**Software details- system requirements and installation guide**

All software used in this manuscript was run using Microsoft Windows 11 Business Version 10.0.26100 Build 26100. As described in the Methods section, images were first acquired using Slidebook and then processed in ImageJ Fiji using specific macro scripts available on our GitHub link or Matlab using custom-written scripts previously published, as cited in the Methods section. The output data was exported to Excel and analyzed using Excel, Python, and Jupyter Notebook. The analyzed data were plotted using OriginPro.

The following table shows the details of the software and their purpose in this manuscript:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Name** | **Version** | **License** | **Installation Instructions** | **Purpose** |
| Slidebook | 6, 2023 and 2024 | 3i Intelligent Imaging Innovations Academic License purchased by the Lab under a strict confidential agreement | https://www.intelligent-imaging.com/slidebook  Installed after purchase under a License agreement- not open for public use | Image Acquisition |
| Image J Fiji | ImageJ 1.54p | GPLv3+, Open for public use | https://imagej.net/downloads  Open for public use | Image Processing and Analysis |
| Image J Fiji Time series Analyzer | 3.0 | Author Balaji J, open for public use | https://imagej.net/ij/plugins/time-series.html  Open for public use | Image Processing and Analysis |
| Image J Fiji StackReg | 2.0.1.jar | GPLv3+, Open for public use | https://imagej.net/imagej-wiki-static/StackReg  Open for public use | Image Processing and Analysis |
| Python | 3.12.3 | PSF v2, GPL compatible, Open for public use | https://www.python.org/downloads/  Open for public use | Data Analysis |
| Jupyter Notebook | 7.2.0 | 3‑Clause BSD, GPL compatible, Open for public use | https://jupyter.org/install  Open for public use | Data Analysis |
| Matlab | R2023b | MathWorks Inc. Academic License under a strict confidential agreement with the Institute | https://www.mathworks.com/help/install/ug/install-products-with-internet-connection.html  Installed after purchase under a License agreement- not open for public use | Image Analysis |
| Microsoft Excel | 16.69.1 | Proprietary Closed Source purchased by the Institute under a strict and confidential agreement | https://www.microsoft.com/en-us/microsoft-365/download-office  Installed after purchase under a License agreement- not open for public use | Data Export and Analysis |
| OriginPro | 2023b | OriginLab Corporation Proprietary Academic License purchased by the Institute under a strict and confidential agreement | https://www.originlab.com/demodownload.aspx  Installed after purchase under a License agreement- not open for public use | Plotting Analyzed Data |

**Instructions for use: How to run ImageJ Macros and Python codes**

This repository contains ImageJ/Fiji Macros and Jupyter Notebook Python scripts used in: Ghosh, I., Fan, R., Shah, M., Bapat, O., & Rangaraju, V. (2025). Synapses drive local mitochondrial ATP synthesis to fuel plasticity. bioRxiv, 2025-04. We used these codes to analyze the images and data for spine ATP, mito ATP, mito pH, calcium, and spine head width analyses as follows:

**Spine ATP analysis instructions:**

*Figures: 1 B-E, 2 C,D,F, 6E,F,G, S1B-F; S2A,B,C, S6G*

1. Image acquisition was performed using Slidebook as mentioned in the Methods section
2. All image files were exported as .tif and saved under specific folders for each experiment
3. The image file names ended with specific channel names for red Spn-ATP fluorescence 561 (C1) and Spn-ATP luminescence (C0) for easy recognition by the Macro scripts
4. Images were processed using ImageJ Fiji, and the plugins Time series Analyzer and StackReg were used as required
5. Concatenate and align all fluorescence and luminescence image frames:
   1. Open the ImageJ Fiji Macro script: SpnATP\_Concatenate.ijm
   2. Drag and drop any .tif image file, eg, Baseline.tif, and hit Run- the macro script will detect the file path to the folder containing the images, and ask the user to upload the image stacks for baseline, stimulation, and post-stimulation for the fluorescence and luminescence image stacks by detecting the channel names described in point 3 above
   3. Select the specific image stacks, Uncheck ‘open as 4D image’, and hit OK
   4. The Macro will concatenate all frames, SIFT Align them, and the final Image stacks will be saved under an ‘Output’ folder under the main experiment folder containing the raw image files
6. Spine Tracker to measure fluorescence and luminescence intensities:
   1. Open the ImageJ Fiji Macro script: SpnATP\_Spine Tracker\_Fluorescence intensity.ijm for the concatenated fluorescence image stack and SpnATP\_Spine Tracker\_Luminescence intensity.ijm for the concatenated luminescence stack
   2. Drag and drop the aligned fluorescence image stack from the output folder for the fluorescence spine tracker macro and the aligned luminescence image stack for the fluorescence spine tracker, and hit Run
   3. The script will ask the user to draw an ROI (circle for fluorescence and square for luminescence) on the spine that was stimulated and enter the spine number as 1, 2, 3 as applicable
   4. Once the user draws the ROI, the script will automatically track the spine through each frame of the stack and measure the fluorescence and luminescence intensity from each frame
   5. Once the spine tracker macro run is complete, hit OK, and the script will save the fluorescence and luminescence intensity data for each spine as .csv files under the same ‘Output’ folder
7. Background subtraction:
   1. Open the fluorescence and luminescence image stacks on ImageJ Fiji, and open the plugin ‘time series analyzer’
   2. Draw 5-6 big background ROIs on blank areas of the stack using the freehand tool and hit ‘Add’ after drawing each ROI
   3. Once all 5-6 ROIs are added, select all, hit ‘More’, and then ‘OR Combine’ and hit ‘Add’- this will combine all the background ROIs into one ROI and save it as ‘BG’
   4. Select the BG roi and hit ‘Get Average’- this will measure the background intensities from all the frames of the fluorescence and luminescence stacks and save this as.csv under file name ‘F\_BG\_AVG’ for fluorescence background intensities or ‘L\_BG\_AVG’ for luminescence background intensities
8. Python script to calculate L/F, pH, pH correction of F, pH corrected L/F and ATP:
   1. Under the ‘Output’ folder make a sub-folder called ‘For Code’ and save all the exported spine and background fluorescence and luminescence intensity .csv files
   2. Copy and paste the file path of this ‘For Code’ folder next to the ‘Address'- this will retrieve a list of all files and directories for the Python script to analyze
   3. Within the code, enter the specific baseline time points for each experiment. For eg. base\_t = 3 is for the spine plasticity induction experiments, where the baseline was 3 min and base\_t = 10 is for the resting state experiments, where the baseline was 10 min
   4. For the ATP calculation, enter the specific Km and Vmax for each calibration 1 or 2. For eg. Calibration 2 resulted in Km = 1.446 and Vmax = 1.649
   5. Hit Run- the output .csv file will be saved as ‘All\_ATP’. This file will contain the background-subtracted luminescence and fluorescence intensities for all time points, raw L/F, pH, pH corrected F, pH corrected L/F, and ATP (mM)
9. Plotting: Plot the Spine ATP data on OriginPro

**Mito ATP analysis instructions:**

*Figures: 3 C, 6 C,D, S3 A, S6 C,F*

1. Image acquisition was performed using Slidebook as mentioned in the Methods section
2. All image files were exported as .tif and saved under specific folders for each experiment
3. The image file names ended with specific channel names for red mito-ATP fluorescence 561 (C1), mito-ATP luminescence (C0), and GCaMP6s fluorescence for Calcium 488 (C2) for easy recognition by the Macro scripts
4. Images were processed using ImageJ Fiji, and the plugins Time series Analyzer and StackReg were used as required
5. Concatenate and align all fluorescence and luminescence image frames:
   1. Open the ImageJ Fiji Macro script: MitoATP\_Concatenate.ijm
   2. Enter the main file path for the folder containing baseline and post-stimulus frames under ‘Main’
   3. Define ‘Input’ and ‘Output’ folders
   4. Define image file names as ‘BASE’ for baseline, ‘AFTERSTIM’ for post-induction as applicable for the experiment
   5. Hit Run- the macro script will identify all defined image frames, stack them into fluorescence and image stacks containing frames from all time points as defined in the macro by the user
   6. Alignment- Open each stack, open the plugin ‘StackReg’, click on ‘Rigid body’ to align all the frames and hit ‘Save’
6. Fluorescence measurement from concatenated stack:
   1. Open the Calcium stack and mark the spine using the plugin time series analyzer with a circular roi (5 px or 1 µm diameter)
   2. Open the mito-ATP red fluorescence stack, project the spine roi and use the line function to draw a 2 um line at the base of the spine on the mitochondrial compartment compartment
   3. Use the freehand selection tool to trace the mitochondrial compartment along the length of this line and save the rois as RoiSet
   4. Select only the freehand mito roi, click get average on time series analyzer, and save the .csv file in the ‘output’ folder under the main experiment folder
7. Luminescencemeasurement from concatenated stack:
   1. Open the luminescence stack from the ‘output’ folder and project the mito roi
   2. Select only the freehand mito roi, click get average on time series analyzer, and save the .csv file in the ‘output’ folder under the main experiment folder
8. Background subtraction: same method as spine ATP above
9. Calculation of L/F, L pH corrected and LpHcorrected/F on Excel
   1. Copy and paste all fluorescence, luminescence, and background intensities into Excel
   2. Subtract the background intensities and calculate L/F (see Methods section)
   3. Make a fresh column for pH at each time point and conditions as applicable (see mito pH analysis below)
   4. Calculate pH-corrected L using the formula mentioned in the Methods section
   5. Calculate LpHcorrected/F
10. Plotting: Plot the Mito-ATP data on OriginPro

**Mito pH analysis instructions:**

*Figure: S3B*

1. Image acquisition was performed using Slidebook as mentioned in the Methods section
2. All image files were exported as .tif and saved under specific folders for each experiment
3. The image file name ended with specific channel names for green mito-pHluorin 488 (C1) for easy recognition by the Macro scripts. The NH4Cl stack was kept as is.
4. Images were processed using ImageJ Fiji, and the plugins Time series Analyzer and StackReg were used as required
5. Concatenate and align all fluorescence and luminescence image frames:
   1. Same method as mito-ATP with the same macro script MitoATP\_Concatenate.ijm- without the luminescence part
   2. Save Stack as pH\_Stack
   3. Alignment- Open the stack, open the plugin ‘StackReg’, click on ‘Rigid body’ to align all the frames and hit ‘Save’
6. Fluorescence measurement from concatenated stack: same method as mito ATP above for the pH and NH4Cl stacks
7. Background subtraction: same method as mito ATP for the pH-Stack and NH4Cl stack
8. Mito pH calculation on Excel:
   1. Copy and paste the fluorescence intensities from the pH stacks and subtract the backgrounds
   2. Copy paste the NH4Cl peak intensity average
   3. Use the Henderson-Hasselbach equation for pH calculation as mentioned in the Methods section
9. Plotting: Plot the Mito-ATP data on OriginPro

**Mito ATP plot profile analysis instructions:**

*Figure: 3D*

1. The control images from Fig. 3C were used to measure the plot profile
2. The image file names ended with specific channel names for red mito-ATP fluorescence 561 (C1), mito-ATP luminescence (C0), and GCaMP6s fluorescence for Calcium 488 (C2) for easy recognition by the Macro scripts
3. Open the ImageJ Fiji Macro script: MitoATP\_Plot Profile Generator.ijm
4. Add the path to the Main Folder where the Slidebook exported images are located under ‘Main’
5. Define the ‘Output’ folder location and hit ‘Run’
6. The script will ‘average project’ the Ca stack and then ask the user to mark the spine that was stimulated with a circle roi. Once marked, the script will mark a segmented line every 0.2 um with a total length of 40 um on either side of the spine as mentioned in the Methods section using the Ridge Detection algorithm, and save all the ridge ROIs.
7. The script will then automatically open the fluorescence and luminescence stacks from the input folders, project the ridge rois, measure the intensities along the ridge from all the stacks, and export the data as .csv files into the same ‘output’ folder
8. Background subtraction: the same background intensities of the images in Fig. 3C control were used- copy and paste the background .csv files into this ‘Output’ folder
9. Calculation of ΔL and plot profile generation:
   1. Open the Python Jupyter Notebook code: MitoATP\_PlotProfile.ipynb
   2. Define the file path where the above Image .csv output files are located in the ‘Address’ section
   3. Define the baseline frames: base\_df = df\_output[df\_output['TimePoint'] < 10]. In this case, it is 10 because the first 9 frames were the baseline 20sx9 = 3 min, and 10th frame was post-induction 20s for which we need the plot profile
   4. The code will calculate the ΔL from the luminescence intensities calculated by the above ImageJ script, and export the data as .csv files into the user-defined file path where the ImageJ exported .csv files are located
10. In Excel, calculate the rolling average by sliding a 5 µm window along the data and computing the average value within each window at every position.
11. Further rearrange the output summary data in Excel and plot in Origin.

**Calcium analysis instructions:**

1. Fig 4C, 5B - I; Supplementary Fig 4, 5, 6A - B Calcium ΔF/F and peak quantification.
   1. Export single-channel image files from SlideBook software. Split the two channels into two different folders. In this case, “C0” will be 488-channel images and “C1” will be 561-channel images.
   2. Open macro *time\_series\_merge\_channels.ijm*. Specify the directory of the two input folders containing single-channel images in the macro, where channel 1 should be ended with suffix “C0” and channel 2 should be ended with suffix “C1”. Specify the output directory as well.
   3. Run macro *time\_series\_merge\_channels.ijm*. Two-channel merged image will be generated in the output folder specified.
   4. Open each two-channel merged image and draw ROIs (Region of Interests) corresponding to the stimulated spine, the mitochondrial/ER region underneath the spine and background regions. Add the ROIs to the ROI Manager and rename them as “x\_sp” for spine, “x\_stim” for mitochondria or ER and “x\_bg” for the background, where x is the number of the spine being stimulated at this imaging round (most of the time is 1). Perform “Image >> Overlay >> From ROI Manager” and save the file so that the ROIs will be saved in the image file as an overlay.
   5. After the ROIs are annotated in all the images in the file, open macro *getaverage.ijm* and specify the input directory containing the ROI-annotated two-channel images and the output directory for the csv files.
   6. Run *getaverage.ijm*. The output csv files will be the input of the next step.
   7. Open the Jupyter Notebook *Getting\_file\_info.ipynb.* Run according to the instructions specified in in the notebook. The folder containing the csv files will be the source folder.
   8. Open the Jupyter Notebook *Calcium normalization and peak detection.ipynb*. Run according to the instructions specified in in the notebook.
   9. Further re-arrange the output summary data in Excel and plot in Origin if needed.
2. Fig 4D Calcium profile quantification.
   1. Export single-channel image files from SlideBook software. Split the two channels into two different folders. In this case, “C0” will be 488-channel images and “C1” will be 561-channel images.
   2. Open macro *time\_series\_merge\_channels.ijm*. Specify the directory of the two input folders containing single-channel images in the macro, where channel 1 should be ended with suffix “C0” and channel 2 should be ended with suffix “C1”. Specify the output directory as well. This will be the input of the next step.
   3. Open macro *average\_project\_for\_plot\_profile.ijm*. Specify the input and output directories. Run the macro.
   4. Open each output image from last step and draw polyline ROIs along the stimulated dendrite. Use the Segmented Line tool, set the width to 5 pixel (~1 m), draw a polyline along the dendrite where the stimulated spine is located, starting from the stimulated spine to the proximal dendrite up to ~ 40 µm long. Add the polyline ROI to the ROI Manager and rename it as “x\_n”, where x is the number of the spine being stimulated at this imaging round (most of the time is 1). Do the same for the distal side of the dendrite and rename the ROI as “x\_p”. Draw background ROIs around the dendrite, add to the ROI Manager and rename as "x\_bg”.
   5. Open the macro *plot\_profile.ijm*. Specify the input and output directory and run the macro.
   6. Open the Jupyter Notebook *Plot profile.ipynb.* Run according to the instructions specified in in the notebook.
   7. In Excel, calculate the rolling average by sliding a 5 µm window along the data and computing the average value within each window at every position.
   8. Further re-arrange the output summary data in Excel and plot in Origin if needed.

**Spine head width analysis instructions:**

*Figures: 2E, S6 E,H,*

A custom-written MATLAB 2023b script was used to calculate the spine-head width. This script was previously published as cited in the manuscript- Rangaraju, V., Lauterbach, M. & Schuman, E. M. Spatially Stable Mitochondrial Compartments Fuel Local Translation during Plasticity. Cell 176, 73-84 e15, doi:10.1016/j.cell.2018.12.013 (2019).

1. Open Matlab and drag the folder with the .tiff files to the command window
2. ‘Set path’ to the location of the folder and ‘save’
3. Then type the following to mark a spine: a = spine \_mark('\*.tiff').
4. The image file should appear.
5. To adjust the contrast: Right click on the color bar -> Interactive colormap shift. -> click and drag in the color bar. You can also zoom with the magnifying glass (top row of buttons). Once you finish adjusting the contrast, right click on the interactive colormap shift. again (to uncheck it).
6. Go to the image field and draw a line across the spine of interest.
7. To continue type on the command line: dbcont
8. It will ask if you want to add another spine, respond yes (1) or no (0), or redo the previous line (9)
9. For fitting, type: spine\_fit('\*LP.mat ')
10. To show lines of the marked spines, type: spine\_show(‘FileName\_LP.mat’)