Image Processing and Analysis Protocol for Fluorescent Calcium Imaging (Recommended for use with MATLAB R2024b)

Prerequisites:

- Software and Tools:
  - FIJI (ImageJ)
  - MATLAB with the following custom scripts (make sure to include folders and all subfolders in MATLAB path):
    - Correct_movements Script
    - Neuropil Script
    - Thresholding Script

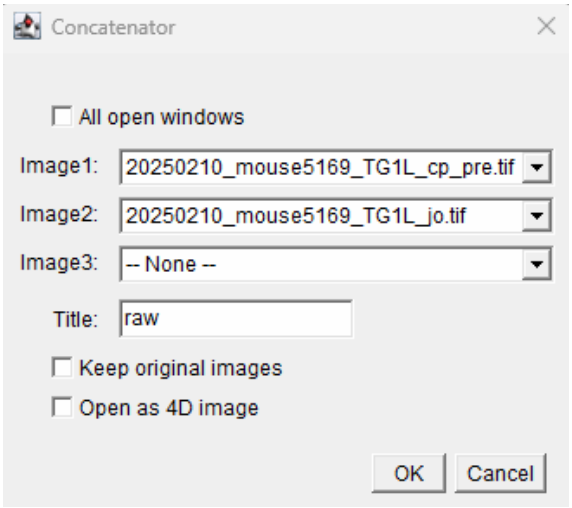
Steps:

Initial Setup:

- Open your acquired image set in FIJI (ImageJ).

Concatenate Images:

- Drag and drop all relevant `.tif` files into ImageJ.
- Go to `Image > Stacks > Tools > Concatenate...`
  - Confirm that all images are listed in the correct order.
  - Uncheck “open as 4D image” option.

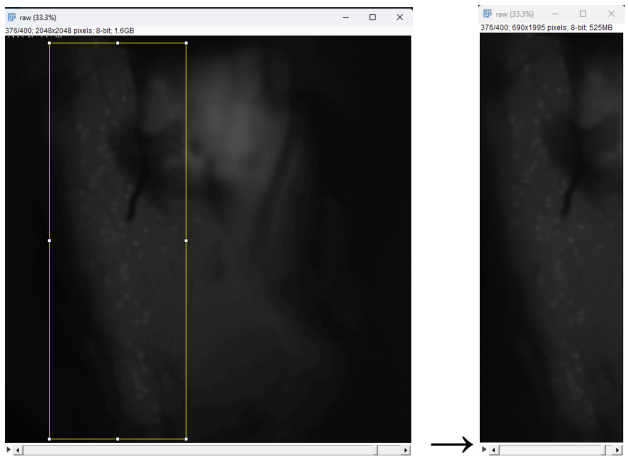


- Save the concatenated stack as `raw.tif` in an empty folder named “Analysis.”

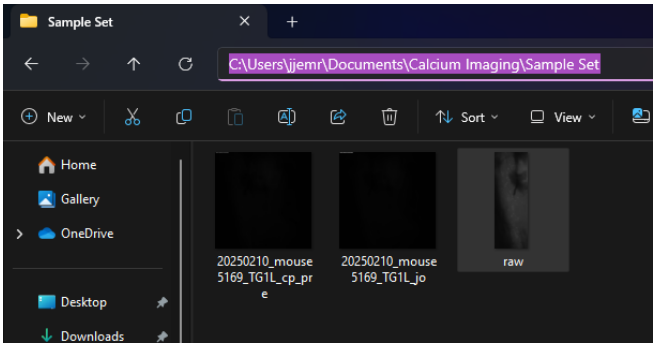
Image Alignment with MATLAB:

- Ensure live data is first converted to 8-bit using ImageJ.

- Before converting to 8-bit, ensure signal is visible: `Image > Adjust > Brightness/Contrast`
- Convert to 8-bit: `Image > Type > 8-bit`
- Crop the image using the Rectangle selection tool, ensuring the signal image remains in frame throughout the image stack
- `Image > Crop`



- Download ‘Image Processing Toolbox’ in MATLAB
- Copy the image location by right-clicking the folder icon in the file explorer and selecting "Copy Address as Text."

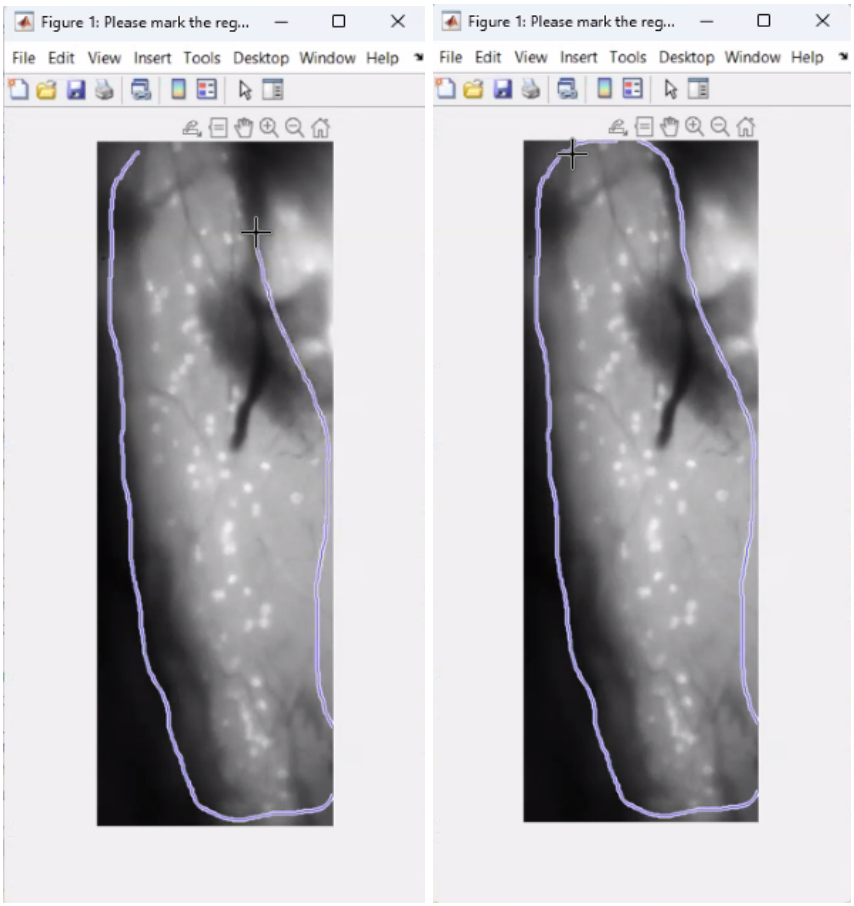


- Open “correct_movements.m” in MATLAB.
- Paste the copied image address into the script under the ‘filename’ section for user parameters. Ensure to replace only the relevant text.
- Add `\raw.tif` to the end of the path.
- Modify the “stacks” line to reflect the number of stacks in your `raw.tif` image.

```
function correct_movements

% setting user parameters:
filename = ['C:\Users\jjemr\Documents\Calcium Imaging\Sample Set\raw.tif'];
stacks = [1,200; 201,400];
```

- Press the run button in the MATLAB editor.
- Follow the on-screen prompts to circle an ROI mask when the max intensity image pop-up.



- The program runs at about 1s per frame and outputs `raw_aligned.tif` into the same folder where your `raw.tif` is located.

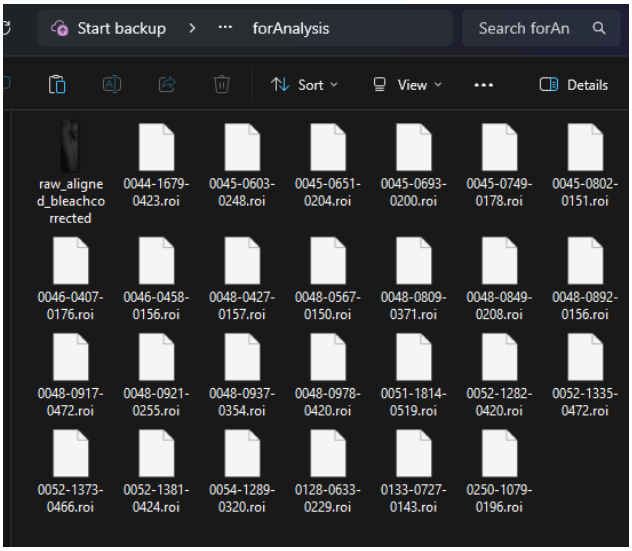
Bleach Correction and Cropping:

- Open the aligned stack in ImageJ.
- Confirm that the alignment was successful by scrolling through the stack.
- Create two subfolders inside the “Analysis” folder, titled “forAnalysis” and “done”
- Crop the aligned stack to remove black borders generated during alignment.
- Use `Image > Adjust > Bleach Correction > Exponential Fit`. Save the bleach-corrected stack (DUP_raw_aligned.tif) to the “forAnalysis” folder.

        - *it is important to not apply bleach correction before image alignment

ROI Selection with ImageJ:

- You can directly circle ROIs of interest using the Freehand selection tool.
- For each ROI selected, add it to the ROI Manager by pressing “T”.
- Toggle “Show All” to visualize all selected ROIs and avoid duplicates.
- After selecting ROIs, save the ROI set as a .zip file in the “Analysis” folder using `More > Save...` in the ROI Manager.
- Unzip the ROIs to the “forAnalysis” folder.



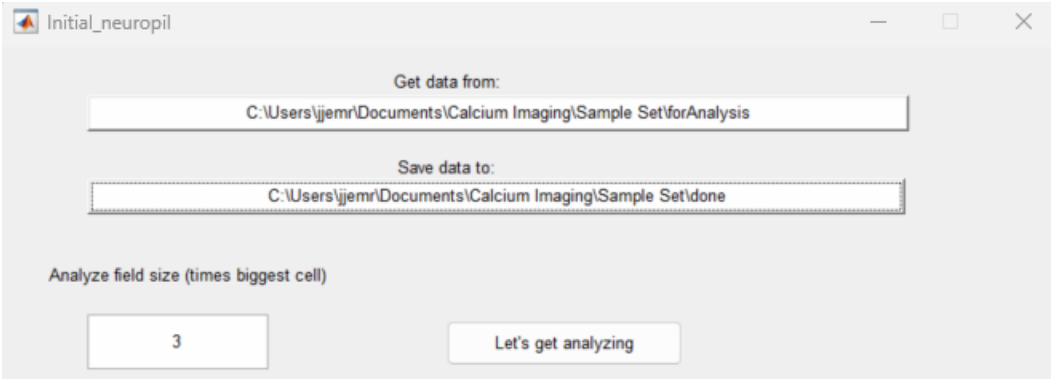
Optional: Noise Filtering to Visually Identify Signals of Interest

- Convert the file type to 32-bit using `Image > Type > 32-bit`.
- Go to `Image > Stacks > Z-Project...` and set:
    - Start Slice = 1
    - End Slice = 10
    - Projection Type = Average Intensity
- Rename the resulting image as "F0".
- Use `Process > Image Calculator` with subtraction: Image1 = aligned, Image2 = F0. Check 32-bit (float) result.
- Select ‘Yes’ to Process Stack (all xxx images).
- Rename the resulting stack "deltaF".
- Convert the file type to 8-bit: `Image > Type > 8-bit`.
- Adjust brightness and contrast via `Image > Adjust > Brightness & Contrast`.
- The output should be a black background with flickering white neural signals.

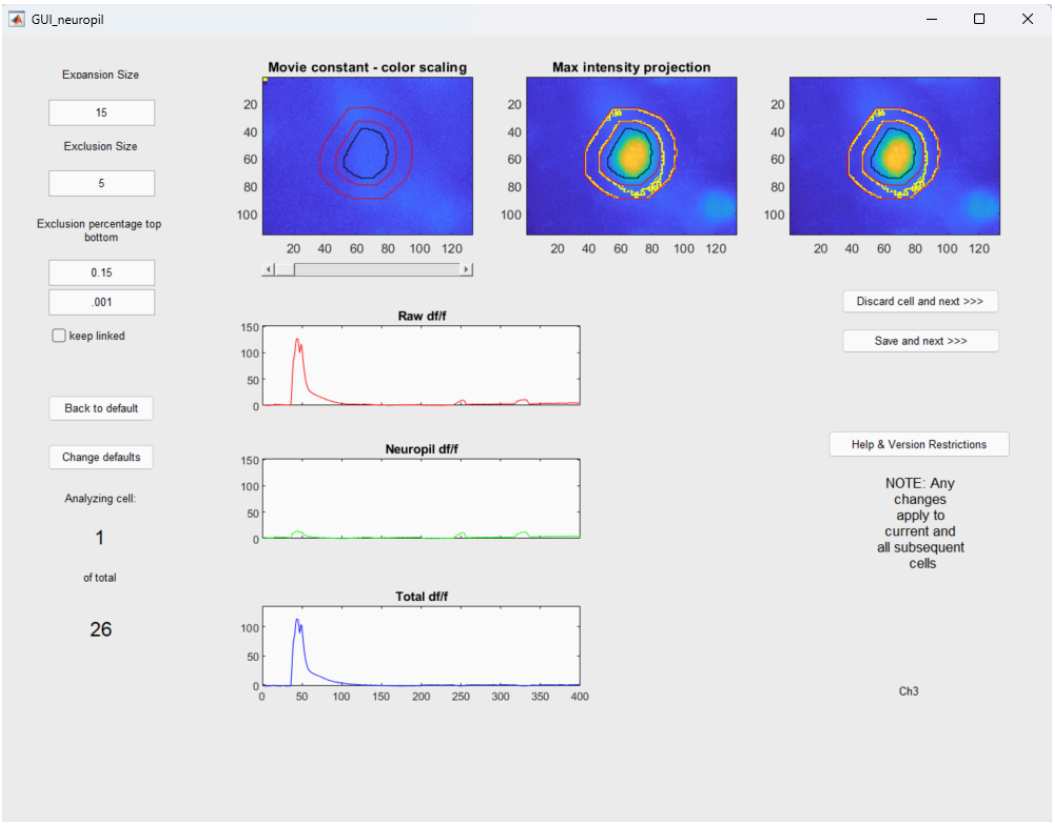
- This processing aids in identifying neurons that fluoresce above baseline.

Delta F Calculation and MATLAB Processing:

- Open the Neuropil Script in Matlab by locating and selecting “main_neurop_sub.m”
- Ensure the folders “geom2d” and “PolyGeom” are added to the Matlab path.
- Run the Neuropil Script.
- When prompted, select the `forAnalysis` folder for input, containing the aligned `.tif` and ROIs.
- Set the output path to the “done” folder.



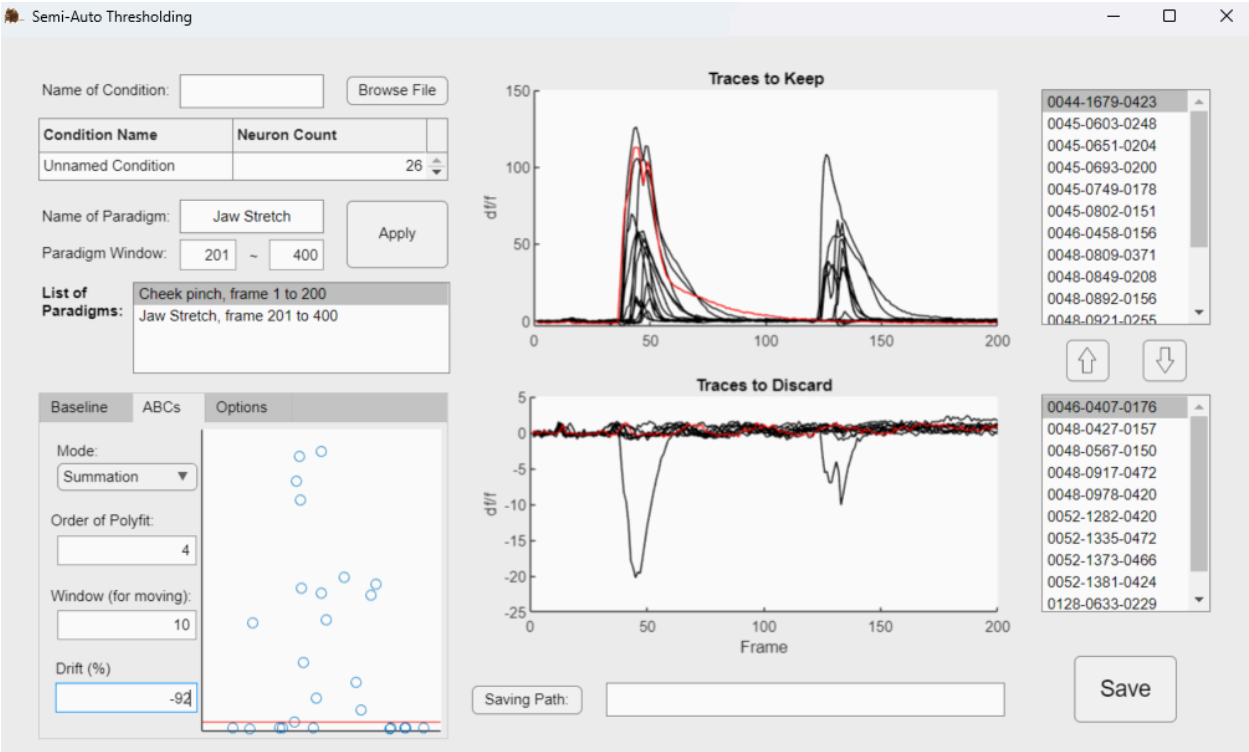
- Configure these settings before analysis:
  - Expansion size = 15
  - Exclusion size = 5
  - Uncheck "keep linked box"
  - Top exclusion percentage = 0.15
  - Bottom exclusion percentage = 0.001



- The script will perform Delta F calculations and process the data.
- “Save and next >>>” for all ROIs to be kept
- After processing, inspect results in the Done folder.
- Convert output file “neuro_corr_traces” from `.csv` to `.xlsx` extension.

Thresholding Script and AUC Calculation:

- Tool to exclude inappropriate waveforms from analysis
- Open the Thresholding.mlapp. (Downloand Add-on ‘Curve Fitting Toolbox’)
- Load the intensity data obtained from the Neuropil processing step (neuro_corr_traces`.xlsx`). Enter name of data set for “Name of Condition:”
- Follow the on-screen instructions to specify the baseline and event windows (i.e. ‘Name of Paradigm = cheek pinch; Paradigm Window = 1 ~ 200’).
- Exclusion Threshold % removes traces that reach X% of maximum value of trace during defined baseline period
- ABCs window: Alter drift based on the ‘Traces to Discard’ and decrease up to -98% to capture ‘Traces to Keep.’
- The GUI uses Area Under the Curve (AUC) to exclude traces that exceed boundaries set for responders.
- Once calculated, save the binned traces as needed for further analysis or reporting (‘Saving Path’).



Heatmap Generation:

- Import the `.csv` in Matlab.
- Use `imagesc(raw(:,2:end)',[1.0,40])` for generating a heatmap.
- Adjust the colors using the Colormap Editor.
- Save the heatmap as a `.png` or `.fig` file.

Final Notes:

- Conduct quality checks after each step.
- Document any anomalies for future reference.
- Regular backups are recommended to maintain data integrity.