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Automated analysis of dynamic Ca2+ signals in image sequences.

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Abstract:	Intracellular Ca2+ signals are commonly studied with fluorescent Ca2+ indicator dyes and microscopy techniques. However, quantitative analysis of Ca2+ imaging data is time consuming and subject to bias. Automated signal analysis algorithms based on region of interest (ROI) detection have been implemented for one-dimensional line scan measurements, but there is no current algorithm which integrates optimized identification and analysis of ROIs in two-dimensional image sequences. Here we describe an algorithm for rapid acquisition and analysis of ROIs in image sequences. It utilizes ellipses fit to noise-filtered signals in order to determine optimal ROI placement, and computes Ca2+ signal parameters of amplitude, duration and spatial spread. We have implemented this algorithm as a freely available plugin for ImageJ (NIH) software. Together with analysis scripts written for the open source statistical processing software R, this approach provides a high-capacity pipeline for performing quick statistical analysis of experimental output. We suggest that use of this analysis protocol will lead to a more complete and unbiased characterization of physiologic Ca2+ signaling.
Author Comments:	
Additional Information:	
Question	**Response**

Dear editors,

We are submitting this manuscript entitled “Automated analysis of dynamic Ca^{2+} signals in image sequences” for publication in JoVE. This work concerning an algorithmic approach to region of interest analysis of dynamic biological signals may benefit greatly from being published in the unique JoVE format. The experimental protocol implemented in this technique is best communicated through the video and animated medium, rather than pure text. Video formatting of this content also allows the reader to develop an intuitive approach to protocol application, and a quick visualization of the primary input data (confocal image sequences). Thank you for considering our work for publication in your journal.

Sincerely,

Author contributions:

Francis, Michael: development of algorithm, primary author of manuscript

Waldrup, Josh: performed experiments demonstrated within the manuscript

Qian, Xun: beta testing and development of the algorithm

Taylor, MS: development of algorithm and experimental techniques, secondary author of manuscript, manuscript edits.

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Keywords:

signaling, ImageJ, detection, microscopy, algorithm, calcium

Short Abstract:

Here a novel region of interest analysis protocol based on sorting best-fit ellipses assigned to regions of positive signal within two dimensional time lapse image sequences is demonstrated. This algorithm may enable investigators to comprehensively analyze physiological Ca^{2+} signals with minimal user input and bias.

Long Abstract:

Intracellular Ca^{2+} signals are commonly studied with fluorescent Ca^{2+} indicator dyes and microscopy techniques. However, quantitative analysis of Ca^{2+} imaging data is

time consuming and subject to bias. Automated signal analysis algorithms based on region of interest (ROI) detection have been implemented for one-dimensional line scan measurements, but there is no current algorithm which integrates optimized identification and analysis of ROIs in two-dimensional image sequences. Here an algorithm for rapid acquisition and analysis of ROIs in image sequences is described. It utilizes ellipses fit to noise-filtered signals in order to determine optimal ROI placement, and computes Ca^{2+} signal parameters of amplitude, duration and spatial spread. This algorithm was implemented as a freely available plugin for ImageJ (NIH) software. Together with analysis scripts written for the open source statistical processing software R, this approach provides a high-capacity pipeline for performing quick statistical analysis of experimental output. The authors suggest that use of this analysis protocol will lead to a more complete and unbiased characterization of physiologic Ca^{2+} signaling.

Introduction:

Ca^{2+} is a ubiquitous second messenger signaling molecule and cytosolic Ca^{2+} levels are highly regulated. Intracellular Ca^{2+} signals are complex and include isolated transients, oscillations, and propagating waves[1–4]. Spatial and temporal control of Ca^{2+} is thought to underlie physiological signal specificity, and therefore the analysis of Ca^{2+} signal patterns is of considerable interest to investigators in multiple fields[5].

Ca^{2+} indicator dyes such as Fluo-4 and Fura-2 are commonly employed to measure intracellular Ca^{2+} signals with fluorescence microscopy[5–12]. Typically, temporal Ca^{2+} signals are evaluated as time-dependent changes in mean fluorescence within a user-defined area, or region of interest (ROI)[5,6,13–16]. Currently, manual ROI analysis is both time consuming and labor intensive because it requires users to identify many ROIs and perform repetitive computations[17–19]. These techniques may also be subject to considerable user error, including introduction of artificial signal modes and exclusion of low amplitude or diffuse signals[18,20].

Automated ROI detection algorithms have previously been implemented using a variety of statistical approaches to determine optimal ROI placement, but they have generally been limited to analysis of line scan or pseudo-line scan images, which restricts analysis to a single spatial dimension in time[17,19–22]. Additionally, many existing algorithms are not adequate to encompass the diversity of Ca^{2+} release events which range from periodic, localized transients to propagating waves[23,24]. Comprehensive evaluation of physiological Ca^{2+} signals is often further complicated by the presence of significant image artifact that confounds signal to noise discrimination in many experimental systems.

Previously, an automated ROI detection algorithm solution to Ca^{2+} signal transient detection, implemented as a plugin for NIH ImageJ software (National Institutes of Health, Bethesda, MD), was developed and validated[25,26]. This algorithm, called LC_Pro, was designed to identify and analyze ROIs encompassing Ca^{2+} signal transients in 2-dimensional time lapse image sequences. Here a practical experimental protocol and representative demonstration of an application of the algorithm in porcine coronary artery endothelium is provided, with additional post-processing using the open source statistical processing software R to generate usable graphical output.

Protocol:

1. Vessel Dissection and Imaging

1.0.1) Harvest tissue from domestic juvenile pigs as described in Martens *et al*.[27] Place harvested swine right ventricles into a polydimethylsiloxane (PDMS)-bottom dissection dish containing HEPES buffered physiological saline solution (PSS).

1.0.2) With the aid of a stereomicroscope, dissect and remove a segment of the left anterior descending coronary artery (~8 mm length, 0.5 mm diameter) from surrounding tissue using forceps and spring scissors by removing the vessel segment from the surrounding cardiac tissue layers. NOTE: take care to not puncture the vessel wall.

1.0.3) Place a PDMS block (1 x 0.5 x 0.5 cm) into the dissection dish, and use a needle to pin it to the bottom. Cut an approximately 40 m-diameter tungsten wire into twelve ~0.3-cm length segments, forming micro-pins, and place these in the dish. Using forceps, secure one end of the vessel segment to the block with a micro-pin.

1.0.4) Carefully insert small spring scissors into the vessel lumen, and cut longitudinally down one side of the vessel to open it completely. Orient the opened vessel segment with the endothelium up.

1.0.5) Use the remaining micro-pins to secure the borders of the opened vessel segment to block such that the vessel forms a flat rectangle. NOTE: Micro-pin tops should be bent to 90˚ and inserted until flush with the block surface. Care should be taken to stretch the vessel preparation without overstretching; final width should be ~1.5 times the starting unstretched width.

1.0.6) Prepare a small volume (~2 mL) of Fluo-4 AM loading solution by mixing Fluo-4 AM (10 µM) dissolved in DMSO with pluronic (0.03 %) in a HEPES-buffered PSS (containing in mM: 134 NaCl, 6 KCl, 1 MgCl, 10 HEPES, 10 glucose), and insert the entire block into the loading solution for approximately 40 min at room temperature in the dark.

1.0.7) After loading, wash the block in HEPES-buffered PSS for 5-10 minutes.

1.0.8) Mount the block onto 50 - 100 µm thick spacers in a coverglass bottom chamber containing HEPES-buffered PSS. NOTE: metal pins can be used as spacers and ensure the vessel segment is facing down and not touching the spacers.

1.0.9) Place the chamber on the stage of an inverted microscope equipped for confocal imaging, and focus on the endothelial cell layer.

1.1.0) Capture time lapse image sequences of basal relative fluorescence for ~3 min at 20 x magnification and a frame rate of ~8 frames per second using confocal image sequence aquisition software.

1.1.1) After ~ 3 min of recording basal fluorescence, replace HEPES-buffered PSS solution with the same volume (~2 ml) of Substance P (100 pM) dissolved in HEPES-buffered PSS, and record for an addtional 3 min.

2. Automated Analysis

2.1) Render image sequence(s) of fluorescent calcium activity from confocal acquisition software as 8 bit, grayscale ".tif" format files with no scale information.

2.2) Open imageJ, click "open" in the file menu, and select the appropriate image sequence in the explorer window to view the image sequence(s) in imageJ.

2.3) Determine an appropriate ROI diameter by using the rectangular ROI tool to estimate the upper and lower boundaries of the spatial spread of activity within the image sequence(s). NOTE: The mean rectangular diameter is a suitable choice for ROI diameter.

2.4) Create a new folder on the computer hard drive, and add the image sequence(s) into the folder directory.

2.5) In imageJ, click the "plugins" window, then click "LC_Pro" to start the analysis.

2.6) Enter the ROI diameter value and select the filter threshold value (either 0.05 or 0.01).

2.7) Click the "drug treatment" checkbox and enter the time point values immediately before and after the drug was added (in seconds).

2.8) Click "ok", and enter the image sequence directory into the file explorer window.

3. Graphical Output

3.1) Download R version 3.0.2. from: http://www.r-project.org and install.

3.2) Open the 32 bit version of R.

3.3) Click “file”, then "open script" and select the "traceplot.R" R script for generating graphical experimental reports.

3.4) Install the calibrate, and gplots packages by selecting “packages”, “install packages” and selecting the appropirate packages.

3.5) Click “file” then "change directory" command and use the explorer window to select the directory that corresponds to the LC_Pro analysis output.

3.6) Click on the script window and then the "run all" command to run the script.

Representative Results

A custom algorithm, LC_Pro, was developed and implemented in order to perform automated analysis of Ca^{2+} dynamics on confocal image sequences. As depicted in Fig 1, the algorithm utilizes sequential processing modules that A) detect and track sites of dynamic Ca^{2+} change above statistical ($p < 0.01$) noise, B) define regions of interest (ROI) automatically at active site centers, and C) calculate average fluorescence intensities at ROIs to determine specific event parameters. A graphical overview of the algorithm is shown using computer generated Gaussian pulses of

known intensity and location (Fig 2). Signal pulses (Fig 2 A and B) were converted to binary using the z-score for a standard normal distribution,and best-fit ellipses were assigned to pixel loci above signal threshold (Fig2 C). An ellipse sorting algorithm was used to determine optimal ROI placement (Fig 2 D). ROI mean intensity versus time was then measured and signal parameters of amplitude, duration and spatial spread were computed (Fig 2 E). This analysis approach was applied to assess cellular Ca^{2+} dynamics in intact vascular endothelium. Specifically, confocal imaging was performed in opened swine coronary arteries as described in Fig 3, and the algorithm was employed offline to quantify distinct Ca^{2+} parameters. For these experiments, continuous recordings were made before and after addition of the endothelial stimulus, substance P (SP; 100 pM), and LC_Pro analysis was subsequently performed. For scaling signal within each ROI, baselines were derived as linear regressions of ROI intensity over the experimental time course (Fig 4). Mean signal intensity values were computed for each ROI (Fig 4A), and values above the mean were truncated to the mean and a linear regression was performed to approximate signal baseline (Fig 4B). Finally, raw intensity values were divided by the value of the regression line to convert values to fold change over baseline (Fig 4C). Figure 5 shows a representative experiment, including images of Ca^{2+} dependent fluorescence in the endothelium (Fig 5A), accumulate binary masks of total Ca^{2+} signal detected within the sampled field (Fig 5B), and recordings of average fluorescence at each ROI (Fig 5C) before and after SP treatment. Subsequent parameter analysis was performed using R software. Resulting histograms show the amplifying effect of SP on event amplitude, duration, and spatial spread (Fig 6).

Figure Legends

Figure 1. Signal flow charts of algorithm processes (this figure was obtained with permission from Francis et al.)[24]. The algorithm was organized into three sections: Image processing, event processing, and region of interest (ROI) processing. Image sequences are input into the flow chain, and event statistics are generated as final output. The image processing (A) subroutine of the algorithm converts the input image sequence into a list of best-fit ellipses by thresholding, using the z-score for a standard normal distribution and ImageJ particle analysis. Event processing (B) is a sorting subroutine used to determine optimum ROI position by organizing ellipse locations into event “sites” by time. After mean intensity measurements are taken at each ROI, statistical parameters for each event and site are calculated by the ROI processing subroutine (C) to generate the final output. BKGD, background; AVG, average.

Figure 2. Demonstration of automated ROI acquisition and signal detection using a computer-generated Gaussian pulse (this figure was adapted with permission from Francis et al.) [24]. A single computer-generated signal pulse (A) was embedded in random background noise. The gray scale image sequence was filtered to remove static background pixel values (B) and converted to binary using threshold pixel intensity values of $P < 0.05$ calculated by the standard score (C). ImageJ particle analysis algorithms were then applied to the image sequence to assign best-fit ellipses to pixel loci within each frame. A novel algorithm was used to group ellipses into discrete temporal “events” and determine the optimal position for each ROI based on the mean ellipse center (D). An ROI of user defined radius is

then placed at each position (dotted circle). Mean intensity values within an ROI are calculated for each frame and scaled using a linear baseline approximation. Peak amplitude is identified as local maxima above $P < 0.05$ as defined by the standard score for a corresponding ROI tracing (E).

Figure 3. Porcine coronary artery dissection protocol. Approximately 0.5 mm diameter x 8 mm length branches of the left anterior descending coronary artery (1st image, dotted circle) were dissected from the surrounding myocardium. Vessel segments were trimmed of surrounding adventitial tissue and cut lengthwise with small scissors (2nd image). Next, the opened vessels were pinned flat to a PDMS block using fine tungsten wires (3rd image). Mounted vessel segments were loaded with Fluo-4 Ca^{2+} indicator dye, washed, and then placed in a coverglass-bottomed imaging chamber. Images were collected at 20x magnification (488 ex. 510 em.) at 8.0 frames per second using an inverted confocal microscope.

Figure 4. ROI mean intensity baseline approximation. Following mean intensity versus time measurements (solid lines) for each ROI, the raw intensity values are scaled to F/F0 using the following baseline approximation method. The mean signal intensity (dashed line) during the control period is computed from user defined control and treatment interval values (A). The time-dependent signal intensity curve is then truncated above the mean control intensity to the mean control intensity to filter activity from baseline approximation, and a linear regression is performed on the resultant curve (dotted lines) (B). Finally, the raw intensity values are scaled to the computed linear baseline (C).

Figure 5. Representative results from LC_Pro analysis of basal and stimulated Ca^{2+} dynamics. Time lapse images of an input image sequence of a basal sampling interval (A, left panel) followed by a Substance P-stimulated sampling interval (A, right panel) are shown in grayscale. Time lapse image sequences of best fit ellipses for basal and stimulated intervals (B) were rendered by LC_pro. Finally, time-dependent scaled intensity curves from each automatically positioned region of interest (ROI) are shown (C).

Figure 6. Histograms of parameter distributions from a representative experiment (Figure 4). Histograms from parameters of peak amplitude (F/F0), duration at ½ max (s), and maximum spatial spread (μm^2) for both control (left column) and Substance P-stimulated (right column) events from a representative experiment were rendered using R to graphically process the output from LC_pro. Notably, Substance P stimulation expanded the number of events, and caused a significant right shift in the median amplitude and duration by Mann-Whitney U test ($p < 0.01$).

Discussion

Decoding complex Ca^{2+} signals at the cellular and multicellular level will require rigorous experimental and analytical approaches. Here, an approach is described in which time-resolved confocal image sequences of Ca^{2+} dependent fluorescence are subjected to an automated analysis that identifies and quantifies statistically relevant Ca^{2+} signals within intact cellular fields In the specific case presented, an artery segment was isolated from pig heart, pinned opened to expose the endothelium, loaded with the Ca^{2+}indicator Fluo-4 AM, subjected to confocal imaging, and

evaluated with the custom algorithm LC_Pro. This algorithm is designed to 1) detect and track sites of dynamic Ca^{2+} change above statistical ($p < 0.01$) noise, 2) define regions of interest (ROI) automatically at active site centers, and 3) analyze average fluorescence intensities at ROIs to determine specific event parameters. The approach overcomes major limitations of biological signaling research by reducing user error and bias, greatly reducing offline analysis time, and providing a complete index of signals across a field to discern representative profiles or patterns. Output from LC_Pro is further processed through R scripts, providing full parameter reports for individual experiments, including high quality graphical output.

Several steps are important for proper performance of the described technique. The tissue dissection procedure (step 1.0.2) must be performed carefully in order to avoid denudation or damage of the endothelial cell layer. Additionally, image sequences must be in the proper format (step 2.1) for the automated region of interest algorithm to work correctly. For example, if there is scale information associated with the file, regions of interest will be incorrectly placed according the pixel locations, rather than the scaled unit locations. Also, choosing a suitable region of interest diameter is critical to correctly performing automated analysis of the fluorescent activity within an image sequence. Too small a diameter value may result in redundant measurements, whereas too large a diameter will result in exclusion of subtle signals.

Potential pitfalls of this technique primarily relate to sensitivity of the automated analysis to x-y shifts in recorded image sequences and to signalsaturation and/or bleaching, which may result in false positive or false negative results. Dimensional drifts or shifts may be addressed directly through use of stack registration software and accurate designation of intervals where perturbations are introduced. Gray levels and ROI diameter parameters may also be optimized for a given preparation in advance of data collection in order to standardize raw data sets.

Because it serves as a standard approach for any dynamic fluorescence signal, the analysis described here is suitable for a diverse body of applications. In the vasculature, this method is being employed to define characteristic physiological and pathophysiological signal modalities in multiple vascular beds under basal and stimulated conditions[25,26]. It is also being applied in the automated tracking of Ca^{2+} waves. Ultimately, such global automated analysis will be a crucial linchpin in deciphering spatially and temporally complex bio-signals and in establishing new cell and multi-cell models.

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Disclosures
The authors have nothing to disclose.

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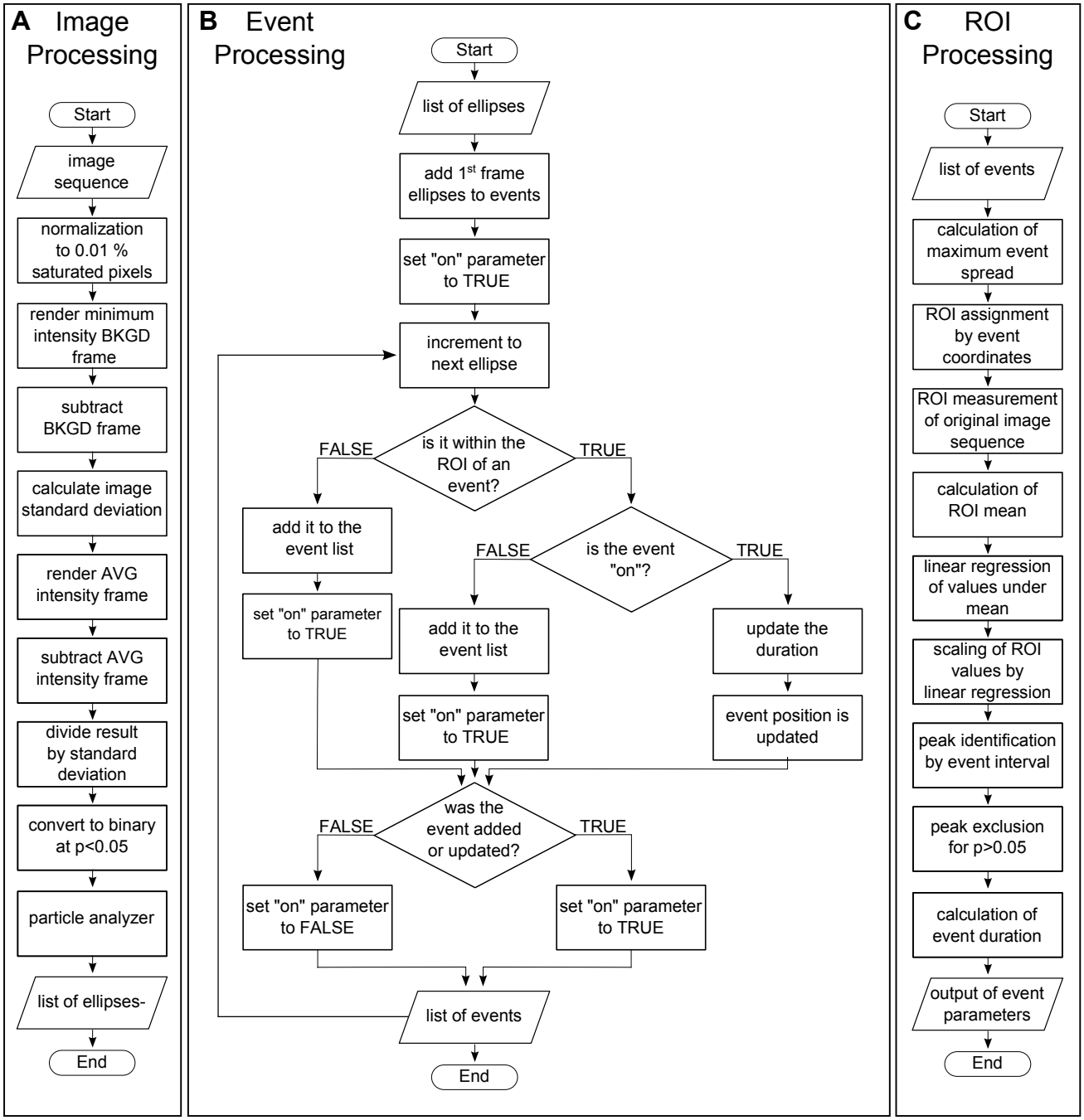

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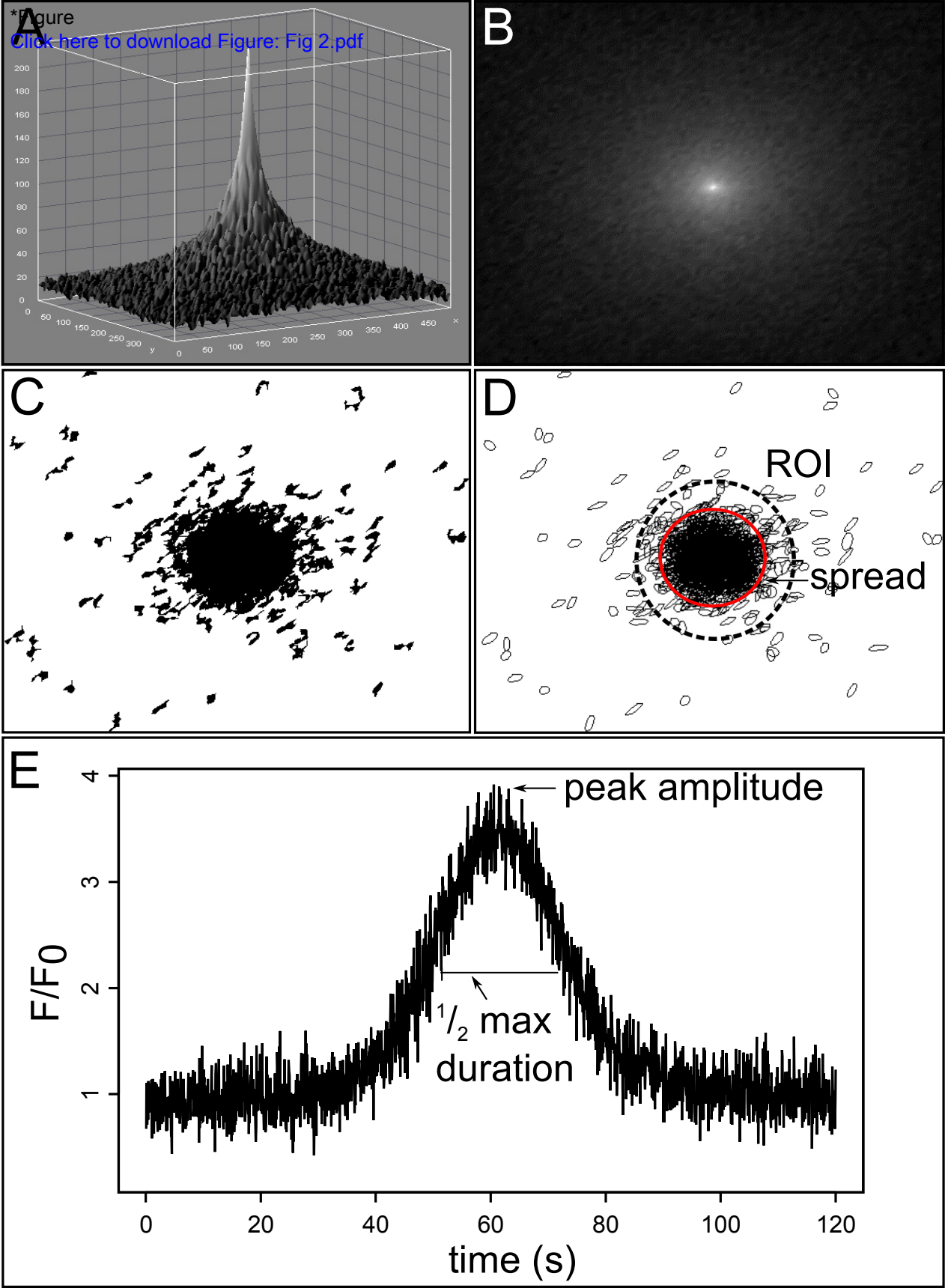

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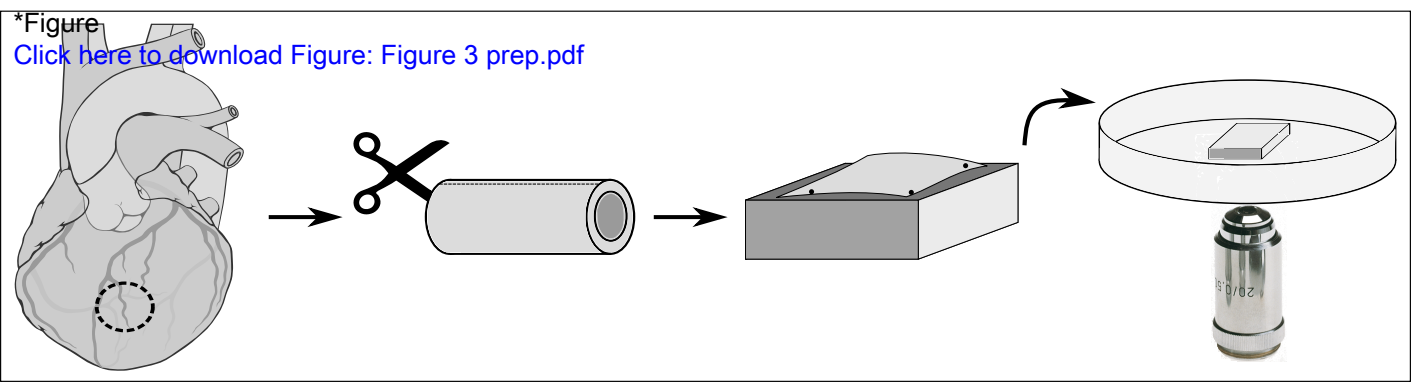

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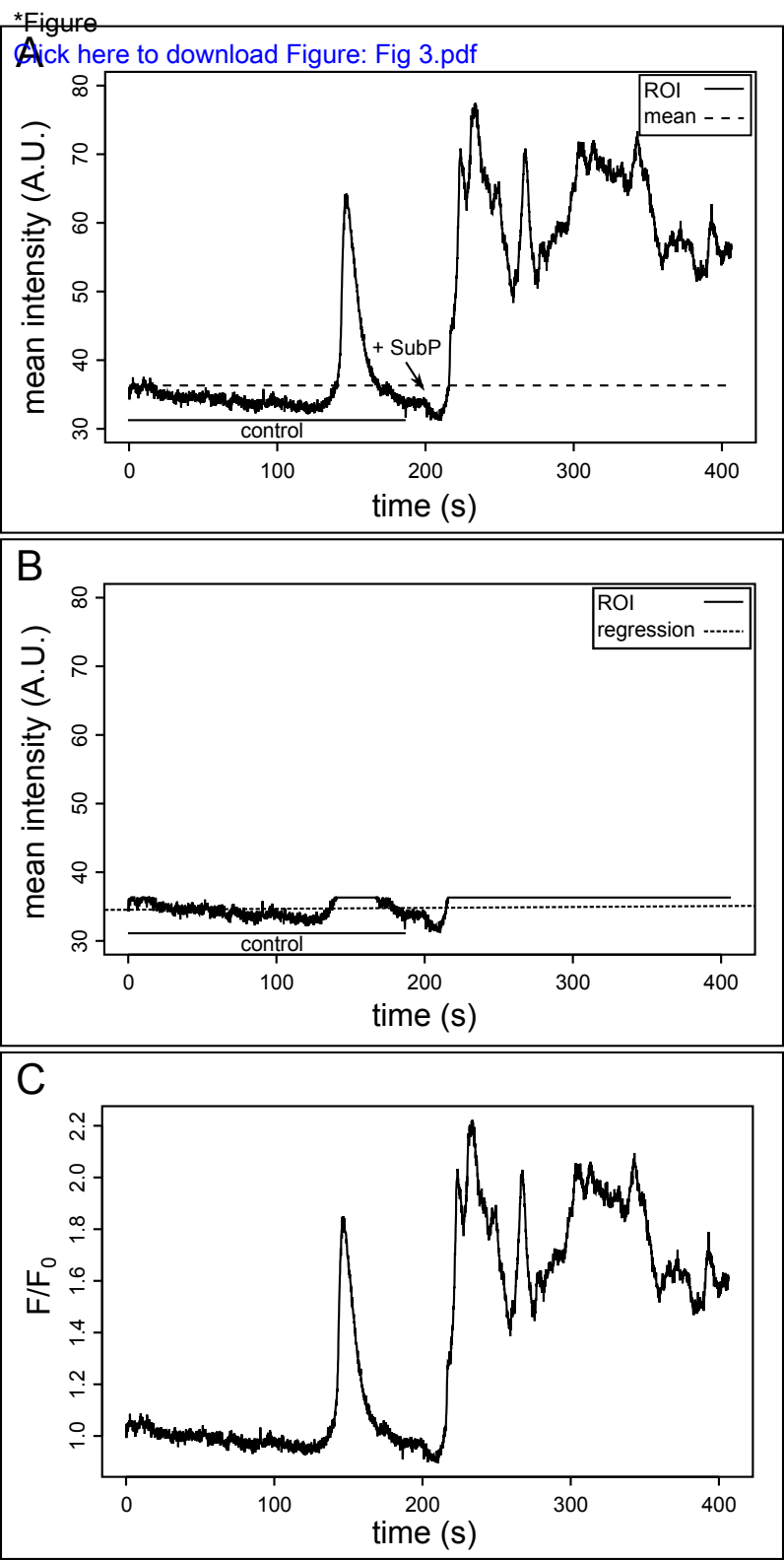

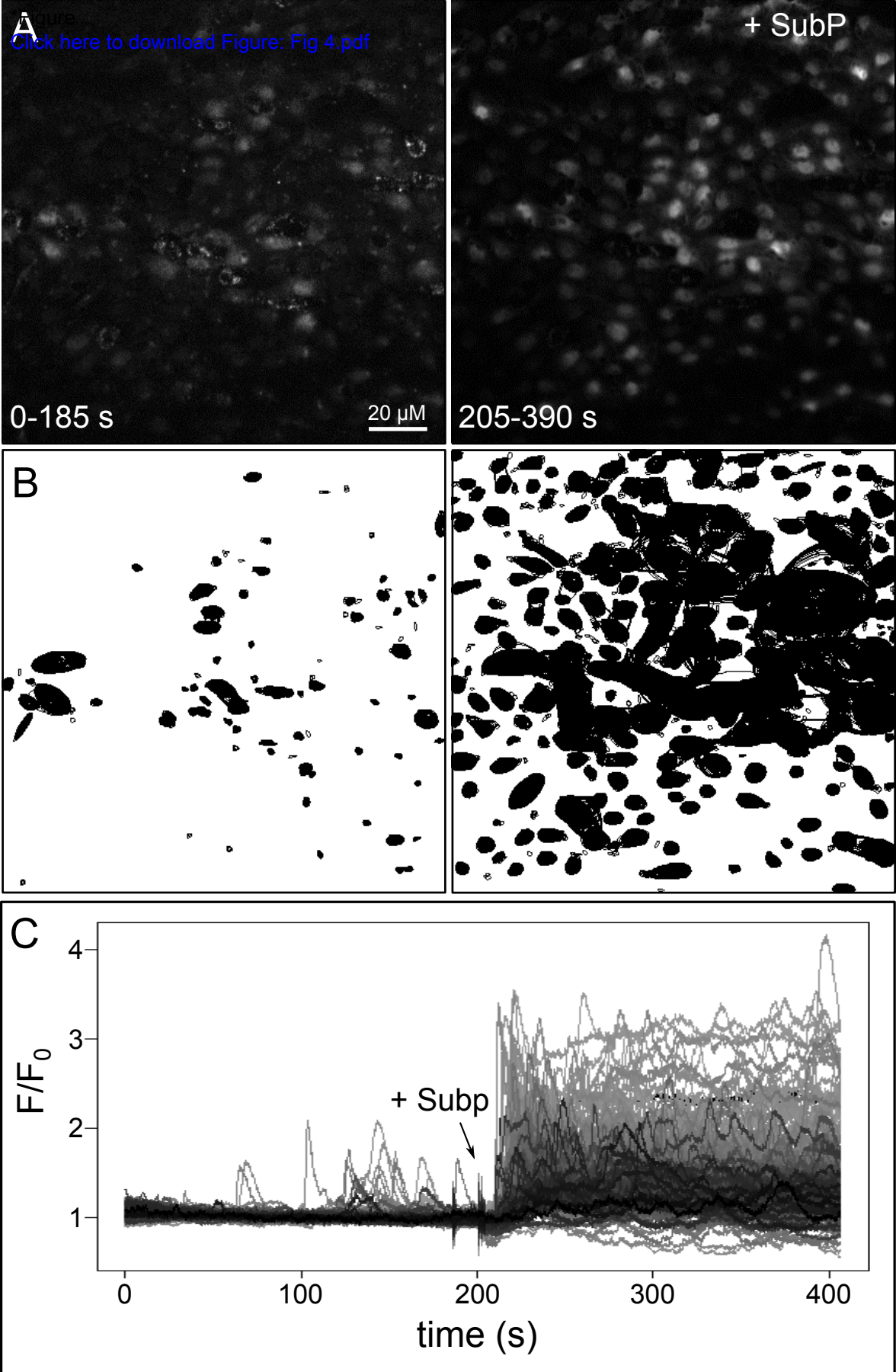

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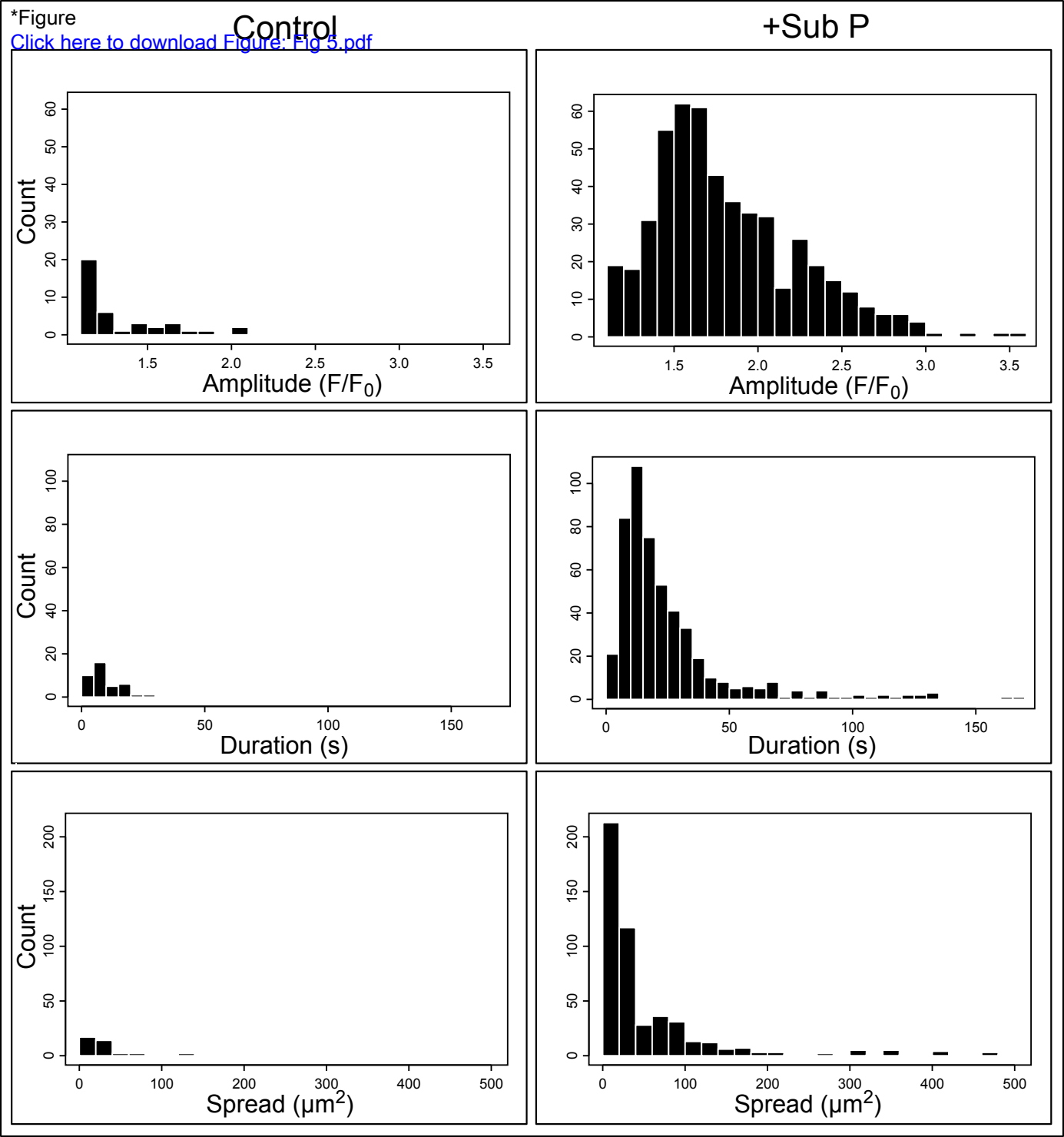

*Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: JoVE_Materials R2.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
dissection dish	Fisher Sci	#08-772-70	
polydimethylsiloxane (PDMS)	Fisher Sci	#NC9644388	elastomer kit, must be molded into dishes
HEPES-buffered PSS	Sigma	#H3375-250G	HEPES acid
stereomicroscope	Nikon Inst.	#MNA42000	
forceps	Fine Science Tools	#11223-20	
spring scissors	Fine Science Tools	15003-08	
tungsten wire	Scientific Inst Svcs	#406	
Fluo-4 AM	Life Tech.	#F-14201	
pluronic F-127	Life Tech.	#P3000MP	
metal pins	Fine Science Tools	#26002-10	
cover-glass bottom chamber			Custom designed
spinning disc confocal microscope	Perkin Elmer	RS-3	
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R software			download at: http://www.r-project.org/
R traceplot script			download at: https://docs.google.com/file/d/0B-PSp1D9e2fjV3NIcGp

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Author(s): Michael Francis

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The authors thank the editor and the reviewers for their helpful comments. Author responses to reviewer comments are enumerated below:

1. With regards to editorial comments: the latest manuscript word file was downloaded from the file inventory and checked for spelling and grammar. Each panel of each data figure was checked for text reference.

2. Reviewer 1 major concern: The reviewer requested a more complete description of the installation of R and imageJ, such that a new end user would be able to implement the analysis scripts for R and LC_pro for imageJ as referenced in the manuscript. The authors included a more complete description of the software use and installation by describing a naive installation of R (lines 147-155).

3. Reviewer 1 minor concern: The reviewer asked for clarification as to what is meant by the standard score. This text was modified to clarify that the standard score is the z-score for a standard normal distribution (line 188).

4. Reviewer 2 minor concern: The reviewer asks for details of the software, packages and scripts used during the graphical output analysis steps, with detail for users with minimal programming knowledge. This comment was addressed as part of the response to reviewer 1's major concern.

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