Automatic quantitative analysis of t-tubule organization in cardiac myocytes using ImageJ

Côme Pasqualin, François Gannier, Claire O. Malécot, Pierre Bredeloux, and Véronique Maupoil
Laboratoire CNRS ERL 7368, Signalisation et Transports Ioniques Membranaires, Equipe Physiologie des Cellules Cardiaques et Vasculaires, Tours, France

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Pasqualin C, Gannier F, Malécot CO, Bredeloux P, Maupoil V. Automatic quantitative analysis of t-tubule organization in cardiac myocytes using ImageJ. Am J Physiol Cell Physiol 308: C237–C245, 2015. First published November 12, 2014; doi:10.1152/ajpcell.00259.2014—The transverse tubule system in mammalian striated muscle is highly organized and contributes to optimal and homogeneous contraction. Diverse pathologies such as heart failure and atrial fibrillation include disorganization of t-tubules and contractile dysfunction. Few tools are available for the quantification of the organization of the t-tubule system. We developed a plugin for the ImageJ/Fiji image analysis platform developed by the National Institutes of Health. This plugin (TTorg) analyzes raw confocal microscopy images. Analysis options include the whole image, specific regions of the image (cropping), and z-axis analysis of the same image. Batch analysis of a series of images with identical criteria is also one of the options. There is no need to either reorientate any specimen to the horizontal or to do a thresholding of the image to perform analysis. TTorg includes a synthetic “myocyte-like” image generator to test the plugin’s efficiency in the user’s own experimental conditions. This plugin was validated on synthetic images for different simulated cell characteristics and acquisition parameters. TTorg was able to detect significant differences between the organization of the t-tubule systems in experimental data of mouse ventricular myocytes isolated from wild-type and dystrophin-deficient mice. TTorg is freely distributed, and its source code is available. It provides a reliable, easy-to-use, automatic, and unbiased measurement of t-tubule organization in a wide variety of experimental conditions.

transverse tubules; ImageJ; automatic analysis; cardiac myocytes

THE TRANSVERSE axial tubular system is a complex network of invaginations of the plasma membrane in cardiac myocytes (CM). It is involved in excitation-contraction coupling, mediating the rapid and homogeneous calcium signal throughout the CM. Calcium channels, highly localized in t-tubules (TT), trigger calcium-induced calcium release beginning predominantly in dyads localized close to TT. In healthy ventricular myocytes, TT are well organized transversally along z-lines, thus forming a striation pattern throughout the CM. Any disorganization of the transverse-axial tubular system impairs the intracellular calcium movements, decreasing the calcium transient amplitude and speeding up its time course, thus leading to asynchronous and inhomogeneous calcium increases within the CM. These disorders impair contraction and may be responsible for some arrhythmias (11). Irregularity of TT has been described in atrial CM of several mammals (1) and occurs in a number of pathologies, including human heart failure (8, 10), atrial fibrillation (5), and chronic ischemia (3). Therefore, the measure of the TT organization level represents an important issue for a better understanding of the physiopathology of these cardiac diseases.

To observe TT and their distribution in CM, fluorescent dyes such as di-8-ANEPPS or di-4-ANEPPS are used in living cells, and wheat germ agglutinin or antibodies against caveolin-3 in fixed tissues and cells. High spatial resolution images are recorded with confocal laser scanning or two-photon microscopy (4, 9).

To quantify TT organization, authors use fast Fourier transforms (FFT) of the cell image. The regularity of striations and TT within the cell results in the emergence of a peak in the FFT spectrum of the image. This incidence of the peak is used to determine the TT period, and its amplitude is considered as indication of a TT “organization index” (2, 10, 11). Algorithms have been developed to automate the analysis of TT organization (2, 5, 12). However, the absence of the source codes makes it difficult to understand exactly how the analysis is performed, and, to our knowledge, the analyses have not been validated on “synthetic” images with controlled features. Moreover, these analyses are performed with scripts in licensed languages such as Matlab or IDL, and require human intervention (such as cell rotation) and sometimes several other software.

Our objective was to develop an open-source plugin for ImageJ and Fiji [the freely available software developed by the National Institutes of Health (NIH)] for the automatic and unbiased analysis of most formats of raw confocal microscopy images of TT. This plugin should have an easy graphical user interface to configure analysis parameters and be able to work on Windows, Macintosh, and Linux operating systems. Finally, it should be validated with synthetic images corresponding to different experimental conditions and on real images of CM with different organization indexes. Here we describe TTorg, a plugin for automatic or semiautomatic two-dimensional analysis of the organization index of TT. This software allows the processing of any microscope image format (TIF, OIB, OIF, etc.) with the plugin Bio-Formats (6). TTorg is available in the plugin section of the ImageJ website.

Compared with existing plugins, the main strengths of TTorg lie in the possibility to analyze any image, irrespective of the orientation of the cell, making analysis quicker and easier, and the lack of human intervention during the process avoids any bias, particularly when thresholding or rotation of the image is required. Moreover, TTorg source code is available in the plugin folder for an exact understanding of how it works.

METHODS

TT Organization Analysis Algorithm

Algorithms to calculate the transverse organization level of a TT network are based on the calculation of the peak amplitude in the Fourier spectrum of the image at the TT frequency. The amplitude of this peak is called TT power and usually considered as the best
indicator of the transversal organization level in the transverse-axial tubular system (2, 10, 11). Figure 1 represents the flow diagram of the TTorg algorithm.

The “Preprocessing” phase consists of a $3 \times 3$-pixel median filtering followed by a histogram normalization of the image to correct any difference in the labeling levels between images. Then the multiplication by a Hanning window minimizes the noise induced in the FFT spectrum by image edges. Finally, the “analysis” phase starts by performing the 2D FFT. Background noise of the spectrum is cleared with a high-pass filter before searching peaks. These procedures have been chosen to match the largest number of experimental conditions. Peak detection used the built-in peak detection function of ImageJ. A peak is detected when its amplitude is greater or equal to a specific value. This value is defined by the Sensitivity and Specificity of Peak Detection (SSPD) setting. An empiric value of 28 is recommended (see RESULTS). Peaks in the FFT spectrum are searched at the frequency interval specified by the user. If Remove duplicate peaks is unchecked in the option menu, peaks are identified in the real and the imaginary part of the FFT. The maximum amplitude of each peak is measured and reported in a table with its corresponding striation period and accuracy and angle relative to the horizontal. Finally, the original image multiplied by the Hanning window and the FFT spectrum with detected peaks are displayed.

Setup and Use

Setting up. Unzip the file TTorg.zip into the plugin folder of ImageJ. To load the menu, click on ImageJ > Plugins > TTorg > Striation Analysis Menu: a <<TT>> icon will appear in the main menu of ImageJ (Fig. 2A).

Menu’s main functions. The menu (Fig. 2) is divided into three sections as follows.

MODES OF ANALYSIS. The different modes of analysis are as follows. 1) Analysis on the entire image: performs an analysis on the entire open image(s) (Fig. 2B); 2) Analysis on crop: performs an analysis on selected parts of the image, such as a cell or a part of a cell; 3) Batch mode: performs an identical analysis to that “on entire image” but on all images contained in the specified folder; and 4) Analysis on all slices: performs an analysis like Analysis on the entire image but on all z-slices of a single image.

SELF-VALIDATION OF THE PLUGIN. Tools for self-validation of the plugin are as follows. 1) Create Test Image: creates a synthetic image with striation pattern of known features (Fig. 2D); 2) Set Display Mode Color: allows selection of the channel to analyze. Use this mode on composite image; and 3) Analyze background noise level: analyzes the selected part of background in the active image and displays mean, SD, and minimum and maximum pixel values.

GENERAL PARAMETERS FOR THE PLUGIN. General parameters for the plugin are as follows. 1) Verbose (TT Options menu): when checked, displays performed analysis operations in real time in a verbose window; 2) Summarize (TT Options menu): when checked, displays main information (image file name, measured power, period and accuracy, angle) from the analysis in a new window (Fig. 2C). This table can either be saved by “File > Save as” as a text or Excel file, or copied to the clipboard and pasted into another program for further analysis. Summarize is automatically activated in batch mode and the window’s content is saved as an .xls file in the working directory; and 3) Select a working directory... choice of a working directory where all analysis output will be saved.

Fig. 1. Flow diagram of the TTorg algorithm. Analysis settings are selected via the graphical user interface. All other steps are automatically performed. A: an example of an original image multiplied by the Hanning window. B: 2D fast Fourier transform (FFT) spectrum of image A with its gray level profile; detected maxima are indicated by crosses on the 2D FFT spectrum and arrows on gray level profile.
Details of main functions. ANALYSIS ON THE ENTIRE IMAGE. The function Analysis on the entire image performs an analysis of all periodic image signals within the range defined by the user. The entire frame is analyzed without the need of selecting the cell. To set analysis parameters, either load a parameter file from the working directory or enter them manually in the dialog box (Fig. 2B). If Save parameters is checked, these settings and pixel size are saved as a text file in the working directory and can be loaded for other analyses. When analyzing multiple images, select Save list of images to keep the list in the directory.

SSPD value sets the sensitivity and specificity of peak detection in the FFT spectrum. A low SSPD value increases sensitivity and decreases specificity; a high SSPD value will have the opposite effect. Minimum and maximum spacing of TT defines edges within the FFT spectrum where peak detection works. We recommend using a 200-nm margin around the supposed period value. Analysis is started by clicking the “OK” button.

The output consists of two images: the original image multiplied by the Hanning window (see algorithm description), and the FFT spectrum with detected maxima. The resulting log window: “image-name_log.txt” is an array containing peak amplitudes, their corresponding periods with accuracy of the measurement, and the angle of the sinusoidal signal relative to the horizontal. This file is automatically saved in the working directory.

ANALYSIS ON CROP. The use of this mode is required in some cases, e.g., when there are several CM in the image, if the CM is bent or when CM widths are significantly different between compared groups. It can also be used in particular cases. For example, if the organization level seems highly heterogeneous within the CM it is possible to repeat the analysis on different parts of the cell, and likewise, if the cell needs to be analyzed without its membrane. One disadvantage of this mode lies in the necessary rotation of the image that cancels the benefit of having no pixel interpolation.

Except for the image rotation, analyses on crop are performed like Analysis on entire images, but three additional settings appear in the dialog box: the crop width, height, and angular tolerance. The dialog box will automatically propose the best crop size for an optimal FFT analysis.

However, users are free to choose their own crop size. Angular tolerance corresponds to the maximum angular error with respect to the perpendicular of TT once the crop selection is done (see below).

The crop section needs a selection using either “Line tool” (default tool), or “Segmented Line tool” along its longitudinal axis, i.e., perpendicular to TT with a maximal angular error corresponding to Angular tolerance. If the CM is bent, it will automatically be straightened by TTorg (native function of ImageJ) along its longitudinal axis before analysis. ANALYSIS OF ALL Z-SLICES. This mode is useful if the image contains several z-slices. Analysis is the same as Analysis on entire image, but it is done on all z-slices of the chosen channel of the opened image(s). It allows the user to test if one z-slice can be considered as representative of the whole cell. This mode is not available for “crop.”

BATCH MODE. This mode is optimized to work on many images in automatic mode. The images are opened and analyzed one at a time, thus limiting the use of the computer’s memory. Once the analysis is launched, no intervention from the operator is required.

To use it, select a folder containing all image files to be analyzed. All images are automatically opened, analyzed with the same parameters, and closed, and the output is saved in a file in the image folder. The mode proposes the same functions as Analysis on entire image, adding the option Select analyzed slice that allows selection of the slice to analyze on each image. This mode is not available for “crop.”

Results are automatically displayed into a summary window (SMZ, Fig. 2C), and its content is saved in the working directory.

ALGORITHM FOR IMAGE SYNTHESIS OF CELLS SHAPES WITH TT LIKE STRIATIONS. To test the efficiency and the limits of the analyzer, a module creating synthetic images has been implemented within this plugin. It allows the creation of “realistic” images of CM with transverse-axial tubular system either well organized or not. This mode can help with the validation of one’s own experimental conditions and illustrates the influence of different conditions on the measured parameters.

The 16-bit images of a simulated cell with TT pattern are obtained using a mathematical model based on a sinus wave (see APPENDIX).

Size, striation interval, thickness of TT, spacing between TT, angle of
parameters: SSPD = 28; Min spacing = 1.6 μm; Max spacing = 2 μm.

DETERMINATION OF THE BEST VALUE FOR SSPD PARAMETER. First, the optimum value for SSPD parameter was determined. It allows performance of the analysis with the best sensitivity and specificity for peak detection in the FFT spectrum. At the peak search in the FFT spectrum, SSPD should allow the finding of TT power peaks of CM with the lowest TT organization, without detecting “false positive” peaks in other CM with higher organization levels.

Two groups of 10 images each, the first containing CM presenting a low organization value of 30, the second with a high organization value of 80, were created (group 30 and group 80). For analysis of each group, different SSPD were tested. For each of them, specificity and sensitivity were measured. Sensitivity was defined by the number of detected peaks divided by the total number of peaks and specificity by the inverse of the number of false-positive peaks (FPF) found plus one, i.e., 1/(FPF + 1).

STRENGTH TEST OF THE ANALYSIS WITHIN SEVERAL SIMULATED EXPERIMENTAL CONDITIONS. Potential bias in the measurement of FFT spectrum peak amplitude of TT power and period due to modifications in the experimental conditions was then checked. For each tested parameter, several groups of cell images were created. All groups included CM with defined TT organization, and each tested group contained 10 images and presented common values (see Table 1) except for the tested parameter. The images were then embedded in the same Gaussian noise corresponding to a signal-to-noise ratio of 3. When modified, parameters were changed within a realistic range of values compared with our experimental confocal images results. These groups were compared with each other using an ANOVA test.

VALIDATION OF THE ABILITY OF TTORG TO DETECT DIFFERENCES BETWEEN TT ORGANIZATION INDEXES. To show TTorg’s ability to underline possible TT power differences, we analyzed four groups of 10 images presenting different organization levels. These images were generated with decreasing organization value from 80 to 20, all other parameters presenting common values (see Table 1).

Measurement of TT organization in WT and mdx cardiac myocytes to validate analysis on real confocal microscopy images. CM of mdx mice present membrane alterations due to dystrophin deficiency. Their TT organization is less regular than wild-type mice CM (C57 BL10 Scn Mice). The original study of TT organization was performed with an IDL macro and highlights a difference within TT power between wild-type and mdx CM (7). To validate our algorithm on real images, 10 ventricular CM images of mdx and WT mice were analyzed. These images have been recorded with an Olympus FV1000 confocal microscope using a ×60 1.4-NA oil-immersion objective. TT were labeled with di-8 ANEPPS.

Statistics

Results are shown as means ± SE. All statistics have been performed with R and SigmaStat. Differences are considered significant when the P value is inferior to 0.05. For the comparison between

Table 1. Tested image parameters for the simulation of different experimental conditions to control the efficiency of the TTorg measurements of TT power and period

<table>
<thead>
<tr>
<th>Image Parameters</th>
<th>Common Values</th>
<th>Modified Values</th>
<th>P Value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (length × width), μm</td>
<td>100 × 20</td>
<td>110 × 20, 90 × 20, 100 × 25, 100 × 15</td>
<td>TT power</td>
</tr>
<tr>
<td>TT period, μm</td>
<td>1.8</td>
<td>1.750, 1.850</td>
<td>0.023*</td>
</tr>
<tr>
<td>Angle of the cell longitudinal axis, °</td>
<td>324</td>
<td>10, 55, 100, 145, 190</td>
<td>0.228</td>
</tr>
<tr>
<td>Position of the cell in the image</td>
<td>Center</td>
<td>High, low</td>
<td>0.957</td>
</tr>
<tr>
<td>Added Gaussian noise SD (gray levels)</td>
<td>0</td>
<td>10, 20, 50, 100</td>
<td>0.73</td>
</tr>
</tbody>
</table>

TT, transverse tubules. “Common Values” are the values of parameters that are common to all images groups. Each group corresponds to different experimental conditions, except for the tested parameter. The values of the latter for each group are listed as “Modified Values.” Two last columns show the P values of ANOVA tests on each image group of modified parameter. *P < 0.05, †P < 0.01; n = 10 for each group; organization value = 80.
groups, either Student’s t- or ANOVA tests were performed. Student-Newman-Keuls has been used as post hoc of ANOVA test. Pearson correlation tests have been used to check the potential existence of a linear correlation between two variables.

RESULTS

Context That Led to Development of a Novel Algorithm for the Measurement of TT Organization

TTorg was developed to overcome common biases encountered with previously described TT power measurement algorithms (2). Global thresholding distorts the power measurement over- or underestimating the TT area and the skeletonization of the thresholded image after background subtraction and local adaptive noise removal has been proposed to overcome this bias (2).

To examine the thresholding effect, a real TT network image has been set at two different brightness levels (Fig. 4A). Then, an automatic threshold has been applied to each image with the MaxEntropy thresholding function of ImageJ. These binary images were then skeletonized with the Skeletonize ImageJ function, and their two-dimensional FFT spectra have been calculated and compared. Even though the variability of the TT power measurement is reduced, we showed that the TT power values remain dependent on the brightness of the image (Fig. 4A). Indeed, thresholding deletes some TT labeling in low-fluorescent areas and raises noise in high ones. It follows differences in image skeletons and resulting FFT spectrum and measured TT power. Moreover, the cell longitudinal axis needs to be parallel to the x-axis of the image. If not, the image should be rotated and if the rotation needed does not correspond strictly to a multiple of 90°, pixel interpolation is also required. To examine the effect of image rotation, two skeletons of TT networks were obtained from the same original image of a CM horizontally oriented and then 45° clockwise-rotated with a bilinear interpolation mode. Then the two-dimensional FFT spectrum of each skeleton were calculated and compared. As shown in Fig. 4B, this interpolation modifies the FFT spectrum, and therefore biases the TT power measurement. TTorg was developed to be, to our knowledge, the first software to overcome such biases by providing an analysis without thresholding, whatever the orientation of the cell.

Validation on Synthetic Images Representing CM and TT System

SSPD value determination. Comparison of the TT low-organization group (group 30) with the TT high-organization

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**Fig. 4.** A: images A and B were obtained from the same myocyte with 2 different brightness levels. An automatic threshold was applied to both images before the skeletonization. Skeletons of images A and B are shown at right with their corresponding FFT spectrum profiles. B: skeletons C and D were derived from the same original image. Skeleton C was obtained from the raw image, and skeleton D was obtained from the 45° clockwise-rotated image. Their FFT spectrum profiles are shown at right. Arrows in FFT spectrums indicate the peaks used to determine the TT organization level or TT power.
group (group 80) showed, in each group, the existence of a SSPD range corresponding to a sensitivity and specificity of 100% (Fig. 5). These ranges correspond to values of SSPD from 26 to 40 for group 30 and 28 to 52 for group 80. The intersection of these ranges gave an optimal interval of SSPD between 28 and 40.

Thus, for the following analyses, a SSPD value of 28 corresponding to the smallest value of this interval has been used.

Specific effect of the background noise SD on the TT power and accuracy of striation interval measurement. The shape of TT in synthetic images is dependent on a random process, leading to a small variation in the measured TT power despite an identical organization value. This variation enables the reproduction of realistic experimental results, thus allowing the comparison of different groups of images. On 10 analyzed images, all presenting the same common values (see Table 1), the mean TT power measured was 79 ± 1.5 AU (see APPENDIX), and the period was 1.798 ± ~0. µm. These measured power and period values were then compared with those measured in the other groups of images, presenting one modified parameter.

Validation on synthetic images of CM recorded within different simulated experimental conditions. CM images realized with a confocal microscope are not all perfectly graded with the same size cells placed exactly at the center of the image, and absolutely horizontal. Moreover, the background noise may vary from one record to another. To overcome the consequences of these variations in the experimental conditions, we tested the influence of the parameters listed in Table 1 on TT power and period measures as reported in Fig. 6.

For TT power (Fig. 6, Aa–Ea), no significant differences were observed when these parameters were changed, except for CM width (Fig. 6Da). TT power is significantly greater when CM width is set at 25 µm instead of 15 µm. Hence, we

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**Fig. 5. Sensitivity and specificity of TTorg analysis for different values of the Sensitivity and Specificity of Peak Detection (SSPD) parameter. A: analysis of TT with high organization value (group 80). B: analysis of TT with low organization value (group 30); n = 10 in each group. Vertical bars delimit best SSPD range for each group.**

**Fig. 6. Aa–Ea: results of TT power (gray bars) analysis in batch mode for different values of a modified image parameter (x-axis). Ab–Eb: results of TT period (open bars) analysis in the same conditions as TT power analysis. F: measured TT power for increasing values of cell width. G: TT power analysis on crop for cell widths of 15 and 25 µm. *P < 0.05. Characteristics of synthetic CM are common values (see Table 1) except for the studied parameter.**
tested the correlation between CM width and TT power. Widths from 10 to 30 μm were tested, and no correlation was highlighted ($P = 0.275$) (Fig. 6F). Then, analyses on crop were performed on 25- and 15-μm-width CM groups with a crop width of 15 μm, corresponding to the narrowest CM, and a crop length of 70.6 μm, corresponding to the recommended value for an optimal analysis, smaller than CM lengths. This time, no significant differences were found ($P = 0.627$) (Fig. 6G). Therefore, if CM width between two compared groups is significantly different, analysis on crop mode is recommended.

Some significant differences were found between groups for a given TT period value (Fig. 6, Cb and Eb, and Table 1). However, they represent <10% of the pixel size (FFT analysis allows such a resolution), thus being considered as negligible.

**Ability to detect differences between various organization levels.** Having shown that TTorg does not detect significant TT power differences when there are no actual differences, we created synthetic images of CM differing only by the level of TT organization (20, 40, 60, 80) to analyze TTorg’s response (Fig. 7A).

As expected, for the four different organization levels tested, TTorg detected significant differences between all groups ($P < 0.001$; Fig. 7B). Moreover, no significant differences have been found within measured TT periods ($P = 0.242$; Fig. 7C).

We showed that TTorg is able to highlight TT power differences when they actually exist, without impairing the measure of the period.

**Validation on Real Images of mdx and WT Mice Cardiac Myocytes**

To test its practicability on original experimental data, we worked on images of isolated mice CM, with labeled TT. The analysis was realized on two CM populations, the first presenting a well-organized TT network (WT mice), and the other a low-organization level of TT network due to dystrophin deficiency (mdx mice).

The analysis of TT power of mdx and WT mice CM with TTorg (Fig. 8) showed that WT mice have a better TT organization index (74.1 ± 1.6 AU) than mdx mice (64.6 ± 2.6 AU) ($P < 0.01$) in agreement with the results of Lorin et al. (7).

**DISCUSSION**

Interest in the study of TT is growing because of their implication in pathologies such as atrial fibrillation, heart failure, and arrhythmias. To efficiently and objectively analyze the regularity of TT networks, i.e., to give an indication of their organization, a parameter preferably measured without any subjective intervention of the operator on the image is needed. Diverse data-processing tools have been developed to analyze TT networks, but only one Matlab program has been published so far (2).

The TTorg plugin we developed presents several advantages. It is written for ImageJ/Fiji, the free and open-source NIH software for analysis of microscope images. Thanks to its interface, and also to the plugin Bio-formats, TTorg can analyze most microscope image formats without one having to manually enter image-acquisition settings. TTorg is user-friendly, enabling a steady and completely automated analysis of images without any human intervention after selection of the analysis parameters, thus overcoming any subjective bias.

During our validation test, we observed that image rotation frequently induces the apparition of spurious frequencies in the signal due to the pixel interpolation, which twists the TT power measure. Therefore we designed TTorg to analyze a cell whatever its orientation in the image, avoiding rotation and pixel interpolation bias. Moreover, TTorg runs without performing a thresholding of the image for the analysis. This allows the exploitation of all of the information contained within the image without deleting any data which could be particularly useful in cases of heterogeneous labeling or photobleaching.

The module for the creation of synthetic images of CM with (dis)organized TT networks that we integrated to the plugin allows the testing of its validity in users’ own experimental conditions. With this tool, we showed that measures of TT power and TT period were not affected by either the cell position within the image, its angle, the presence of noise, or the interval between TT. However, we observed that the cell width could impact the TT power measure. Even though there is no evidence of a link between cell width and TT power, it is recommended to use Analysis on crop when the widths of CM
of compared groups are significantly different: the crop width then needs to be that of the width of the narrowest cell. This analysis, despite limiting to the crop of the image, enables one to overcome any problem with CM size variations. Regarding the measure of a TT period, it was extremely precise and accurate for all tested experimental conditions, with a resolution of 10 nm for a pixel size of 138 nm. The accuracy of the measure is indicated for each analysis, allowing the user to frame the real value.

The practicability of TTorg was tested with experimental data obtained with a preparation of dystrophin-deficient and WT mice ventricular myocytes, the first being known to have a disorganized TT network compared with the second (7). As expected, significant differences were highlighted between these two groups of mice CM, demonstrating the efficiency of TTorg on real experimental data. However, to minimize the sources of variation and reinforce the analysis efficiency, it is recommended to work with the same acquisition parameters, material, and tonal resolution.

Finally, TTorg also proposes other useful options, such as automatic analysis of all z-slices of an image, analysis on crop to enable the analysis of a single cell within a tissue slice, or analysis of bent cells, frequent in the rodent atrial CM.

Conclusions

TTorg showed its efficiency and steadiness in the measurement of TT organization and period, with an accurate and adaptable validation method. It provides an easy graphical user interface to configure analysis parameters and output results. This open source plugin runs with ImageJ and Fiji and can be used with any Windows, Macintosh, or Linux operating system. It is freely available, and the source code is apparent. It is easy to use, and versatile, and thus could be used to standardize the measurement of TT organization index and period. Moreover, this plugin is adapted for other measurements such as the space between sarcomeres, for example, to characterize the cell’s contracture level, or any other ultrastructure distribution with a striation pattern such as ryanodine receptors or calcium channels.

APPENDIX

Arbitrary Unit

“Power” values are log-scaled depending on the minimum and maximum of the energetic spectral density (EDS). EDS values could be obtained directly from raw spectrum in ImageJ. Units are given in [intensity^2/μm^2] where Intensity is the brightness of each pixel on a gray scale (for 16-bit images, each pixel is coded on 65,536 gray levels). This removes any significant variation in brightness or background illumination from one image to another and allows the comparison of images with brightness coded on 8, 16, or 32 bit. Power is given by the following equation: “power” = 1 + [(log(R) - log(Mn) × Raw_Scale), where R = values from EDS; logMn = log(Min of raw spectrum); logMx = log(Max of raw spectrum); Raw_Scale = 253.999/(logMx - logMn), and (logMx - logMn) should not be lower than 50, or otherwise the difference is set to 50.

Creation of Synthetic Cell With Transverse Tubule System

The algorithm to create synthetic cells is defined to obtain a realistic image of the TT system from a cardiomyocyte. It is based on the regularity of the TT system along the z-line (forming dyads), which we can reproduce by a sinusoidal function of the length Y(l) = B + A·sin[2π·f·l], where l = length, B = basal level added to the sinus function, A = amplitude = peak deviation from zero, and f = ordinary frequency, i.e., number of oscillations per unit of length.

Basal level and amplitude values modulate the sinus wave to obtain lines with the desired thickness. Basal level can be a negative value. To reproduce different levels of organization of the TT, we apply to the sinus wave signal an algorithm of randomization based on the “organization level” value (in percent). For a value of 100%, the sinus wave signal is not changed. Depending on the “organization level” value, a variable percentage of pixels will have their value reversed or substituted by a randomly defined Gaussian noise value. The presence of this sinus wave signal is restricted to the dimension and the position selected for the cell. Y(l) is then encoded on a scale of 65,536 values to create a 16-bit image. After that, the resulting image is rotated if needed, and a random Gaussian noise background is applied.

Then a median filter followed by a mean filter is applied. Finally, the value of the SD of the background noise is subtracted from all pixels.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.P., F.G., P.B., and V.M. conception and design of research; C.P. performed experiments; C.P. analyzed data; C.P. and F.G. interpreted results of experiments; C.P. prepared figures; C.P. drafted manu-

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