Video Article

Clock Scan Protocol for Image Analysis: ImageJ Plugins

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Abstract

The clock scan protocol for image analysis is an efficient tool to quantify the average pixel intensity within, at the border, and outside (background) a closed or segmented convex-shaped region of interest, leading to the generation of an averaged integral radial pixel-intensity profile. This protocol was originally developed in 2006, as a visual basic 6 script, but as such, it had limited distribution. To address this problem and to join similar recent efforts by others, we converted the original clock scan protocol code into two Java-based plugins compatible with NIH-sponsored and freely available image analysis programs like ImageJ or Fiji ImageJ. Furthermore, these plugins have several new functions, further expanding the range of capabilities of the original protocol, such as analysis of multiple regions of interest and image stacks. The latter feature of the program is especially useful in applications in which it is important to determine changes related to time and location. Thus, the clock scan analysis of stacks of biological images may potentially be applied to spreading of Na^{+} or Ca^{++} within a single cell, as well as to the analysis of spreading activity (*e.g.*, Ca^{++} waves) in populations of synaptically-connected or gap junction-coupled cells. Here, we describe these new clock scan plugins and show some examples of their applications in image analysis.

Video Link

The video component of this article can be found at https://www.jove.com/video/55819/

Introduction

The goal of this work is to present a Clock Scan protocol that is platform-free and freely available to any researcher interested in this type of image analysis. The Clock Scan protocol was originally developed in 2006[1], with the goal of improving existing methods of pixel intensity quantification within convex-shaped regions of interest (ROI), a method which has better integrative capacity and improved spatial resolution. During the acquisition, the protocol sequentially collects multiple radial pixel-intensity profiles, scanned from the ROI center to its border, or to a predetermined distance outside the ROI for the purpose of measuring the "background" pixel intensity. The protocol scales these profiles according to the cell radius, measured in the direction of the scan. Thus, the distance from the center to the ROI border of each individual radial scan is always 100% of the X scale. Finally, the program averages these individual profiles into one integral radial pixel-intensity profile. Because of scaling, the mean pixel-intensity profile, produced by the "Clock Scan" protocol, depends neither on the ROI size nor, within reasonable limits, on the ROI shape. This method allows direct comparison or, if required, averaging or subtraction of profiles of different ROIs. The protocol also allows correction of the integral pixel intensity profiles, of any object for background noise, by a simple subtraction of the average intensity of pixels located outside the object. Although it has only been tested in biological samples, our protocol provides a valuable addition to other existing image analysis tools used in studies of images of physical or chemical processes that are arranged around a point of origin (such as diffusion of substances from a point source)[1].

However, the major limitation of the original image analysis method was that the protocol was developed as a Visual Basic 6 (VB6) (code, and therefore, it was platform-dependent and difficult to distribute (requiring VB6). To address this problem and to join similar recent efforts by other investigators[2], we converted the VB6 Clock Scan program code into two Java-based plugins, compatible with the NIH-sponsored and freely-available open-source and platform-independent image analysis programs, ImageJ[3] and Fiji ImageJ[4]. Furthermore, these plugins have now several new functions that expand the capability of the original protocol to process multiple ROIs and image stacks. Many image analysis applications are not user-friendly, with regards to performing statistical analysis of multiple objects, and thus, often only representative data are shown. With the multi Clock Scan ImageJ plugin, it is possible to facilitate the analysis of multiple objects simultaneously. A robust statistical evaluation of microscopy data, with regards to signal intensity distribution in single cells/objects, is now possible with this plugin extension. Here, we describe the Clock Scan plugins and show examples of their applications in image analysis.

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Protocol

1. Software Installation

1. Install the latest versions of bundled Java and either ImageJ or Fiji ImageJ as recommended on the respective websites (see materials table for links to the corresponding websites). In the text below, both programs are referred to as "ImageJ".
2. Copy "Clock_Scan-1.0.1. jar" and "Multi_Clock_Scan-1.0.1.jar" plugin files using the link provided in materials table and paste them into the ImageJ plugin directory. Alternatively, use "Plugins | Install plugin" menu option to install these files after they have been saved on the computer hard drive.

2. Clock Scan analysis

1. **Standard Clock Scan plugin (Figure 1):**
    1. Use the ImageJ "File | Open" menu command to open an image of interest.
    2. Click on the 'polygon' tool, or 'segmented line selection' tool, and then draw on the image to outline the entire ROI or a segment of this region. See **Figure 1A** for an example of polygon selection (inner dashed outline).
    NOTE: Other selection tools, available in the software (rectangular, oval and freehand line selection), may also be used.
    3. Select "Plugins | Clock Scan" from the menu to open the standard clock scan protocol pop-up option window. Note that this command will also open the ROI Manager window with the outline automatically added to it.
    4. Use the plugin option window to do the following.
        1. Review and change the X and Y coordinates of the ROI center (automatically-calculated as coordinates of the physical mass center) by using scroll bars or changing the values in the corresponding input boxes. See **Figure 1B**.
        2. Depending on how much of the background region outside of the object should be covered by scanning, adjust the scan limits by using the "scan limit" scroll bar. See **Figure 1A**.
        NOTE: Scan limit is the fractional number representing how far the scan should proceed beyond the objects' border in any given direction; the default value is 1.20, indicating that the scan length will be 20% longer than the object radius in the direction of scan; see **Figure 1A**, outer dashed line).
        3. Modify the output of the plugin using "real radius", "subtract background", "polar transform" and/or the "plot with standard deviation" check-boxes.
        4. Click "OK" to run the plugin. See **Figure 1C-H**.
        NOTE: Examples of the output of the protocol with "plot with standard deviation" and "polar transform" or "real radius" and "polar transform" options selected are shown in **Figure 1C** and **1D** and **Figure 1E** and **1F**, respectively. Note that the calculated standard deviation (SD) values represent the variation between individual radial pixel intensity scans of the object. Also note the "ROI selection length" line in the plugin window, which displays the information on the ROI outline length measured in pixels.
    5. In the generated "Clock Scan Profile Plot", use the "List" command to plot values displayed in two, X and Y columns of data for grey scale images and in X and four Y columns of data for RGB images, of which Y0, Y1, Y2 and Y3 columns will be filled with integral and individual (red, green and blue) color channel pixel intensity values.
2. **Multiple ROI Clock Scan plugin - working with multiple ROI (Figure 2):**
    1. Open an image containing multiple ROI.
    2. Open the ROI Manager by clicking "Analyze | Tools | ROI Manager".
    3. Sequentially outline (see step 2.1.2) and add each ROI to the ROI Manager by clicking "Add" in the ROI Manager window; do this for all ROIs within the image. Use the "Analyze| Measure" command if ROI metrics are of interest.
        1. See **Figure 2A** for an example of multiple segmented line selections and **Figure 2E** for an example of multiple polygon selections.
    4. Select "Multi Clock Scan" in the "Plugins" menu to open the protocol options pop-up window.
    5. Use the protocol option window to do the following.
        1. If needed, reset the scan limit as per step 2.1.4.2; default value is 1.20.
        2. If needed, select the option to plot the mean clock scan profile with SD bars by checking the "Plot with standard deviation" box. See **Figure 2C** and **D**.
        NOTE: The calculated SD values will represent variation between integral clock scan profiles of different objects. Also, note the line in the plugin window displaying information on the "number of selected ROIs".
        3. Click "OK" to run the protocol.
    6. In the generated "Clock Scan Profile Plot", use the "List" command to plot the values displayed in the "Plot Values" window. See the "Multi Clock Scan Profile Plot" window legend for column designation by color channel.
    7. Note that the ROIs are numbered and their clock scan profiles for any given color channel are plotted in the same sequence in which the ROIs were outlined and added to the "ROI Manager".
3. **Multiple ROI Clock Scan plugin - working with an image stack (Figure 3):**
    1. Open an image-stack of interest.
    2. Open the ROI Manager by clicking "Analyze | Tools | ROI Manager".
    3. Outline the ROI of the images within the stack and add it to ROI manager as described in steps 2.1.2 and 2.2.3. Use the "Analyze | Measure" command if the ROI metrics are of interest.
    4. Select "Multi Clock Scan" in the "Plugins" menu to open the protocol options pop-up window.

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5. Use the protocol option window to do the following.
    1. Reset the scan limit as described in step 2.1.4.2; default value is 1.20.
    2. Select the option to plot the mean clock scan profile with SD bars by checking the 'Plot with standard deviation" box.
    NOTE: The calculated SD values will represent variation between different instances of the object selected in the image stack. Also, note the line in the plugin window displaying information on the "number of images in the stack".
    3. Click "OK" to run the protocol.

6. In the "Clock Scan Profile Plot" window, click "List" to plot the values displayed in the "Plot Values" window, where the Y column number represents the image position within the stack - 1.

Representative Results

The images that are used here for illustration purpose, are taken from databases created during our previous cell and tissue biological studies[5,6,7] and from the Allen Mouse Brain Atlas[8]. Both plugins were successfully tested using ImageJ 1.50i/Java 1.8.0_77, ImageJ 2.0.0-rc-44/1.50e/ Java 1.8.9_66 and Fiji ImageJ 2.0.0-rc54/1.51g/Java 1.8.0_66 program environment.

Figure 1 shows representative results of image analysis with a standard Clock Scan plugin. For both plugins, the basic code and major steps of the clock scan procedure are essentially the same as described in the original protocol[1]. Briefly, after the ROI or a segment of the ROI is outlined on the image (**Figure 1A**, inner yellow outline) and a center of outline is determined (automatically or manually, using the plugin option window; **Figure 1B**), the radial scanning of pixel intensity starts in a direction from the center to the first pixel of the cell outline and continues clockwise pixel-by-pixel along the outline (**Figure 1A**; straight vector and curved arrow, respectively) until all the ROI radii are scanned. To quantify the ROI background intensity, the length of each radial scan may be set to exceed the radius of the ROI in the direction of the scan by a preset fractional number (0.2 or 20% of the radius in default for the Clock Scan plugin's value, outer yellow line in **Figure 1A**). Collected radial profiles are then aligned by scaling to the corresponding radii and averaged to produce the integral clock scan intensity profile in 256 intensity levels of grey scale units (**Figure 1C**). For RGB images, both plugins automatically produce independent integral radial pixel intensity profiles for each color channel (256 intensity levels of red, green and blue colors) in addition to a combined color profile.

By default, the x-scale of the clock scan pixel intensity profile represents normalized ROI radius, with 100% of the scale representing pixels located at the border of ROI (**Figure 1C**). The profile shown in **Figure 1C** was generated with the "plot with standard deviation" option selected, and therefore, the graph also displays the SD calculated for each data point along the X-scale of the profile. When the "subtract background" option is selected, the entire intensity profile is corrected for background noise by point-by-point subtraction of the mean intensity of pixels located between the ROI border and the scan limit border (outer yellow line in the **Figure 1A**; data not shown). If the "polar transform" option is selected, the clock scan plugin generates an additional output window. It contains a polar transform of the image of the selected region including the scan limit territory, in which the image is modified in each radial scan direction in such a way that the distance from the center to the border of the objects is always normalized to 100% and represented by 100 pixels. Regardless of the actual size of the object, the vertical and horizontal dimensions of its polar transform image are two times the scan limit in pixels (240 pixels x 240 pixels in the example shown in **Figure 1D**). Finally, selecting the "real radius" option will result in the generation of the clock scan profile and a polar transform image, scaled per the actual mean radius of the object and in the units of spatial calibration of the original image (**Figures 1E** and **F**, respectively).

Figures 1G and **H** illustrate additional image analysis options using the object size- and shape-independent polar transform and the integrated ImageJ commands and tools. Examples of commands, which might be considered useful for certain types of image analysis, are the segmented line tool and "Analyze| Plot Profile" command (**Figure 1G**) and the "Analyze | Surface Plot" command (**Figure 1H**).

Figures 2 and 3 show representative results of image analysis with the Multi Clock Scan plugin. The output of the multi clock scan plugin consists of two graphs: the first graph shows individual clock scan profiles of the selected objects (**Figure 2C**), and the second graph displays the mean of these individual clock scan profiles (± SD, optional; **Figure 2D**). For RGB images (**Figure 2E**), the clock scan profile calculated for each individual color channel is also displayed for each selected ROI (**Figure 2F**), and the mean is calculated within a given channel for all selected objects (**Figure 2G**). Similarly, the individual and mean clock scan profiles for objects in the image stack are displayed after performing the clock scan analysis of the stack (**Figures 3A-3D**, the mean clock scan profile is not shown). As stated before, the numerical data are used to generate these plots by executing the plot "List" command.

Figure 4 illustrates one additional application of the polar transform option in the Clock Scan plugin: its suitability for image registration and overlay operations. In this figure, ROI size- and shape-independent polar transformations were used to compare the distribution of fluorescence labeling of neurons expressing α3 sodium/potassium-ATPase pump between different mouse cortical regions, with the atlas image showing the borders and the anatomical organization of these regions (**Figures 4A-4B**). With a clock scan protocol, the registration of reference (atlas) and the actual images required for such comparison are limited to a simple procedure of aligning the images, outlining the structure of interest in both images, and then generating ROI size- and shape-independent polar transforms. In the example shown in **Figure 4**, a comparison of polar transformations clearly demonstrates a non-uniform distribution of labeled cells in the mouse brain cortex, with their density being specifically high in superficial areas of layer 2/3rds of the motor cortex, dorsal part of the agranular insular cortex, the lateral orbital cortex, and in deep layers of the motor cortex (**Figures 4C-4D**).

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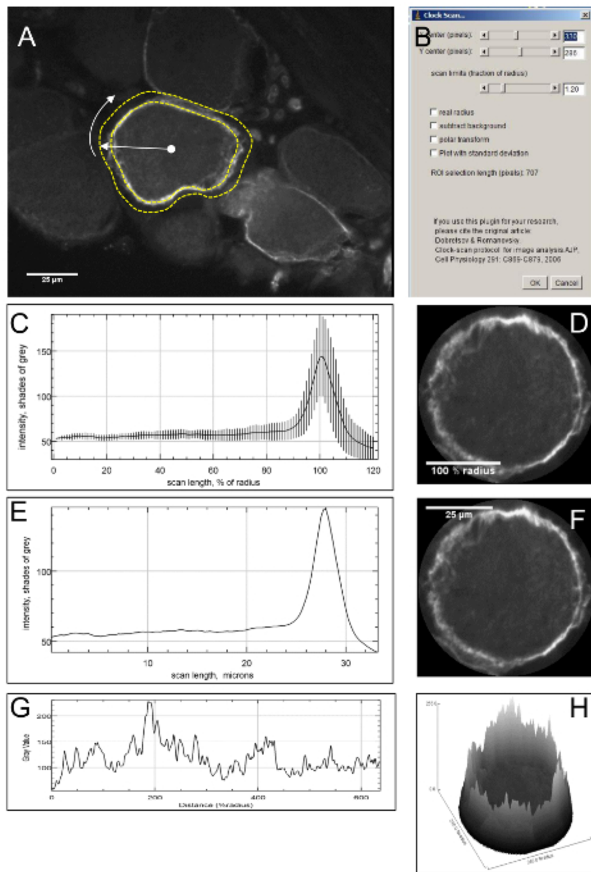

Figure 1: Representative Example of Application of the Clock Scan Plugin for Image Analysis. (**A**) Fluorescent light image of a section of a rat dorsal root ganglion immunostained for the α3 isoform of the Na+/K+- ATPase (α3 NKA; see Schneider *et al.*[3] for the details of tissue processing and staining). One of the neuronal profiles, with its border heavily labeled for α3 NKA (white), is outlined using a polygon line tool (inner yellow line). Radial scan (white arrow) limits (outer yellow line) were set to 120% of the object radius, from the object center (white dot) to the first pixel of the outline, as shown in panel B (scan limit scroll bar). (**B**) Screenshot of the main-option window of the Clock Scan plugin. (**C**) Plot of integral pixel-intensity profile of the cell shown in panel A (mean of 706 radial scan profiles, see outline length in B; vertical bars are SD bars). (**D**)- Polar transform image of the studied cell profile. (**E**) Clock-scan profile of the same cell obtained with the "real radius" option selected. Note that unlike the profile shown in C, the x-scale of this profile displays real spatial calibration units (µm). (**F**) Polar transformation of the same cell obtained with the "real radius" option selected. Note that the scale of this transformation is now in real spatial calibration units (µm). (**G**) The border of the polar transformation, shown in D, was outlined using the segmented line tool (line thickness was set to 10 pixels or 10% of radial scan length) and analyzed. "Analyze | Plot Profile" command was executed to measure the changes in mean labeling intensity along the border of the object (each data point of the graph represents the mean intensity of all pixels across the selection line width). (**H**) The "Analyze | Surface Plot" command was applied to the polar transform image shown in panel D to create 3-D representation of the labeling intensity of the object. Please click here to view a larger version of this figure.

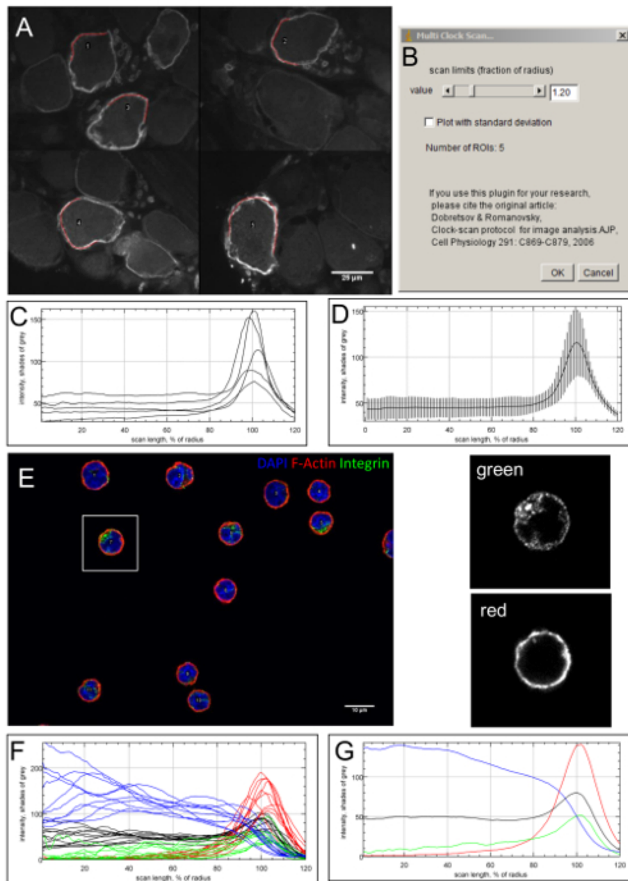

Figure 2: Representative Example Application of using Multi Clock Scan Plugin for Image Analysis. (**A**) Four fields of view were captured within the section of rat dorsal root ganglion immunostained for α3 NKA (see **Figure 1A** legend). For simplifying the use of the multi-clock scan plugin, these images were placed in a stack and then converted into a single image using "Image | Stacks | Make Montage" command. Red lines and numbers indicate a segmented line selections of five regions of interest in this image. (**B**) A screenshot of the Multi Clock Scan window is shown when the plugin is used to analyze a grey-scale image. (**C**) Individual clock-scan profiles of five ROIs' shown in panel A. (**D**) Average clock-scan profile for selected ROIs (panel A) with SD bars (option "plot with standard deviation" selected). (**E**) RGB image of cultured mouse preBI-lymphocytes, labeled with 4,6-Diamidino-2-phenylindole (DAPI, nuclear stain, blue) and with fluorescently-labeled antibodies for β1-integrin (green) and F-actin (red; see Dobretsov *et al.*[7] for the cell culture technique and Yuryev *et al.*[11] for staining details). Eleven cells (see number labels) were outlined using the ImageJ polygon selection tool. Panels on the right show green and red channel view of the cell #7 (rectangular selection on the left panel) after the "Image | Color | Split Channels" menu function was executed. (**F**) Individual cell clock-scan profiles (composite and red, green and blue color channel profiles are shown by black, red, green and blue lines, respectively). (**G**) Mean clock-scan profiles for all eleven ROIs selected in panel E. Color designations as in panel G (no plot with standard deviation option was used during the Multi Clock Scan procedure). Please click here to view a larger version of this figure.

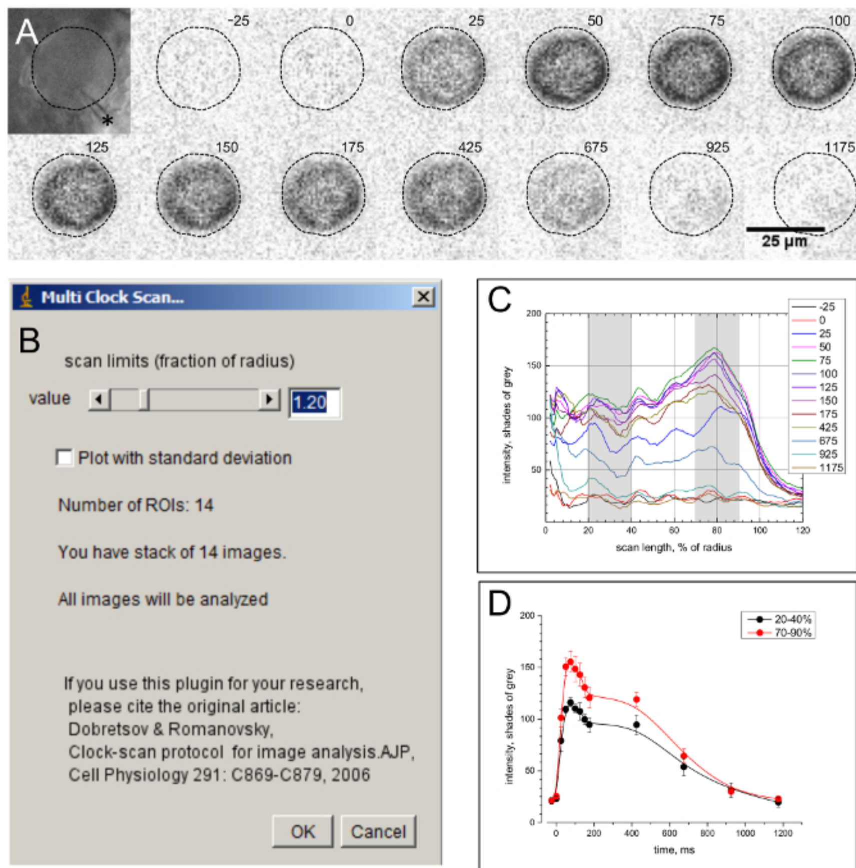

Figure 3: Multi Clock Scan Plugin and Analysis of Image Stacks. (**A**) Montage of selected and "saved as stack" image frames. An image of a dorsal root ganglion neuron captured with differential interference contrast (DIC) microscopy is shown in the first frame. Subsequent frames were acquired using epi-illumination fluorescence microscopy to monitor intracellular calcium concentration at different time intervals, before and after electrical stimulation of the cell. Numbers next to the respective image indicate time in ms[6]. The border of the cell was outlined using the DIC image of the stack (top-left frame; asterisk indicates the patch-clamp pipette used for recording and filling the cell with the calcium-sensitive dye Oregon Green BAPTA-1 (OGB-1)), and then used to run the Multi Clock Scan procedure on the remaining images. (**B**) Screenshot of Multi Clock Scan window, when the program is run on a stack of images. (**C**) Clock-scan profiles of the OGB-1 fluorescent signal at different distances from the cell center (% of radius) and at different times before and after electrical stimulation (legend, in ms). To prepare these graphs professional graphing software was used. (**D**) Changes in the intensity of the OGB-1 signal with time in sub-membrane and deeper cytoplasmic cell regions (red and black circles and lines, respectively). To obtain these data, the mean and SD were calculated for each data point located between 20-40% and 70-90% of the x-scale of each clock-scan profile shown in panel C (shaded areas). Please click here to view a larger version of this figure.

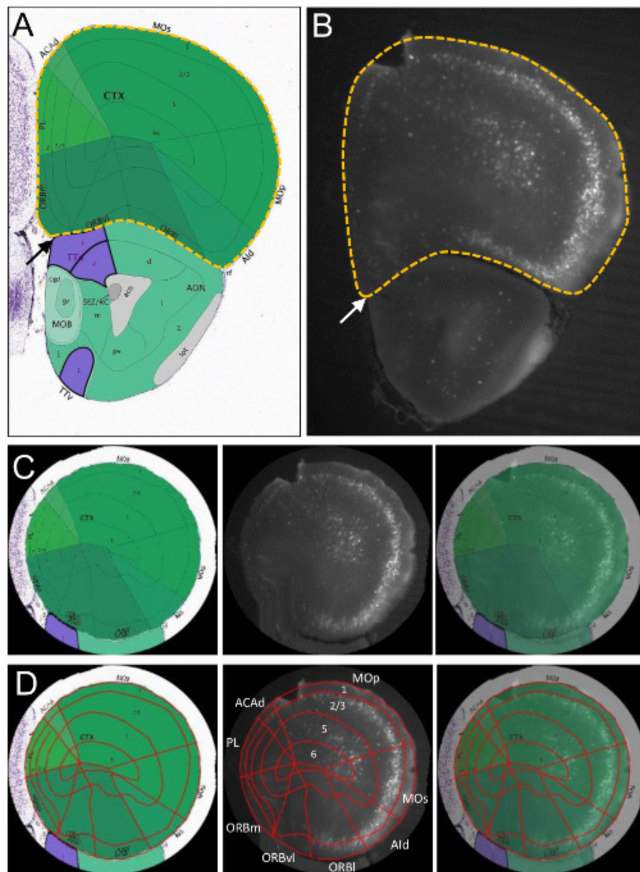

Figure 4: Example of using the Clock Scan Plugin in Image Registration and Overlay. (**A** and **B**) Screenshots of plate 29 from the coronal section (Allen Mouse Brain Atlas) and a 200 µm thick vibratome section from the gelatin-embedded mouse brain cut at approximately the same level as the atlas image. The transgenic mouse used in this example was expressing ZsGreen-fluorescent protein under the promotor of α3NKA, to identify α3NKA-expressing neurons[2]. To determine the cortical regions that are specifically enriched with these neurons (bright dots on the image in panel B), the entire cortical area was outlined (yellow dashed lines) starting in both images with the same reference point (mid-border between the cortex and the olfactory bulb; arrows). (**C**) Panels represent (from left to right): Clock Scan polar transforms the ROI, selected within the atlas image (panel A), within the image of mouse brain section (panel B) and overlay of these two transform images ("Image | Overlay | Add Image" command with 50% opacity setting). (**D**) Same images as in panel C but with the borders of the major cortical regions (as shown in the atlas) outlined in two other transform images using the ImageJ polygon, segmented line selection tools and the "Analyze | Tools | Synchronize Windows" command. Abbreviations are the same as in the original brain atlas image: Motor primary and secondary (MOp, MOs), agranular insular, dorsal part (AId), orbital lateral, ventro-lateral and medial (ORBl, ORBvl, ORBm), prelimbic (PL), anterior cingulate, dorsal part (ACAd) cortex. Numbers in the MOs area refer to the main cortical layers, which can be distinguished in the mouse motor cortex at the appropriate coronal brain level. Please click here to view a larger version of this figure.

Discussion

Clock Scan Protocol: The Clock Scan protocol is a fast and simple tool of image analysis. The advantages of this protocol, compared to existing common approaches of image analysis (such as linear pixel intensity scans or calculation of mean pixel intensity of the ROI), have been described in details in previous publications[1,9]. Briefly, this protocol allows the generation of integral radial pixel-intensity profiles by quantifying the intensity of pixels located at different distances from the ROI center such as the object's border, or a pre-determined location outside the object (background). Because of the latter, the clock scan profiles of each ROI can always be corrected for its immediate background, which (in biological applications) makes this profile less dependent on local, within-the-sample or sample-to-sample, non-uniformities in labeling/staining, as well as instability in the intensity of the microscope light source or fluorescent light exposure times. The object size and shape independence of the clock scan profiles further expand the area of application of this protocol by enabling the comparisons of different objects, as well as correction by point-by-point subtraction of profiles of "positive" and "negative" control objects.

Clock Scan Plugins: The major limitation for distributing and sharing the original protocol was the platform-dependence of its code which was developed with Visual Basic 6.0 (VB)[1,9]. This problem has been recently addressed by one of the research groups at the Leibniz Institute of Molecular Pharmacology, Germany, by developing a similar Fuji ImageJ Clock Scan plugin[2]. The Leibniz Institute's plugin reproduces the original clock scan basic functionality in its ability to generate integral radial scan profiles for the enclosed convex-shape ROI, and in addition, it can process segments of outlines (arcs). However, the scan limit of the profile generated by their plugin can only be set to 100% (the object's border), which means, that the background pixel intensity cannot be quantified. Moreover, it has no capacity to generate polar transforms, to work with different color channels in RGB images, or to work with stacks of images and to process multiple ROIs. By comparison, the two new plugins, described here, fully reproduce the capability of the original VB code (*i.e.*, generation of integral clock scan pixel intensity profiles

with optional display of SDs and/or background subtraction, as well as processing different color channels of RGB images). In addition, they can analyze a segment/ arc-shaped ROI (functionality introduced in the Fuji ImageJ plugin developed at the Leibniz Institute of Molecular Pharmacology[2]). Furthermore, these plugins expand the utility of previous programs by generating ROI size- and shape-independent polar ROI image transformations, which can be used in applications that require image registration. Finally, the multi-clock scan plugin effectively facilitates clock scan of multiple ROIs located within the same image or in an image stack. The latter new feature of the program is especially useful in applications in which it is important to determine changes related to time and location.

Limitations and troubleshooting: The major limitation of the Clock Scan method is the requirement to select a convex-shaped ROI. The clock scan profile would be meaningless in situations when any of radial scans cross the ROI outline more than once. This would make the normalization of the length of such radial scan with respect to the distance from the center to the ROI border impossible. Another limitation is that the clock scan profile information is progressively decreased in ROI lacking radial symmetry. However, at least in part, these two limitations may be overcome by the analysis of selected segments (arcs) of complex-shaped and asymmetric ROIs. Using the segment scan is also recommended in cases when sections of the background territory contain labeled features, which may affect the background subtraction procedure (see **Figure 2A** for an example of selection for analysis of those cell segments that are not facing other labeled cells). Finally, if analysis of composite images containing more than 3 color channels is required, the color channels of these images should be split before running the plugin.

Future directions: Future improvement in the functionality of these plugins will include, but are not limited to updating the code to combine the functionality of clock-scan and multi-clock-scan plugins into one plugin. Color co-localization algorithms (such as algorithms based on calculations of Pearson's correlation or Manders split coefficients), and the development of the plugin to become capable of working with multiple ROIs that are selected in different images or in different slices in an image stack (current version of the plugins allows analysis of several ROIs selected within one image or one ROI selected for all images in the stack), will be implemented. The authors will also appreciate any suggestions from the plugin users and reports of any problems encountered during the use of existing plugins.

Conclusion: The clock scan analysis is a promising tool for imaging studies in many areas of biology, from the analysis of static cell labeling with various markers to studies of spreading of Na^+ or Ca^{++} , within a single cell as well as for the analysis of spreading activity (*e.g.*, Ca^{++} waves) in populations of synaptically-connected cells[10,11] or gap junction-coupled cells[12]. Other potential areas of application of the clock scan analysis include medical image analysis (ultrasound images of blood vessels, CT-scan images and bone cross-sections), astronomy (spiral and radial galaxy imaging), chemistry (diffusion from a point source), physics (diffraction pattern analysis), forestry (tree stem ring analysis to determine age of the tree as well as periods of dry weather and poor fertilization), engineering (metal pipe corrosion) and climatology (weather radar image analysis).

Disclosures

The authors declare that they have no competing financial interests or other conflicts of interest.

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