**CALIMA 2.0 setting parameters**

**Fluorescence signals**

The relation between the intensity of the fluorescent signal and the concentration of free calcium in the cell at any given moment in time is given by

[Ca2+]*i*(*t*) = *Kd*(*F*(*t*)−*Fmin*)/(*Fmax*−*F*(*t*))*,* (1.1)

with [Ca2+]*i*(*t*) representing the concentration of free calcium in the cell in [M] at a time instance *t*, *Kd* the dissociation constant of the fluorescent dye, *F*(*t*) the time-dependent fluorescent signal [a.u.], and *Fmin* and *Fmax* the intensity of the fluorescent signal in the absence of free calcium and in the situation the dye is saturated with calcium [a.u.]. Rewriting equation (1.1) under the assumption that [Ca2+]*i << Kd* leads to   
  
*F*(*t*) = (*Fmax*/*Kd*)[Ca2+]*i*(*t*) + *Fmin.* (1.2)

Due to local variations in dye distribution and illumination intensity, not every cell displays a signal of equal brightness. Therefore, the calcium signals are often normalized per region of interest to their fluorescence in rest *F*0. In order to do so, *Fmin* is estimated and subtracted from the fluorescence traces. Afterwards, the fractional increase in free calcium can be written per ROI as

([Ca2+]*i*(*t*) *−* [Ca2+]0)/[Ca2+]0 = (*F*(*t*) *− F*0)/*F*0 = Δ*F*/*F*0*.* (1.3)

An example of a *F*0-normalized trace can be seen in *Figure 1*. Note that

calcium spikes typically last seconds, while action potentials only

last a few milliseconds.

A part of the processing pipeline of CALIMA involves estimating *Fmin* and

the resting fluorescence *F*0 per ROI. Furthermore, the resting fluorescence

can change over the duration of an experiment as e.g. dye leaks from the

cell, and therefore *F*0 needs to be re-estimated per frame.

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| *Figure 1: An example of an F0-normalized fluorescence trace with clearly distinguishable spikes, obtained from rat cortical cells.* |

**Workflow**  
*Contrast enhancement*  
CALIMA accepts a variety of image (PNG, JPEG, BITMAP, GIF, TIFF) and video

formats (including Quicktime and ISO/IEC Base Media File Format). Visual information provides an important indicator to whether the ROIs found in a later processing stage concur with the user’s demands. To enhance the features of the cells in the displayed image, the image is contrast stretched. The contrast stretched image ***I***[*n*] of the selected frame *n* follows from

***I***[*n*] = (***O***[*n*] *−* min(***O***[*n*]))/(max(***O***[*n*]) *−* min(***O***[*n*]))*,* (1.4)

with ***O***[*n*] a 2D-matrix containing the raw data of frame *n*. The normalized

image ***I***[*n*] is displayed, but the original footage stack ***O*** is used as input for

the other processing steps.

This step is automated.

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| *Figure 2: The workflow of CALIMA 2.0.* |

*DOG Filter*The cells glow more brightly than their surroundings in the images-to-be processed,

and therefore an difference of Gaussiancs (DoG) edge detection algorithm is used to discover the ROIs. This algorithm enhances the features of the image at edges to discover locations with sharp dark-to-bright boundaries.

The image stack is first averaged per pixel and stretched to enhance its features using formula (1.4), resulting in image ***I****avg*. A Gaussian blur filter is given by

***G***(*σ*) = 1/(2*πσ2)* exp(*−(x2* + *y2)/*2*σ2),* (1.5)

with ***G*** representing a matrix with a Gaussian kernel with variance *σ*, and *x* and *y* representing spatial coordinates. The result of the DoG operation ***D*** is hence found by

***D***(*σa, σb*) = ***I****avg ∗* ***G***(*σa*) *−* ***I****avg ∗* ***G***(*σb*)*.* (1.6)

Here, *σa* and *σb* represent the variances of the DoG filter which are set by

the user (see Figure 1.2), with *σa < σb*.

From trials of the DoG procedure in several data sets, it was found that both *σa* and *σb* should typically be 3-10 pixels in size. Furthermore, setting *σb* a factor 1.6 larger than *σa* results in the optimal edge detection. Additionally, setting *σb* to a high value would result in drawing the ROI boundaries at a smooth edges (a gradual change from the dark background to the bright cell), setting it to a low value would result in the ROI boundaries to be placed at the sharp edges (e.g. at abrupt changes in brightness from a very dark background to a very bright cell in a few pixels). The threshold to detect edges is dependent on both *σa* and *σb . ThDoG* =0.01 (*σb/ σa*) appears to be a good starting estimate. Lowering *ThDoG* results in more and larger ROIs to be found.

*Extract mean signals*To find the Δ*F*/*F*0 traces mentioned in Formula (1.3), the minimum fluorescence *Fmin* is automatically estimated by averaging the 1% lowest-valued pixels of the first frame.  
This step is automated.

*F0 normalization*The fluorescence traces *F*(*t*) are found by first averaging the pixels per ROI per frame. Next, a sliding window algorithm estimates *F*0 by taking the mean of the lowest values of thelatest values of the fluorescence trace. The algorithm is applied to the previous frames, and the number of previous frames used is the user-set window length *K.* The lowest values are found from averaging all values in the last *K* frames that are below a user-set threshold *F0th.*The value of *K* should be chosen longer than the duration of one full-length calcium spike, so at least 2.5 seconds regarding the example in *Figure 1*, but not much longer. The value *F0th* should be chosen sufficiently high such that multiple samples are used to estimate *F*0, but well below 50% to prevent overestimation of *F*0 in case a calcium spike is present in the previous frames. Think in the range of 10-20%. When *K* is increased, *F0th* can be lowered.

*Spike detection*The peaks in the Δ*F*/*F*0 traces are found with a so-called robust sliding Z-score algorithm. This variation of the Z-score algorithm uses a sliding window to determine the signal mean and standard deviation locally. Furthermore, it reduces the influence of peaks on the mean and standard deviation with a low-pass filter. The robust Z-score can be described by the following set of equations:

*μf* [*t*] = mean(*Fbuff* [*t − Lf −* 1 : *t −* 1])*,* (1.8)

*SDf* [*t*] = SD(*Fbuff* [*t − Lf −* 1 : *t −* 1])*,* (1.9)

*Zf* [*t*] = (Δ*F*/*F*0[*t*] *− μf* [*t −* 1])/*SDf* [*t −* 1]*,* (1.10)

*Pf* [*t*] = 1 if *Zf* [*t*] *> ThZ*,

= 0 otherwise, (1.11)

*Fbuff*[*t*] = *jin*Δ*F*/*F*0[*t*] + (1 *− jin*)*Fbuff* [*t −* 1] if *Pf* [*t*] = 1,

=Δ*F*/*F*0[*t*] otherwise, (1.12)

with *Fbuff* representing the low-pass filtered fluorescence signal, *Zf* [*t*] the Z-score at time instant *t*, *Pf* the time instances with activity higher than the threshold *ThZ*, and *jin* the smoothing factor of the filter.

The user can determine the sliding window length *Lf* , the peak height threshold *ThZ* and the smoothing of the filter *jin*. Ideally, *Lf* is longer than the expected duration of a calcium spike and could be set to match *K*. Note, however, that calcium spikes occurring in the first *Lf* frames might be missed due to the absence of valid estimates for the signal mean and standard deviation. The peak height threshold *ThZ* determines the minimum peak height in terms of standard deviations, and setting the value to 3 or higher should suffice in most cases. The smoothing factor *jin* should be set to a small value (typically 0.1-0.2) to ensure that spikes do not contaminate the sliding window estimates. However, setting *jin* to 0 must be avoided to prevent filling the buffer with identical numbers in the case of long-lasting calcium activity, resulting in a standard deviation *SDf* of 0 (see Equation (1.12) and (1.10)).

Correlation analysis  
The correlations between the Δ*F*/*F*0 traces or the detected calcium spikes over the different ROIs are calculated through Pearson cross-correlations. Regarding single-ROI analysis, the Pearson correlations between selected ROIs are given in bar graphs for different delays between the signals. A correlation map provides the user with information to the location of ROIs spiking at a user-set time delay according to a selected ROI. Furthermore, a cross-correlation matrix displays the correlations between all ROIs and a heatmap identifies the most active areas in the cell culture. A spatio-temporal map labels the occurence of the first spike per ROI with a certain color to help identify the response to a stimulus progressing from ROI to ROI. Lastly, an outline map draws lines between the ROIs with a minimum user-chosen Pearson

correlation.

Taking insight from previous neuronal cell culture studies from in the literature into account, neurite growths is limited per day to several micrometres, hence a subroutine is introduced in the software that calculates the user-chosen Pearson correlations between ROIs that are within a certain distance of one another. The information is then processed in the images as line connections between the ROIs. Note, however, that this subroutine is useful only in the study of network development in early-stage cell cultures, and that the interpretation of these results should be done with care.  
  
The user-set values here are free, and largely depend on the information one is seeking.