



PAT-GEOM

User Guide

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Ian Z.W. Chan, Martin Stevens, Peter Todd

For more info: ian@ianzwchan.com, www.ianzwchan.com

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About PAT-GEOM

Animal colour patterns can be decomposed into colour, brightness, polarisation properties and the underlying pattern, i.e. the spatial arrangement of the former properties. Whilst there are many techniques to quantify colour (e.g. using RGB values or reflectance spectra), the same is not true for the underlying pattern. PAT-GEOM is our attempt to correct this deficiency and to equip researchers in this field with a useful tool.

PAT-GEOM is a free-to-use collection of seven ImageJ macros that measure seven properties of patterns: marking shape, marking size, pattern contrast, marking distribution, marking randomness, marking shape directionality and marking distribution directionality. These properties and how they are measured are illustrated in **Fig. 1** and explained in **Table 1**; they are not meant to be a comprehensive list of what can be measured in a pattern, but were selected because we judged that they would be useful to the scientific community based on what researchers are currently doing and what they might want to do in future.

Finally, the program is designed to work as macros in ImageJ (Schneider et al. 2012) and so require ImageJ to be installed on your computer (see the Installation section for installation instructions). They work with any type of image that ImageJ can open, but were designed specifically with multispectral (.mspec) images in mind. These .mspec images are part of the Multispectral Image Calibration and Analysis (MICA) toolbox created by Troscianko & Stevens (2015). The toolbox corrects for different light conditions and uneven camera sensor responses to light of different wavelengths, producing linearised luminance values which accurately represent the light coming from the item of interest. It also allows images in both the human visible and ultraviolet range to be stitched together for analysis. The technique is slightly involved but well worth the time and effort to learn because it is a rigorous way of analysing patterns (and colour). Its use is recommended for anyone who wishes to analyse image data robustly (not just for PAT-GEOM users).

Citing PAT-GEOM

To cite PAT-GEOM, please use: [\[as of 2 Dec 2018, PAT-GEOM has been accepted for publication by *Methods in Ecology and Evolution*, this information will be updated when a citation and doi become available\]](#)

Reporting Bugs And Other Feedback

To report bugs or give any other form of feedback, please email ian@ianzwchan.com. Although the programs have been tested on as many image types and analysis situations as we could think of, bugs will still arise when using them in situations that were not foreseen. We also anticipate and hope that PAT-GEOM's use will not eventually be limited to these seven properties or even the study of animal behaviour. If you are a botanist, molecular biologist, landscape ecologist or a

researcher from any other field and have suggestions, please do get in touch and share your ideas with us as we would love to improve PAT-GEOM and make it more useful for the scientific community. All constructive feedback is most welcome and much appreciated, so thanks in advance!

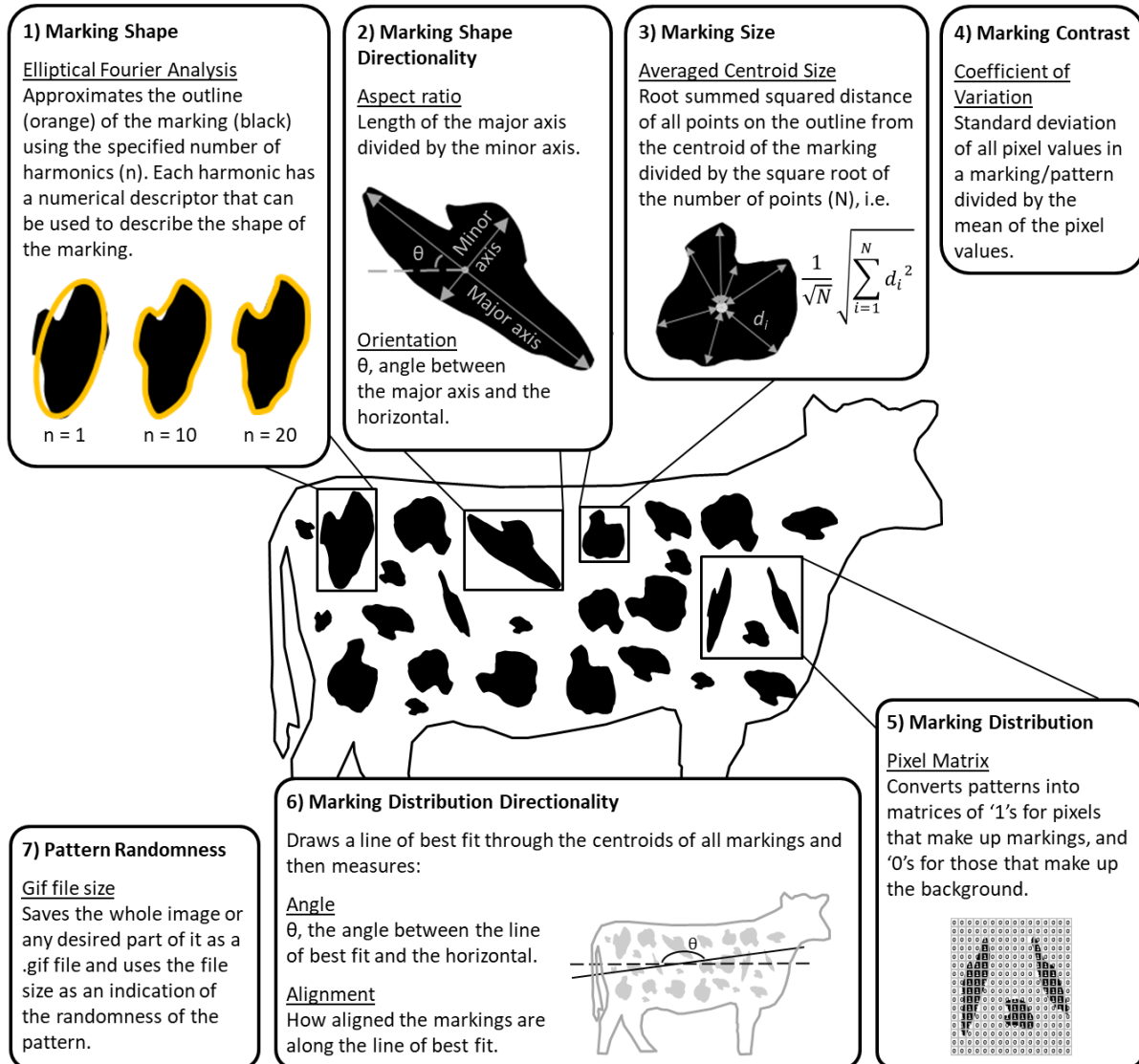



Fig. 1. An illustration (using black markings on an imaginary white cow) of the seven properties and how they are measured using PAT-GEOM. Further details on each property and measurement technique can be found in Table 1.

Installation

Install ImageJ before installing PAT-GEOM.

Installing ImageJ (if you have already installed ImageJ, skip to “Installing PAT-GEOM”)

<i>For Windows</i>	<i>For macOS</i>
<p>1) Go to [https://imagej.nih.gov/ij/download.html].</p> <p>2) Click and download “ImageJ bundled with 64-bit Java 1.8.0_112” (under Windows).</p> <p>3) Extract the contents of the downloaded file and note its location¹, e.g. if on the desktop, it will be C:\Users\[yourName]\Desktop\ImageJ.</p> <p>Note: Windows users may also choose to install FIJI (ImageJ bundled with some useful plugins) instead of base ImageJ. There is no difference with the installation process or running of PAT-GEOM. FIJI can be downloaded from: [https://imagej.net/Fiji/Downloads].</p>	<p>You may install either:</p> <ul style="list-style-type: none">- base ImageJ, a smaller installation but it may require more steps to set up. <p>OR</p> <ul style="list-style-type: none">- FIJI, simply ImageJ bundled with a few useful plugins, a larger installation but potentially simpler to set up. <p>(FIJI is recommended)</p> <p><u>Option 1: base ImageJ</u></p> <p>1) Go to [https://imagej.nih.gov/ij/download.html].</p> <p>2) Click and download “ImageJ bundled with Java 1.8.0_172” under Mac OS X.</p> <p>3) Note the location where you saved the file.</p> <p><u>Option 2: FIJI</u></p> <p>1) Go to [https://imagej.net/Fiji/Downloads].</p> <p>2) Click the Apple icon  to start the download.</p> <p>3) Note the location where you saved the file.</p> <p>Useful instructions for known issues on macOS, see: [https://imagej.nih.gov/ij/docs/install/osx.html].</p>

ImageJ/FIJI is now installed; there is no need to go through an installation process. However, we suggest to wait till PAT-GEOM is installed to open ImageJ/FIJI.

Note: full installation instructions, together with known issues and their fixes are available in the online ImageJ documentation^{2,3}.

¹ The ImageJ developers suggest you do not put it into the Program Files folder, put it into another folder that you have write access to, e.g. in My Documents. See: [https://imagej.nih.gov/ij/docs/install/windows.html].

² The ImageJ user guide can be found here: [https://imagej.nih.gov/ij/docs/guide/user-guide.pdf]. Basic Concepts, Guided Tutorials/Examples and a helpful community can be found at: [https://imagej.nih.gov/ij/docs/index.html].

³ Full installation instructions for Linux, macOS and Windows: [https://imagej.nih.gov/ij/docs/install/index.html].

Installing PAT-GEOM

- 1) Go to [<http://ianzwchan.com/home/my-research/>].
- 2) Download the PAT-GEOM.zip file and open it.

<i>For Windows</i>	<i>For macOS</i>
<p>3) Locate the Plugins folder of your ImageJ installation, e.g. if ImageJ is on your desktop, the plugins folder will be: [C:\Users\[yourName]\Desktop\ ImageJ\plugins].</p> <p>4) Open the Plugins folder.</p> <p>5) Copy the entire PAT-GEOM folder (in the PAT-GEOM.zip file from in step 2).</p> <p>6) Paste it into your ImageJ Plugins folder (in step 3).</p>	<p>3) Locate and right click on the ImageJ/FIJI file.</p> <p>4) Click “Show Package Contents”.</p> <p>5) Navigate to the “Plugins” folder. OR Set the Finder window to Icons or List mode (not Column mode). See: [https://imagej.net/MacOSX_tips#Accessing_the_plugins_and_macros_folders].</p> <p>6) Copy the entire PAT-GEOM folder (in the PAT-GEOM.zip file from step 2) into your ImageJ Plugins folder (in step 3).</p>

For BOTH Windows and Mac operating systems:

- 7) Download the “fourier2.5_.jar” file (for the Elliptical Fourier Analysis) from [http://imagejdocu.tudor.lu/doku.php?id=plugin:analysis:fourier_shape_analysis:start]).
- 8) Place it into the ImageJ/FIJI Plugins folder.

PAT-GEOM should now be in the Plugins menu of your ImageJ installation (**Fig. 2**).

Starting ImageJ and PAT-GEOM

- 1) Start ImageJ/FIJI by:

If not already open, double click the downloaded ImageJ/FIJI file. For windows, this is the ImageJ.exe or FIJI.exe file; For macOS, this is the ImageJ.app or FIJI.app file⁴.

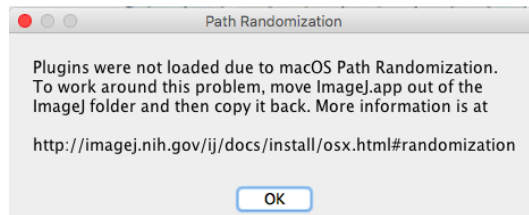
OR

If ImageJ/FIJI is already open, click Help>Refresh Menus (this seems to work only with Windows) OR close and restart ImageJ/FIJI.

⁴ You may have to change your Security Preferences to allow the programme to be opened. For Windows: click Allow on the dialogue box. For macOS: [https://support.apple.com/kb/PH25088?locale=en_US].

Note: ImageJ and FIJI require Java to run. If Java is not installed on your system, you may be prompted when first starting the programme to download and install Java. Follow the on-screen prompts and links.

Note for macOS users: depending on the macOS version you are running, you may also be required to solve a “Path Randomisation” issue. If so, the dialogue box below will appear. Follow the instructions in the dialogue box that pops up or at. There are no updates yet regarding known issues for the newest Mojave version of the macOS.



2) Start PAT-GEOM: click Plugins>PAT-GEOM>PAT-GEOM (**Fig. 2**).

Congratulations! PAT-GEOM should now be running.

Fig. 2. The PAT-GEOM Selection Window (Below) and how to access the PAT-GEOM programs from the ImageJ Plugins menu (Right).

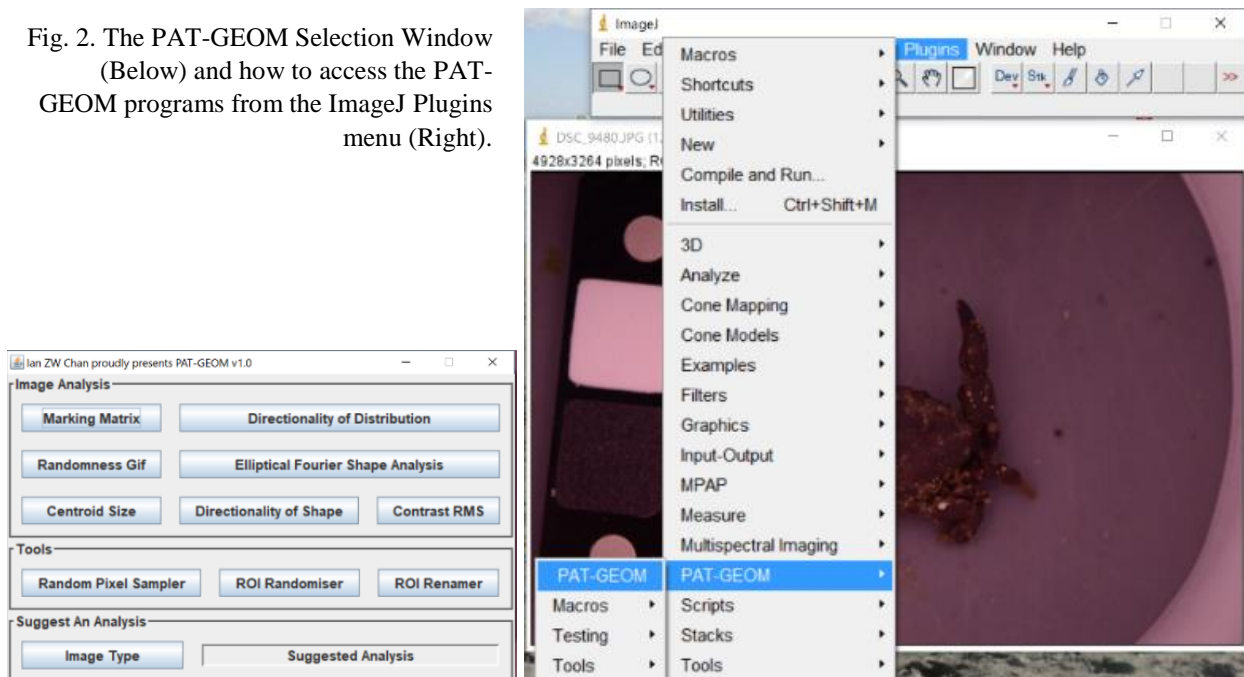


Image Analysis

Overview

The analysis process is relatively simple and is shown in the flowchart in **Fig. 3**. The raw data are digital images (more specifically, the pixel values therein) and the first (and perhaps hardest) step is deciding what properties to measure. Thereafter, the actual analysis is simply a matter of following the directions provided within ImageJ. Detailed instructions for each step are provided in the subsequent sections.

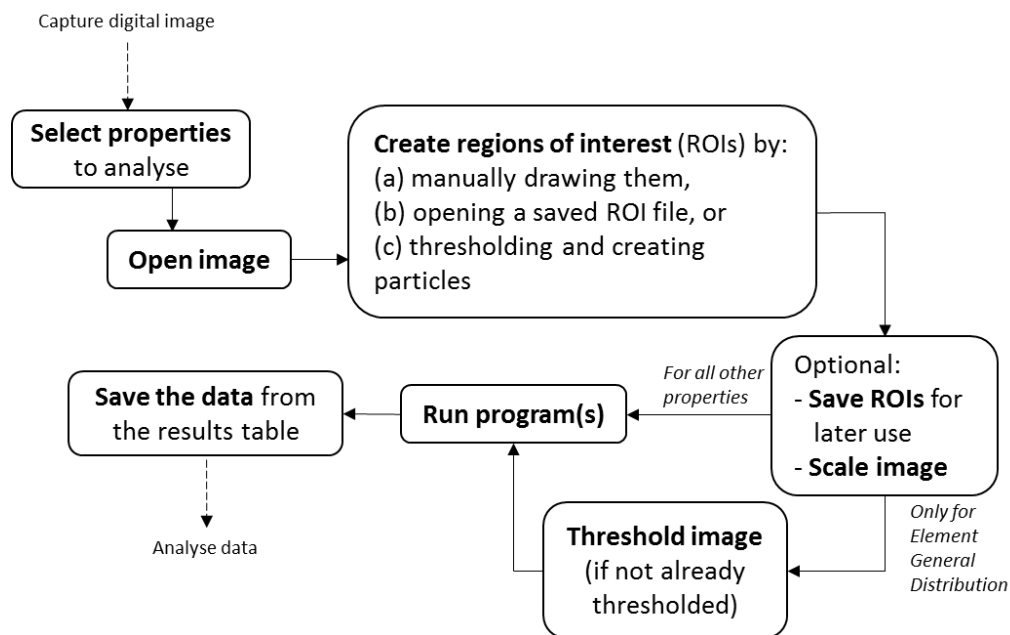


Fig. 3. An overview of the steps in the image analysis process.

Capturing a digital image

Digital cameras perform a lot of behind-the-scenes image processing and the data (i.e. pixel values) on the resulting image may not correspond to the actual spectrum being emitted from or reflected by the sample. Stevens et al. (2007) and Troscianko & Stevens (2015) set out a rigorous technique to capture and reproduce accurate digital images and are highly recommended. We realise, however, that it may not always be possible to apply their technique. Hence, **PAT-GEOM works with any type of digital image (.jpg, .tif, .gif, .png, etc.) captured in any manner.**

Deciding what properties to analyse

PAT-GEOM is able to analyse seven different properties of morphological patterns (illustrated in Fig. 1), but there is no need to measure all properties, all the time. The right property to measure for each study will depend on the nature of the pattern being studied (e.g. measuring marking distribution directionality makes more sense in a striped zebra than a spotted hyena) and the

research question (e.g. if you are only comparing the contrast of two zebras, it is not necessary to measure the shape and size of their stripes).

In writing this manual, we provide some example situations describing properties to measure (in the Worked Examples section); however, it would be impossible to cover all types of situations. Hence, this manual instead gives information about each property and provides examples of when it would be advisable to measure them. This should better equip users to decide which properties would be useful to measure for their specific study and is found in **Table 1**.

Opening an image

Once you have already opened ImageJ, there are two main ways of opening an image:

Option 1: drag and drop an image file onto the ImageJ main window (**Fig. 4**).

Option 2: click [File>Open] and choose your image file in the dialogue that opens.

If you are trying to open an .mspec file (Troscianko & Stevens 2015), you will either have to generate or load a multispectral image (see the MICA toolbox user guide). If you are simply trying to open .raw files in ImageJ, one way to do this is to download the DCRAW plugin package, copy it to the ImageJ plugins folder, restart ImageJ and use it from the Plugins menu.

Creating regions of interest

Regions of interest (ROIs) specify to the programs the areas of the image you want to measure. These could be an entire pattern or animal (e.g. the whole zebra) or the markings therein (e.g. the stripes on the zebra). There are three main methods to create ROIs in ImageJ:

Option 1: manually draw ROIs.

- 1) Select a selection tool (e.g. polygon tool for complex shapes, see **Fig. 4**).
- 2) Draw a selection on the outline of the region (e.g. **Fig. 5**).
- 3) Click [Edit>Selection>Add to Manager] (or press Ctrl+T) to add the ROI to the ROI Manager⁵.

Option 2: open a previously saved ROI (.roi) file or ROI zip (.zip) file (see the section on saving ROIs for the difference between the two).

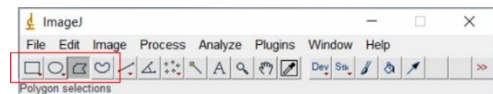


Fig. 4. The ImageJ main window with the selection tools (on the left of the window, below “File”) highlighted by a red box). The polygon selection tool is activated.

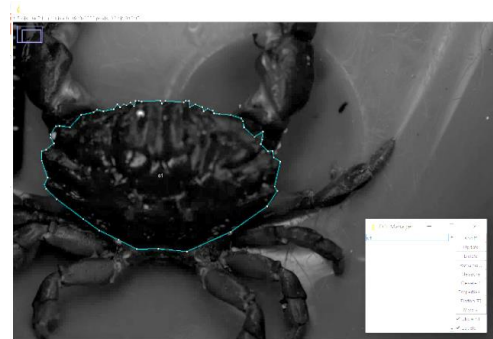


Fig. 5. A polygon selection drawn on the outline of a crab carapace and saved in the ROI Manager window (bottom right) as “c1”.

⁵ In .mspec files, you can simply press any alphabet (except “s” which is saved for scale bars).

- 1) Drag and drop the .roi or .zip file onto the ImageJ main window.

Stop here if opening a .zip file, continue if opening an .roi file:

- 2) Click [Edit>Selection>Add to Manager] (or press Ctrl+T) to add the ROI to the ROI Manager¹.

OR

- 1) Click [Edit>Selection>Add to Manager]> (or press Ctrl+T) to open the ROI Manager.

- 2) In the ROI Manager window, click [More>Open], and choose the .roi or .zip file in the dialogue that opens.

Option 3: threshold to automatically draw ROIs. Thresholding converts a colour or grayscale image to a binary black-or-white image, e.g. all pixels above a certain threshold set by you are changed to black and all pixels below it are changed to white, or vice versa. It is an extremely powerful tool used in many image analyses (e.g. shape and size analysis and identifying ROIs, see **Fig. 6**). Thresholding is too complex to be explained in detail here, but multiple guides are available online⁶, and it is well worth the time and effort to master.

- 1) Threshold the image by clicking either:
 - a. [Image>Adjust>Threshold] (or press Ctrl+Shift+T) if your image is a black-and-white type (e.g. 8-bit); or
 - b. [Image>Adjust>Color Threshold] if your image is a colour image (e.g. 8-bit Color or RGB Color).

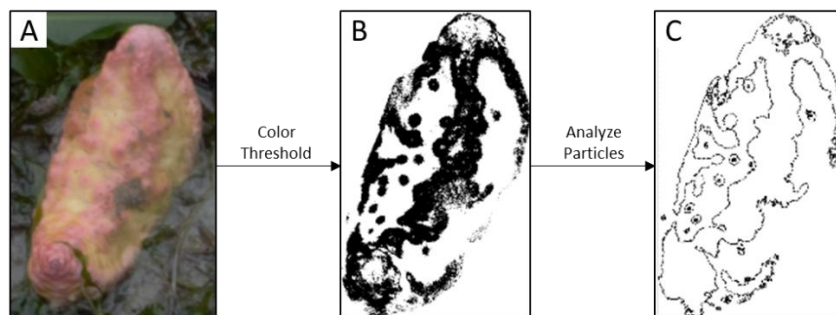


Fig. 6. A colour photo of a sea cucumber (A) is Color Thresholded so that the pixels corresponding to pink markings are changed to black and the yellow background changed to white (B). Thereafter, the Analyze Particles function is used to draw ROIs around the black markings.

⁶ As a starting point, see: [http://imagej.net/Particle_Analysis], [<http://imagej.net/Segmentation>], [<https://imagej.nih.gov/ij/docs/guide/146-28.html#toc-Subsection-28.2>], [<http://occm.otago.ac.nz/resources/ImageJ-Thresholding.pdf>], and Sezgin & Sankur (2004).

Table 1. Information on the different properties that can be measured using PAT-GEOM.

Property	Technique	Description	Data Output	Usage Examples
Marking Shape				
General Shape	Elliptical Fourier Analysis (Kuhl & Giardina 1982)	Represents the shape of a region of interest using descriptors calculated from harmonically-related trigonometric functions (based on the plugin by Boudier & Tupper, 2016 which must first be installed). The functions approximate shape, and using more harmonics produces a more detailed approximation. The mean of the descriptors of each harmonic of all markings in a pattern gives an indication of the “average marking shape”.	One descriptor per harmonic. The user defines how many harmonics to use for each region of interest.	<ul style="list-style-type: none"> - Delineating two species using their markings. - Identifying unique individuals using their markings. - Can be used for any pattern.
Directionality	Aspect Ratio and Orientation	Measures how elongated a marking is and the direction in which it is oriented (in terms of the angle of the major axis of the marking). The mean and standard deviation of these values for all the markings in a pattern gives an indication of marking shape directionality in that pattern.	Two measurements per region of interest: aspect ratio and angle of major axis.	<ul style="list-style-type: none"> - Comparing the markings on an animal to its background. - More useful for patterns with elongated markings, e.g. stripes.
Marking Size	Averaged Centroid Size (Bookstein 1991)	The only independent measure of size, divided by the square root of the number of points to control for different numbers of points in different markings; can be used to measure the size of any region of interest.	Two measurements per region of interest: averaged centroid size and size in square pixels.	<ul style="list-style-type: none"> - Can be used for any pattern.
Marking Contrast	Root Mean Square Contrast (Peli 1990)	Measures the standard deviation of all pixel values in the region of interest. Can be used to measure contrast in a single marking or over the entire pattern.	One measurement per region of interest: Root Mean Square contrast.	<ul style="list-style-type: none"> - Comparing the contrast of an animal to that of its background. - Can be used for any pattern.
Marking Distribution				
General Distribution	Element Matrix (Todd et al. 2005)	Each pixel in the digital image is an element of the matrix, and markings are represented by ‘1’s and the background by ‘0’s. Combining multiple matrices gives information on a group of markings or patterns.	A matrix of ‘1’s and ‘0’s whose size is the dimension of the image in pixels.	<ul style="list-style-type: none"> - Comparing the distribution of markings between different populations of patterns. - Can be used for any pattern.
Marking Distribution Directionality	Angle and Alignment	Measures the directionality of the location of the markings in a pattern by drawing a line of best fit through their centroids and reporting the angle and R^2 value (representing alignment) of the line.	Two measurements for each pattern (i.e. group of markings): angle and R^2 value.	<ul style="list-style-type: none"> - Comparing the directionality of markings on different individuals. - Can be used for any pattern.
Randomness	Gif File Size (Sprott et al. 2002)	Saves the region of interest as a Gif file and measures the randomness of the markings in it by the size of the file. This uses the fact that Gif files are optimally compressed and a random pattern would hence require a larger Gif file.	One measurement per region of interest: Gif file size.	<ul style="list-style-type: none"> - Comparing the markings of an animal to its background. - Can be used for any pattern.

- 2) Adjust the thresholding settings to obtain a match to your original pattern, determine these settings either:
 - a. Automatically using the “Sample” option (available in colour thresholding only) which allows you to indicate the foreground and background areas on the image itself; or
 - b. Manually adjusting settings. For a guide on the effects of the options, see [<https://imagej.nih.gov/ij/docs/guide/146-28.html#toc-Subsection-28.2>]. Though this is more tedious, for technical reasons, the best judge for whether the settings are correct remains the user; but see Sezgin & Sankur (2004) for a good discussion on avoiding user bias.

Important Notes:

- Thresholding settings should remain consistent if any comparison is going to be done, e.g. comparing an animal with its background.
 - PAT-GEOM programs assume that you set the foreground (i.e. markings) to black ('1') and the background to white ('0')⁷.
- 3) Optional: record the thresholding settings for future use.
 - 4) Optional: save the thresholded image as a new image by clicking [File>Save As] in the ImageJ main window.
 - 5) Draw ROIs around each marking by clicking [Analyze>Analyze Particles].

- 6) Adjust the settings in the Analyze Particles window that opens (**Fig. 7**) so that your ROIs match your markings. A guide to the settings is found at [http://imagej.net/Particle_Analysis]. Commonly-used settings include:
 - **“Add to Manager”**: must be checked to add the ROIs to the ROI Manager window.
 - **“Size”**: e.g. adjust the lower limit to omit extremely small markings.
 - **“Exclude on edges”**: to exclude or include markings on the outline of your pattern.
 - Whether to summarise and display results in the ImageJ Results window.

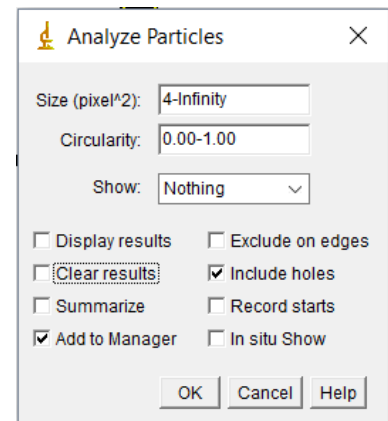


Fig. 7. The Analyze Particles window with its various options for drawing ROIs.

⁷ To change foreground and background colours, click: [Edit>Options>Colors].

Saving regions of interest (optional)

In certain cases, it may be useful to save ROIs for later use, e.g. comparing an animal to animal-shaped patches of its background. ImageJ allows users to both save ROIs individually (as a .roi file), and to save all the ROIs in an image together (as a .zip file) so that they may be collectively reopened with one click⁸. It is useful to know that if you save all the ROIs as a .zip file, you can still open them individually if you wish: simply go into the .zip file and open the specific .roi file you want. For instructions on opening .roi and .zip files, see Option 2 in the section on “Creating regions of interest”.

Saving ROIs individually:

- 1) In the ROI Manager window, click on the ROI you wish to save
- 2) Ensure that the ROI is highlighted on the image
- 3) In the ROI Manager window, click [More>Save] and save the .roi file as desired.

Note: In the Save Selection dialogue, ensure that the file name automatically generated by ImageJ **ends with “.roi”** (Fig. 8). The ROI Manager tends to be a little buggy when selecting ROIs, and may save all ROIs as a .zip file even if you only selected one ROI. If the name ends with “.zip”, close the dialogue and start again from Step 1 (usually works).

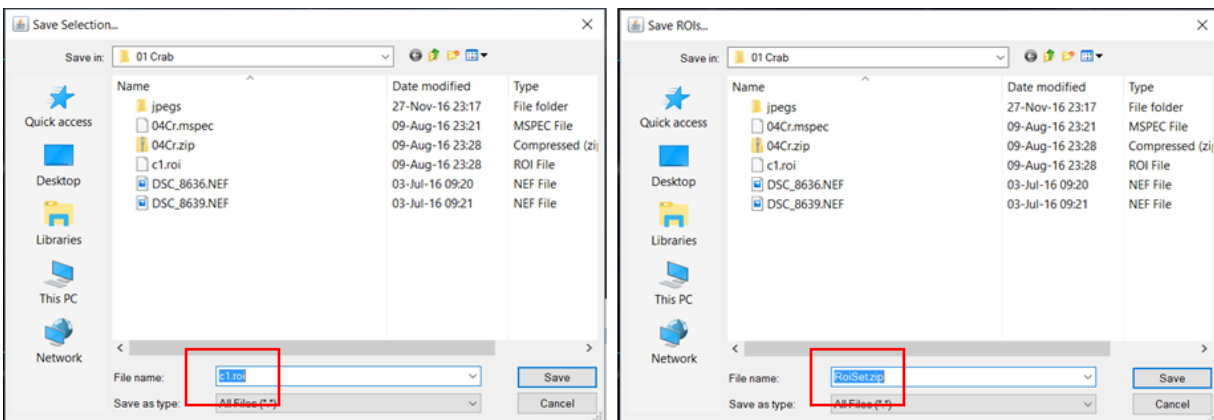


Fig. 8. The correct Save Selection dialogue for saving an individual ROI (left) and all the ROIs in an image collectively (right). Note that the name in the dialogue on the left (highlighted with a red box) ends with “.roi” whereas that on the right ends with “.zip”.

Saving ROIs collectively:

- 1) In the ROI Manager window, select all the ROIs by clicking on the first ROI, holding shift, and then clicking on the last ROI

⁸ If you are working with .mspec images, Jolyon has included a very useful shortcut for saving all ROIs as a .zip file, simply press “0” (the number).

- 2) Ensure that all ROIs are highlighted on the image
- 3) In the ROI Manager window, click [More>Save] and save the .zip file.
 Note: In the Save Selection dialogue, ensure that the file name automatically generated by ImageJ **ends with “.zip” (Fig. 8)**. If the name ends with “.roi”, close the dialogue and start again from Step 1 (usually works).

Scaling an image (optional)

When analysing morphological patterns (which are spatial), it makes sense to report data in terms of real lengths. However, it is **not necessary to scale your images** before running PAT-GEOM as the data produced is in pixel units. Users can then go on to convert the data into the length unit of their choice (which means that there should still be a scale in the images). Nevertheless, if the user intends to use ImageJ’s built-in Measurement function, it is useful to scale the image first using [Analyze>Set Scale]. Guides can be found at [<http://imagej.net/SpatialCalibration>] and [<https://imagej.nih.gov/ij/docs/guide/146-30.html#toc-Subsection-30.8>].

Thresholding the image (if not already done; only needed for Marking Distribution)

The Pixel Matrix technique requires a binary black-and-white image to construct the pixel matrix, so images should first be thresholded. If thresholding was previously used to create ROIs, this step may be skipped. If not the steps are identical to the previous subsection on thresholding:

- 1) Threshold the image by clicking either:
 - a. [Image>Adjust>Threshold] (or press Ctrl+Shift+T) if your image is a black-and-white type (e.g. 8-bit); or
 - b. [Image>Adjust>Color Threshold] if your image is a colour image (e.g. 8-bit Color or RGB Color).
- 2) Adjust the thresholding settings to obtain a match to your original pattern, determine these settings either:
 - a. Automatically using the “Sample” option (available in colour thresholding only) which allows you to indicate the foreground and background areas on the image itself. This is still [quote] experimental [unquote], so use with care; or
 - b. Manually adjusting settings. For a guide on the effects of the options, see [<https://imagej.nih.gov/ij/docs/guide/146-28.html#toc-Subsection-28.2>]. Though this is more tedious, for technical reasons, the best judge for whether the settings are correct remains the user; but see Sezgin & Sankur (2004) for a good discussion on avoiding user bias.

Important Notes:

- Thresholding settings should remain consistent if any comparison is going to be done, e.g. comparing an animal with its background.
- PAT-GEOM programs assume that you set the foreground (i.e. markings) to black ('1') and the background to white ('0')⁹.

- 3) Optional: record the thresholding settings for future use.
- 4) Optional: save the thresholded image as a new image by clicking [File>Save As] in the ImageJ main window.

Running the program(s)

Once the image to analyse is open and the preceding steps are performed, you should be ready to perform the analysis. Check if your open image meets all the requirements for the respective program listed in **Table 2**. Thereafter, run the appropriate PAT-GEOM program:

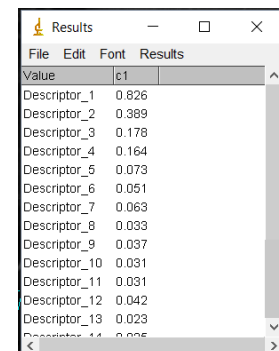
- 1) On the ImageJ main window, click [Plugins>PAT-GEOM>PAT-GEOM] and a Selection Window will open from which you can choose the program(s) you want to run (**Fig. 2**).
- 2) Select the analysis options desired, available options for each analysis are listed and briefly discussed in **Table 2**.
Note: to run the Shape analysis, you must first download the Elliptical Fourier Analysis plugin by Boudier & Tupper (2016) (i.e. the "fourier2.5_.jar" file from http://imagejdocu.tudor.lu/doku.php?id=plugin:analysis:fourier_shape_analysis:start), place it in the ImageJ Plugins folder and click [Help>Refresh Menus] or reopen ImageJ.
- 3) Analysis results will be displayed in the ImageJ Results window (**Fig. 9**).
Note: this may take some time, especially if you are running centroid size or Elliptical Fourier Analysis measurements with multiple and complicated ROIs.

Saving the data

There are two ways to save data from the ImageJ Results window:

Option 1: through the Results window menu. This allows you to save the data with the row and column names as a file type of your choice, e.g. .xlsx or .csv.

- 1) On the Results window, click [File>Save As].



Value	c1
Descriptor_1	0.826
Descriptor_2	0.389
Descriptor_3	0.178
Descriptor_4	0.164
Descriptor_5	0.073
Descriptor_6	0.051
Descriptor_7	0.063
Descriptor_8	0.033
Descriptor_9	0.037
Descriptor_10	0.031
Descriptor_11	0.031
Descriptor_12	0.042
Descriptor_13	0.023
Descriptor_14	0.005

Fig. 9. The ImageJ Results window with data from an Elliptical Fourier Analysis.

⁹ To change foreground and background colours, click: [Edit>Options>Colors].

- 2) Choose the desired file type and location.

Option 2: manually copying and pasting the results.

- 1) Click on any row of data in the Results window.
- 2) Press Ctrl+A to highlight all the data.
- 3) Copy the data and paste it into your desired location (e.g. an .xlsx file).

Analysing the data

It would not be possible to list all the different ways to handle the data produced by PAT-GEOM. We suggest some common ways the data may be used and analysed in **Table 2** below, but it is by no means comprehensive, and it is up to researchers to determine how best to use the data to answer their research question.

Table 2. The data output, analysis options and requirements for each analysis technique, and the possible uses statistical analyses for the data produced.

Property	Technique	Data Output	Options	Requirements Checklist	Possible Uses of the Data	Stats Analyses†
General Marking Shape	Elliptical Fourier Analysis	Descriptors for all ROIs in the image.	- The desired number of descriptors.	- First install Boudier & Tupper (2016) plugin. - ROIs must be open and must be either polygon, freehand or traced types. - No other ROIs (e.g. scale bars) in the image.	- The mean of each descriptor (e.g. finding the average of the first descriptor of all markings) can be used to describe the “average marking shape” of a pattern. - The variance or standard deviation of the descriptors can similarly be used to describe the variation of marking shapes.	- PCA - CDA - MANOVA
Marking Shape Directionality	Aspect Ratio & Orientation	The aspect ratio and orientation for all ROIs in the image.	Nil	- ROIs must be open. - No other ROIs in the image.	- The mean represents how elongated the markings in the pattern are on average. - The mean angle can be used as an indication of the overall orientation of the markings.	- Wilcoxon signed rank test
Marking Size	Averaged Centroid Size	The centroid size for all ROIs in the image divided by the square root of the number of points in each ROI.	Nil	- ROIs must be open. - No other ROIs in the image.	- The mean represents the average marking size. - The variance or standard deviation represents the variation of the markings in terms of their size.	- Wilcoxon signed rank test
Marking Contrast	Coefficient of Variation	The standard deviation divided by the mean of the specified part of the image.	- To measure the whole image, all ROIs combined as one or individual ROIs. - If multiple slices are present: slices to analyse.	- ROIs must be open. - If either of the ROI options are to be selected: no other ROIs in the image.	- Measuring the contrast of the area in the animal only or in the background only gives data on how contrasting the pattern in that area is. This can then be compared. - Also possible to compare an area of the background with the animal in it to an equivalent area without the animal.	- Wilcoxon signed rank test
Marking Distribution Directionality	Angle & Alignment	The angle (Fig. 1) and R ² value for the best fit line of all the ROIs in the image.	Nil	- ROIs must be open. - No other ROIs in the image.	- The mean angle and alignment (controlled for orientation) can be calculated for different populations or species and used to compare them.	- Wilcoxon signed rank test
General Element Distribution	Pixel Matrix	A pixel matrix that either represents the whole image or is a cumulative matrix of all the ROIs.	- To measure the whole image, or all the ROIs combined. - To save the results as an excel file.	- ROIs must be open and must be identical in size. - No other ROIs in the image.	- The cumulative matrix of a population (preferable normalised to between 0 and 1) can be used to compare two populations or species.	- Permutational MANOVA
Element Distribution Randomness	Gif File Size	The Gif file size of the specified part(s) of the image (corrected for the size of the header).	- To measure the whole image or individual ROIs. - If multiple slices are present: slices to analyse. - To save the gif files.	- ROIs must be open. - No other ROIs in the image.	- The mean file size (controlled for image dimensions) can be a measurement of how different the patterns of two populations or species are in terms of randomness.	- Wilcoxon signed rank test

† These are only some examples of analyses that may be applied to the data. Other analyses may also be used depending on your study's specific aim.

PAT-GEOM Tools Package

Over the course of writing and testing PAT-GEOM, we encountered a few menial but tedious and repetitive tasks; and wrote macros to automate them. Just in case you run into the same tasks, we have included these macros (listed below) in PAT-GEOM, and describe what they do in the following sections.

Macro Listing:

- 1) Random Pixel Sampler
- 2) ROI Randomiser
- 3) ROI Renamer
- 4) ROI Detector (in development)

To use these macros:

- 1) Ensure that PAT-GEOM is installed.
- 2) In the ImageJ main window, click [Plugins>PAT-GEOM>Tools], and choose the desired macro.

Random Pixel Sampler

Purpose: randomly samples the pixel values in an area and outputs their coordinates and values to the ImageJ Results window.

Options: the user may select

- The area to sample from;
- The number of samples needed;
- The size of each sampling (e.g. each sampling may be a single pixel, or the average of a 10×10 box around the pixel);
- The slices to sample from (if there are multiple slices).

ROI Randomiser

Purpose: creates scaled and randomly-placed copies of a previously saved ROI (i.e., a .roi file) around the whole image or in a specified area of the image.

Options: the user may select

- The area to create the ROIs in;
- The number of ROIs to create;
- The amount of scaling to be done, e.g. if the ROI was created in a picture with a different pixel:length scale. All created ROIs will be scaled by this same amount.

ROI Renamer

Purpose: renames all the ROIs in the ROI Manager so that they are sequentially numbered.

Options: the user may select

- A desired prefix for the number;
- A desired postfix for the number;
- The number to start numbering from.

ROI Detector

Purpose: automatically draws an outline around a marking based on its difference in colour from the background. Also allows easy saving of ROIs with a customised name (circumventing the need to rename the saved ROIs). Note that this works better for clearly defined, contiguous markings that have a clearly distinct colour from their background.

Options: the user selects

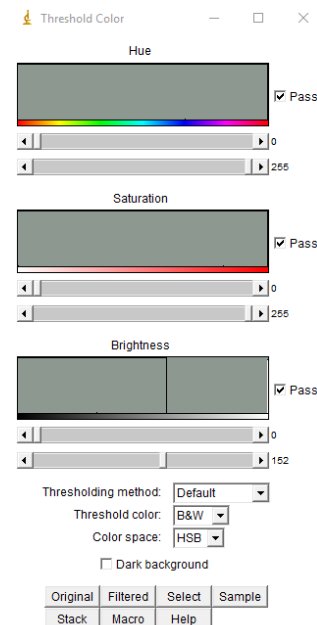
- The reference marking colour by clicking on a pixel within the marking;
- The tolerance value (a measure of how much the colour of a pixel can differ from the reference colour before it is no longer considered part of the marking).

Worked Examples

Example 1

This example demonstrates measuring Marking Shape, Shape Directionality, Size, Contrast, Distribution Directionality & Randomness of a cow's spots, also illustrating how to work with normal .jpeg images, threshold images and automatically generate Regions of Interest. The relevant files can be found in the "Example1" folder within the "User Guide.zip" file.

- 1) Start ImageJ.
- 2) Open the "Eg1_Image.jpg" file in ImageJ: "File>Open" OR drag and drop the file onto ImageJ).
- 3) Colour Threshold the image:
 - a. Select Image>Adjust>Color Threshold.
 - b. Choose these settings:
 - i. All "Pass" checkboxes: CHECKED.
 - ii. The Slider settings will automatically be set after step (vi) below and can be ignored.
 - iii. Thresholding method: "Default".
 - iv. Threshold color: "B&W".
 - v. Color space: "HSB".
 - vi. Dark Background: UNCHECKED (this should automatically set the values for the Sliders).



Checkpoint 1: At this point, the settings should be as shown in Fig. E1-1. There is no need to click any button to implement the threshold, it was being implemented in real time as you change settings. The image you now see is the thresholded image (clicking "Original" takes you back to the original).

Fig. E1-1. Colour threshold settings used to convert the image to a thresholded (i.e. purely black or white) image.

- c. Close the "Threshold" window.
- 4) Create Regions of Interest (ROIs) for each marking:
 - a. Select Analyze>Analyze Particles.
 - b. Choose these settings:
 - i. Size: "0-Infinity".
 - ii. Circularity: "0.00-1.00".
 - iii. Show: "Nothing".
 - iv. Exclude on edges: CHECKED.
 - v. Add to Manager: CHECKED.
 - vi. All other checkboxes: UNCHECKED.
 - c. Click "OK".

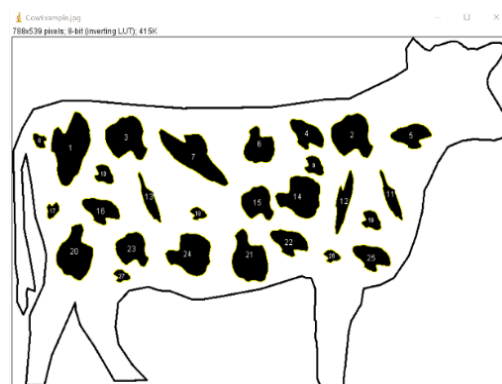


Fig. E1-2. The sample image (a cow) with each marking surrounded by a yellow outline, indicating Regions of Interest (ROIs) drawn automatically using the Analyze Particles function in ImageJ.

Checkpoint 2: You should now see yellow (the default colour) outlines surrounding each marking (Fig. E1-2). These are the ROIs which should have been automatically added to the ROI Manager (which should also have been automatically opened) and can be saved if required (see the section on “Saving regions of interest”). For your reference, these ROIs are also provided in the “Eg1_ROIs_Reference.zip” file.

- 5) Close the thresholded image.
- 6) Re-open the “Eg1_Image.jpg” file in ImageJ.

Note: ROIs are not tied to any particular image, so the ROIs you created using the thresholded image (which lost all its colour information) can now be applied to the original image (which still has its colour information).

- 7) Convert the image to an RGB Stack: click Image>Type>RGB Stack.

Checkpoint 3: The RGB image should now be a “stack” (i.e. three images stacked up on top of one another). Each image is now called a “slice” in the stack. Use the slider bar at the bottom to scroll through the three slices. The first slice represents pixel values from the Red channel, the second slice represents the green channel, and the third slice the Blue channel. This is a good way of preserving information from each channel and measuring them independently.

- 8) Open PAT-GEOM, select Plugins>PAT-GEOM>PAT-GEOM (note: if ImageJ tells you “Jython.jar” is not found at this point, click “OK” on the dialogue box to download it).
- 9) Measure Marking Shape:
 - a. Ensure the Elliptical Fourier Analysis plugin has been installed (see steps 5 and 6 in the “Installing PAT-GEOM” section).
 - b. In the new PAT-GEOM window: click “Elliptical Fourier Shape Analysis”.
 - c. In the dialogue window:
 - i. Number of descriptors, key in (as an example): “3”.
 - ii. Click: “OK” (a few windows will open and close rapidly at this point).
 - d. The Results window in Fig. E1-3A should appear, listing three descriptors for all 27 ROIs. The full results output can be found in the “Shape” tab of the “Eg1_Results.xlsx” file provided.
- 10) Measure Marking Shape Directionality:
 - a. In the PAT-GEOM window: click “Directionality of Shape”.
 - b. The Results window in Fig. E1-3B should appear, listing Aspect Ratio and Angle for all 27 ROIs. The full results output can be found in the “Shape Directionality” tab of the “Eg1_Results.xlsx” file provided.
- 11) Measure Marking Size:
 - a. In the PAT-GEOM window: click “Centroid Size”.
 - b. The Results window in Fig. E1-3C should appear, listing Centroid Size and Size in Square Pixels for all 27 ROIs. The full results output can be found in the “Size” tab of the “Eg1_Results.xlsx” file provided.
- 12) Measure Contrast:
 - a. In the PAT-GEOM window: click “Contrast CoV”.

- b. Select the following options:
 - i. “ROIs Individually”
 - ii. All three slices (i.e. “Red”, “Green” and “Blue”): CHECKED.
 - iii. Click “OK”.
 - c. The Results window in Fig. E1-3D should appear, listing the contrast measurement for the area defined by all the ROIs on each slice and also the Mean of the slices. The full results output can be found in the “Contrast” tab of the “Eg1_Results.xlsx” file provided.
- 13) Measure Distribution Directionality
 - a. In the PAT-GEOM window: click “Directionality of Distribution”.
 - b. The Results window in Fig. E1-3E should appear, listing the angle, alignment and equation of the line of best fit and a plot of the centroids of the markings in Fig. E1-3F. The full results output can be found in the “Distri Directionality” tab of the “Eg1_Results.xlsx” file provided.
- 14) Measure Randomness
 - a. In the PAT-GEOM window: click “Randomness Gif”.
 - b. Select:
 - i. In the drop-down menu: “ROIs”.
 - ii. All three slices (i.e. “Red”, “Green” and “Blue”): CHECKED.
 - iii. Do you want to keep the gif file(s) created: UNCHECKED.
 - iv. Click “OK”.
 - c. The Results window in Fig. E1-3G should appear, listing the Randomness for each ROI. The full results output can be found in the “Randomness” tab of the “Eg1_Results.xlsx” file provided. Note that if the intention was to compare the markings, they should first have been adjusted so that they are all equally sized.

Note: ImageJ can be buggy with intensive programmes like the Elliptical Fourier Analysis and Randomness macros. If an error occurs, save the ROIs (see section on “Saving Regions of Interest (optional)”), restart ImageJ, re-open the image and ROIs and redo the analysis.

End point: each of these measurements can be saved (see “Saving the data”). Data from multiple images (e.g. of animals from the same population) can be extracted in the same manner and used to run statistical analyses, such as t-tests, (generalised) linear models and PCAs.

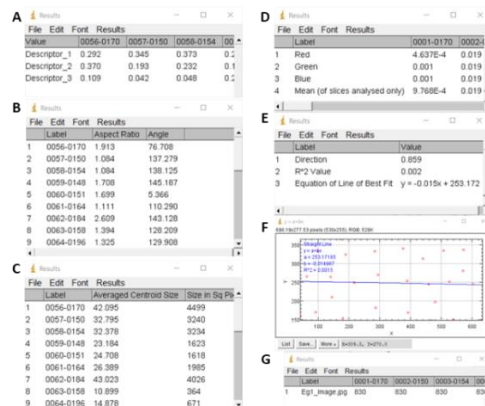


Fig. E1-3. The Result windows that will be output for:

- (A) Marking Shape (step 9),
- (B) Marking Shape Directionality (step 10),
- (C) Marking Size (step 11),
- (D) Contrast (step 12),
- (E & F) Distribution Directionality (step 13), and
- (G) Randomness (step 14).

Example 2

This example demonstrates measuring Marking Distribution in sea cucumbers, illustrating how to produce a heat map and cumulative matrix from multiple images. The relevant files can be found in the “Example2” folder within the “User Guide.zip” file.

- 1) Start ImageJ.
- 2) Open PAT-GEOM: click Plugins>PAT-GEOM>PAT-GEOM.
- 3) In the PAT-GEOM window: click “Marking Matrix”.
- 4) In the Options window:
 - a. Select “Whole Image”.
 - b. Select “Multiple Images”.
 - c. Output options:
 - i. “Individual Matrices”: UNCHECK.
 - ii. “Cumulative Matrix”: CHECK.
 - iii. “Heat Map”: CHECK.
 - d. Click “OK”.
- 5) In the next Options window:
 - a. Select “All images in a folder”.
 - b. Click “OK”.
- 6) Navigate to the “Example2” folder, click on it, and click “Select” (note: it needs to be extracted from the zip file to be detected by ImageJ).

Processing may take up to 2 minutes depending on your computer. When complete, a heat map (Fig. E2-1) will open (the heat map legend is also output separately). The following files will also be saved and should have now appeared in a subfolder of the Example 2 folder: the heat map (“all_images_Heat_Map.jpg”), the heat map legend (“all_images_Heat_Map_Legend.jpg”) and a cumulative matrix (“all_images_cumulative.xls”).

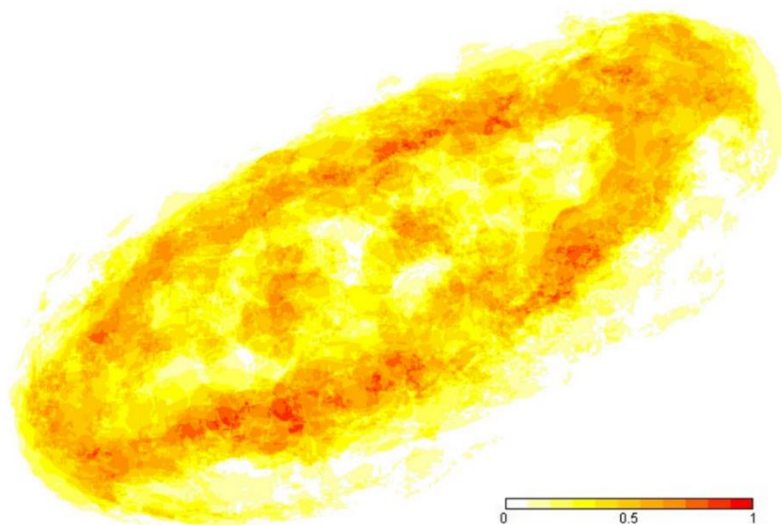


Fig. E2-1. Heat map of the 10 images in the Example 2 folder, output after step 6.

Example 3

This example demonstrates extracting markings from a thresholded crab carapace and measuring Marking Shape and Centroid Size. The relevant files can be found in the “Example3” folder within the “User Guide.zip” file.

- 1) Start ImageJ.
- 2) Open the “Eg3_Image_Thresholded.jpg” file in ImageJ (this image has been thresholded from the crab shown in Fig. E3-1 using Colour Thresholding, demonstrated in Example 1).
- 3) Open the “Eg3_carapace.roi” file by dragging and dropping it onto the main ImageJ menu window (NOT the image). This outlines the crab’s carapace.
- 4) Create ROIs for each marking on the carapace:
 - a. Select: Analyze>Analyze Particles.
 - b. Choose these settings:
 - i. Size: “0-Infinity”.
 - ii. Circularity: “0.00-1.00”.
 - iii. Show: “Nothing”.
 - iv. Exclude on edges: CHECKED.
 - v. Add to Manager: CHECKED.
 - vi. All other checkboxes: UNCHECKED.
 - c. Click “OK”.



Fig. E3-1. The original, non-thresholded image of the furrowed crab used in this example.

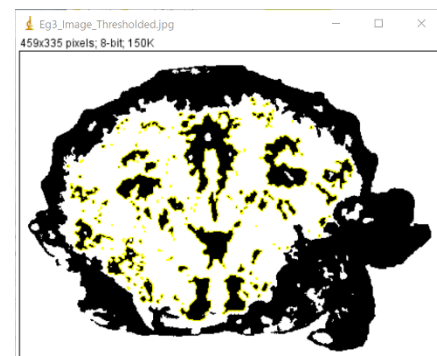


Fig. E3-2. The thresholded crab with its markings, detected using the Analyze Particles function in ImageJ, outlined in yellow.

Checkpoint 1: All the markings on the crab carapaces should now be outlined in yellow as shown in Fig. E3-2. These markings are also saved in the “Eg3_markings.zip” file.

- 5) Measure Marking Shape:
 - a. Ensure the Elliptical Fourier Analysis plugin has been installed (see steps 5 and 6 in the “Installing PAT-GEOM” section).
 - b. In the new PAT-GEOM window: click “Elliptical Fourier Shape Analysis”.
 - c. In the dialogue window:
 - i. Number of descriptors, key in (as an example): “5”.
 - ii. Click: “OK” (a few windows will open and close rapidly at this point).
 - d. The Results window in Fig. E3-3A should appear, listing five descriptors for all 146 ROIs. The full results output can be found in the “Shape” tab of the “Eg3_Results.xlsx” file provided.
- 6) Measure Marking Size:
 - a. In the PAT-GEOM window: click “Centroid Size”.
 - b. The Results window in Fig. E3-3B should appear, listing Centroid Size and Size in Square Pixels for all 146 ROIs. The full results output can be found in the “Size” tab of the “Eg3_Results.xlsx” file provided.

End point: this demonstrates how Marking Shape and Marking Size measurements can be taken for a single organism. This can be used, for example, to compare two populations of organisms by measuring more individuals and visualising the data, e.g. with PCA for Marking Shape and with boxplots for Marking Size.

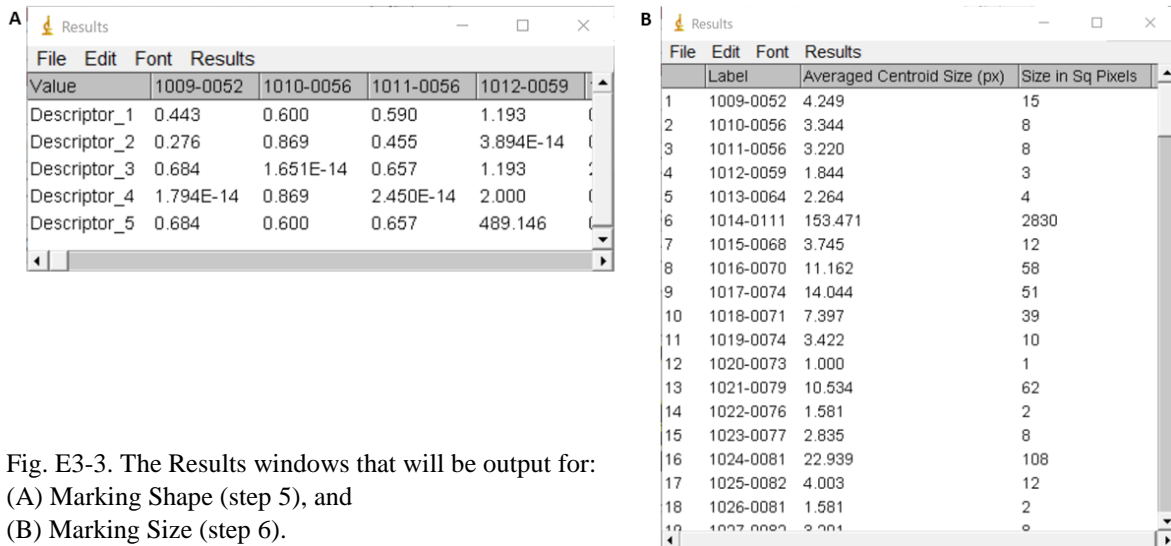


Fig. E3-3. The Results windows that will be output for:
 (A) Marking Shape (step 5), and
 (B) Marking Size (step 6).

FAQs

Questions

- 1) Why can't I get the Marking Shape macro to work?
 - 2) How can I trust the data that PAT-GEOM produces?
 - 3) What are slices and how does PAT-GEOM handle images with multiple slices?
 - 4) How do I pre-process my images (e.g. filtering to sharpen them) before running PAT-GEOM? Should I?
 - 5) Can I use a normal image with PAT-GEOM, for example a .jpg?
 - 6) What is thresholding and how do I threshold my images?
 - 7) How do I make all my images the same size?
 - 8) I'm not familiar with ImageJ or FIJI, what do I need to know to run PAT-GEOM?
 - 9) How do I uninstall ImageJ/PAT-GEOM?
 - 10) Will PAT-GEOM work with ImageJ2?
-

1) Why can't I get the Marking Shape macro to work?

The most common reason is because the Elliptical Fourier Analysis plugin by Boudier & Tupper (2016) has not been installed. You must first download the "fourier2.5_.jar" file from http://imagejdocu.tudor.lu/doku.php?id=plugin:analysis:fourier_shape_analysis:start, place it in the ImageJ Plugins folder and click [Help>Refresh Menus] or reopen ImageJ.

2) How can I trust the data that PAT-GEOM produces?

The analyses performed in PAT-GEOM use established measurement techniques from published papers and we have been careful to test the programs on datasets. The program has also been published in [as of 2 Dec 2018, PAT-GEOM has been accepted for publication by *Methods in Ecology and Evolution*, this information will be updated when a citation and doi become available].

3) What are slices and how does PAT-GEOM handle images with multiple slices?

Some images types in ImageJ are made up of multiple slices stacked on top of each other, e.g. RGB Stack files have three slices (one each for the red, blue and green channels) and .mspec files are designed to have five (visible red, blue and green, and UV blue and red). There are two ways to measure images with slices.

The first is to measure each slice individually and then calculate the average of the slices from the data. For images with multiple slices, PAT-GEOM allows you to select the slices you want to

analyse and then outputs the measurement(s) for each slice and the average for the slices measured.

The second is to average the pixel values in the different slices to first form a new image, and then measure that “average image”. For example, converting an RGB Color image to an 8-bit image using ImageJ’s file type converter (Image>Type>8-bit) will average the red, green and blue pixel values. This can be done using ImageJ’s built-in type converter.

ImageJ’s built-in file type converter

- 1) Open the image to be converted.
- 2) Click [Image>Type] and choose the file type to convert the image to.
Note: it is often most useful to convert images with multiple slices to 8-bit. Guides on allowed conversions and other settings (using and adjusting weighted averages) can be found at [<https://imagej.nih.gov/ij/docs/guide/146-7.html>].

4) How do I pre-process my images before running PAT-GEOM? Should I?

A good introduction to pre-processing can be found here: [http://imagej.net/Particle_Analysis]. PAT-GEOM can certainly work with pre-processed images, however, it is difficult to give a universally applicable yes/no answer. In some cases, pre-processing might make the images clearer, but it is difficult to judge the effects this may have on your data. Hence, we suggest erring on the side of caution and recommend that you do not pre-process your images.

5) Can I use a normal image with PAT-GEOM, for example a .jpg?

Yes you can. Simply open it up in ImageJ where most “normal images” will be opened as an RGB image. We suggest that each channel (i.e. Red, Blue and Green) is analysed separately, and this can be done by converting the RGB image to an RGB Stack using the Image>Type>RGB Stack function (Worked Examples, Example 1, step 7).

6) What is thresholding and how do I threshold my images?

Steps to threshold images are covered in the section on “Creating regions of interest” and an example is shown in “Worked Examples, Example 1, step 3). In summary, thresholding converts a colour or grayscale image to a binary black-or-white image (e.g. all pixels above a certain cut-off value (i.e. threshold) set by you are changed to black and all pixels below it are changed to white, or vice versa) in order to identify regions of interest. Broadly, three types of thresholding can be done in ImageJ:

- Global greyscale thresholding: this is applied to greyscale images where all the pixels take a value of 0 to 255 (i.e. it only works on 8-bit images). The cut-off value is a value between 0 to 255 and this same cut-off is used throughout the entire image.

- Global colour thresholding: this is applied to colour images that have different RGB channels on each pixel. The cut-off value is a colour (which can be defined, e.g., as an RGB or HSB value) and this is used throughout the entire image.
- Local greyscale thresholding: this is applied to greyscale images where all the pixels take a value of 0 to 255 (i.e. it only works on 8-bit images). The cut-off value is a value between 0 to 255 but the cut-off used on each pixel depends on the value of the other pixels around it. For example, it could be the average pixel value of all the pixels less than 5 pixels away. The user can adjust these parameters as necessary.

Thresholding is a complex subject that is still being developed and it would not be possible to cover all aspects of it in this short FAQ. Guides are available online, and a good starting point can be found at [<http://occm.otago.ac.nz/resources/ImageJ-Thresholding.pdf>] and the ImageJ documentation.

7) How do I make all my images the same size?

Use the Image>Scale function in ImageJ. This will stretch/compress your image to any size you wish.

8) I'm not familiar with ImageJ or FIJI, what do I need to know to run PAT-GEOM?

ImageJ is a powerful image analysis software that allows you to open many different types of images, indicate areas that you want to measure (known as regions of interest or ROIs), perform many different types of image processing (e.g. rotating, resizing, rescaling, changing formats, thresholding, watershed analysis and filtering) and take many different measurements. Its strength is its graphic user interface and flexibility: the community can contribute plugins to expand on ImageJ's built-in functions. FIJI, for example, is simply ImageJ packaged together with some plugins that have been found to be very useful.

To run PAT-GEOM, you need to be familiar with only some basic tools in ImageJ, and these are introduced when they are needed as you progress along the step-by-step instructions in this User Guide: installing ImageJ (page 3), installing plugins and macros within ImageJ (page 4), opening images (page 7), scaling and resizing images (pages 12 and 26), using selection tools to create regions of interest (ROIs; page 7), saving and re-opening ROIs (pages 7, 11–12), various thresholding and marking detection techniques (pages 8–10, 12–13 and 25–26), working with Slices (page 24–25) and saving data from the Results window (pages 13–14).

If you would like to find out more about what ImageJ can do, there is comprehensive documentation online at the following links:

- Basic concepts: [<https://imagej.nih.gov/ij/docs/concepts.html>].
- Tutorials and Examples: [<https://imagej.nih.gov/ij/docs/examples/index.html>].
- Installation instructions and known issues for Linux, macOS and Windows: [<https://imagej.nih.gov/ij/docs/install/index.html>].

- Full PDF user guide: [<https://imagej.nih.gov/ij/docs/guide/user-guide.pdf>].
- Online user guide: [<https://imagej.nih.gov/ij/docs/guide/>].
- Forum, FAQs and other resources: [<https://imagej.nih.gov/ij/docs/index.html>].

9) How do I uninstall ImageJ/PAT-GEOM?

Simply delete the ImageJ/PAT-GEOM folders and zip files. No uninstallation process is needed.

10) Will PAT-GEOM work with ImageJ2?

Yes. The ImageJ developers have assured users that one of ImageJ2's Mission Statements is to "Maintain backwards compatibility with existing ImageJ functionality".

Further Reading

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