Below are step-by-step instructions for using the three modules of EZcalcium toolbox.

Motion Correction

1) From the initial GUI, click on the box titled *Motion Correction* to load the Motion Correction GUI **(Figure S5)**

2) In the Motion Correction GUI, click the Load button to choose any number of videos you would like to load. You can drag a box or use the select or *shift* key to select multiple files with the mouse. If you close the file select window and decide to add more files later (e.g., from a separate folder), they will appear at the bottom of the list. If you accidentally try to add the same file multiple times, it will not be added to the list again and an error will display. Compatible file types include multipage 8- or 16-bit .tif, .avi, and .mat in which the largest variable in the .mat workspace is a three-dimensional matrix of *xyz* dimensions. Files used need to be single-channel images. .tif files greater than 4 GB need to be in an uncompressed format. If your data is of an incompatible file type, it can likely be converted to .tif or .avi with free software such as ImageJ, available at https://imagej.nih.gov/ij/

3) To remove a file that has been added to the *Files to Process* list, select the file with the mouse and click the *Remove* button.

4) Choose a template source from the list *Template Source.* Choosing *Previous Frame* will align each frame, starting with the second frame, to its previous frame. *Mean* and *Median* calculate the mean and median intensity value for each pixel, respectively. *Max Projection* creates a projection of the highest intensity value of each pixel. *Brightest Frame* detects the frame with the overall highest pixel intensity. Two other options are also included here and are based on the same principles as above but designed for working on a system with low RAM.

5) Choose the number of frames from which to create the template under *Template Frames.* If a large section of the video is stable and has a representative amount of fluorescence signal, but other sections exhibit a lot more motion artifact, it may be useful to restrict *Template Frames* to the stable frames of the video. Checking the box *Use All Frames* will automatically detect and use all the frames in any given video and ignore the input values.

6) Set the number of *Blocks*, which refers to the number of blocks (spread vertically) that the image will be divided into prior to alignment. To register the image as a single block, enter a *Blocks* value of 1. Generally, using a larger number of blocks processes faster than using fewer blocks, due to the ease of aligning several smaller blocks instead of larger more complex images. However, it may also introduce artifacts on otherwise steady videos if blocks are too numerous. For example, a *Blocks* value of 8 for a 256x128 will break down into a total of 64 32x16 blocks in an 8x8 configuration (**Suppl. Fig 1c**). *A* recommended value for *Blocks* would be less than the square root of the number of pixel rows (video height).

7) Select a format for the corrected video to be saved under *Output.* 8-bit or 16-bit should be chosen to match the bit depth of the image acquisition. Although .mat files will load quickly for ROI selection, they are not viewable in ImageJ and other software packages. In EZcalcium, saving as a .mat is currently the only way to save a compressed version of .tif files over 4 GB.

8) Lossless compression options for saving .tif files are available under *Compression.* These can reduce file size without losing any data but will likely take longer to save. These do not apply to .mat or .avi files. *Deflate* generally results in the smallest file size when saving 16-bit .tiff files smaller than 4 GB. *Packbits* is usually the fastest form of compression but it may not reduce file size as much as other methods. If the user selects *None*, this will result in no compression and will

save in the fastest time, although this will also result in larger file sizes. For EZcalcium, *None* must be selected for saving .tif files over 4 GB.

9) *Background Subtraction* will remove elements of the images determined to be related to background changes in fluorescence intensity. The choice of *Minimum* removes the minimum value of each pixel from the pixel. This is useful for removing constant sources of brightness, such as bleed-through fluorescence, without impacting changes in imaged activity.

10) *Iterations* refers to the number of repeated times the user wishes to perform motion correction. For a *Template Source* such as *Mean*, multiple iterations are useful so that the mean image projection used for the template will change with each iteration and result in improved motion correction. *Iterations* should generally be less than 7.

11) The *Save Template* option saves the template that was generated and used for alignment, for further reference. *Save Max Projection* saves a maximum intensity projection of all frames, no matter what template source was used.

12) *Low RAM Batch* is used when a *Low RAM* template source is selected. These sources separate the videos into "batches" to prevent overloading of the computer's RAM. The *Low RAM Batch* number indicates the number of frames to be placed in each batch.

13) The *Save Settings* button allows the user to save all settings under a specific name of your choosing. Settings are saved as .mat files. These include all settings in the *Settings* section as well as timing data that records how long it took to go through the main steps of the previous five video alignments.

14) The *Load Settings* button allows one to load all saved settings in future sessions.

15) Once all settings are configured, click the *Run Motion Correction* button. Immediately after attempting to start motion correction, and autosave file is automatically generated and updated as

progress continues. This will automatically load the last used settings when the application is next used. Progress of motion correction will display the current iteration of motion correction, the current progress of the current iteration, and the overall progress of the all the files listed in the *Files to Process* list. *Est. Time Remaining* displays the estimated time remaining for all the files to be motion corrected. Time estimates may not be updated under when loading or saving .mat files or when loading uncompressed .tif files.

16) After motion correction is completed, the new file generated will be added to the bottom of the *Processed Files* list. Original files will not be overwritten. A new file will be created with *_mcor* and the number of iterations appended to the filename.

17) The *Open* button will open the selected file in the default program set by the operating system.

18) *Clear List* clears the entire list of *Processed Files*.

19) The *Reset Timing* button can be used when creating a new configuration or working with files of a different size. It will reset all timing information on how long individual steps typically take that are used for calculating *Est. Time Remaining*. After running motion correction five times on a new configuration, *Est. Time Remaining* should be the most accurate. In the event that stored timing information is corrupted and *Est. Time Remaining* displays as "NaN", click the *Reset Timing* button to clear it.

ROI Detection

1) From the initial GUI, click on the icon titled *Automated ROI Detection* to load the *ROI Detection* GUI **(Fig. S6)**.

2) In the *Automated ROI Detection* GUI, click the *Load* button to choose any number of videos you would like to load. Compatible file types include multipage .tif, .avi, and .mat in which the largest variable in the .mat workspace is a 3-D matrix of xyz dimensions. One can drag a box or use the select or shift key to select multiple files with the mouse. The user closes the file select window and decides to add more files later, such as from a separate folder, the files will appear at the bottom of the list. If the user accidentally tries to add the same file multiple times, it will not be added to the list again and an error will display. File type requirements for ROI Detection are similar to that of Motion Correction. When troubleshooting and testing motion correction for the first time, it is advisable to start with correcting a single file and testing the settings.

3) To remove a file that has been added to the *Files to Process* list, select the file with the mouse and click the *Remove* button.

4) *Initialization* methods for providing an initial estimate of spatial and temporal components: *Greedy* is recommended for videos of neuronal somata. It relies heavily on spatial components and generally runs much faster; *Sparse NMF* is recommended for more complex structures, such as dendrites, dendritic spines, or axons.

5) *Search Method* determines the spatial components (location) of ROIs: *Ellipse* assumes components have an ellipsoid shape, such as for neuronal somata; *Dilate* can be used with either ellipsoid or non-ellipsoid ROIs, but generally takes longer.

6) *Deconvolution* determines the method for translating activity-induced changes in the fluorescence intensity of the indicator into approximate firing rates. If you are imaging an organism that does not produce action potentials, set this to the fastest setting available. Noise-constrained deconvolution methods include *SPGL1 - Constrained foopsi* and *CVX - Constrained foopsi*. Both are also available through https://github.com/epnev/constrained-foopsi. *SPGL1 -*

Constrained foopsi works well even with medium-to-low signal-to-noise traces. *CVX - Constrained foopsi* requires CVX and is not available in the standalone version of EZcalcium. It is typically the fastest method of deconvolution when working with high signal-to-noise traces. It is available at http://cvxr.com/cvx/doc/install.html. *MCMC* is a fully-Bayesian deconvolution method that is computationally-intensive and is recommended when higher precision is required. *MCEM* alternates between the listed *Constrained foopsi* deconvolution and *MCMC* to update time constants. It is significantly faster than *MCMC* alone and is generally recommended when deconvolving calcium signals from cell somata.

7) *Autoregression* is used to estimate the calcium indicator kinetics. *Rise and Decay* estimates both the rise and decay kinetics of the calcium indicator and incorporates them when extracting fluorescence traces and deconvolving the signal. Due to the difficulty in detecting fast rise times, using *Rise and Decay* may result in overfit data if the imaging was performed at low temporal resolution (<16 Hz). *Decay* estimates just the decay kinetics of the calcium indicator and is the recommended setting for lower temporal resolution imaging. *No Dynamics* will produce only raw traces and will not perform deconvolution.

8) Choose the number of frames to analyze under *Frames*. Checking the box *Use All Frames* will automatically detect and use all the frames in any given video and ignore the inputted values.

9) *Manual Initial Refinement* adds an additional step following initialization to manually add or remove ROIs. ROIs can also be removed in the step *ROI Refinement*. To fully automate the process, it is recommended to optimize your settings to slightly overestimate the number of ROIs and then remove erroneous ROIs later. This step is included as an option for particularly troublesome files and for those who prefer semi-automated ROI selection. Initial spatial components will be displayed in a new figure. The center of estimates in ROIs is highlighted with

a magenta circle, surrounded by the boundary of the ROI. To manually add an ROI, left click with the mouse where you want to add the center of an ROI. The boundary of the ROI will be automatically computed and drawn. To manually remove an ROI, right click on any ROI center. Hit the enter key to continue ROI detection.

10) Choose how you would prefer to export the data. The program automatically exports a .mat file that is necessary for the final step, *ROI Refinement. Output to .csv* exports the same data as *Save to .mat*, but to a .csv file that can be opened in a variety of programs outside of MATLAB.

11) Choose which additional figures you want generated. When batch processing many files, it is not recommended to use these. *Display Components* shows extracted raw fluorescence data, the inferred trace generated, and the ROI shape and location. *Display ROI Centers* generates a map showing the centers of all ROIs following initialization. *Display ROI Map* generates a map with all the ROI boundaries, each labeled with the same ROI number as was used in the data. *Display Merging Example* shows an example of how several components were merged to form a single ROI. This is useful when optimizing *Merge Threshold* and *Component Width. Save PDFs* saves .pdf files displaying traces of the analyzed data, with each PDF showing 10 ROIs. *Open PDFs* automatically opens the .pdf files generated by *Save PDFs* upon completion of the ROI detection.

12) *Estimated Components* is the estimated maximum number of components in the field of view. This must be set to a minimum value of 1. If you want to perform fully manual ROI selection, set *Estimated Components* to 1 and check the box to enable *Manual Initial Refinement*. When the manual refinement step starts, delete the initial automatically detected ROI. If no components are determined to be similar enough to be merged, the most likely result is that the number set for *Estimated Components* is the number that will be initially detected.

13) *Merge Threshold* is the threshold at which two components will be merged into a single ROI. Components that share a correlation coefficient above *Merge Threshold* will be merged into a single ROI.

14) *Component Width* is the estimated width, in pixels, of your components. If you have a simple ROI shape, such as a cell soma, you can use the width of the entire ROI as your component width.

15) *Fudge Factor* is useful for estimating time constants of very noisy data, in particular those with low temporal resolution (slow frame rate). The value indicates a multiplicative bias correction for time constants of each ROI during deconvolution. *Fudge Factor* should generally be set to 0.95-1. A value of 1 indicates that no bias correction will be performed.

16) *Spatial Downsampling* will downsample the spatial resolution of a video by a factor set here. The value entered should be a positive integer. A value of 1 means that no downsampling will be performed. This is useful for rapidly troubleshooting the settings on videos with a very large field of view.

17) *Temporal Downsampling* is similar to Spatial Downsampling, except it downsamples the temporal resolution. This is useful for optimizing settings on very long, high frame rate (>15 Hz) videos.

18) *Temporal Iterations* is the number of iterations that will be performed to calculate the temporal components of ROIs. This should be set to at least 2 when not rapidly testing other parameters.

19) *Save Settings* allows you to save all settings under a specific name of your choosing. Settings are saved as .mat files.

20) *Load Settings* allows you to load any previously saved settings.

21) Once all settings are configured, click the *Run ROI Detection* button. Immediately after attempting to start ROI detection, an autosave file is automatically generated and updated as progress continues. This will automatically load the last used settings when the application is next used. Status of ROI detection will be displayed in the *Status* box.

22) After ROI detection is complete, the new file generated will be added to the bottom of the *Processed Files* list. A .mat file will be created with the complete set of data generated during The *Open* button will open the selected .mat file into a MATLAB workspace, if running source scripts.

23) *Clear List* clears the entire list of *Processed Files*.

ROI Refinement

1) From the initial GUI, click on the icon titled *ROI Refinement* to load the ROI Refinement GUI **(Figure 3)**. In the ROI Refinement GUI, click the *Load* button to choose any number of data files generated by *Automated ROI Detection* you would like to load. All files should be .mat format files. You can drag a box or use the select or shift key to select multiple files with the mouse. If you close the file select window and decide to add more files later, such as from a separate folder, they will add to the bottom of the list. If you accidentally try to add the same file multiple times, it will not be added to the list again and an error will display.

2) To remove a file that has been added to the *Files to Process* list, select the file with the mouse and click the *Remove* button.

3) To view an ROI, select an ROI by clicking on it, and then click the button *View ROI*. The left arrow button (<) will automatically select and load the previous ROI (lower in ROI number). The right arrow button (>) will automatically select and load the next ROI (higher in ROI number).

4) The ROI field is displayed in the top left corner. Isolated ROIs are displayed in the smaller figure on the right side of the screen. Below the ROI figures are the extracted traces. The upper trace is the fluorescence trace and the bottom trace is the deconvolved trace.

5) Set *Automated Exclusion* criteria. *dF/F Activity* can be set to include ROIs that surpass a chosen activity threshold for a given number of consecutive frames. This threshold can be set in the *Value* box in units of dF/F, and the chosen number of consecutive frames can be set in the *Frames* box. *Baseline Stability* is used to check if an ROI has a stable baseline throughout the imaging session by comparing the baseline at the beginning of recording with the baseline at the end. *Baseline location* determines the percentage of the data from the beginning and end of the recording that will be considered when determining the baseline activity level; for example, if this is set to 25%, the first 25% and the last 25% of the total frames will be used to determine the baseline values. EZcalcium will find the minimum value within these frames and use the surrounding frames to calculate a mean baseline value. *Window* determines the number of frames to be averaged to find this baseline value. The *dF/F Baseline Stability* represents the absolute value of the difference between the baseline values from the beginning and end of the data. *Roundness* measures how similar an ROI is to a circle. This is useful when you are looking exclusively for neuron somata or other round ROIs. *Oblongness* is a measure of the ellipsoid shape of an ROI. It is calculated by dividing the length of the major axis of the ellipse by the length of the minor axis. This is useful in excluding overly oblong ROIs that are not likely to be neurons.

6) *Borderline %* can be set to allow an ROI to have criteria slightly outside of the desired range. If an ROI has a number of criteria within the Borderline range equal to or less than the *Borderline Allowance*, the ROI will be included.

7) *Saturated Frames* can be used to set a maximum number of frames in which the ROI can be fully saturated. This can also be input in terms of the percentage of total frames.

8) The next section allows the user to set minimum and maximum values for a variety of parameters. *Area* measures the area of an ROI and is useful for excluding ROIs that are too small or too large. *Width* indicates the mean width of an ROI and the relative size of the ROI. It is useful for removing ROIs that result from having too many components coalesce together into a single profile. *Skewness* and *Kurtosis* are used to describe the probability distribution of either the dF/F data or the deconvolved data for an ROI. *Skewness* measures the left- or right-skewness of the data. *Kurtosis* measures the likelihood of finding outliers in a distribution. For example, a cell with large, fast, and frequent spikes in calcium levels will have a higher kurtosis value than cells with a more moderate distribution of slow activity [1]. *Skewness & Kurtosis Data* is used to select whether the dF/F or deconvolved data will be used as the basis of the exclusion criteria for these two parameters.

9) Click the *Run Refinement* button to automatically exclude ROIs based on your chosen criteria.

10) To manually exclude an ROI, select an ROI and click the *Exclude ROI* button. After being excluded, the ROI number in the list will be stricken out and the *Exclude ROI* button will become an *Include ROI* button.

11) Click the *Export* button to export your data in your selected format.

References

1. Ringach, D.L. *et al.* Spatial clustering of tuning in mouse primary visual cortex. *Nature communications* **7**, 12270 (2016).