HHS Public Access

Author manuscript

J Vis Exp. Author manuscript; available in PMC 2023 December 16.

TACI: an ImageJ Plugin for 3D Calcium Imaging Analysis

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Abstract

Research in neuroscience 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 obtain reliable results, but many laboratories struggle to adopt computational methods when updating protocols to meet modern standards. Difficulties arise due to the lack of programming knowledge and paywalls for software. In addition, cells of interest display movements in all directions during calcium imaging. Many approaches have been developed to correct the motion in the lateral (x/y) direction.

This paper describes a workflow using a new ImageJ plugin, TrackMate Analysis of Calcium Imaging (TACI), to examine motion on the z-axis in 3D calcium imaging. This software identifies maximum fluorescence values from all z-positions a neuron appears and uses it to represent the neuron's intensity of corresponding t-positions. Therefore, this tool can separate neurons overlapping in the lateral (x/y) direction that appear on distinct z-planes. As an ImageJ plugin, TACI is a user-friendly, open-source computational tool for 3D calcium imaging analysis. We validated this workflow using fly larval thermosensitive neurons that displayed movements in all directions during temperature fluctuation and a 3D calcium imaging dataset acquired from the fly brain.

SUMMARY:

TrackMate Analysis of Calcium Imaging (TACI) is an open-source ImageJ plugin for 3D calcium imaging analysis that examines motion on the z-axis and identifies the maximum value of each z-stack to represent a cell's intensity at the corresponding time point. It can separate neurons overlapping in the lateral (x/y) direction but on different z-planes.

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A complete version of this article that includes the video component is available at http://dx.doi.org/10.3791/64953.

DISCLOSURES:
The authors have no conflicts of interest to disclose.

INTRODUCTION:

The level of intracellular calcium is a precise marker for neuronal excitability. Calcium imaging measures the changes in intracellular calcium to understand neuronal activities[1]. Studies in neuroscience have increasingly used this method due to the development of techniques for measuring intracellular calcium concentration, including genetically encoded calcium indicators (GECIs), such as GCaMP[2,3], which can be noninvasively expressed in specific sets of neurons through genetic approaches. The lower costs of lasers and microscope components have also increased the use of calcium imaging[4]. Importantly, calcium imaging allows for recording and studying single neurons as well as large neuron populations simultaneously in freely moving animals[5].

Nevertheless, the analysis of calcium imaging data is challenging because (1) it involves tracking changes in fluorescence of individual cells over time, (2) the fluorescence signal intermittently disappears or reappears with neuronal responses, and (3) neurons may move in all directions, specifically in and out of a focal plane or appearing on multiple planes[4,6]. Manual analysis is time-consuming and becomes impractical as the length of recordings and the number of neurons increases. Various software programs have been developed to accelerate the process of analyzing calcium imaging. Previously, software was designed in a limited experimental context, making it difficult for other laboratories to adopt it. Recent efforts to meet modern standards for software sharing have led to the development of several tools that can consistently analyze calcium imaging data across different groups[7–19]. However, most of these tools require programming knowledge and/or depend on commercial software. Lack of programming knowledge and software paywalls deter researchers from adopting these methods. Moreover, many of these tools focus on correcting x/y motion, though motion on the z-axis also needs to be explicitly diagnosed and corrected[6]. There is a need for a computational tool to analyze 3D calcium imaging that focuses on neurons exhibiting z-drift and appearing on multiple z-planes. Ideally, this tool will use open-source software and not require programming knowledge to allow other laboratories to readily adopt it.

Here, we developed a new ImageJ plugin TACI to analyze 3D calcium imaging data. First, the software renames, if needed, and organizes 3D calcium imaging data by z-positions. Cells of interest are tracked on each z-position, and their fluorescence intensities are extracted by TrackMate or other computational tools. TACI is then applied to examine motion on the z-axis. It identifies the maximum value of a z-stack and uses it to represent a cell’s intensity at the corresponding time point. This workflow is suited to analyze 3D calcium imaging with motion in all directions and/or with neurons overlapping in the lateral (x/y) direction but appearing on different z-positions. 3D calcium imaging datasets from fly larval thermosensitive neurons and mushroom neurons in the brain were used to validate this workflow. Of note, TACI is an open-source ImageJ plugin and does not require any programming knowledge.

PROTOCOL:

1. Calcium imaging

1.1. Fly larvae preparation

NOTE: Flies and larvae are maintained at 25 °C under a 12 h:12 h light:dark cycle.

1.1.1. Anesthetize flies by CO_2. Sort 20–45 males and 20–45 females in each fly vial and give them at least 24 to 48 h to recover from CO_2 exposure.

NOTE: Fly exposure to CO_2 should last for the shortest amount of time possible.

1.1.2. To synchronize larvae age, tap over the flies into new vials containing yeast granules and allow them 4–8 h to lay eggs. Remove the flies by flipping them into new vials.

1.1.3. Collect larvae at 72 h using 10 mL of 20% w/v sucrose solution.

1.2. Microscope and temperature control setup

1.2.1. Perform the imaging on a confocal microscope (see the Table of Materials) and a z-axis piezo stage with stage insert using the following settings: **laser, Argon**; **scan mode, frame**; **frame size, 512 × 512**; **speed, maximum**; **channels/bit depth, 1/8 Bit**; **zoom, 1.5**; **z-stack, slice =1 5, Keep = slice**; **focus devices and strategy, Definite Focus**; **focus, Definite focus on**.

1.2.2. Attach a Peltier cooling module to a heat sink by the heat transfer compound to build a thermoelectric cooler. The Peltier is powered by a 2A power supply.

1.2.3. Attach a thermocouple microprobe to a data acquisition device to record the temperature.

1.3. Calcium imaging

1.3.1. Rinse the larvae 3x in 1x phosphate-buffered saline (PBS).

1.3.2. Pipette 75 µL of 1x PBS on the center of a glass slide.

1.3.3. Put one or two larvae in 1x PBS and place the thermocouple microprobe near the larvae.

NOTE: The distance between larvae and the microprobe should be ~5 mm. Larvae may move if the microprobe is positioned too close to them. A great distance could result in inaccurate temperature readings.

1.3.4. Cover the larvae and thermocouple microprobe with a glass coverslip. Seal the coverslip with nail polish.

1.3.5. Place the slide on the microscope stage, find the focus using 25x objective, and place the thermoelectric cooler on the slide.

NOTE: The Peltier is placed directly on the slide where the larvae are to deliver temperature stimuli.

1.3.6. In confocal software, focus on fluorescent cells of interest. Adjust the laser power to avoid oversaturation. Set the first and last slice positions in the z-stack setting.

NOTE: Use the lowest possible laser power before recording to prevent photobleaching.

1.3.7. Start the z-stack scanning and temperature recording at the same time.

1.3.8. Control the power supply that powers the Peltier to change the Peltier surface temperature. Turn on the power supply to decrease the temperature and turn it off to increase the temperature.

1.3.9. Stop the z-stack scanning and temperature recording.

2. Analysis of 3D calcium imaging data

2.1. Export calcium imaging data to TIFF files and save them in a folder with the same name as the base name of the TIFF files inside.

NOTE: The filename **must not contain any commas**.

2.1.1. Use the following parameters to export calcium imaging data from ZEN (black edition): **file type, TIFF**; **compression, LZW**; **channels, 2**; **Z-position, all**; **Time, all**; **phase, 1**; **region, full**.

2.2. Installation

2.2.1. Download TACI-Calcium_Imaging.jar.

2.2.2. Install the plugin in FIJI by clicking **Plugins** in the **menu bar** and then clicking **Install** in the dropdown menu (**Plugins | Install**). Then, restart FIJI.

NOTE: Do **NOT** use **Plugins | Install Plugin**.

2.2.3. Run the plugin by clicking **Plugins** and then choosing **TACI-Calcium Imaging** (**Plugins | TACI-Calcium Imaging**).

2.3. Use the **RENAME** function to convert TIFF filenames to the required structure.

NOTE: The tool uses filename_h#t#z#c#.tif as the default structure (h# and c# are optional; #: a positive integer). If image filenames are not the default structure, the **RENAME** function must be executed.

2.3.1. Choose the folder in which TIFF images need renaming by clicking **Browse Folders**.

2.3.2. Fill in the **parameter information**. Five parameters are listed, including **Filename**, **Phase**, **Max T-Position**, **Max Z-Position**, and **Channel**. Each parameter has three values: **Preceding Text**, **Parameter Value**, and **Order**.

NOTE: The information is **case-sensitive**. If image filenames contain **a phrase before a parameter**, fill the phrase as the **Preceding Text** of the corresponding parameter. If image filenames contain a **phrase after all parameters**, fill the phrase as the **Post Text**.

2.3.2.1. Be sure to enter **Filename**, **Max T-Position**, and **Max Z-Position** and that the parameter values for **Max T-Position** and **Max Z-Position** include **all digits**.

2.3.2.2. Wait for the **Filename** to be automatically filled using the folder name.

2.3.2.3. If TIFF filenames do not include phase and channel, leave the corresponding parameter value blank and choose **Na** in **Order**.

2.3.3. Click **Rename** to create a folder with the same name and _r. Observe that in the folder, the TIFF filenames have been restructured to be compatible with the **ORGANIZE** function.

NOTE: _r has been added to the filenames of TIFF images.

2.4. Use the **ORGANIZE** function to save TIFF images from the same z-position in one folder.

NOTE: The folder name must have the **same name** as the **base name of TIFF files inside** and must **NOT have any commas**.

2.4.1. Choose the folder in which TIFF images need organizing by clicking **Browse Folders**.

2.4.2. If the parameter CSV file (param.csv) exists, wait for the parameter values to be filled in automatically.

NOTE: The param.csv file has a required format. Parameters, including filename, phase, position_t, position_z, channel, and is_gray, must be filled in row 1 from left to right starting from column A. Corresponding values for each parameter must be filled in row 2.

2.4.3. If the parameter CSV file (param.csv) does not exist, manually fill in the parameter values. Ensure that the parameter values of **Phase** and **Channel** include **letters**, while the parameter values of **T Position** and **Z Position** should be the **largest numbers** of t-

and z-positions. If image filenames do not include phase or channel, enter **Na**.

2.4.4. Create grayscale TIFF images when needed. Leave the box of **Are images gray?** unchecked to grayscale the images.

2.4.5. Click **Organize** to create a folder with the same name and _gray_stacks and to generate folders with the same name and _# (#: z-positions) in the folder. Observe that the TIFF files are sorted into corresponding folders by z-positions and that a file named param.csv is generated, in which the parameters and their values can be found.

2.5. Use TrackMate in FIJI to extract the fluorescence intensities of cells of interest from each z-position.

NOTE: This step can also be accomplished by other imaging software.

2.5.1. Open TIFF images in a z-position folder by FIJI.

2.5.2. Run TrackMate by clicking on **Plugins | Tracking | TrackMate** and adjust the following parameters if necessary.

2.5.2.1. Use **DoG** or **LoG detectors**.

2.5.2.2. Change the **blob diameter**, **threshold**, and **median filter**. Adjust the **blob diameter** to be similar to the diameter of the cells. If cells are oval, adjust the **blob diameter** to be similar to the minor axis. Increasing the **threshold** helps avoid background noise being picked up as signals without affecting signal intensities (Supplementary Figure 1). However, an increase in the **threshold** may miss true signals (Supplementary Figure 1). If signals are strong, we recommend using the **median filter** to decrease the Salt and Pepper noise.

2.5.2.3. Set the filters to remove some, if not all, irrelevant signals. **Filters X** and **Y** are spatial filters. Use **filters X** and **Y** to remove the irrelevant signals that are distant from the real signals.

NOTE: When filters are set on one image, it is crucial to check all other images to ensure that the real signals are not removed.

2.5.2.4. Set **linking max distance**, **gap-closing max distance**, and **gap-closing max frame gap**. Set the **linking max distance** and **gap-closing max distance** to be **3–5x the blob diameter**, especially when samples move significantly over time, to help

decrease the number of tracks. Set **gap-closing max frame gap** to the **number of images in the stack**.

2.5.2.4.1. Export the fluorescence intensities of the regions of interest (ROIs) to a CSV file. If an old TrackMate version is used, choose **Export all spots statistics** in the **Select an action** window. If the TrackMate version is 7.6.1 or higher, choose the **Spots** in the **Display options** window. Export or save as the interactive files to CSV files.

NOTE: Both files are interactive with the image window: highlighting an ROI displayed the corresponding ROI in the image window. These files include mean intensities (MEAN_INTENSITY or MEAN_INTENSITY_CH1) of cells of interest at corresponding time points (POSITION_T). The same TRACK_IDs should represent the same ROIs at different time points. However, this is not always true and may need to be corrected manually, when necessary. If TrackMate does not recognize the ROI at some time points, the time points are not displayed.

2.6. Extract background fluorescence intensity and create the Background_list.csv file.

NOTE: In this study, the background intensity for each z-position is estimated by using the average value of three to five, neighboring, same-size blobs that do not contain fluorescence signals and are from different time points.

2.6.1. The Background_list.csv file has a required format: each column contains the information of one neuron, starting from Neuron 0. Fill neuron numbers, such as Neuron 0, in row 1. Then provide the background intensity for each z-position analyzed—if five z-positions are analyzed for Neuron 0, fill five background intensity values below Neuron 0.

NOTE: The Background_list.csv file is required for the TACI **EXTRACT** function. If the background is negligible, the

Background_list.csv with the zero-background intensity of every neuron must be provided.

2.7. Use the **EXTRACT** function to identify the maximum fluorescence intensities at each t-position and calculate $\Delta F/F_0$ as shown in equation (1).

$$\frac{\Delta F}{F_0} = \frac{F - F_0}{F_0} \quad (1)$$

2.7.1. Create a folder and name it using the neuron number, starting from Neuron 0. Save CSV files containing fluorescence information of the corresponding neuron in the folder. Name CSV files as Mean_Intensity#.csv (# represents z-position, so the number of the CSV files equals the number of z-positions analyzed.) and include at least two columns: POSITION_T and MEAN_INTENSITY or MEAN_INTENSITY_CH1.

NOTE: Create a folder for every cell of interest.

2.7.2. Save the Background_list.csv file and folders created in step 2.7.1 in one folder. Choose this folder by clicking **Browse Files**.

NOTE: The number of background values in the Background_list.csv file must match the number of the Mean_Intensity#.csv files for each neuron.

2.7.3. The Background File is automatically filled in. Fill the largest number of t-positions to the **Number of T Positions**.

2.7.4. Click **Extract** to create a results folder, including CSV files and plots for each neuron. The CSV files include information of the maximum fluorescence intensity and $\Delta F/F_0$ at each t-position. The plots are line charts of $\Delta F/F_0$ over t-positions.

NOTE: $\Delta F/F_0$ was calculated using equation (1). The first value of each z-position was used as F_0. If this F_0 is not appropriate[20], the plugin provides files including raw data for each neuron in the python_files folder.

2.8. Use the **MERGE** function to average each neuron's $\Delta F/F_0$, calculate SEM, and plot the average $\Delta F/F_0$ over t-positions.

2.8.1. Choose the results folder created by the **EXTRACT** function by clicking **Browse Files**.

2.8.2. Fill the **Number of T Positions** with the largest number of t-positions.

2.8.3. Click **Merge** to create a merged_data folder, including a merged_data.csv file and an Average_dF_F0.png plot. The CSV

file includes the information on average and SEM $\Delta F/F_0$ at each t-position. The plot is a line chart of average $\Delta F/F_0$ over t-positions.

REPRESENTATIVE RESULTS:

A workflow of 3D calcium imaging analysis.

In this study, we developed a new ImageJ plugin TACI and described a workflow to track z-drift and analyze 3D calcium imaging that pinpointed responses of individual cells appearing in multiple z-positions (Figure 1). This tool has four functions: **RENAME**, **ORGANIZE**, **EXTRACT**, and **MERGE**. First, if image names are not compatible with the **ORGANIZE** function, the **RENAME** function can convert image names to the required structure. Then, the **ORGANIZE** function grayscales (if needed) and organizes 3D calcium imaging TIFF data by z-positions. Images from the same z-position are saved in one folder. Next, different imaging analysis tools can be used to detect and track ROIs and extract their fluorescence intensities over time for every z-position. An ImageJ plugin, TrackMate, was used to accomplish this step. TrackMate is an open-source ImageJ plugin for tracking single particles[21]. It has been widely used to track particles in various biological studies involving live-cell imaging, including calcium imaging[11,21–23]. TrackMate, in an automated manner, tracks cells in the lateral (x/y) direction, detects ROIs, and extracts signals from a live-imaging data set[4,12]. For every cell of interest, the **EXTRACT** function sorts fluorescence intensities from all z-positions by t-positions, identifies the maximum values of each t-position, subtracts the background, and calculates and plots $\Delta F/F_0$. Last, the **MERGE** function calculates and plots the average of $\Delta F/F_0$ of multiple cells.

Calcium responses of fly larval cool neurons to temperature changes

We validated this method using the calcium changes to temperature fluctuations in fly larval cool neurons. A genetically encoded calcium indicator, GCaMP6m[24], was expressed in larval cool neurons by *Ir21a-Gal4*[25]. When exposed to approximately 27 °C, the neurons had low intracellular calcium levels (Figure 2A and Figure 3). When the temperature was decreased to approximately 10 °C and held, the intracellular calcium levels rapidly increased and sustained (Figure 2B and Figure 3). The calcium levels rapidly dropped when the temperature was increased (Figure 3).

Analyzing a fly brain 3D calcium imaging dataset

We also validated this method using a fly brain 3D calcium imaging dataset[11]. The imaged transgenic flies (*VT50339-Gal4;UAS-GCaMP6f*) expressed GCaMP6f in the mushroom body in the brain[11]. Data from 45 z-positions (spaced at 1.5 μm intervals) were collected at 50 Hz for 225 s (250 time points), and the first half of the dataset (125 time points) was analyzed. When the recording began, seven neurons had obvious fluorescence, and four of them were analyzed (Figure 4A). The intensities in these neurons decreased over time (Figure 4B). When octanol was applied (Figure 4C), multiple neurons brightened. The fluorescence of 10 neurons was found to increase simultaneously at time point 92 (Figure 4D), suggesting that these mushroom neurons respond to octanol odor. Although octanol was applied for 5 s, high fluorescence in these neurons was observed in only one time point (0.9 s) and then quickly dropped, suggesting the response is phasic and transient.

Separation of overlapping cells

Figure 5A was a maximal projection image of three neurons. The white arrowhead pointed to two neurons that overlapped in the x/y plane but were separate in the ortho view (blue and orange arrowheads in Figure 5B), indicating that these neurons appeared in different z-positions: the orange cell had the strongest signal on z7 (Figure 5C), while the blue cell had the strongest signal on z10 (Figure 5D). The tool distinguished these two cells and revealed the delayed but strong activation of the orange cell (Figure 5E).

DISCUSSION:

This study developed a new ImageJ plugin TACI and described a workflow analyzing 3D calcium imaging. Many currently available tools focus on correcting x/y motion, though motion on the z-axis also needs to be explicitly diagnosed or corrected[6]. During image acquisition in a live organism, movement on the z-axis is unavoidable even when it is immobilized, and some stimuli, such as temperature change, often cause significant z-drift. Increasing the height of z-stacks will record cells of interest during the whole imaging process; however, it is not trivial to analyze motion on the z-axis, especially when individual cells appear on multiple z-positions. If such movement is ignored, researchers will not obtain the precise calcium responses of these cells. TACI corrects z-drift by extracting fluorescence signals from every z-position. A critical step of TACI is to sort the maximum value at each time point and use it to represent a cell's intensity. Therefore, it is key to include all z-positions of cells of interest during the imaging process. We recommend a cell appearing on five or more z-positions so that z-distances and z-positions won't affect the maximum value (Supplementary Figure 2A, B). In addition, TACI allows for the separation of cells that overlap in the lateral (x/y) direction but appear on different z-positions.

z-drift can also be corrected by extracting fluorescence intensities from 3D ROIs[8,11,29]. We compared TACI with two 3D-ROI methods. First, Klein et al. created a custom software written in IGOR Pro that uses the brightest 100 pixels in each 3D ROI to generate the raw signal[29]. This method and TACI got similar results (Supplementary Figure 3A). Second, we applied IMARIS (version 9.8.2), a commercial software, to model 3D ROIs and extract their mean intensities. Although the results from TACI and IMARIS displayed similar trends, the fold changes were different (Supplementary Figure 3B). This discrepancy may be due to algorithms. Of note, the maximum value extracted by TACI is proportional to the ground truth intensity (Supplementary Figure 2C).

Although this workflow is semi-automatic and still requires manual efforts from researchers, it provides a computational approach for 3D calcium imaging analysis. Importantly, this workflow is based on ImageJ and does not require commercial software or programming knowledge. Limitations and potential solutions for this workflow are as follows. First, TACI only accepts TIFF files and a specific file name structure: filename_h#t#z#c#.tif (h# and c# are optional). However, other file formats compatible with ImageJ can be easily converted to TIFF files by ImageJ. Moreover, the plugin has a **RENAME** function that converts image names to the required structure so that it is compatible with calcium imaging data obtained from different systems.

Second, the plugin is designed for calcium imaging data with constant backgrounds. Subtracting the corresponding background information from ROI intensities at each time point is one way to correct fluctuating backgrounds. The tool provides ROI intensities at each time point in the python_files folder. The background intensities could be represented by (1) the images' mean intensities or (2) the mean intensities of ROIs with no active cells. ImageJ provides methods to obtain the images' mean intensities (by clicking on **Image | Stack | Measure Stack**) and mean intensities of random ROIs (by clicking on **Analyze | Tools | ROI Manager | Multi Measure**). If photobleaching happens during calcium imaging, the **Bleach Correction** function (by clicking on **Image | Adjust | Bleach Correction**) may be run before TrackMate.

Finally, cell registration across z-positions needs to be included in this workflow if using TACI to analyze a large number of neurons simultaneously. Accordingly, an additional function needs to be developed that organizes the information of fluorescence intensities to the data structure required by the **MERGE** function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS:

A Zeiss LSM 880 in the Fralin Imaging Center was used to collect calcium imaging data. We acknowledge Dr. Michelle L Olsen and Yuhang Pan for their assistance with the IMARIS software. We acknowledge Dr. Lenwood S.Heath for constructive comments on the manuscript and Steven Giavasis for comments on the GitHub README file. This work was supported by NIH R21MH122987 (https://www.nimh.nih.gov/index.shtml) and NIH R01GM140130 (https://www.nigms.nih.gov/) to L.N. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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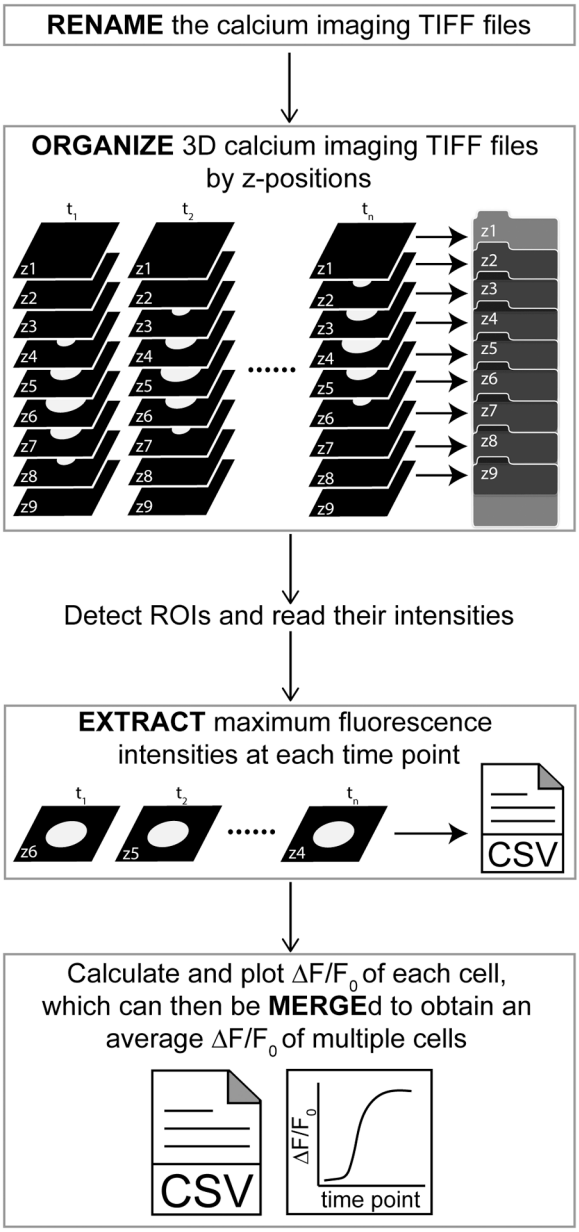

Figure 1: Workflow using TACI to analyze 3D calcium imaging.

TACI has four functions: **RENAME**, **ORGANIZE**, **EXTRACT**, and **MERGE**. First, if TIFF names are not compatible with the **ORGANIZE** function, the **RENAME** function can convert image names to the required structure. Then, the **ORGANIZE** function grayscales (if needed) and organizes 3D calcium imaging TIFF data by z-positions. Images from the same z-position are saved in one folder. Next, different imaging analysis tools can be used to detect and track ROIs and extract their fluorescence intensities in every z-position. For every cell of interest, the **EXTRACT** function sorts fluorescence intensities by the corresponding time points, identifies the maximum values of each t-position, subtracts the background, and calculates and plots $\Delta F/F_0$. Last, the **MERGE** function calculates and plots the average of $\Delta F/F_0$ of multiple cells. Abbreviations: TACI = TrackMate Analysis of Calcium Imaging;

ROIs = regions of interest; $\Delta F/F_0$ = ratio of change in fluorescence to initial fluorescence intensity.

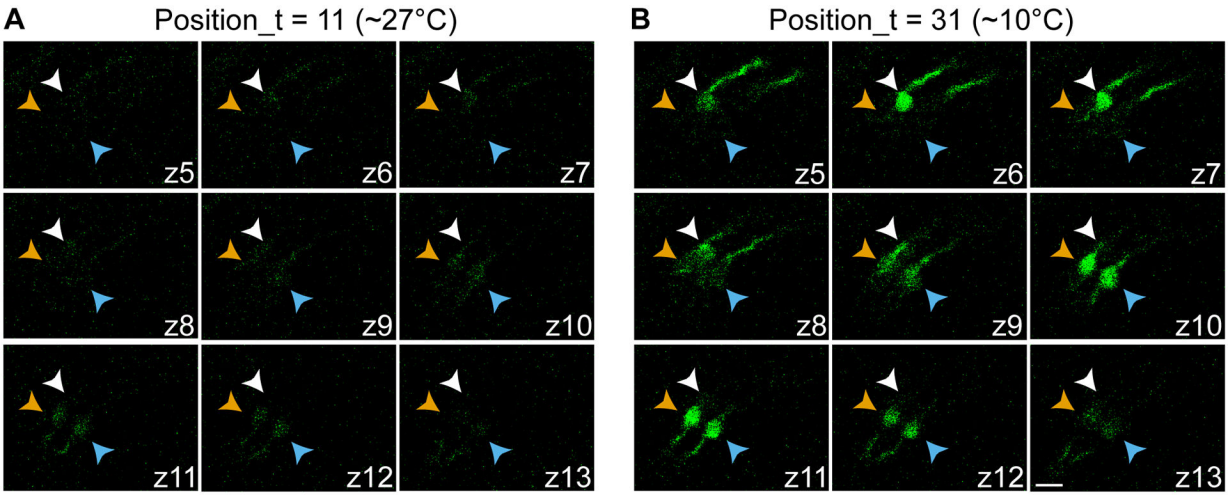

Figure 2: Calcium imaging of fly larval cool cells in inactive and active states.

(**A**) Cells are barely visible in the inactive state. (**B**) Cells are strongly fluorescent in the active state. Different color arrowheads indicate different cells. The genotype is *Ir21a-Gal4;UAS-GCaMP6m*. z5–13: images at z-positions from 5 to 13. In **B**, the cell indicated by white arrowheads is shown on z5 to z8; the cells dictated by orange and blue arrowheads are shown on z8 to z13. Scale bar = 10 μm.

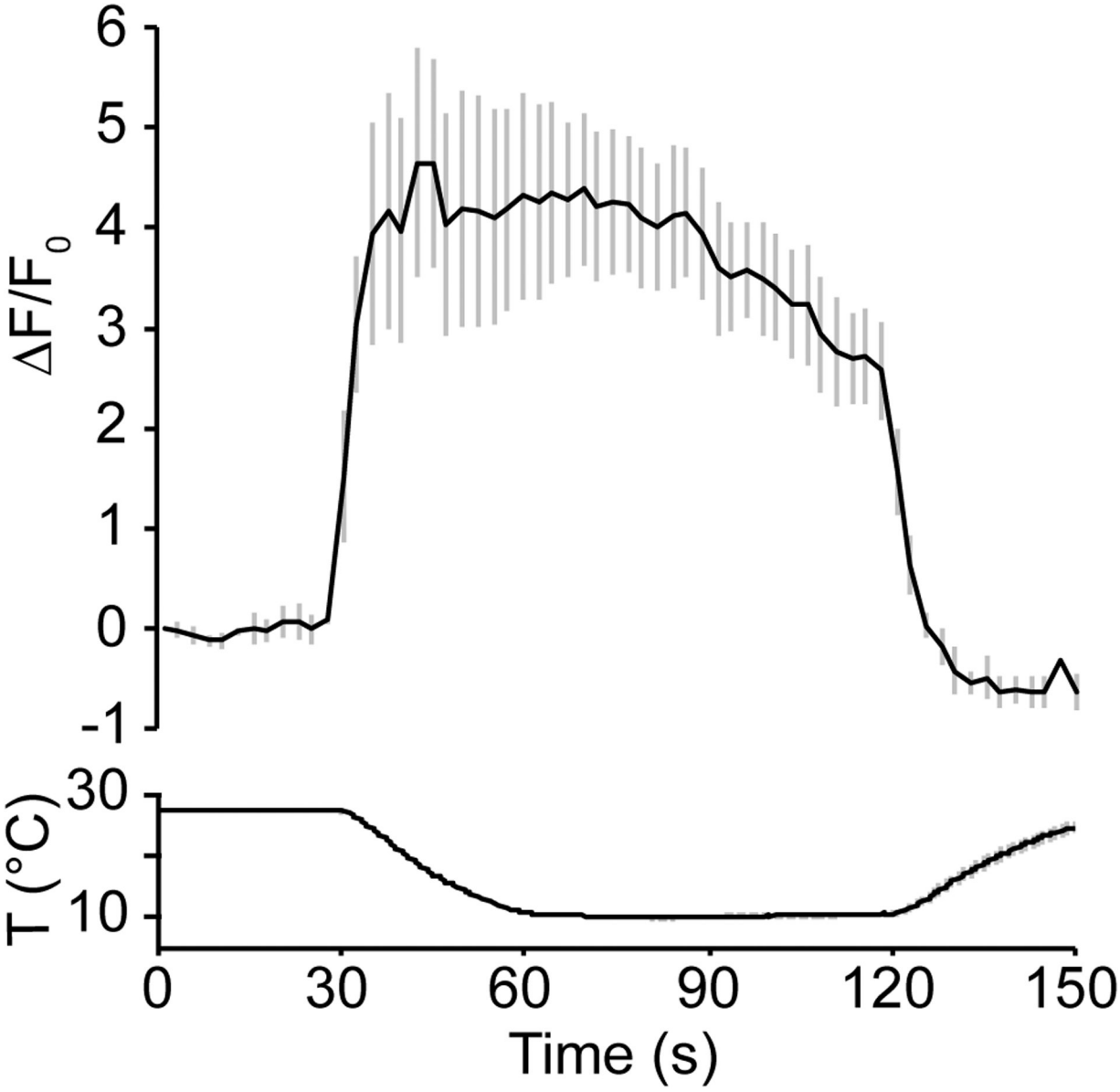

Figure 3: Quantification of fluorescence as the change in fluorescence intensity (ΔF) compared to the initial intensity (F_0).

The genotype is *Ir21a-Gal4;UAS-GCaMP6m*. n = 7 cells from 3 animals. Traces, mean ± SEM.

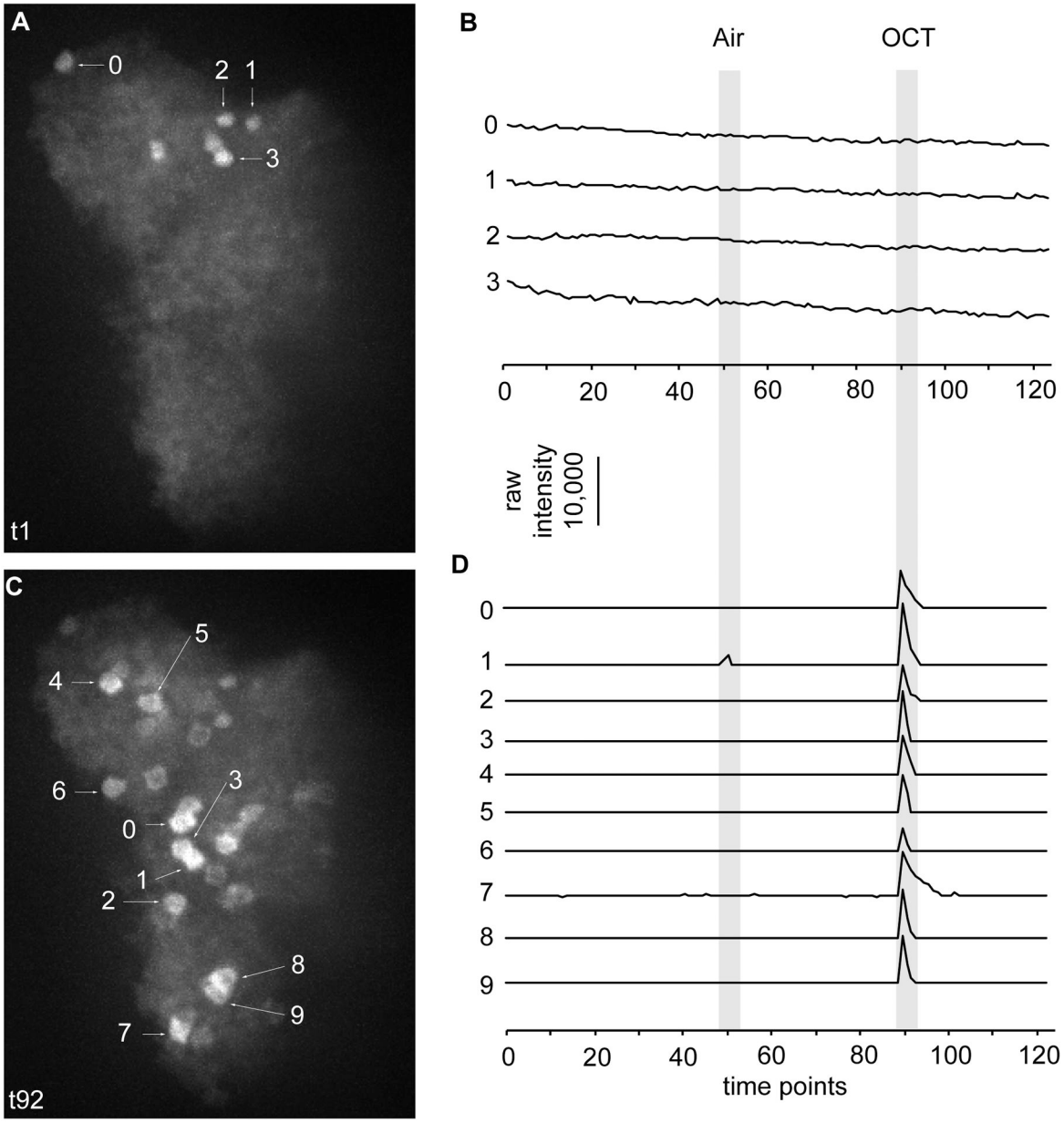

Figure 4: Analyzing a fly brain 3D calcium imaging dataset.

(**A**) The maximal projection image at time point 1 (t1). 0–3 dictate the analyzed four neurons. (**B**) Fluorescence changes of neurons 0–3 in **A** during time points 0 to 125. (**C**) The maximal projection image at time point 92 (t92). 0–9 dictate the analyzed ten neurons. (**D**) Fluorescence changes of neurons 0–9 in **C** during time points 0 to 125. Five seconds of air or 5 s of octanol were applied as shown in gray. Scale bar = 10,000 units of raw fluorescence intensity. Abbreviation: OCT = octanol.

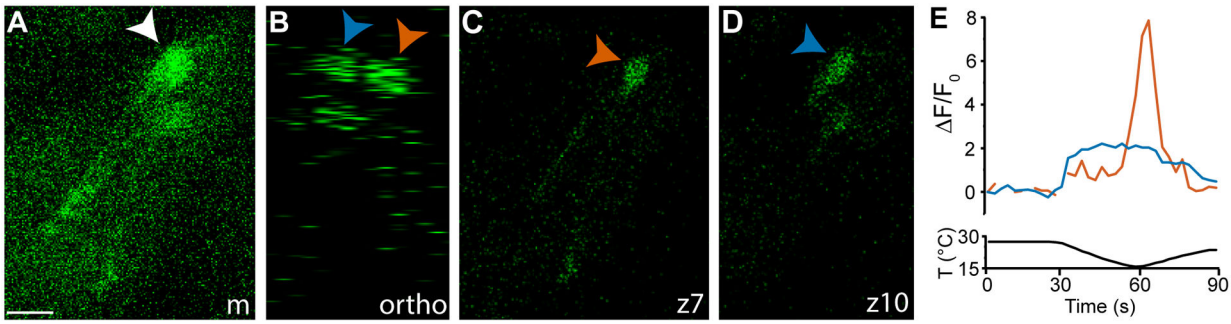

Figure 5: Separation of overlapping cells based on maximum value.

(**A**) Two neurons are overlapped (white arrowhead) in a maximal projection image (m). The genotype is *Ir21a-Gal4;UAS-GCaMP6m*. (**B**) These neurons are separate in the ortho view (blue and orange arrowheads). (**C, D**) The orange cell appears on z7 (**C**), while the blue cell appears on z10 (**D**). Scale bar = 10 μm. (**E**) Fluorescence changes of orange and blue cells are quantified using maximum values from individual z-positions. Abbreviation: $\Delta F/F_0$ = ratio of change in fluorescence to initial fluorescence intensity.