An open-source method for analysis of confocal calcium imaging with sparse cells

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10 # Highlights:

11 - TrackMate is applied to analyze calcium changes at single-cell resolution.
12 - This method depends on an open-source Fiji software, ensures reproducibility, and
13 requires no coding ability.
14 - Practice images and a step-by-step guide are provided to implement this method in
15 various cell types and animal models.

Abstract:

Background

The research in the neuroscience field has evolved to use complex imaging and computational tools to extract comprehensive information from data sets. Calcium imaging is a widely used technique that requires sophisticated software to get precise and reproducible results. Many laboratories struggle to adopt computational methods due to the lack of computational knowledge and paywalls for software.

New Method

Here we propose a calcium imaging analysis method using TrackMate, an open-source Fiji plugin, to track neurons at single-cell resolution, detect regions of interest (ROIs), and extract fluorescence intensities. For confocal images, this method uses the maximal value to represent the cell intensity for each z stack. This method can be done without coding or be combined with Python or Jupyter Notebook scripts to accelerate the analysis.

Results

This method is validated in fly larval cool neurons, whose calcium changes in these neurons respond to temperature fluctuation. It also identifies potential problems in approaches that extract signals from maximal projection images.

Comparison with existing methods

This method does not depend on programming knowledge and/or commercial software but uses open-source software and requires no coding abilities. Since TrackMate automatically defines ROIs, this method greatly avoids human error and increases reproducibility. In addition, practice

images and a step-by-step guide are provided to help users adopt this method in their experiments.

Conclusions

Therefore, this open-source method allows for the analysis of calcium imaging data at single-cell resolution with high reproducibility and has the potential to be applied in various cell types and animal models by researchers with different programming abilities.

43 # Introduction:

44 Intracellular calcium level is a precise marker for neuronal excitability. Calcium imaging
45 measures the changes in intracellular calcium to understand neuronal activities [1]. Neuroscience
46 studies have increasingly used this method with the development of techniques for measuring
47 intracellular calcium concentration, including genetically encoded calcium indicators (GECI), such
48 as GCaMP [2], which can be expressed in specific sets of neurons noninvasively by genetic
49 approaches. Lower costs of lasers and microscope components have also increased the use of
50 calcium imaging [3]. Importantly, this method allows for recording and studying single neurons
51 or large neuron populations simultaneously in freely moving animals [2, 4, 5].

52 However, the analysis of calcium imaging data is challenging because (1) it involves
53 tracking changes in fluorescence of individual cells over time; (2) the fluorescence signal
54 intermittently disappears or reappears with neuronal responses; and (3) neurons may shift in all
55 directions, specifically in and out of the focal plane [3]. Many laboratories perform calcium
56 imaging analysis manually, which is a time-consuming process and becomes impractical as the
57 length of recordings and the number of neurons increases. Manual analysis also introduces
58 operator bias, is prone to error, and affects the replicability of experiments [5].

59 Various software has been developed by individual laboratories to accelerate the process
60 of calcium imaging and increase reproducibility. Previously, software was designed in a limited
61 experimental context making it difficult for other laboratories to adopt them [6]. Recent efforts
62 to meet modern standards for software sharing have led to the development of several tools to
63 analyze the calcium imaging data consistently across different groups [5-11]. Many of these tools
64 still require programming knowledge and/or depend on commercial software. Importantly, most

of these tools focus on calcium imaging data that include hundreds or thousands of neurons. The lack of programming knowledge and paywalls of software deter researchers from adopting these tools, especially when they record sparse neurons that can be analyzed manually. Thus, there is a need for an open-source method that is easy to understand, suitable to analyze less complex calcium imaging data, accelerates the analysis process, and increases reproducibility.

TrackMate is an open-source Fiji plugin for tracking single particles [12]. It has been widely used in tracking particles in various biological studies involving live-cell imaging, including calcium imaging [11-14]. Generally speaking, calcium imaging analysis includes three steps: motion correction, region of interest (ROI) detection, and signal extraction [3, 5]. TrackMate can automatically perform all these three steps and thus it can significantly reduce operator error and increase reproducibility in calcium imaging analysis.

Here, we propose a method that uses TrackMate to analyze calcium imaging data with sparse cells. This method is particularly apt to analyze confocal calcium imaging captured in high magnification so that each cell appears on more than one image in a z stack. Instead of using the maximal projection, the method extracts fluorescence signals from every image and uses the maximal value of each z stack to represent the cell intensity [15]. Additionally, TrackMate automatically tracks cell motion and defines the ROI, which significantly reduces human error and increases reproducibility. This method does not require computing knowledge as every step can be performed manually. To accelerate analysis, Python and Jupyter Notebook scripts and a step-by-step guide are provided. We also include practice images to help new coders to understand how to use these scripts [6]. Last but not least, this method is compatible with standard laptop and/or desktop computers.

Materials and methods:

2.1 Fly strains

Ir21a-Gal4 [16] and *UAS-GCaMP6m* (*P{20XUAS-IVS-GCaMP6m}attp2*) [17] were previously described.

2.2 Calcium imaging

Calcium imaging data for S4_Figure in the previous study [18] were reanalyzed using the current method. Briefly, in fly larvae, dorsal organ cool neurons expressed the calcium indicator, GCaMP6 [17], by *Ir21a-Gal4* [16]. Three-day-old larvae were immobilized between a glass slide and a glass coverslip with 1 x PBS. The temperature was held at about 27°C for 30 seconds and decreased to about 10°C for 30 seconds. Then, the temperature was held at about 10°C for about 60 seconds and returned to about 27°C for 30 seconds.

2.3 Statistical analysis

Statistical details of experiments are mentioned in the legend of Fig 3D-3G. The normality of distributions was assessed by the Shapiro-Wilk W test ($p \leq 0.05$ rejected normal distribution). Statistical comparisons of normally distributed data were performed by Welch's t test or paired t test. For data that did not conform to a normal distribution, statistical comparisons were performed by the Wilcoxon test. Data analysis was performed using GraphPad Prism 9.

2.4 Data and code availability

Python and Jupyter Notebook scripts, as well as practice folders, have been deposited in GitHub and can be accessed at: https://github.com/niflylab/CalciumImagingAnalysis.

Original statistics and raw data are available at: https://doi.org/10.7910/DVN/AXEVQT.

Results:

This analysis process includes four steps (Fig 1). Step 1 is to convert confocal images to .tif files and organize them. Step 2 is to identify z positions including cells of interest. Step 3 is to use TrackMate to track cells and extract their intensities. Step 4 is to choose the maximal value at each time point and use it to represent the cell intensity. Further analysis, such as to calculate $\Delta F/F_0$, may be necessary. While all steps can be done manually, Python and Jupyter Notebook scripts are provided for Step 1 and Step 4 to accelerate analysis.

3.1 Organize .tif files

For the convenience of the following steps, we recommend exporting calcium imaging files to .tif files. A folder titled Practice 1 is provided for practice purposes on GitHub. We recommend using the name of the calcium imaging file to name this folder. For example, if the name of the calcium imaging file is Neuron0to2.czi (.czi is the file extension of Zeiss images), the folder name should be Neuron0to2. Within the Practice 1 folder, there is a folder titled Neuron0to2 that contains 21 images (Fig 2A). The format of their names is Neuron0to2_h#t#z#c#.tif. h is the phase. The number after h is the phase number. One phase is analyzed at a time. All images in this folder were taken in phase 01. t is the time point or the z stack. Twenty-one images in Fig 2A were taken at 3 time points: 01, 02, and 03. z represents the z position. Each time point contains 7 z positions: 01 to 07. c is the channel. Channel 2, c2, is used to take these images. Of note, the example is a Zeiss format. Other confocal software may give

127 different name formats. The images from the same z position are recommended to be saved in
128 the same folder (Fig 2B).

129 Next, the z positions including cells of interest need to be identified manually. At each
130 time point, a single cell always appears in multiple z positions (Fig 3A, 3B). All z positions
131 containing the cell should be analyzed. For the cell dictated by white arrowheads, z positions of
132 5 to 8 should be analyzed. For the cells shown by orange and blue arrowheads, z positions of 8
133 to 13 should be analyzed. It is important to note that a z position may contain more than one cell
134 of interest (Fig 3B).

135 Since TrackMate requires grayscale images, these .tif files need to be converted to
136 grayscale images. While Nikon NIS elements viewer and Leica LAS X export grayscale .tif files,
137 Zeiss ZEN lite exports color images. When necessary, we recommend using the Channels Tool in
138 Fiji (Image > Color > Channels Tool) to convert color images to grayscale images to avoid losing
139 the fluorescence information.

140 This process can be done by the Python or Jupyter Notebook script named
141 gray_sort_tiff_1, which is provided on GitHub.

142 3.2 Extract the fluorescence intensity

143 TrackMate, a Fiji plugin, combines the functions of motion correction, ROI detection, and
144 extraction of the fluorescence intensities of cells of interest [12, 14]. Fiji is an open-source image
145 processing program for analyzing scientific multidimensional images
146 (https://imagej.net/Fiji/Downloads). The folder titled Practice 2 on GitHub is provided for
147 practice. It is recommended to be downloaded onto the Desktop. In this folder, there are 7
148 folders named Neuron0to2_1 to Neuron0to2_7. These 7 folders represent 7 z positions. Each

149 folder contains 3 images that are from the same z position but different time points (Fig 2B). Of
150 note, the cell dictated by white arrowheads in Fig 3B appears in z positions of 1 to 3. So, the
151 images in folders of Neuron0to2_1 to Neuron0to2_3 are used to analyze this cell. The cells
152 dictated by orange and blue arrowheads in Fig 3B appear in z positions of 4 to 7. So, images in
153 folders of Neuron0to2_4 to Neuron0to2_7 are used to analyze these two cells.

154 Fiji can open all images in a folder and present them as one stack in order. For example,
155 when the folder Neuron0to2_1 is opened, all images in this folder are opened in order as a stack.
156 Then, TrackMate is run. We recommend adjusting the following parameters in TrackMate.

157 1. Use the DoG detector. DoG detector works best for small spot sizes (Fig 4A).
158 2. Change the blob diameter, threshold, and median filter. Adjust the blob diameter based on
159 the sizes of cells. The blob diameter should be similar to the diameter of cells. If cells are oval,
160 the blob diameter should be similar to the minor axis. An increase of the threshold and use
161 of the median filter can help avoid background noise being picked up as signals. For the
162 practice images, we recommend setting the blob diameter to 22, the threshold to 1, and using
163 the median filter (Fig 4B).
164 3. When irrelevant signals are picked up, set filters can help remove some, if not all, irrelevant
165 signals. Filters X and Y can easily remove the irrelevant signals that are distant from the real
166 signals. When filters are set on one image, it is crucial to check all other images to make sure
167 the real signals are not removed. In practice imaging, there are three cells of interest and two
168 of them appear on the same z positions. We recommend analyzing them one by one. In Fig
169 4C, to analyze the left cell, the right cell (arrowhead) and irrelevant signal (arrow) (Fig 4C1)
170 can be removed by setting filters X and Y (Fig 4C2).

171 4. We recommend setting linking max distance and gap-closing max distance to be 3 – 5 times
172 of the blob diameter, especially when samples are moving over time. This can help decrease
173 the number of tracks. We recommend setting gap-closing max frame gap to the number of
174 images in the stack. For the practice images, we recommend setting linking max distance and
175 gap-closing max distance to 110 and gap-closing max frame gap to 3 (Fig 4D).
176 5. To export the data, we recommend choosing the option of Export all spots statistics (Fig 4E)
177 and pressing the Execute button. The All Spots statsitics.csv is created. In this file, each row
178 shows the information of a ROI. Highlighting a ROI in the All Spots statistics window in Fiji
179 shows the corresponding ROI in green in the Fiji image. The same TRACK_ID is supposed to
180 represent the same ROI at different time points. However, this is not always true and needs
181 to be checked manually. For code users, we recommend setting a folder for each cell and
182 saving the All Spots statsitics.csv file as Mean_Intensity#.csv (#: the z position of the images)
183 in the corresponding folder.
184 In the All Spots statistics file, the column POSITION_T shows the time point and
185 MEAN_INTENSITY shows the mean intensity of the cell of interest. These values include
186 background. So, a background value needs to be extracted and subtracted from the
187 MEAN_INTENSITY. It is recommended to calculate the background intensity by using the
188 mean value of 3-5 nearby same size blobs that don't contain fluorescence signals and are
189 from different time points for each z position. Once subtracted, this difference is used to
190 represent the intensity of the cell of interest at this z position for each time point. All of the z
191 positions that contain the cell of interest need to be analyzed. The maximal value at each

192 time point is then chosen to represent the fluorescence signal [15]. Further analysis, including
193 to get $\Delta F/F_0$, can be performed manually.
194 6. If there are multiple cells of interest, repeat steps 1 to 5 to get the information of intensity
195 change over time for each cell.
196 After getting the All Spots statistics files by TrackMate, Python and Jupyter Notebook
197 scripts are provided for the following analysis.

198 3.3 A step-by-step guide for new coders

199 This guide is for new coders with no or limited coding experience. Jupyter Notebook
200 scripts are provided on GitHub and will be used as an example. The file extension for Jupyter
201 Notebook is .ipynb. For experienced Python coders, .py scripts are also provided on GitHub.
202 The Practice 3 folder is provided for practice purposes on GitHub. We recommend
203 downloading this folder onto the Desktop. In this folder, there is an Analysis folder and a
204 Neuron0to2 folder (Fig 5A).
205 In the Analysis folder, there is a file named Background_list.csv and three folders named
206 Neuron 0, Neuron 1, and Neuron 2. For practice purposes, background intensities are listed in
207 Background_list.csv. Each column represents a cell of interest, starting from Neuron 0. As
208 mentioned, a background intensity needs to be extracted in every z position. In this practice set,
209 3 z positions include Neuron 0 (white arrowheads in Fig 3B) and 4 z positions include Neuron 1
210 and Neuron 2 (orange and blue arrowheads in Fig 3B). Thus, there are 3 background values for
211 Neuron 0 and 4 background values for Neuron 1 and Neuron 2. Neuron 1 and Neuron 2 share
212 identical background values because they appear on the same z positions. Moreover, TrackMate
213 data have been saved in folders of Neuron 0, Neuron 1, and Neuron 2. The name of the All Spots

statistics.csv files exported from Fiji needs to be changed to Mean_Intensity#.csv and saved in corresponding folders. # is the number of the z position. For example, in the Neuron 0 folder, there are 3 .csv files named Mean_Intensity01, Mean_Intensity02, and Mean_Intensity03. They are Neuron 0's All Spots statistics.csv in z position 1 to 3, respectively. Similarly, in folders of Neuron 1 and Neuron 2, there are 4 .csv files, each containing information for Neuron 1 and Neuron 2 extracted by TrackMate from z positions 4 to 7.

In the Neuron0to2 folder, there are 21 .tif files. These images are from 3 time points (position_t) and each time point contains 7 z positions (position_z).

3.3.1 Preparation

To run the script, the following files and software must be downloaded and/or installed.

1. Download the .ipynb files from GitHub and save them on the Desktop.
2. Download and install Individual Edition Anaconda from https://www.anaconda.com/products/individual.
3. Open Anaconda-Navigator and launch Spyder.

   The screen in Spyder contains 3 parts. The bottom right corner is the iPython console. In this Guide, three packages, opencv-python, tifffile, and imagecodecs, will be installed in the iPython console.

   To install opencv-python, input the following code line in the iPython console and press Enter/return.

```
pip install opencv-python
```

Install tifffile by inputting the following code line in the iPython console and press Enter/return.

```
pip install tifffile
```

Use the following code line to install imagecodecs.

```
pip install imagecodecs
```

The system will check the existence of these packages before installing. Thus, this step will not pose any problems even the packages have already been installed.

4. Quit Spyder and launch Jupyter Notebook from Anaconda-Navigator.

3.3.2 Run gray_sort_tiff_1.ipynb

This file is used to convert color images to grayscale images, if they are not grayscale, and save the copies into appropriate folders. gray_sort_tiff_1.ipynb is recommended to be saved on the Desktop. In this file, there are three boxes.

Box 1 functions to import os, cv2, and tifffile. It should not be edited.

Box 2 functions to convert the calcium imaging .tif files to grayscale files and save them in proper folders. If the original files are grayscale, they will be copied and sorted into proper folders. The original files will not be changed. If the calcium imaging is obtained from a Zeiss confocal, box 2 may not need to be edited (but, we still recommend checking the file name format and, if necessary, change box 2 as described below). If the calcium imaging is obtained from a confocal other than Zeiss, please change the following code line based on the information in the paper. This code line appears 4 times in the box 2 and all 4 lines must be changed.

```
file = f'{file_name}_{phase}t{t}z{z}{channel}.tif'
```

This code line includes 5 variables for the name of the file. {file_name} is the file name and should not be edited. _ is automatically added by the Zeiss software when converting the calcium imaging to .tif files. {phase} is phase. If the name has no phase, 'Na' should be input in box 3 and it will be skipped. Of note, it must not be deleted. {t} is t position of the image. {z} is z position. Each image has a unique combination of t position and z position. {channel} is channel. If the name does not include channel, 'Na' should be input in box 3 and it will be skipped.

When the name format is different, this line must be changed to match for the file names. For example, if the name of the calcium imaging is Neuron0to2 and the name of .tif files are Neuron0to2Z#T#.tif, all 4 lines would be changed to the following:

```
file = f'{file_name}{phase}Z{z}T{t}{channel}.tif'
```

The letters in the curly brackets following the Z and T are also changed to match their variables defined by the original script. In addition, phase and channel must not be deleted but set 'Na' in box 3.

Box 3 is used to input the information of the calcium imaging .tif files. The following information is needed: (1) The location of the folder in which the calcium imaging .tif files are saved. The location can be found by dragging the folder to a browser search bar, such as Google Chrome and Firefox. (2) The phase to be analyzed. (3) The number of time points (position_t). (4) The number of z positions (position_z). (5) The channel to be analyzed. (6) Whether the files are grayscale. The following box is box 3 from a Mac computer.

```
gray_sort_tiff (r'/Users/LinaNi/Desktop/Practice 3/Neuron0to2',
```

```
                phase = ‘h01’,
                position_t = 3,
                position_z = 7,
                channel = 'c2',
                is_gray = ‘No’
        )
```

The following box is box 3 from a Windows computer.

```
gray_sort_tiff (r'C:\Users\LinaNi\Desktop\Practice 3\Neuron0and1',
                phase = ‘h01’,
                position_t = 3,
                position_z = 7,
                channel = 'c2',
                is_gray = ‘No’
        )
```

In line 1, input the location of the folder between single quotation marks. Quotation marks must not be deleted or added. On Mac computers, the file location is: /Users/LinaNi/Desktop/Practice 3/Neuron0and1. On Windows computers, a C: needs to be added at the beginning and thus it is: C:\Users\LinaNi\Desktop\Practice 3\Neuron0and1. The file location must be identified on the computer that is used to run the script. So, the example of the file location only works on the author’s computer and will cause errors on other computers. Any slash (/) or backslash (\) at the end of the file location must be deleted.

In line 2, input the phase name that is to be analyzed. In the example, the phase is ‘h01’. If the file name does not include the information of the phase, input ‘Na’. Of note, it is case sensitive. Thus, the input of ‘na’, ‘NA’, or ‘H01’ will cause an error. The quotation marks are necessary.

In line 3, input the number of time points (position_t) after the equal sign. In the example, the number is 3. Since this is a number value, quotation marks are not needed.

309 In line 4, input the number of z positions (position_z) after the equal sign. In the example,
310 the number is 7.
311 In line 5, input the channel name that is to be analyzed. If the file name has no information
312 about the channel, input 'Na'. In the example, the channel is 'c2'.
313 In line 6, input whether the .tif files are grayscale. If the .tif files are grayscale, input 'Yes'.
314 If the .tif files are not grayscale, enter 'No'.
315 To run this file, select box 1 and click the Run button in the menu bar. After it is done, box
316 2 is automatically selected. Click Run to execute box 2. Then, box 3 is selected, and click Run.
317 In the Practice 3 folder, another folder named Neuron0to2_gray_stacks is created. In this
318 folder, there are 7 folders, each containing 3 grayscale images (Fig 5B). These 7 folders represent
319 the 7 z positions. Within each folder, there are 3 images from the same z position but at different
320 time points.
321 Now the original calcium imaging .tif files have been converted to grayscale images and
322 sorted into proper folders according to their z positions. They are ready to be analyzed by
323 TrackMate. To minimize modification of the following scripts, TrackMate data and background
324 intensities are recommended to be saved as shown in Fig 5A.

325 3.3.3 Run individual_dFoverF0_1.ipynb

326 This file is used to extract the intensities, calculate $\Delta F/F_0$ for individual cells at each time
327 point, and generate a plot to show the intensity change over time. individual_dFoverF0_1.ipynb
328 is recommended to be saved on the Desktop. In this file, there are three boxes.
329 Box 1 functions to import os, shutil, pandas as pd, numpy as np, and matplotlib.pyplot as
330 plt. It should not be edited.

Box 2 has 3 functions. 1) It merges the columns of the MEAN_INTENSITY from all Mean_Intensity#.csv files for each neuron. 2) It subtracts the background values from the MEAN_INTENSITY and determines the maximal value at each time point. 3) It calculates $\Delta F/F_0$ and plots $\Delta F/F_0$ against the t position. It should not be edited either.

Box 3 is to input information of TrackMate data and background intensities. The following information is needed: (1) The location of the folder in which TrackMate data are saved. (2) The location of the Background_list.csv file. (3) The number of time points (position_t). The following box is box 3 from a Mac computer.

```
loop_fluorescence_extract (r'/Users/LinaNi/Desktop/Practice 3/Analysis',
                           background_file =r'/Users/LinaNi/Desktop/Practice
                           3/Analysis/Background_list.csv',
                           position_t = 3
                           )
```

The following box is box 3 from a Windows computer.

```
loop_fluorescence_extract (r'C:\Users\LinaNi\Desktop\Practice 3\Analysis',
                           background_file = r'C:\Users\LinaNi\Desktop\Practice
                           3\Analysis\Background_list.csv',
                           position_t = 3
                           )
```

In line 1, input the location of the folder between two single quotation marks. On a Mac computer, the file location is: /Users/LinaNi/Desktop/Practice 3/Analysis. On a Windows computer, the file location is: C:\Users\LinaNi\Desktop\Practice 3\Analysis.

In line 2, input the location of the Background_list.csv file between two single quotation marks. On a Mac computer, the file location is: /Users/LinaNi/Desktop/Practice

357 3/Analysis/Background_list.csv. On a Windows computer, the file location is:
358 C:\Users\LinaNi\Desktop\Practice 3\Analysis\Background_list.csv

359 In line 3, input the number of time points (position_t) after the equal sign. In the example,
360 the number is 3.

361 Run boxes 1 to 3 one by one.

362 In each of the Neuron 0, Neuron 1, and Neuron 2 folders, a folder named python_files is
363 created. In this folder, the intensity information of the corresponding neuron in different z
364 positions is combined and saved (Fig 5C). In the Analysis folder, a folder named results is created.
365 In this folder, the intensity information and plots for Neuron 0, Neuron 1, and Neuron 2 can be
366 found (Fig 5C).

367 **3.3.4 Run merge_dFoverF0_1.ipynb**

368 This file is used to combine the $\Delta F/F_0$ information of all cells, calculate the average and
369 standard error of the mean (SEM), and export a plot. merge_dFoverF0_1.ipynb is recommended
370 to be saved on the Desktop. In this file, there are three boxes.

371 Box 1 functions to import os, pandas as pd, numpy as np, matplotlib.pyplot as plt, and
372 from matplotlib.ticker import MaxNlocator. It should not be edited.

373 Box 2 functions to merge the $\Delta F/F_0$ information of all neurons, calculate the average and
374 SEM at every t position, and plot the mean $\Delta F/F_0$ against the t positions. It should not be edited
375 either.

376 Box 3 is to input the information of individual cells. This information is saved in the results
377 folder (Desktop > Practice 3 > Analysis > results). The following information is needed: (1) The

location of the results folder. (2) The number of time points (position_t). The following box is box 3 from a Mac computer.

```
merge_data (r'/Users/LinaNi/Desktop/Practice 3/Analysis/results',
            position_t = 3,
            plot_title = 'Average \u0394F/F0')
```

The following box is box 3 from a Windows computer.

```
merge_data (r'C:\Users\LinaNi\Desktop\Practice 3\Analysis\results',
            position_t = 3,
            plot_title = 'Average \u0394F/F0')
```

In line 1, input the location of the results folder between two single quotation marks. On a Mac computer, the file location is: /Users/LinaNi/Desktop/Practice 3/Analysis/results. On a Windows computer, the file location is: C:\Users\LinaNi|Desktop\Practice 3\Analysis\results.

In line 2, input the number of time points (position_t) after the equal sign. In the example, the number is 3.

Line 3 should not be edited.

Run boxes 1 to 3 one by one.

In the folder of results, another folder named merged_data is generated (Fig 5D). This folder contains a .csv file that include the $\Delta F/F_0$ information of all cells, the average $\Delta F/F_0$ and SEM at each time point, as well as a plot to show the change of mean $\Delta F/F_0$ over time.

3.4 Fly larval cool neurons respond to temperature changes.

The calcium changes in fly larval cool neurons responding to temperature fluctuation is used to validate the method. A genetically encoded calcium indicator, GCaMP6m [17], was expressed in larval cool neurons by *Ir21a-Gal4* [16]. When exposed to about 27°C, the neurons

403 had a low intracellular calcium level (Fig 3A, 3C, and 3D). When the temperature was decreased
404 to about 10°C and held, the intracellular calcium level was greatly increased and sustained (Fig
405 3B, 3C, and 3D). The calcium level rapidly dropped with the increase of temperature (Fig 3C). Of
406 note, a similar plot to Fig 3C can be generated by the Python or Jupyter Notebook Scripts.

407 In the previous study, maximal projection images were used to extract the fluorescence
408 intensities [18]. Though the trends of $\Delta F/F_0$ over time were similar, the plateau was higher from
409 maximal projection images. To understand the cause of this difference, we compared
410 background intensities that were used in both two studies. Background intensities from
411 individual z positions were significantly lower than that from maximal projection images (Fig 3E).
412 The cell intensities at the position_t = 11 and position_t = 31 were also compared (Fig 3F, 3G). No
413 difference was observed at the position_t = 11 when cool cells were exposed at about 27°C and
414 they were inactive (Fig 3F). When cool cells were exposed at about 10°C at the position_t = 31,
415 cell intensities from individual z positions were significantly higher than that from maximal
416 projection images (Fig 3G). These data suggest that extraction of fluorescence signals from
417 maximal projection images may overestimate the background intensities and/or underestimate
418 cell signals, both of which affect $\Delta F/F_0$.

419 **Discussion:**

420 This paper describes a code-free method that uses TrackMate, a Fiji plugin, to analyze
421 confocal calcium imaging with one or a few cells of interest. This method is particularly apt for
422 calcium imaging that is captured using high magnification and most cells appear on several z
423 positions. In addition, Python and Jupyter Notebook scripts are provided to accelerate analysis.
424 A step-by-step guide and practice files are also included for new coders. Since Fiji, Python, and

425 Jupyter Notebook are all open-source software, this method is completely free. Moreover, it is
426 compatible with standard laptop and desktop computers.

427 This method uses TrackMate to track cells, identify ROI, and extract the fluorescence
428 intensities of cells. Many studies, including a recent study from our lab [18], manually analyze
429 calcium imaging. This manual process is time consuming and prone to human bias and error.
430 TrackMate achieves automation of motion correction, ROI identification, and data extraction. For
431 motion correction, TrackMate is used to track the movement in the lateral (X-Y) direction. During
432 image acquisition in a live organism, such movement is unavoidable even when it is immobilized.
433 In addition, some stimuli, such as temperature change, cause significant z drifting. Increasing the
434 height of z stacks can resolve this issue because it includes the cells of interest in the imaging. To
435 track the changes in the z direction, all z positions that include the cells of interest need to be
436 analyzed and the maximal values are used to represent the signal intensities at corresponding
437 time points. Importantly, when using the same parameter settings, fluorescence extraction by
438 TrackMate is consistent from one computer or operator to another and therefore increases
439 reproducibility. Another challenge for calcium analysis is that cells disappear without stimulation
440 and reappear with stimulation. TrackMate can track the cells when they reappear.

441 However, this is not a bias-free method. We realize two steps may introduce variations.
442 (1) The background intensities may cause variation. Operators are unlikely to pick the same
443 region to extract background intensities. To minimize the variation, we recommend extracting
444 background intensities using a nearby same size blob that does not contain any fluorescent cells.
445 Another recommendation is to use the average of 3-5 background intensities from different time
446 points and using the mean value as the background intensity for the corresponding z position. (2)

447 Weak signals may also introduce variation. When the signals are weak, TrackMate may not be
448 able to recognize the cells of interest or may mistake noise as the signals of the cells. Researchers
449 have to manually decide whether these signals are correct.

450 In summary, this paper provides an open-source analysis method for calcium imaging that
451 is captured using high magnification and most cells appear in several z positions. It will help
452 researchers or laboratories who have limited or no programming knowledge to accelerate the
453 analysis process and increase reproducibility.

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Figure 1

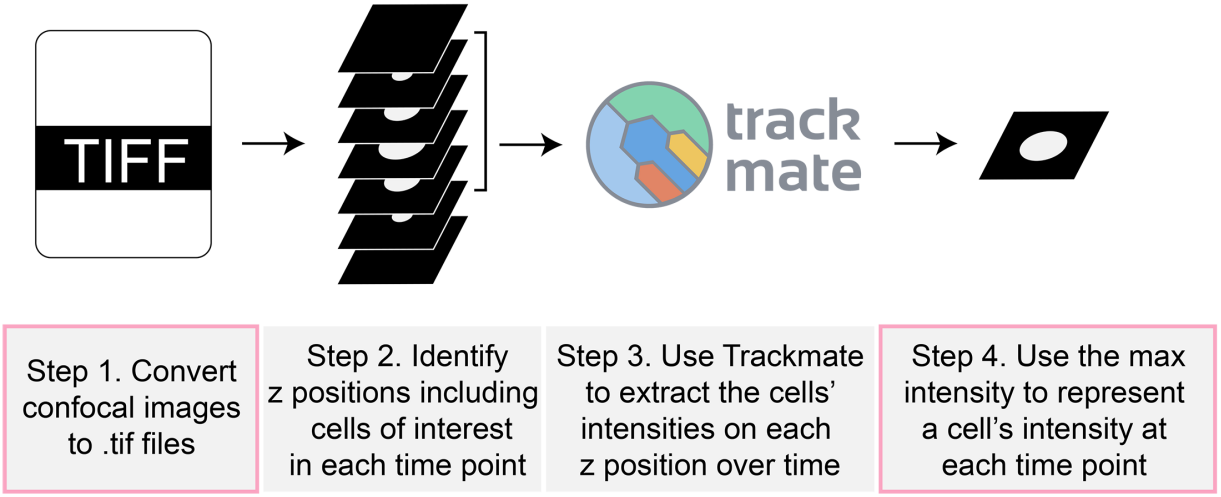
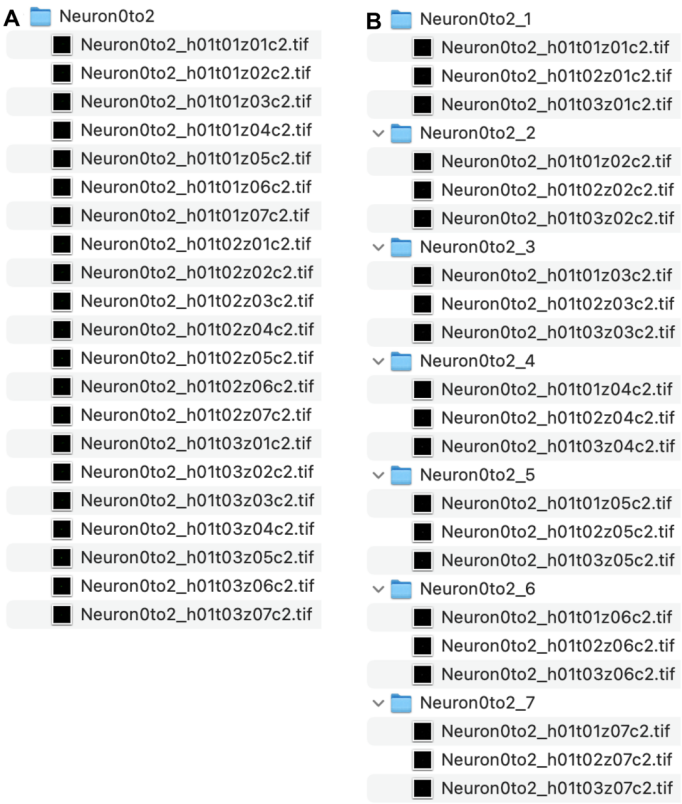


Fig 1. Workflow of confocal calcium imaging analysis with sparse cells. This analysis includes four steps. The first step is to convert confocal images to .tif files. This step can be done by confocal software or Fiji. Images from the same z position over time are recommended to be saved in the same folder for analysis. The second step is to identify z positions including cells of interest at each time point. At each time point, a cell usually appears on multiple z positions; different cells may appear on the same or different z positions. Only the z positions that include cells of interest need to be analyzed. The third step is to use TrackMate, a plugin in Fiji, to extract cells' intensities in each image. Images from the same z position over time are recommended to be analyzed as a stack. All z positions including cells of interest need to be analyzed. The fourth step is to determine the maximal intensity of each cell at each time point and use it to represent the intensity of this cell at this time point. All steps can be done manually. Python and Jupyter Notebook scripts are available for Step 1 and Step 4 to accelerate the process.

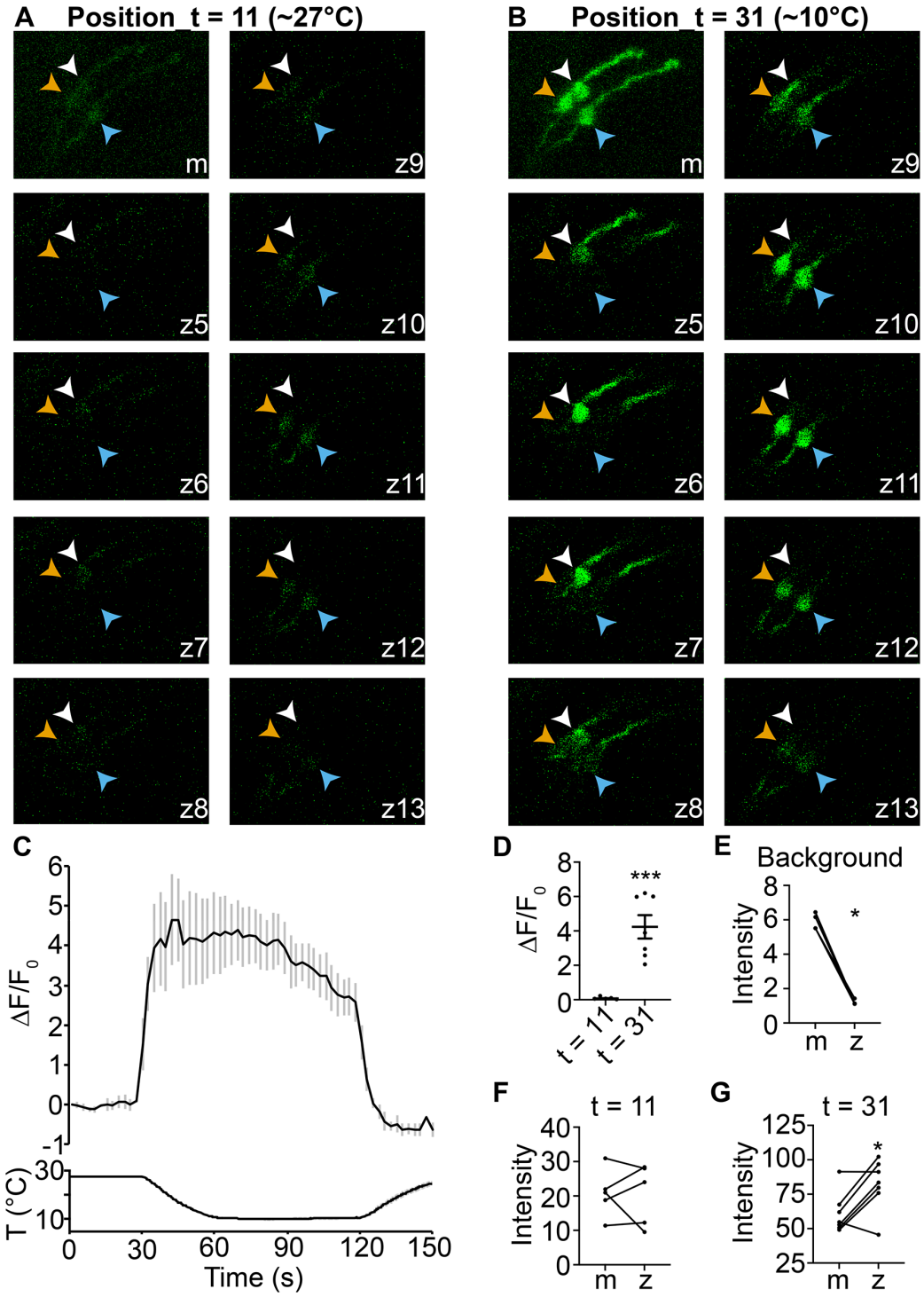
517 Figure 2



518

519 **Fig 2. An example of the organization of the .tif files.** (A) The folder name must be the same as
520 the name of the calcium imaging. Neuron0to2 is the name of calcium imaging file and _h#t#z#c#
521 is the specific information of each .tif file. (B) The images from the same z position must be saved
522 in the same folder. The folder name is the name of calcium imaging file followed by the number
523 of the z position.
524

525 Figure 3



526

527 **Fig 3. Calcium imaging of fly larval cool cells in the inactive and active states.** (A) Cells are barely
528 visible in the inactive state. (B) Cells are strongly fluorescent in the active state. Different color
529 arrowheads dictate different cells. m: maximal projection. z5-13: z positions from 5 to 13. In (B),

530 the cell dictated by white arrowheads is shown on z5 to z8; the cells dictated by orange and blue
531 arrowheads are shown on z8 to z13. (C) Fluorescence is quantified as the change in fluorescence
532 intensity compared to the initial intensity. n = 7 cells from 3 animals. Traces, mean ± SEM. (D) The
533 changes in fluorescence intensities compared to the initial intensity at indicated time points.
534 Welch's t test, *** $p < 0.001$. (E) Background intensities from maximal projection images (m) and
535 from individual z positions (z). Wilcoxon test, * $p < 0.05$. (F, G) Fluorescence intensities from
536 maximal projection images (m) and from individual z positions (z) at indicated time points. Paired
537 t test, * $p < 0.05$.
538

Figure 4

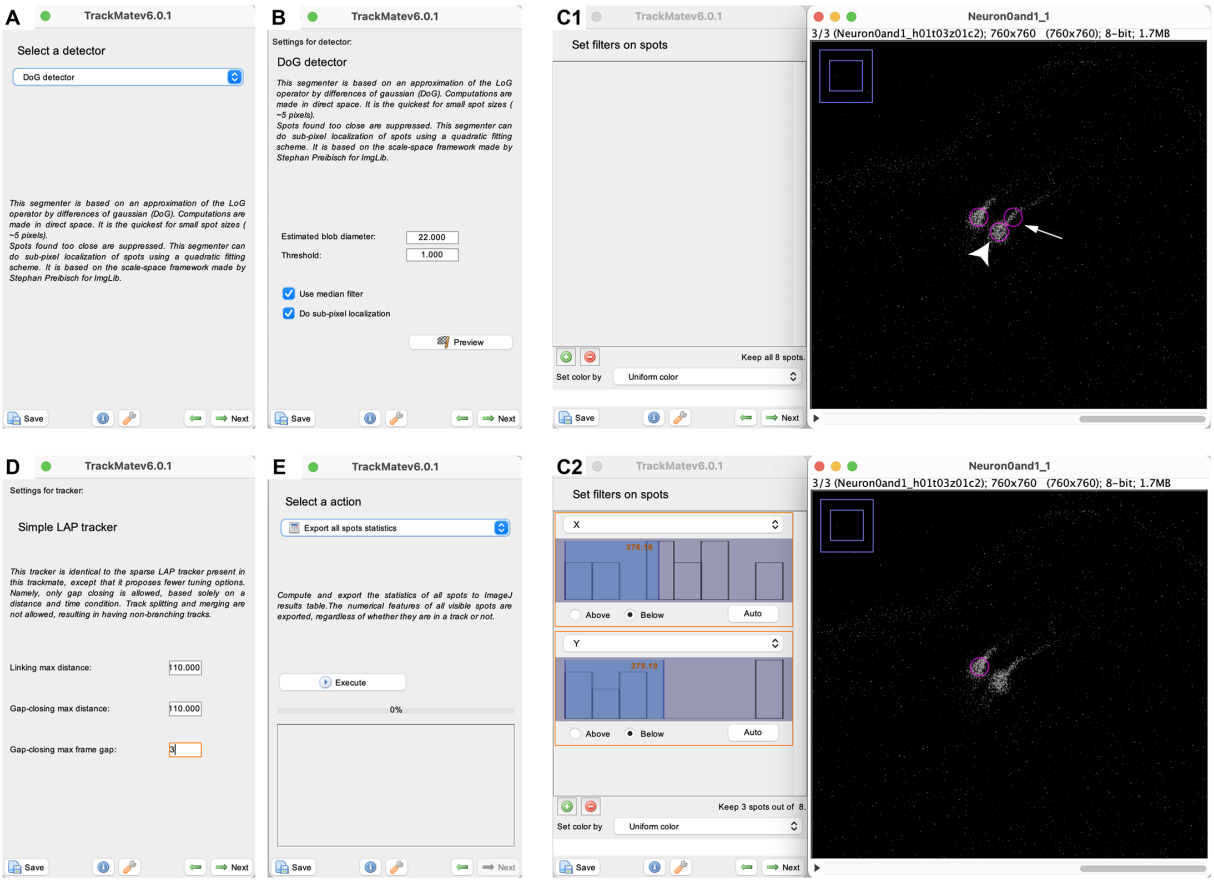


Fig 4. Use Trackmate, a Fiji plugin, to extract cells' intensities. The following parameters are recommended to be input. (A) Select a detector. We recommend using the DoG detector that works best for small spot sizes. (B) DoG detector. We recommend changing the blob diameter based on the sizes of cells. The blob diameter should be similar to the diameter of cells. If cells are oval, the blob diameter should be similar to the minor axis. Increasing the threshold can help decrease the effects of the background noise. If signals are strong, we recommend using the median filter that can help decrease the Salt and Pepper noise. (C) Set filters on spots. When irrelevant signals are picked up, filters can help remove some, if not all, irrelevant signals. Filters X and Y can easily remove the irrelevant signals that are distant from the real signals. When filters are set on one image, it is crucial to check all other images to make sure the real signals are not removed. In (C1), two cells of interest and one irrelevant signal (arrow) are picked up (magenta circles). To analyze the left cell, the right cell (arrowhead) and irrelevant signal (arrow) can be removed by setting filters X and Y (C2). (D) Simple LAP tracker. We recommend setting linking max distance and gap-closing max distance to be 3 – 5 times of the blob diameter, especially when samples are moving over time. This can help decrease the number of tracks. We recommend setting gap-closing max frame gap to the number of images in the folder. (E) Select an action. We recommend choosing the option of Export all spots statistics.

Figure 5

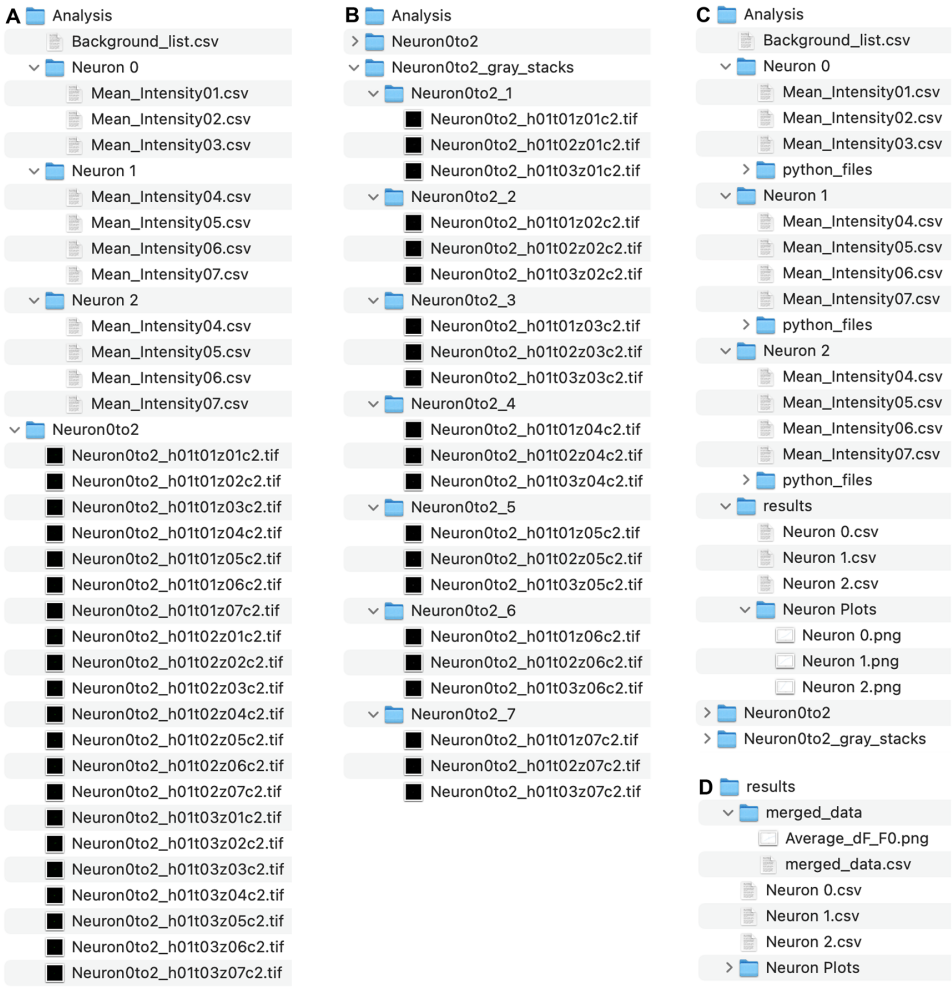


Fig 5. The information in the folder of Practice 3 before and after analysis using Jupyter Notebook scripts. (A) Before analysis, in the folder of Practice 3, there is an Analysis folder and a Neuron0to2 folder. In the Analysis folder, there is a file named Background_list.csv and three folders named Neuron 0, Neuron 1, and Neuron 2. The Background_list.csv includes the background intensities of Neuron 0, Neuron 1, and Neuron 2 in different z positions. The Neuron 0 folder contains three .csv files that represent 3 z positions. In folders of Neuron 1 and Neuron 2, each contains four .csv files that represent 4 z positions. Each .csv file contains intensity information of the corresponding neurons from the same z position. (B) After running gray_sort_tiff_1.ipynb, a folder named Neuron0to2_gray_stacks is created. In this folder, there are 7 folders, each containing 3 grayscale images. These 7 folders represent 7 z positions. In each folder, there are 3 images from the same z position. (C) After running individual_dFoverF0_1.ipynb, in every of the Neuron 0, Neuron 1, and Neuron 2 folders, a folder named python_files is created. The intensity information of the corresponding neuron in different z positions is combined and saved in this folder. In the Analysis folder, a folder named results is created. In this folder, the intensity information and plots for Neuron 0, Neuron 1, and Neuron 2 can be found. (D) After running merge_dFoverF0_1.ipynb, a folder named merged_data is generated in the results folder. This folder contains a .csv file, including the $\Delta F/F_0$

578 information of all cells, the average $\Delta F/F_0$ and SEM at each time point, and a plot to show the
579 change of mean $\Delta F/F_0$ over time.