured or nominal value and indicate any range or uncertainty.
• Clarify that the use of “correlative” in the optogenetics section is not referring to mathematical-based correlation, to distinguish it from correlative fluorescence microscopy techniques.

D. Appropriate Use of Statistics and Treatment of Uncertainties
In general, statistical treatment is appropriate, though some improvements would enhance clarity and rigor:
• In the super-resolution tracking analysis, uncertainties should be reported with a single significant figure (e.g., 25 ± 4 nm, not 24.6 ± 4.3 nm).
• In Figure 3, localization precisions (e.g., 16 nm, 23 nm, etc.) are listed without uncertainties. Please add error ranges.
• The claim of a 2.7× increase in dendrite detection (Figure 6) should be clearly quantified and referenced in the main text.
• When describing acquisition speed (“approaching peak acquisition speed of modern sCMOS cameras”), please include an actual performance value or range for clarity.

E. Conclusions: Robustness and Validity
The conclusions are robust and well supported by data. The biological demonstrations span a range of imaging conditions and objectives, showing that MOSAIC can perform across modes with minimal reconfiguration and consistent image quality.
The system’s long-term stability and ability to maintain registration across imaging paths is impressive. AO implementation appears effective in improving both excitation and detection resolution, and its inclusion across all modes is a major strength.

F. Suggested Improvements
Textual and Structural:
• In the second paragraph of the “Main” section, include a specific biological example to support the argument for needing multiple modalities in a single system.
• In the Discussion, the phrases “…a stable of similarly performative…” and “…extending the use space…” are unclear. Reword these for clarity.
• Suggest moving the second paragraph of the Discussion to the end for a more impactful final impression.
• In paragraph 3 of the Discussion, the authors mention “technological studies” and “biological investigation.” Please add specific examples to help contextualize the system’s future applications.
Figure-Related:
• Figure 1b: Difficult to read. Increase label size and specify “left,” “middle,” and “right” panels using corresponding colors in the caption similar to in text.
• Figure 2: Diffusion histograms are difficult to interpret. Consider stating the binning explicitly and using cumulative distribution functions, as shown in Fig. S9, which presents similar data more accessibly.
• Figure 3: To help verify the claim of nanoscale resolution, including a line section profile and fit to demonstrate measured resolution. Additionally, grey can be challenging to discern, and using more distinct colors may improve accessibility.
• Figure 5b: The layout is difficult to follow. Moving FFT insets to each section individually may help with readability.
• Figure 6: The spatial offset between the “No AO” and “AO + Decon” panels complicates comparison. Explain how the 2.7× dendrite increase was determined and reference it in the main text.
• Figure S21: Consider reorienting the data to a 3-row by 4-column layout to improve visual clarity.
• Figure S23: Include a full comparison of No AO, No AO + Decon, AO, and AO + Decon to allow readers to evaluate individual and combined effects.
Data or Analysis Suggestions:
• Reference the appropriate Supplementary Information section where denoising is discussed in the context of extended SIM imaging.

G. References
The references are appropriate and comprehensive. The authors cite foundational work in light sheet, structured illumination, adaptive optics, and super-resolution microscopy.
Please ensure that the original LLSM paper is cited where prior system designs are discussed.

H. Clarity and Context
The manuscript is well written, with a clear abstract and logically organized structure. The introduction effectively motivates the development of a unified multimodal microscope, and the conclusions summarize the major contributions and future potential.
Minor points for improvement:
• Define acronyms like FUCCI at first mention.
• In the abstract and introduction, consider reinforcing the biological rationale for MOSAIC by briefly previewing some of the key systems demonstrated (e.g., zebrafish embryos, cultured cells, expanded human brain tissue).

Conclusion
This is an impressive technical manuscript describing a flexible, high-performance microscopy platform with significant biological utility. With moderate revisions that improve figure clarity, quantification, and presentation of technical terms and results, the work will make a valuable contribution to the field. The MOSAIC system is well positioned to advance multiscale biological imaging across research disciplines.

Reviewer #3 (Remarks to the Author):

This manuscript from Fu, Liu, Milkie, and Rui et al., provide an overview of the capabilities of a custom-built multi-modal microscope with adaptive optics that can capture exquisite detail of biological specimens. This manuscript represents both a technical advance and extensive, collaborative work by multiple well-respected scientists. Several key differences set this microscope apart from other home-built systems including adaptive optics for all modalities, the ability to image the same specimen with different modalities to perform comparisons and/or collect different levels of spatial and/or temporal data. For the biological imaging community, this microscope has already provided a valuable way to examine a handful of living specimens and will be of interest to developmental biologists and neuroscientist studying in vivo cell behaviors.
The data are eye-catching, quantifiable, and some of the most spatially and temporally resolved images of living cells in organisms I've seen.

Below are some suggestions and minor concerns, which reflect my perspective as a cell and developmental biologist, someone who might lead the writing of a grant to fund the building and maintenance of such an instrument at our institution or who might want to visit to use the instrument.

In its current form, I'm not sure that this reads like a true "Methods" paper. At times, I found myself thinking that I was reading an advertisement for the instrument. Perhaps the authors could specifically discuss some of their design thinking and highlight the different imaging modalities with an initial figure. One possibility would be to combine Fig S1 and S8 (or some version of them) into a didactic Fig 1 to illustrate the optical foundation/basis for the rest of the figures to follow. Along these same lines, there are a lot of abbreviations used to describe the imaging modalities, a text box with key abbreviations described/defined would go a long way to improve readability for a broader audience.

Another important issue is the lack of discussion of the way the massive files are handled (I'm assuming some sort of distributed computing). Data storage, handling, and analysis is a huge issue when single experiments take up tens of TBs of data. Some discussion of this beyond machine learning seems important, especially from a methods and instrument-building side of things.

Finally, I've listed some easily addressable very specific points below:
- I appreciate the multi-functionality and the ability to switch among a suite of imaging modalities, but it wasn't clear to me how the system is controlled and how exactly one switches between modes and/or whether multiple specimens could be imaged during the same experiment.

-In the multimodal live cell super-resolution section, the words "minimal photobleaching" are used. What exactly does this mean and how was it determined?

-On top of page 7, SNR is used for the first time (I think) and not defined. Might be a good idea to double check all other abbreviations (also see comment above about making a text box for key abbreviations)

-I think I probably just missed some citations (there is a lot of data and text here, which is something to consider vis-à-vis publication). For instance, which software was used for image segmentation and data visualizations (image production and plots)?

-Some of the color combinations are colorblind accessible and others aren't. Figure 4 is a good example of clearly accessible color combinations, but some aspects of other figures (e.g., combos of cyan/magenta and green/yellow) are more difficult to distinguish.
Consider viewing images with Color Oracle (https://colororacle.org/) to ensure colorblind accessibility. Other helpful resources include:
Wong, B. Points of view: Color blindness. Nat Methods 8, 441 (2011) ; https://thenode.biologists.com/data-visualization-with-flying-colors/research/; and https://davidmathlogic.com/colorblind/#%23D81B60-%231E88E5-%23FFC107-%23004D40.

Version 1:

Decision Letter:

Our ref: NMETH-A61329A

5th Nov 2025

Dear Gokul,

Thank you for submitting your revised manuscript "A Multimodal Adaptive Optical Microscope For In Vivo Imaging from Molecules to Organisms" (NMETH-A61329A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Methods, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting

requirements within two weeks or so. Please do not upload the final materials and make any revisions until you receive this additional information from us.

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

Best regards,
Nina

Nina Vogt, PhD
Senior Editor
Nature Methods

Reviewer #1 (Remarks to the Author):

The authors have sufficiently addressed previous suggestions and made notable changes to the manuscript, including a decision tree (figure S26) and further discussion on a proposed model to broadly support systems such as MOSAIC.

Regarding "summary statistics of measurements," here is a rewording to clarify the suggestion:
In the Microscope design and characterization, please quantify the bead-based PSFs shown in S8 and list corresponding lateral and axial FWHM for all modalities shown in S2,S3,S4.

Reviewer #2 (Remarks to the Author):

I appreciate the authors taking the time to address my and the other reviewer's suggestions and comments thoroughly. After reading through the new manuscript, I have no further suggestions and recommend publication of this work.

Reviewer #3 (Remarks to the Author):

It seems like the authors have addressed my comments and those of the other reviewers.
My only remaining issue is not a negative comment about the science or the writing. There are over 20 supplemental figures. Many, if not all of the supplemental figures, are essential to the manuscript, especially the new supplemental figure S26, which is a decision tree about which type of microscopy is indicated for different specimens. I agree with reviewer 1 that this is important and it helps the reader consider all of the different imaging modalities offered by MOSAIC. It seems like a shame that this figure is buried in the supplemental figures. I would push to have this figure in the main paper if at all possible.

Version 2:

Decision Letter:

10th Mar 2026

Dear Gokul,

I am pleased to inform you that your Article, "A Multimodal Adaptive Optical Microscope For In Vivo Imaging from Molecules to Organisms", has now been accepted for publication in Nature Methods. The received and accepted dates will be June 2nd, 2025 and March 10th, 2026. This note is intended to let you know what to expect from us over the next month or so, and to let you know where to address any further questions.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Methods style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required. It is extremely important that you let us know now whether you will be difficult to contact over the next month. If this is the case, we ask that you send us the contact information (email, phone and fax) of someone who will be able to check the proofs and deal with any last-minute problems.

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

Best regards,
Nina

Nina Vogt, PhD
Senior Editor
Nature Methods

Your Article, "A Multimodal Adaptive Optical Microscope For In Vivo Imaging from Molecules to Organisms", has now been seen by three reviewers. As you will see from their comments below, while they have no substantive technical criticisms, they have raised a number of concerns about presentation. We therefore invite you to revise your manuscript to address these concerns for what will hopefully be a quick sign off.

We ask that you provide **the decision tree (ref 1)**, **discuss rationale behind MOSAIC design**, and **better discuss complexity and other potential downsides, including big data handling**. We ask that you do this in the spirit of making the paper read a bit less like an ad (ref 3).

We thank the Reviewers for their thoughtful feedback. In response, we:
(i) added the requested decision tree (Fig S26) as a concise flowchart to guide imaging-mode selection;
(ii) clarified the rationale for MOSAIC's design in the Discussion and direct the Reviewer to the details in "Microscope design and characterization" section; and
(iii) expanded the discussion on a "cell observatory" model to handle MOSAIC-scale datasets.

Reviewer #1 (Remarks to the Author):

In this manuscript, TM Fu et. al. present MOSAIC (Multimodal Optical Scope with Adaptive Imaging Correction), a reconfigurable microscope that incorporates multiple imaging modalities (widefield epifluorescence, label-free oblique illumination, lattice light-sheet (LLS), 3D Structured Illumination Microscopy (SIM), LLS-SIM, Image Scanning Microscopy (ISM), 2-photon), all of which are equipped to minimize sample-induced aberrations by applying adaptive optics correction. This work embodies a herculean effort to combine a broad array of microscopy techniques, while sharing key hardware and software modules wherever possible. To demonstrate the utility, the authors showcase MOSAIC across a wide array of imaging scenarios encompassing multiple time and length scales.

We appreciate the positive comments from the reviewer.

Suggested Improvements:
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4. Modalities for correlative imaging (e.g., Oblique Illumination interleaved with LLS)
5. Trade-offs (e.g., with Adaptive Optics ON vs OFF, Denoising for 3D-SIM, acquisition speed, light exposure and such)

- As suggested, we added a decision tree / flowchart as Figure S26 to give the readers and future users general guidance on selecting the appropriate modality for their specimen. The flowchart covers categorical experimental settings including sample category, resolution, imaging speed, choice of using AO or not, etc. On the other hand, specific imaging conditions like FOV, light exposure, acquisition speed, denoising, etc., could be applied to many different imaging modes as warranted by the specimen, fluorescent labels, and the biological questions to be studied. As such, we deemed that the choice of how to set these parameters would be more appropriate to be decided individual users for each specific experiment.

B. I commend the authors' sincerity about the complexity of MOSAIC, not just to assemble one but even to operate across all available modalities. This alone makes MOSAIC challenging for a typical imaging core that needs to serve multiple research projects simultaneously. Further discussion on the challenges presented by a system such as MOSAIC, and the opportunity via an alternative model of an Observatory, as cited in ref 49 (Betzig E., Nature Methods 22, 2025), would help the readers appreciate the possibilities unlocked by this new ecosystem.

- We have expanded the Discussion on the 'Cell Observatory' model that we anticipate will broaden access and support studies that are impractical for individual labs.

C. In the "Microscope design and characterization" section, please provide summary statistics of measurements from Figures S2, S3, S4, and S8

- Figures S2, S3, S4 are optical schematics, as such, statistics of measurements are not applicable. Figure S8 compiles representative PSFs and corresponding OTFs experimentally measured for each modality.

D. In the "Adaptive optical LLSM in living organisms" section, paragraph 2, please quantify the FOV size for which a single isoplanatic patch suffices

- We appreciate the reviewer's request. However, isoplanatic patch size varies widely with tissue composition, even within a single specimen and potentially across time. Reliable determination would require fine-grained volumetric tiling with a Shack–Hartmann wavefront sensor followed by more coarse-grained tiling for acquisition; however, we did not perform this analysis on samples in this paper. Rather, for the AO-LLSM experiments reported here, tile and correction-window sizes followed parameters that we determined previously to achieve reasonable corrections across imaged volume (AOLLSM; 10.1126/science.aaq1392). Patch sizes ranged from a few micrometers to tens of micrometers, depending on the FOV. The total volume sizes and tiling details are listed in Table S1. In separate work, we developed AI methods to estimate patch size from acquired data (AOViFT; arXiv:2503.12593) in optically aberrated samples.

E. Supplementary Fig S2 (c), S3 (b), label deformable mirror. Are all components in magenta shared across modalities? If so, please mention in figure text

- We have added the deformable mirror labels. Not all components in magenta are shared across all modalities, they represent fixed position optical elements as opposed to variable position optical elements (e.g. flip mirrors) which are labeled in orange.

All in all, the authors present a single reconfigurable instrument that not only incorporates multiple microscopy modalities but also extends the utility of these modalities to image multicellular systems by incorporating adaptive optics correction. This work is a treasure trove for tool builders and the nearly 1000 pages of documentation mentioned in the manuscript will be invaluable for any research lab or imaging core interested in replicating MOSAIC in its entirety or as a subset.

Reviewer #2 (Remarks to the Author):

A. Summary of Key Results

The authors present MOSAIC, a reconfigurable and multimodal adaptive optics microscope that integrates multiple advanced imaging modalities: LLSM, SIM (3D and LLS-SIM), SMLM/SPT, ISM, two-photon point scanning, and label-free oblique illumination, within a unified optical platform. The system allows for switching between modalities and incorporates adaptive optics (AO) for all imaging modes.

The manuscript demonstrates MOSAIC's utility across a wide range of biological applications, including long-term live imaging of cell division, optogenetic perturbation, single-molecule tracking, and nanoscale imaging of expanded human tissue. These results span a large spatial and temporal range and convincingly demonstrate the versatility of the system. The final sentence in the "Main" section is especially important, as it emphasizes the platform's integrative power to bridge multiscale biological processes.

- We appreciate the positive comments from the reviewer.

B. Originality and Significance

MOSAIC is a technically innovative and highly significant contribution to the field of optical microscopy, facilitating complex experiments that previously required multiple specialized microscopes. While modular microscopes capable of multiple imaging modes do exist, this platform is unique in its combination of:

• Multiple imaging modalities (SIM, LLSM, SMLM, ISM, two-photon),
• Full integration of adaptive optics,
• Unified control and sample registration,
• Live and fixed imaging across diverse model systems.

This integration is not matched by any existing published system to my knowledge. If comparable systems do exist, the authors should provide a brief comparison in the Discussion to help readers understand how MOSAIC is differentiated.

- To our knowledge, no published system integrates the modalities demonstrated using the MOSAIC with end-to-end adaptive optics, unified control and sample registration, and correlative workflows in a single instrument.

C. Data and Methodology

The optical design, hardware configuration, and control software are well described and technically sound. The Supplementary Information offers extensive detail on the optics, wavefront correction, and imaging paths. The sample preparation and biological protocols are appropriate for the applications shown.

Specific suggestions for improvement include:

• Add a citation to the original LLSM paper when referencing the earlier system using 0.65 NA excitation and 1.1 NA detection objectives.

- We have added this reference.

• Quantify the "modest reduction in resolution" due to design trade-offs. Include lateral and axial resolution comparisons.

- We previously characterized the resolution limits for the objective pairs used here in the MOSAIC (NA_{exc} = 0.6, NA_{det} = 1.0) and the original LLSM (NA_{exc} = 0.7, NA_{det} = 1.1). We have added a reference to this work (DOI: 10.1126/sciadv.ade6623).

• When 3D FOVs are reported, clearly define axis order (e.g., X × Y × Z).
- We have noted the axis order in the first row of Table S1.

• In the “Long-term multimodal imaging” section, specify whether the reported FOV (1.218 × 0.975 mm^2) is a measured or nominal value and indicate any range or uncertainty.
- We specify the FOV as stitched dimensions of the final processed dataset. The stage optical encoders have 1 nm resolution (0.000001 mm), making the readout contribution to FOV uncertainty negligible at this scale.

• Clarify that the use of “correlative” in the optogenetics section is not referring to mathematical-based correlation, to distinguish it from correlative fluorescence microscopy techniques.
- We revised the text to introduce “correlative” as interleaved acquisition of distinct imaging modalities within the same experiment.

D. Appropriate Use of Statistics and Treatment of Uncertainties
In general, statistical treatment is appropriate, though some improvements would enhance clarity and rigor:
• In the super-resolution tracking analysis, uncertainties should be reported with a single significant figure (e.g., 25 ± 4 nm, not 24.6 ± 4.3 nm).
- We revised the analysis to report uncertainties with a single significant figure.

• In Figure 3, localization precisions (e.g., 16 nm, 23 nm, etc.) are listed without uncertainties. Please add error ranges.
- We added the uncertainties to the revised manuscript.

• The claim of a 2.7× increase in dendrite detection (Figure 6) should be clearly quantified and referenced in the main text.
- We added the quantification to the Results and updated the Figure 6 caption.

• When describing acquisition speed (“approaching peak acquisition speed of modern sCMOS cameras”), please include an actual performance value or range for clarity.
- The numbers are added for a clearer performance comparison.

E. Conclusions: Robustness and Validity
The conclusions are robust and well supported by data. The biological demonstrations span a range of imaging conditions and objectives, showing that MOSAIC can perform across modes with minimal reconfiguration and consistent image quality.
The system’s long-term stability and ability to maintain registration across imaging paths is impressive. AO implementation appears effective in improving both excitation and detection resolution, and its inclusion across all modes is a major strength.

F. Suggested Improvements

Textual and Structural:

• In the second paragraph of the “Main” section, include a specific biological example to support the argument for needing multiple modalities in a single system.

- To preserve generality, we did not include a specific biological example in the Main section. Instead, we direct readers to representative, multi-modal use cases (Figs. 2e–f, S11–S13) that combine SIM, LLSM, label-free imaging, and patterned photoactivation. These examples demonstrate how an integrated system enables correlative workflows when the optimal modality is uncertain or when multiple measurements are required.

• In the Discussion, the phrases “…a stable of similarly performative…” and “…extending the use space…” are unclear. Reword these for clarity.

- The phrases have been reworded.

• Suggest moving the second paragraph of the Discussion to the end for a more impactful final impression.

- We evaluated alternative orderings after revision and found that the current structure provides the clearest logic and strongest transitions. As suggested by Reviewer 1, we expanded the Discussion to highlight our forward-looking “cell observatory” initiative – the next stage of our research aimed at efficiently extracting meaningful biological insights from large-scale datasets.

• In paragraph 3 of the Discussion, the authors mention “technological studies” and “biological investigation.” Please add specific examples to help contextualize the system’s future applications.

- We hope that the numerous demonstrations presented within this manuscript are sufficient to illustrate the current (and future) applications of the microscope. In the Discussion, we directed readers to additional cited technological studies and biological investigations, which further highlight the system’s impact. However, due to space constraints, and to keep the focus of the Discussion on the instrument capabilities, we chose not to further describe biological systems studied in each cited reference.

Figure-Related:

• Figure 1b: Difficult to read. Increase label size and specify “left,” “middle,” and “right” panels using corresponding colors in the caption similar to in text.

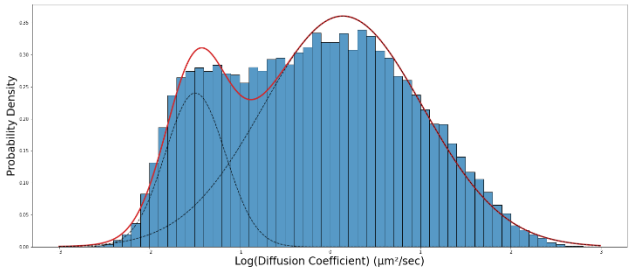
- We increased label sizes in Figure 1b. The text and caption now identify the left, middle, and right panels with their corresponding colors.

• Figure 2: Diffusion histograms are difficult to interpret. Consider stating the binning explicitly and using cumulative distribution functions, as shown in Fig. S9, which presents similar data more accessibly.

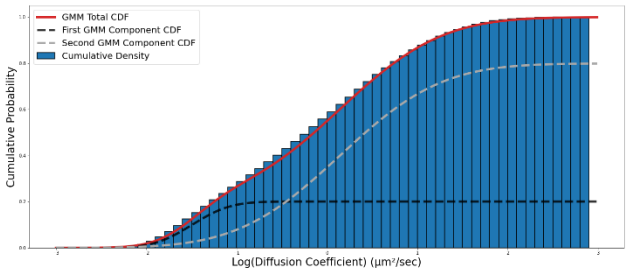
- We thank the reviewer for this comment. We have added the binning parameters used for the histogram to the figure legend for clarity. While we agree that cumulative distributions are powerful tools for comparing and displaying multiple distinct distributions, we believe there may be some confusion regarding the purpose of Figure 2a. In this analysis, we fit a diffusion model to each single-molecule trajectory and extracted the corresponding diffusion coefficient. The histogram in Figure 2a displays the distribution of these extracted diffusion coefficients, which is a widely used approach for visualizing the diffusive behavior of transcription factors in single-molecule tracking experiments (PMID: 24630727, PMID: 27484239, PMID: 34375585). This distribution is well described by a two-component model, consistent with prior literature. The slower-diffusing component is interpreted as representing DNA-bound molecules, while the faster-diffusing component reflects freely diffusing molecules searching for binding sites. In this context, we find that cumulative distribution functions obscure the presence of these distinct diffusive components and make it more difficult to assess their relative contributions.

  See below for the original presentation (as probability density) vs. the suggested revision as (cumulative distribution). We believe that displaying the data as a probability density function, overlaid with the fitted model, provides a more clear and intuitive way to visualize these distinct subpopulations.

  Original figure (probability density):



Cumulative distribution with the fitted model overlaid:



• Figure 3: To help verify the claim of nanoscale resolution, including a line section profile and fit to demonstrate measured resolution. Additionally, grey can be challenging to discern, and using more distinct colors may improve accessibility.

- Nanoscale resolution in this figure derives from single-molecule localization (Figure 3a) and from 4x expansion (Figure 3b–f), both sub-diffraction. Rather than relying on line section profiles, we quantify resolution using bead-based PSF/OTF analyses (Fig. S8) as has been validated in our previous publication DOI: 10.1126/sciadv.ade6623.

• Figure 5b: The layout is difficult to follow. Moving FFT insets to each section individually may help with readability.

- As suggested, we moved the FFT panels as insets to Figure 5b.

• Figure 6: The spatial offset between the “No AO” and “AO + Decon” panels complicates comparison. Explain how the 2.7× dendrite increase was determined and reference it in the main text.

- Small spatial offsets reflect in vivo motion; the display panels are not pixel-registered.
- We clarified the increase in detected events and updated the Results text and the Fig. 6 caption accordingly.

• Figure S21: Consider reorienting the data to a 3-row by 4-column layout to improve visual clarity.

- As suggested, we reorganized Figure S21.

• Figure S23: Include a full comparison of No AO, No AO + Decon, AO, and AO + Decon to allow readers to evaluate individual and combined effects.

- The appropriate comparison is between No AO and AO without decon. Since we know there is sample induced aberration, it is not appropriate to deconvolve with an ideal PSF that is not representative of the recorded sample.
- Our goal is to demonstrate the benefit of AO. Accordingly, Figure. S23 compares the same FOV without and with AO. Deconvolution presumes an aberration-free PSF; in our “No AO” setting, the PSF is aberrated, so “No AO + Decon” would use an incorrect PSF kernel and can lead to artifacts in the reconstructed image. We therefore exclude No AO + Decon, and retain the AO vs. no-AO comparison.

Data or Analysis Suggestions:

• Reference the appropriate Supplementary Information section where denoising is discussed in the context of extended SIM imaging.

- The reference has been added.

G. References

The references are appropriate and comprehensive. The authors cite foundational work in light sheet, structured illumination, adaptive optics, and super-resolution microscopy.

Please ensure that the original LLSM paper is cited where prior system designs are discussed.

- The appropriate reference has been added.

H. Clarity and Context

The manuscript is well written, with a clear abstract and logically organized structure. The introduction effectively motivates the development of a unified multimodal microscope, and the conclusions summarize the major contributions and future potential.
Minor points for improvement:
• Define acronyms like FUCCI at first mention.

- We double-checked and added acronyms at first mention. In addition, we have added a new table (S2) defining all abbreviations used in this manuscript.

• In the abstract and introduction, consider reinforcing the biological rationale for MOSAIC by briefly previewing some of the key systems demonstrated (e.g., zebrafish embryos, cultured cells, expanded human brain tissue).

- Thank you for this suggestion. To conform to journal requirements on manuscript length, we opted to concisely summarize key applications rather than list each specific sample. However, we do note that that the abstract lists “imaging of subcellular dynamics in both cultured cells and live multicellular organisms, nanoscale mapping of molecular architectures across millimeter-scale expanded tissues, and structural/functional neural imaging within live mice”, which we believe captures the spirit of the reviewer request.

Conclusion
This is an impressive technical manuscript describing a flexible, high-performance microscopy platform with significant biological utility. With moderate revisions that improve figure clarity, quantification, and presentation of technical terms and results, the work will make a valuable contribution to the field. The MOSAIC system is well positioned to advance multiscale biological imaging across research disciplines.

- We appreciate the positive comments from the reviewer.

Reviewer #3 (Remarks to the Author):

This manuscript from Fu, Liu, Milkie, and Rui et al., provide an overview of the capabilities of a custom-built multi-modal microscope with adaptive optics that can capture exquisite detail of biological specimens. This manuscript represents both a technical advance and extensive, collaborative work by multiple well-respected scientists. Several key differences set this microscope apart from other home-built systems including adaptive optics for all modalities, the ability to image the same specimen with different modalities to perform comparisons and/or collect different levels of spatial and/or temporal data. For the biological imaging community, this microscope has already provided a valuable way to examine a handful of living specimens and will be of interest to developmental biologists and neuroscientist studying in vivo cell behaviors. The data are eye-catching, quantifiable, and some of the most spatially and temporally resolved images of living cells in organisms I've seen.

- We appreciate the positive comments from the reviewer.

Below are some suggestions and minor concerns, which reflect my perspective as a cell and developmental biologist, someone who might lead the writing of a grant to fund the building and

maintenance of such an instrument at our institution or who might want to visit to use the instrument.

In its current form, I'm not sure that this reads like a true "Methods" paper. At times, I found myself thinking that I was reading an advertisement for the instrument. Perhaps the authors could specifically discuss some of their design thinking and highlight the different imaging modalities with an initial figure. One possibility would be to combine Fig S1 and S8 (or some version of them) into a didactic Fig 1 to illustrate the optical foundation/basis for the rest of the figures to follow.

- We agree that presenting a new instrument requires careful balance. Our goal is to demonstrate MOSAIC's capabilities not as an advertisement, but as a series of biological case studies that quantifiably assess its performance boundaries across a wide range of challenging contexts. We have outlined our design rationale in the 'Microscope design and characterization' section and discuss limitations/challenges with the design in the second paragraph of the discussion.
- While we considered combining Figures S1 and S8, we opted to keep them separate to preserve a clear distinction between the core instrument design supplemental figures S1-S8) from the biological applications. In our view, a single summary figure would be less precise and less technical than the existing supplementary schematics. However, to help readers navigate the instrument's diverse capabilities, we have now added a flowchart designed to guide them in selecting the appropriate imaging mode for their research. For readers seeking implementation details, the complete technical documentation (approximately 1,000 pages) is available, which we anticipate interested readers and prospective builders will consult.

Along these same lines, there are a lot of abbreviations used to describe the imaging modalities, a text box with key abbreviations described/defined would go a long way to improve readability for a broader audience.

- We have added a new table (S2) defining all abbreviations used in this manuscript.

Another important issue is the lack of discussion of the way the massive files are handled (I'm assuming some sort of distributed computing). Data storage, handling, and analysis is a huge issue when single experiments take up tens of TBs of data. Some discussion of this beyond machine learning seems important, especially from a methods and instrument-building side of things.

- In the Discussion and SI, we describe using PetaKit5D (DOI:10.1038/s41592-024-02475-4) as the distributed backbone for real-time acquisition, preprocessing, and analysis of MOSAIC datasets. The framework scales to petabyte-scale studies (DOI:10.1101/2024.08.01.605857). Our PetaKit5D paper details the end-to-end dataflow (acquisition, staging, analysis) and practical considerations for data compression, storage, throughput, and provisioning high performance computing resources. Because the PetaKit5D framework has already been published and since the goal of this manuscript was to describe the MOSAIC instrument itself, we do not elaborate on the

data storage and handling and instead direct readers to prior references for explicit details.

Finally, I've listed some easily addressable very specific points below:
- I appreciate the multi-functionality and the ability to switch among a suite of imaging modalities, but it wasn't clear to me how the system is controlled and how exactly one switches between modes and/or whether multiple specimens could be imaged during the same experiment.

- Thank you for the comment. MOSAIC is controlled by a LabVIEW-based suite (SI: "Microscope Control Software"). Imaging modes are implemented as presets that reconfigure illumination/detection paths, scan patterns, and AO parameters via motorized elements, enabling rapid switching. To make this more explicit, we have added the following text to the SI materials:

  "The software supports multi-position acquisition and user scripting, allowing sequential multi-modal imaging across multiple specimens in a single experiment. Switching between modalities is achieved by opening and closing the motorized flip mirror (Fig. S5c) to direct light to different paths. Multiple specimens can be loaded together in the same experiment, provided that they can fit onto one 25 x 25 mm coverslip and require the same imaging environments (media, temperature, CO2, etc.)"

-In the multimodal live cell super-resolution section, the words "minimal photobleaching" are used. What exactly does this mean and how was it determined?

- We have amended the manuscript to clarify that "minimal photobleaching" was quantified by: tracking the mean integrated FOV intensity over 1,500 time points and showing that it remained within +/- 1 s.d. of the baseline, indicating no measurable bleaching beyond baseline variability.

-On top of page 7, SNR is used for the first time (I think) and not defined. Might be a good idea to double check all other abbreviations (also see comment above about making a text box for key abbreviations)

- We now define signal-to-noise ratio (SNR) at first use and added Supplementary Table S2 listing all abbreviations.

-I think I probably just missed some citations (there is a lot of data and text here, which is something to consider vis-à-vis publication). For instance, which software was used for image segmentation and data visualizations (image production and plots)?

- Details of the software used for segmentation and visualization are provided in the SI: please see "Image Processing", "Visualization and Software", and the last two columns of Table S1.

-Some of the color combinations are colorblind accessible and others aren't. Figure 4 is a good example of clearly accessible color combinations, but some aspects of other figures (e.g., combos of cyan/magenta and green/yellow) are more difficult to distinguish.
Consider viewing images with Color Oracle (https://colororacle.org/) to ensure colorblind accessibility. Other helpful resources include:
Wong, B. Points of view: Color blindness. Nat Methods 8, 441 (2011) ;
https://thenode.biologists.com/data-visualization-with-flying-colors/research/; and
https://davidmathlogic.com/colorblind/#%23D81B60-%231E88E5-%23FFC107-%23004D40.

- Thank you for highlighting this issue. Although we proactively avoided red-green pairings (at least one coauthor is color-blind), we acknowledge that other combinations may be difficult to distinguish. This is mainly because we tried to use distinct color combinations for different datasets presented in one figure to avoid confusion. Because color choices are not critical to our conclusions, we have not modified the figures in this revision. Going forward, we will assess palettes with tools such as Color Oracle and adopt color-blind friendly schemes.

Reviewer #1:

Remarks to the Author:

The authors have sufficiently addressed previous suggestions and made notable changes to the manuscript, including a decision tree (figure S26) and further discussion on a proposed model to broadly support systems such as MOSAIC.

Regarding "summary statistics of measurements," here is a rewording to clarify the suggestion:

In the Microscope design and characterization, please quantify the bead-based PSFs shown in S8 and list corresponding lateral and axial FWHM for all modalities shown in S2,S3,S4.

While FWHM is a common summary statistic, it reduces a 3D PSF to a single 1D scalar value and may be most meaningful when the PSF is close to Gaussian. In our case, several PSFs are engineered, with extended axial structure and side lobes, so a single lateral and axial FWHM is strongly reductive and can be misleading as a resolution metric. More importantly, FWHM does not reflect the frequency-dependent transfer properties that actually determine resolution. It fails to capture side-lobe energy redistribution and the anisotropic 3D frequency support, both of which are critical for understanding image formation and deconvolution performance.

Instead, we quantify performance in the frequency domain using the OTFs shown in Fig. S8. The lateral and axial OTFs provide a direct map of the system's resolving power: they report contrast transfer at all spatial frequencies and the effective cutoff limits imposed by the effective numerical aperture in each modality. This representation is quantitative, comparable across modalities, and directly linked to the resolution relevant for reconstruction. For these reasons, we consider the OTFs in Fig. S8 to be a more informative and appropriate summary than FWHM values.

Reviewer #2:

Remarks to the Author:

I appreciate the authors taking the time to address my and the other reviewer's suggestions and comments thoroughly. After reading through the new manuscript, I have no further suggestions and recommend publication of this work.

Thank you.

Reviewer #3:

Remarks to the Author:

It seems like the authors have addressed my comments and those of the other reviewers.

My only remaining issue is not a negative comment about the science or the writing. There are over 20 supplemental figures. Many, if not all of the supplemental figures, are essential to the manuscript, especially the new supplemental figure S26, which is a decision tree about which type of microscopy is indicated for different specimens. I agree with reviewer 1 that this is important and it helps the reader consider all of the different imaging modalities offered by MOSAIC. It seems like a shame that this figure is buried in the supplemental figures. I would push to have this figure in the main paper if at all possible.

We appreciate the reviewer's emphasis on the importance of the decision-tree figure for guiding modality choice. However, Nature Methods imposes a strict limit of six main figures, which we have already reached, so we are unable to move this figure into the main text. The journal also allows up to ten Extended Data figures, but using this option would distribute the figures across three locations (main figures, Extended Data, and Supplementary Information), making it harder for readers to locate and navigate the material. To keep all non-main figures consolidated and easily accessible, we therefore chose not to use Extended Data figures and to keep all additional material, including Fig. S26, in the Supplementary Information. For these reasons, we have left the figure placement unchanged.