

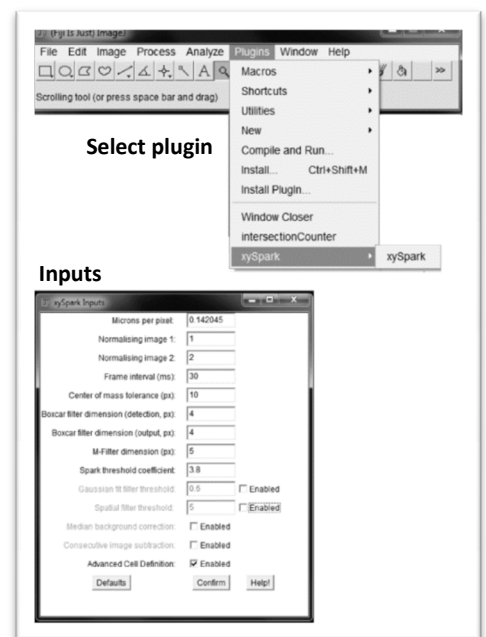
ImageJ update, memory allocation and installation of xySpark plugin

Open ImageJ (or Fiji) then:

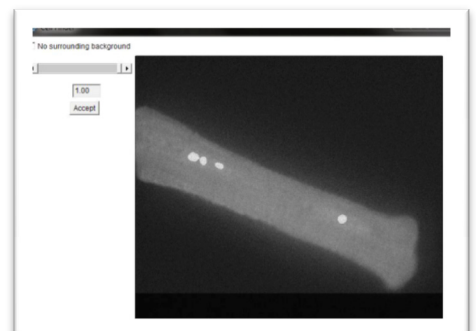
- (i) **Update ImageJ (or Fiji):**
Select 'Help' / Update ImageJ
- (ii) **Configure memory:**
Select 'Edit' / Options / Memory
Allocate 75-80% of your total RAM to ImageJ
(Note: if you have 8GB, allocating 6GB this is sufficient for ~1000 frames 512/512. Memory requirements can be reduced by cropping the stacks to exclude a proportion of the pixels outside the cell boundary. However, reducing the bit depth or rotating the image prior to analysis is not recommended due to loss of precision or changes in pixel values during interpolation. Allocated memory is progressively used by ImageJ/xySpark up to the set limit, but is released when you exit ImageJ. Close as many other applications as possible to free up memory)
- (iii) **Install xySpark:**
Navigate to the 'Plugins' folder within your ImageJ or Fiji installation.
Unzip the provided xySpark folder and place it into the Plugins folder
Restart ImageJ and xySpark it will appear as an option under 'Plugins'

Quick start & demo:

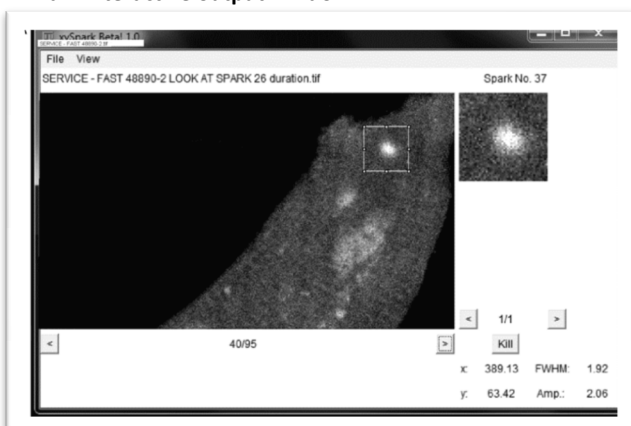
- (i) Drag and drop the provided x-y image stack (demo.tif) onto ImageJ to open. This is a small (256/256) stack of a cardiac myocyte.
- (ii) Select "Plugins"/xySpark/xySpark
- (iii) In the 'xySpark Inputs' box, set the pixel size to 0.28 μm . Press "Confirm" to continue.
- (iv) In the 'Cell finder' window, click the left/right arrows to match the region above threshold (bright region) with the outline of the cell and then click 'Accept'
- (v) The ImageJ progress bar will indicate each stage of analysis
- (vi) Selected outputs will appear, including a window showing the first frame of the original x-y data stack and a window containing the Gaussian fit to a line profile passing through the centre of mass of each spark (used for amplitude and width measurements)
- (vii) In the main interactive window, click the right arrow (bottom) to advance through the stack and see each identified spark highlighted with a yellow box. The corresponding Gaussian fit is presented automatically in the 'Graphs' window.
- (viii) To obtain the table containing the summary data and statistics, select 'View' / Show current table
- (ix) Other outputs are also available under the View menu, including the F/F₀ stack, the binary image showing regions identified as sparks (spark ROIs), the binary cell mask showing the region identified as the cell, a stack containing all of the identified sparks.
- (x) All outputs can be saved together with the same file name (select File/save). Alternatively, the data within the table can be saved (table) 'File'/Save as, or selected and copied to a spreadsheet.



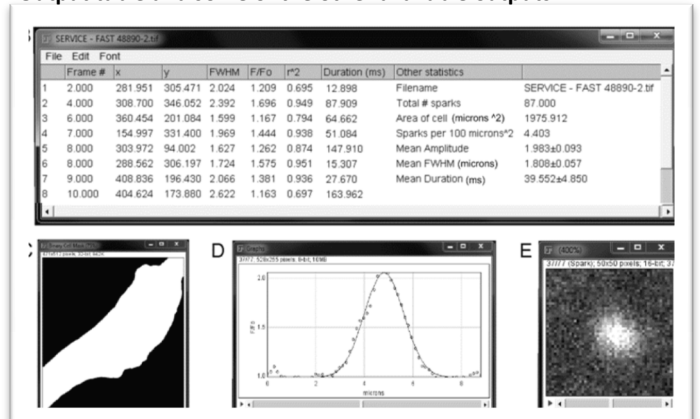
Interactive cell finder window



Main interactive output window



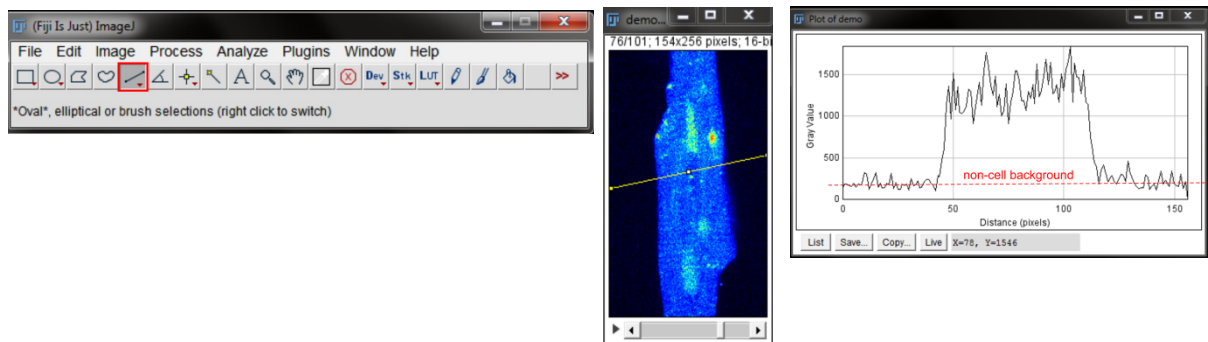
Output table and some of the other available outputs



Using xySpark

For normal use, **BEFORE** running xySpark, do the following:

- (i) **Drag and drop a confocal x-y image stack onto ImageJ or open the stack from the File menu.** Typically this will be an 8, 16 or 32 bit confocal image (512x512, or 256x256 pixels). Depending on the confocal system and the type of file exported by the confocal software, you may have to install a specific filter to import the file into ImageJ (see here for plugins <http://rsbweb.nih.gov/ij/plugins/>).
- (ii) **'Play' the stack (or advance through images) to check if the data are suitable for analysis, i.e.** check that there are no Ca^{2+} waves or corrupted images. If a wave occurs during a long sequence of frames but the data are otherwise suitable, you can remove the frames containing the waves using the built-in ImageJ function (see Image/Stacks/Tools/Slice Remover).
- (iii) **Measure (and note) the background fluorescence outside the cell boundary.** To do this, draw a line across the cell using the 'line' tool (red box below) and then plot the line profile (Analyse/Plot profile). After running xySpark and completing the initial analysis, this background non-cell fluorescence value can be entered (under File, Correct amplitude) to correct the spark amplitudes.

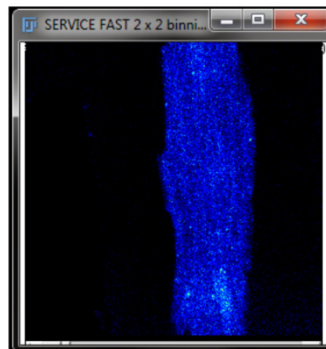


- (iv) **Crop the image, or zero regions of the image stack that are bright and outside the boundary of the cell that you want to analyze,** e.g. dead cells or neighboring myocytes that you aren't interested in. In the example below, there are bright, dead cells to the left of the myocyte. With an image like this, where the cell is vertical or horizontal, cropping is the best option (draw a rectangle using the rectangle tool and then use Image/duplicate to create a new cropped stack). Cropping also reduces processing time. However, if the cell is at an angle, it is usually best to select the region (using the freehand selection tool) are zero the pixels throughout the entire stack. This can be important because xySpark identifies the cell using thresholding. If there is a bright region next to the cell, this will exceed the threshold, causing an error in the cell area calculation (and related calculations, e.g. frequency).

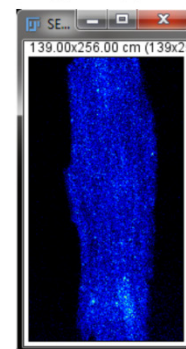
Dead cells encircled using ImageJ freehand tool



Encircled region zeroed using Process/Maths/Subtract



Alternatively, Image cropped



Running xySpark

Having completed the preparation steps above, run xySpark from the ImageJ plugins menu. For a detailed description of how xySpark works and how to configure the inputs, see the following publication ([to be added](#)).

Inputs window

Settings that must be checked or changed each time you run xySpark:

The 'Inputs' window allows the input of calibration information about the image stack, including **pixel size** and the **frame interval**. This should always be checked. It is also necessary to input the frame numbers of 2 "**normalizing images**" (lacking sparks), which are used to divide all other frames, and produce the F/F_0 image stack. The default is frames 1 & 2. If there are sparks on frames 1 or 2, then the plugin will still work, however, it will fail to identify any further sparks at those locations.

Settings that you may not need to change each time you run xySpark:

Tolerance: The '**centre of mass tolerance**' allows for the fact that when a spark appears in successive frames, the calculated centre of mass is unlikely to be exactly the same, and avoids incorrect detection as a new event. The default is 10 and it will generally not need to be changed.

Filtering: The algorithm is designed such that image processing of the F/F_0 stack for optimal detection of Ca^{2+} sparks is distinct from that used during analysis. Hence, there are dimension settings (in pixels) for the **boxcar** & '**M-filter**' used to filter the F/F_0 image stack prior to spark detection and a separate boxcar filter used to process regions identified as Ca^{2+} sparks before analysis. The defaults are: **Boxcar detection: 4, M-filter detection: 5, boxcar analysis: 4**. These defaults work well with in our confocal image stacks, are comparable to the settings used in Sparkmaster (for line-scan images) and may not need to be changed. However, if you have very noisy data, you may get better results by increasing the filtering, particularly during detection.

Threshold for spark detection: The user defined coefficient ' ϵ ' dictates the threshold for spark detection and in normal use is typically in the range 3.4-3.8 (threshold = the mean background fluorescence within the cell + ($\text{SD} * \epsilon$)). The default is 3.8 and again, this works well with most of our stacks. Decreasing towards 3 will detect smaller events, but will also increase the number of false positives.

Excluding events based on the Gaussian fit. A Gaussian fit filter, based on the r^2 value of the curve fitted to a line profile passing through the centre of mass of each identified event, is also provided as an option. This was implemented because it was found that regions occasionally misidentified as Ca^{2+} sparks often had highly irregular, non Gaussian line profiles. excluded. The default value of 0.6 (where a perfect fit = 1), only excludes detected events that are very poor fits. The default is to have this selected.

Excluding events by size: The '**Spatial filter**' allows events greater than a specified width (full width at half max amplitude: FWHM) to be excluded. We found this to be useful because it can be used to automatically exclude sparks that have propagated to form small waves. However, setting the value too low (<5-6) will attenuate the distribution of normal spark widths.

Correction for slow changes in cell fluorescence: We have included methods to correct small systematic changes in background fluorescence (increases or decreases) over time using either 'consecutive image subtraction' (**CIS**) or subtraction of the median pixel value within the cell (**MS**). The default is the combined mode, where CIS is applied during detection and MS during analysis. For further details, see paper ([to be added](#)).

Option to used advanced cell definition routine: The '**advanced cell definition**' option implements an interactive algorithm that enables accurate definition of the region of the image occupied by the cell. The default is to have this selected.

There is a button to 'Confirm' any changes in the settings window and initiate the next stage. Any changes made by the user are reloaded when xySpark is next run, although default values can also be loaded if required ('Defaults' button).

Advanced cell definition window

A stack of modified fluorescence images is presented in the "Cell finder" window, which allows the user to control the region of the image that is above threshold (brighter), the aim being to accurately define the cell and its boundary. The user selects the 'Accept' button to proceed to the analysis stage.

Referencing xySpark

We hope that xySpark will be useful to groups studying local Ca^{2+} regulation in muscle cells. If you wish to reference xySpark, please refer to the associated paper in *Biophysical Journal* ([to be added](#)). The paper also includes a more detailed description of xySpark and characterisation using synthetic data.