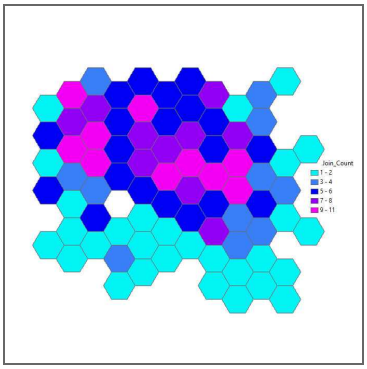

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Geographic Information Systems (GIS)-based spatial analysis of cell distribution V.2

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ABSTRACT

This protocol describes how to perform a Geographic Information System (GIS)-based spatial analysis of cerebellar images focused on the distribution of the Purkinje neurons. It can be used for any biological images to study cellular or molecular spatial distributions, or, more generally, the distribution of any biological feature of interest.
It is based on using ESRI ArcMap to calculate several indexes of *geographical distribution* (**Central Feature**, **Mean Center**, **Median Center**, **Directional Distribution**, and **Standard Distance**), *pattern distribution* (**Average Nearest Neighbor**, **Getis-Ord General G**, **Ripley's K function**, and **Global Moran's I**), and *clusters mapping* (**Anselin Local Moran's I**, and **Getis-Ord G***). It is also shown how to represent the features' distribution graphically with different symbologies.

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We use this protocol and it's working

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Keywords: GIS-based spatial analysis, Average Nearest Neighbor, Spatial Autocorrelation (Global Moran's I), Multi-distance Spatial Cluster Analysis (Ripley's K Function), High/Low Clustering (G tool), Anselin Local Moran's I, Getis-Ord G*, Cerebellum, Purkinje neurons, Development, Organotypic cultures

GUIDELINES

It may be useful to read articles in which ArcGIS has been applied to the study of biological samples, e.g.
https://www.frontiersin.org/articles/10.3389/fevo.2021.642255/full

MATERIALS

Software

ImageJ (Fiji)	NAME
Windows 10	OS
National Institutes of Health (USA)	DEVELOPER
https://imagej.net/software/fiji/downloads	SOURCE LINK

Software

Arc GIS	NAME
Windows 10	OS
ESRI	DEVELOPER

SAFETY WARNINGS

Not applicable

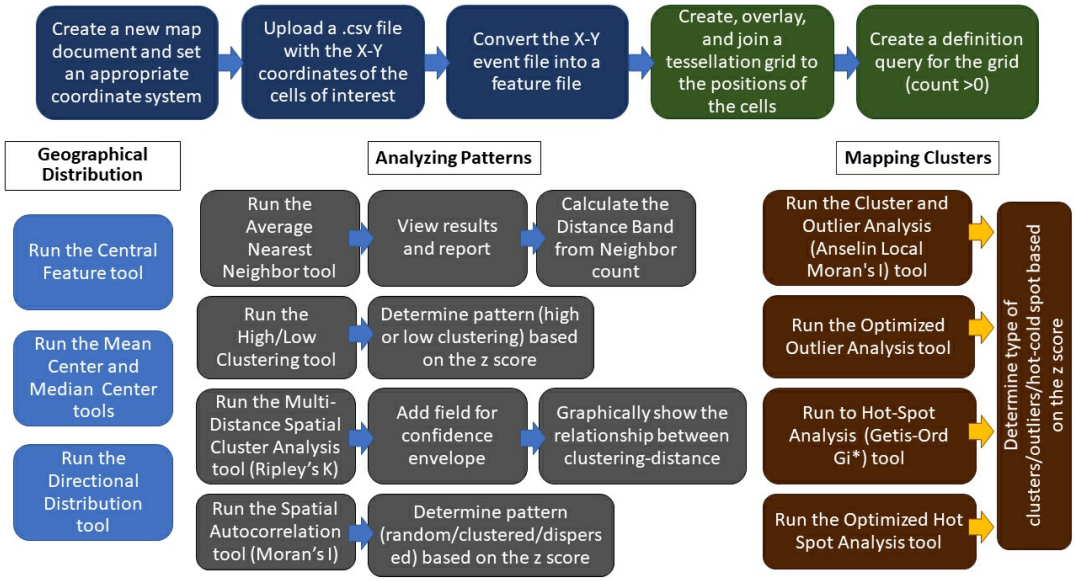
ETHICS STATEMENT

Not applicable

BEFORE START INSTRUCTIONS

Familiarize yourself with the basics of spatial statistics.

GIS-based technologies are used to store, view, analyze, and interpret geographic data. The location of features
(i.e. the objects of interest in a study) is determined by geographic data, often called spatial or geospatial data. As microscopic images can be represented in an X-Y Cartesian coordinate system, GIS can perform spatial analysis of specific biological features, i.e. the positions of the cerebellar Purkinje neurons as described in this protocol. A flowchart of the steps of GIS-based analysis is shown in the Figure below.



Flowchart of the main steps of GIS-based analysis with ArcMap. The top row blocks show the preliminary steps to create a map starting from the X-Y coordinates of the PNs (blue blocks) and the steps of tessellation and joining of Purkinje neuron numbers to tessellated areas (green blocks). The other blocks show the main steps in the use of Geographic Distribution (light blue blocks),

Analyzing Patterns (gray blocks), and Mapping Clusters (brown blocks) tools in ArcMap.

Note

It is possible to use other GIS-based software, e.g. the open-source QGIS (https://www.qgis.org).

Calculation of cells X-Y coordinates

1 Open the image to be analyzed using ***Fiji***: **File → Open**

Note

All images should be of the *same pixel size* and *magnification* if one wants to further compare the analysis results of individual images within a single experimental group and/or between groups.

2 Set the appropriate scale for the image using **Analyze → Set Scale**. In the pop-up window report the distance in pixels related to the known distance using the correct unit of length (µM). Leave the pixel aspect ratio at 1.0.

Note

It is important to set the appropriate scales of the images because it may be possible that not all images are acquired at the same magnification or with the same microscope. Set the unit of length in microns (µm) in the Fiji dialog window.
The ArcMap Coordinate System dialog window allows you to add a new **customized coordinate system** but does not permit you to set the Linear Unit in microns. Setting the Linear Unit of the Microscope Coordinate System in Millimeters is advisable. With these settings, the output of the ArcMap elaborations is nominally in millimeters, but actually in microns.

3 Use the **Multipoint tool** and click with the mouse on the center of each labeled cell. The tool should be configured so that clicked cells are visualized directly on the image. To do so double-click with the mouse on the tool icon and tick the **Label points** box.

4 Set the measurements to be computed using **Analyze → Set Measurements**. In the pop-up window verify that all boxes are not ticked. Set the **Decimal places** box to 3. Use **Analyze → Measure** to calculate the X-Y coordinates. A new window pops up where the results are shown in tabular form.

5 Save data as a **.csv file**.

Initial image elaboration with ArcMap

6 Load all .csv files to an *ad hoc* folder in ArcMap.

7 Open the program and create a new map document: **File → New → New Maps → My Templates → Blank Map**.

8 On the ribbon click **View → Data Frame Properties**. In the pop-up window click **Coordinate System**. Click on the *world icon* and select **New → Projected Coordinate System**. For *Name* type

the name of the new coordinate system e.g. *Microscope Coordinate System*. For *Linear Unit Name* choose *Millimeter.* Leave all other parameters unchanged. Save the new coordinate system: the program creates a new folder named *Custom that contains* the file *Microscope_Coordinate_System*.

Note

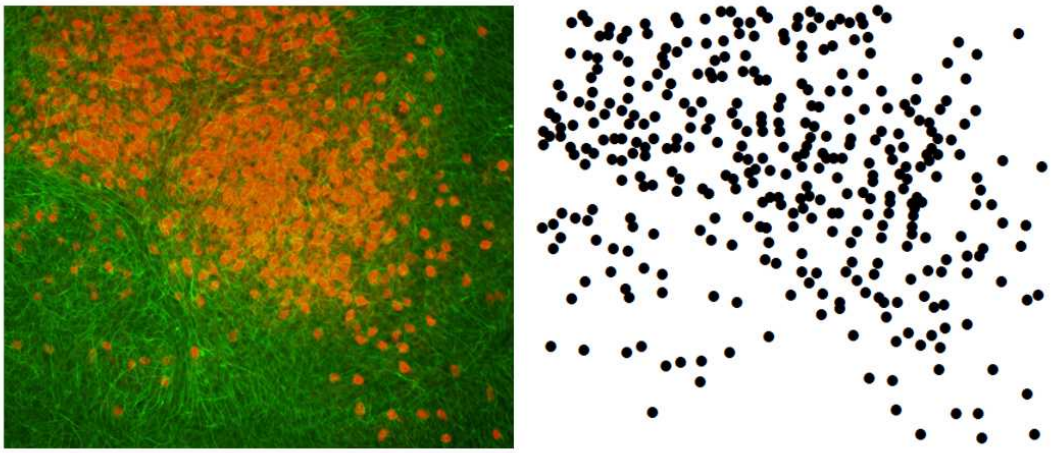
There may be differences in the way the coordinate system is displayed according to the version in use of the software. Be sure that the following parameters are applied:
Projection: Transverse_Mercator
False_Easting: 0.0
False_Northing: 0.0
Central_Meridian: 0.0
Scale_Factor: 1.0
Latitude_Of_Origin: 0.0
Linear Unit: Millimeter (0.001)

9 • Save the *Blank Map* file and name it with the name of the image under investigation, e.g. Image1. The program saves a file named **Image1.mdx**

10 Add the X-Y coordinates of the cells to the map. On the toolbar click the ***Add data icon*** then → **Add Data**. Choose the .csv file with the X-Y coordinates of the cells and upload it. The program creates a new layer on the map with the same name as the .csv file and an *attribute table* containing the cell coordinates. Right-click with the mouse on the new layer and choose **Display XY Data**. In the pop-up window, *be sure that the X and Y fields for the layer correspond to the fields of X and Y coordinates in your .csv file*, and press the **OK** button. A window appears with the warning *Table Does Not Have Object-ID Field*. This is because the layer created so far is an X-Y *event layer* that must be converted into a *feature layer* for further analysis. Press the **OK** button and the positions of the cells will be displayed as in the figure below.

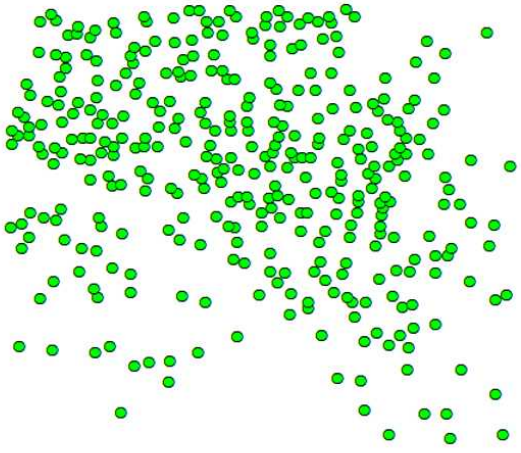
Note

As far as one adds information to the map, the software creates layers whose characteristics depend on the source of the information. Layers can be visualized in the ***Table of content*** window of the program.
The layers created from tabular data such as .csv files containing objects (cells) coordinates are saved as *Event layers*. In ArcMap, ***Event layers*** are based on tables w*ithout ObjectID fields* and cannot be edited in ArcGIS.



Left: Original image of a cerebellar slice from a mutant mouse (*Reeler*) after labeling of the Purkinje neurons (*see* Merighi A and Lossi L. Co-cultures of cerebellar slices from mice with different *reelin* genetic backgrounds as a model to study cortical lamination [version 1; peer review: 2 approved with reservations]. *F1000Research* 2022, **11**:1183 (https://doi.org/10.12688/f1000research.126787.1). ***Right***: X-Y coordinates of the labeled Purkinje neurons as they appear in the data view window of ArcMap. Cells are displayed as black dots but one can choose any type of symbology for their visualization.

11 Convert the X-Y event layer into a feature layer. On the layer right-click → **Data** → **Export Data** → **All features**. Select *Use the same coordinate system as this layer source data* and press **OK**. The program generates a new layer named *Export_Output_#*. By double-clicking with the mouse on the layer name, the *Layer Properties* window opens and it is possible to customize the data by e.g. changing the layer name and using a different symbology to display the cells.



X-Y coordinates of the labeled Purkinje neurons as they appear in the data view window of ArcMap after conversion of the X-Y event layer into a ***Feature layer***. A different symbology has been used to recognize the layer in the map more easily. Features (the objects in the layer, i.e. the labeled Purkinje neurons) can then be processed for further analysis.

Visualization of cell counts and preliminary steps for subsequ...

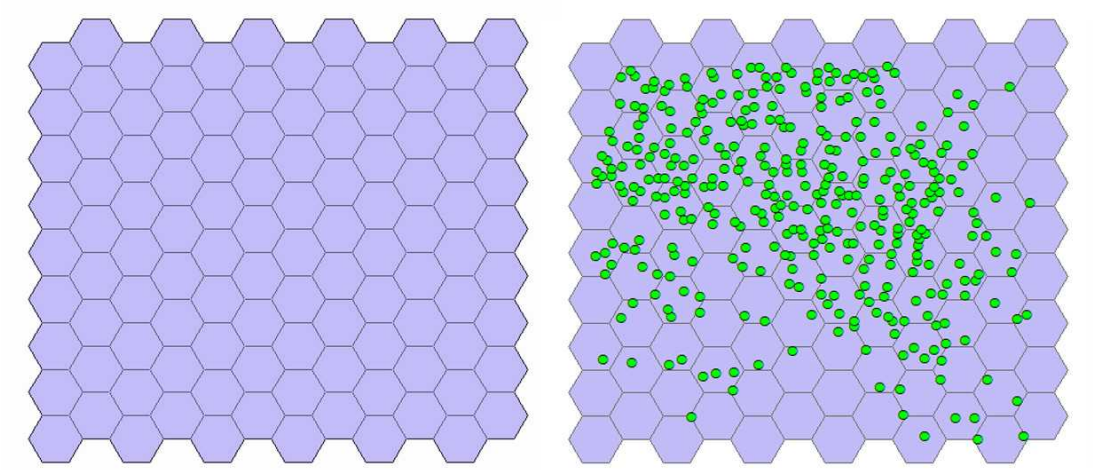
12 ***Graphical visualization of cell counts***

By this approach, it is possible to display graphically on the map a series of polygons (tessellation) over the labeled cells and to join the cell counts to each polygon so that a graphic display of the cell distribution is obtained.

12.1 Generate tessellation. With the mouse select the Export_Output # layer. On the toolbar select the ***Arc Toolbox icon*** then → ***DataManagementTools*** → ***Sampling*** → ***GenerateTessellation***. This tool generates a polygon feature class of a tessellated grid of regular polygons which will entirely cover a given extent. In the pop-up window leave unchanged the path of the Output Feature Class. For Extent click on the folder icon and choose ***Same as layer Export Output #***. Selection of the shape type is optional. Check that HEXAGON is selected by the program. The program creates a new layer named ***Generate Tessellation #*** and the tessellation appears above the cells (see figure below).

Note

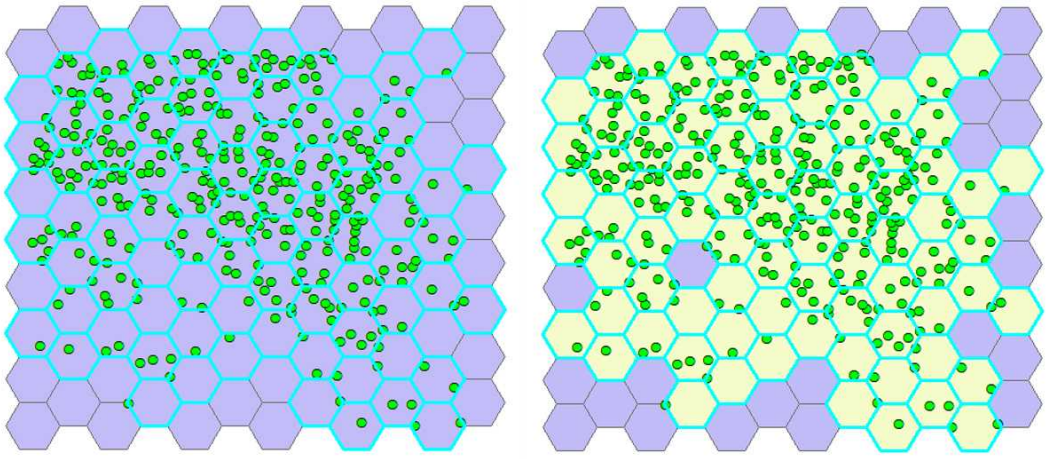
By selecting ***Same as layer Export Output #*** for the Extent of the tessellation, one adjusts the size of the tessellation to that occupied by the cells.



Left: A hexagon tesselation is generated to cover the entire area of the distribution of the cells. ***Right***: combined view of the hexagon tessellation layer and the Export Output layer with cell coordinates.

12.2 Select hexagons with cells. From the ribbon click ***Selection → Selectbylocation***. In the pop-up window, for Target layer(s) select ***Generate tessellation #***, for the Source layer select **Export_Output #**, and for the Spatial selection method for target layer feature(s) choose ***Intersect the source layer feature***. Click OK. The hexagons containing cells are highlighted (see the figure below at left).

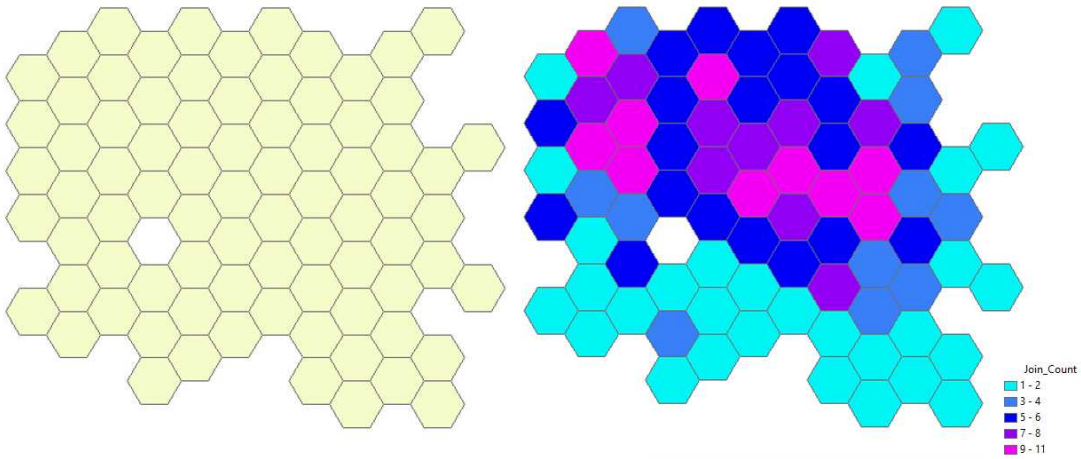
12.3 Join cell positions to selected hexagons. Select the ***Arc Toolbox icon*** on the toolbar→ ***AnalysisTools → Overlay → SpatialJoin***. In the pop-up window, for Target features select ***Generate Tessellation #***, for Join Features select ***Export_Output #***. The program creates a new layer named ***Export_Output #*** **SpatialJoin#** with the hexagons containing cells visualized in a different color than those with no cells (see the figure below at right).



Left: Hexagons containing cells are selected and highlighted in cyan. ***Right***: Cell positions

are joined to the selected hexagons.

12.4 Graphical visualization of the cell counts on the map. Remove the layer ***Generate Tessellation #*** from the map (right-click with the mouse) and turn off the visibility of the layer ***Export Output #***. Only the hexagons with cells remain visible (see the figure below at left). With the mouse right-click on layer → ***Properties*** → ***Symbology*** → ***Show quantities*** → ***Select color ramp*** (e.g. cyan-to-purple) → ***Fields***: ***Value = Join-Count***; ***Normalization = none***. Hexagons on the map are displayed in different colors according to the number of cells that they contain (see the figure below at right).



Left: The cell-containing hexagons are displayed with underlying information on the number of cells in each hexagon (not visible yet). ***Right***: application of a color ramp (cyan-to-purple) to display hexagons containing different numbers of cells in different colors. The color legend is displayed at the bottom right.

Note

Most tools used for spatial analysis require entering an ***Input Field***. In our analysis, after applying the *Spatial Join* tool, this field reports the number of Purkinje neurons per tessellation hexagon. For the analysis of spatial clustering, it is of interest to analyze the density of the Purkinje neurons (number/area) and not their absolute numbers.

Note

For every session of use the program numbers progressively the operations done and the map layers. Layers can be renamed by opening the *Layer Properties* window (mouse right-click) and then selecting *General*.

Analysis of the geographic distribution of the Purkinje neurons

13

Use the tools listed in the table below to analyze some characteristics of the distribution of the Purkinje neurons. These tools are used to calculate a value that represents certain characteristics of
the distribution of the objects under study, such as the center, compactness, or orientation. The *Central Feature tool* identifies the most centrally located feature (i.e. a Purkinje neuron), the *Mean Center tool* identifies the geographic center (or the center of concentration) for the set of Purkinje Neurons, and the *Median Center tool* identifies the location that minimizes the overall Euclidean distance of the Purkinje Neurons. The *Standard Distance tool* measures the degree to which features are concentrated or dispersed around the geometric mean center. The *Directional Distribution tool* creates standard deviational ellipses or ellipsoids to summarize the spatial trend in the distribution of the Purkinje Neurons.

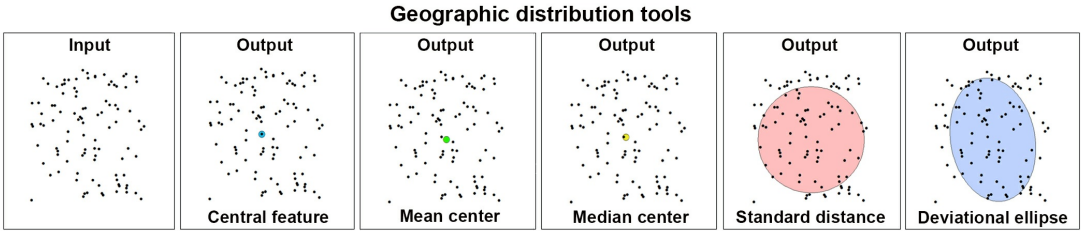
	A	B	C	D	E
	Tools	Input Feature Class	Output Feature Class	Distance Method	Ellipse or Circle Size
	Central Feature	See Step 11 above	Export_Output_# Central Feat	EUCLIDEAN DISTANCE	Not Applicable
	Mean Center		Export_Output_#MeanCenter	Not Applicable	
	Median Center		Export_Output_#Median Center		
	Directional Distribution (Deviational Ellipse)		Export_Output_#Directional		1 STANDARD DEVIATION
	Standard Distance		Export_Output_#StandardDis		

Steps and settings in the use of the Measuring Geographic Distributions tools of ArcMap

Note

If the image scale and the coordinate system are set as indicated above (Step 8) the tool output will be indicated in *millimeters* but it will correspond to *microns*.

The image below shows the results of these tools applied to a set of 100 randomly generated points (cells) in the 2D space.



Geographic distribution tools are applied to a set of 100 randomly generated points (i.e. the centers of mass of 100 cells). The central feature is indicated by the light blue circle, the mean center by the green circle, the median center by the yellow circle, the standard distance is represented by the pink circle, and the deviational ellipse is shown in light blue.

Analysis of the pattern of Purkinje neuron distribution

14 *Average Nearest Neighbor*

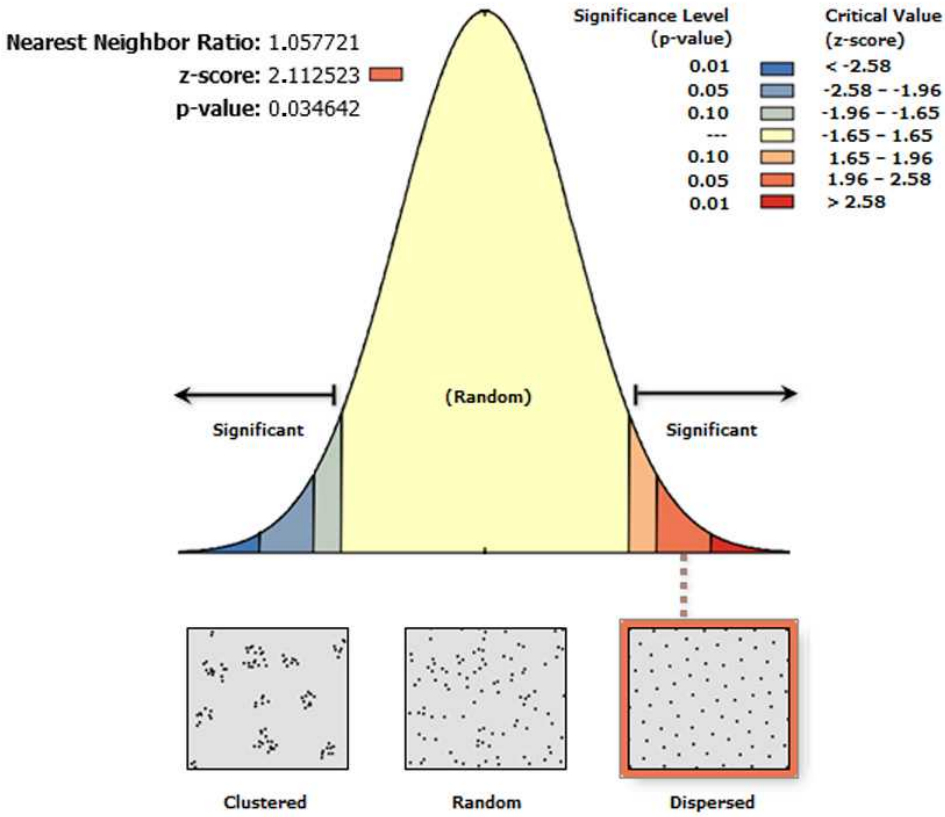
The Average Nearest Neighbor tool calculates the nearest neighbor index based on the average distance from each feature (i.e. a labeled Purkinje neuron) to its nearest neighboring feature. The tool returns five values: *Observed Mean Distance (OMD)*, *Expected Mean Distance (EMD)*, *Nearest Neighbor Index (R)*, *z-score*, and *p-value*. The values are written as messages at the bottom of the ***Geoprocessing pane*** during tool execution and passed as derived output values. With the **Generate Report** box ticked the tool produces an HTML report file with a graphical summary of the results (see figure below).
The nearest neighbor ratio (R) is the ratio between the OMD and EMD among the feature(s) of interest. EMD is the mean distance between the Purkinje neurons calculated on a hypothetical random distribution of the same number of cells covering the same total area. The value of R = 1 indicates randomness; R = 0 indicates maximum aggregation; and R = 2.149 indicates maximum possible dispersion.

Note

If the image scale and the coordinate system are set as indicated above (Step 8) the tool output will be indicated in *millimeters* but it will correspond to *microns*.

14.1 On the toolbar select the ***Arc Toolbox icon*** then → ***Spatial Statistics Tools*** → ***Analyzing Patterns*** → ***Average Nearest Neighbor***.

14.2 In the pop-up windows for *Input Feature Class* select ***Export Output #***, for *Distance Method*, select ***EUCLIDEAN DISTANCE***, and tick the box ***Generate Report***.



Graphic report generated by ArcMap as an output of the ***Average Nearest Neighbor tool***. Given the z-score of 2.11252332149, there is a less than 5% likelihood that this dispersed pattern could result from random chance.

15 ***High/Low Clustering (G tool)***

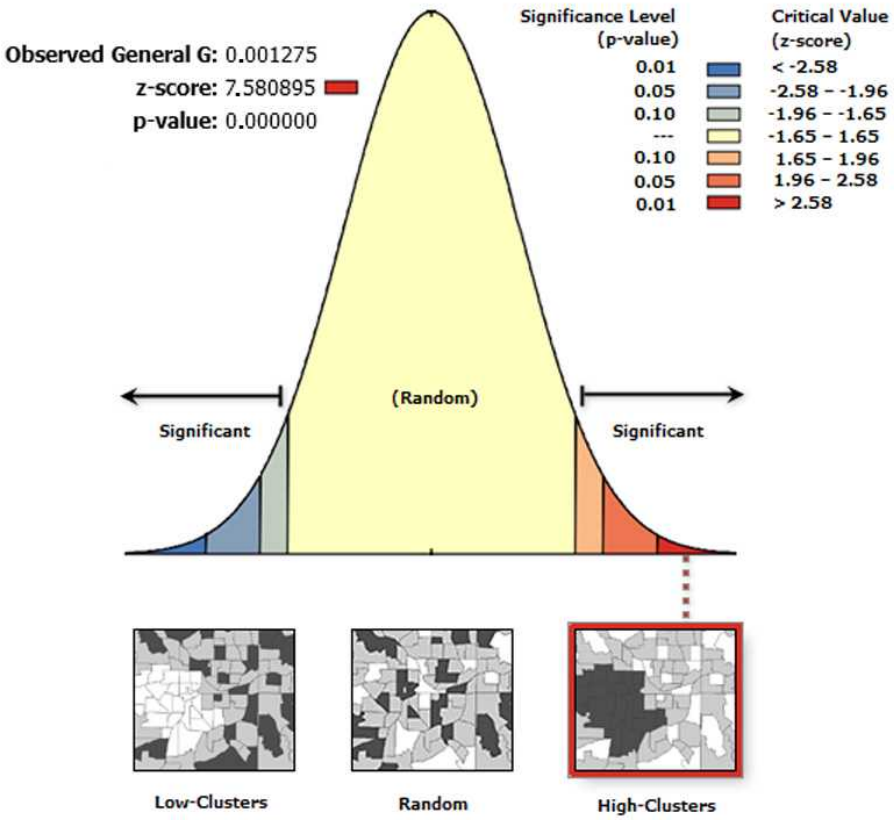
The High/Low Clustering tool measures the degree of clustering for either high or low values using the *Getis-Ord General G statistic*. The High/Low Clustering tool returns four values: *Observed General G*, *Expected General G*, *z-score*, and *p-value*. The values are written as messages at the bottom of the ***Geoprocessing pane*** during tool execution and passed as derived output values. With the **Generate Report** box ticked the tool generates an HTML report file with a graphical summary of results.

Note

If the image scale and the coordinate system are set as indicated above the tool output will be indicated in *millimeters* but it will correspond to *microns*.

15.1 On the toolbar select the ***Arc Toolbox icon*** then → ***Spatial Statistics Tools*** → ***Analyzing Patterns*** → ***High/Low Clustering (Getis-Ord General G)***

15.2 In the pop-up windows for *Input Feature Class* select ***Export Output #***, for *Input Field* select **XM**, for *Conceptualization of Spatial Relationship* select ***INVERSE DISTANCE***, for *Distance Method*, select ***EUCLIDEAN DISTANCE***, for *Standardization* select ***NONE***, and tick the box ***Generate Report***.



Graphic report generated by ArcMap as an output of the ***High/Low Clustering tool (G tool)***. Given the z-score of 7.58089500969, there is a less than 1% likelihood that this high-clustered pattern could result from random chance.

16 Spatial Autocorrelation (Global Moran's I)

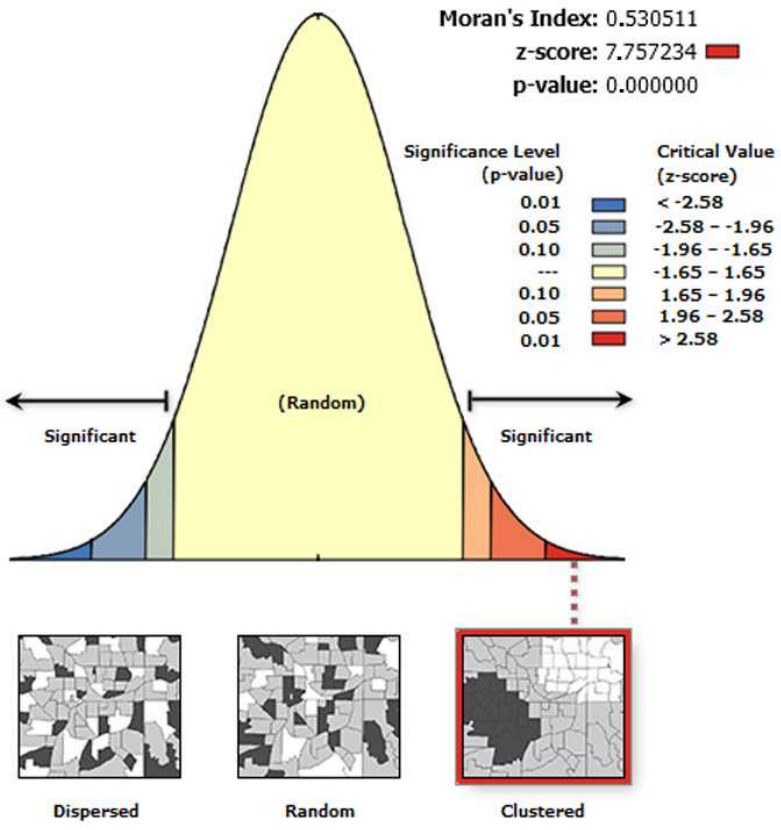
The Spatial Autocorrelation (Global Moran's I) tool measures spatial **autocorrelation based on feature locations and attribute values** using the Global Moran's I statistic.
The tool returns five values: *Moran's I Index*, *Expected Index*, *Variance*, *z-score*, and *p-value.* The values are written as messages at the bottom of the ***Geoprocessing pane*** during tool execution and passed as derived output values. With the **Generate Report** box ticked the tool produces an HTML report file with a graphical summary of results (see figure below).
Moran's I index is a measure of spatial autocorrelation that can vary from -1 to 1, with 0 indicating perfect randomness. When the p-value or z-score indicates statistical significance, a positive Moran's I index value indicates a tendency toward clustering, while a negative Moran's I index value indicates a tendency toward dispersion.

16.1 On the toolbar select the ***Arc Toolbox icon*** then → ***Spatial Statistics Tools*** → ***Analyzing Patterns*** → ***Spatial Autocorrelation (Global Moran's I)***.

16.2 In the pop-up windows for *Input Feature Class* select ***GenerateTessellation#Spati#***, for Input Field select ***Join-Count,*** for *Conceptualization of Spatial Relationship* select ***INVERSE_DISTANCE***, for *Distance Method* select ***EUCLIDEAN DISTANCE***, for *STANDARDIZATION* select ***ROW***. Tick ***Generate Report*** and click **OK**.

16.3 After the tool has run, no layer is added to the map but a report is generated. To view the report in the ribbon, click ***Geoprocessing*** → ***Results***.

16.4 In the results list, expand the Spatial Autocorrelation (Moran's I) folder and click on the Report File to view the report in the browser window (see figure below). The report file is automatically saved as a .html file in the ArcGIS folder of the computer.



Graphic report generated by ArcMap as an output of the ***Spatial Autocorrelation (Global Moran's I) tool***. Given the z-score of 7.75723418722, there is a less than 1% likelihood that this clustered pattern could result from random chance.

17 Multi-Distance Spatial Cluster Analysis (Ripley's K Function)

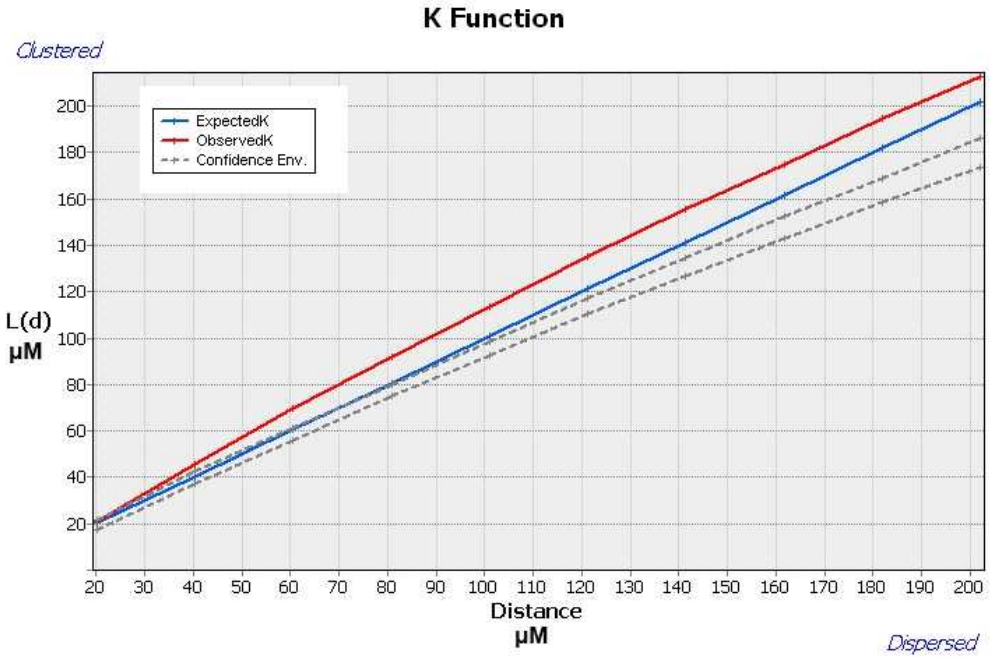
The Multi-Distance Spatial Cluster Analysis (Ripley's K Function) determines whether features or the values associated with features exhibit statistically significant **clustering or dispersion over a range of distances**. The tool output is a table with two fields: *ExpectedK* and *ObservedK* containing the expected and observed K values, respectively. Because the L(d) transformation is applied, the ExpectedK values will always match the Distance value. A field named *DiffK* contains the Observed K values minus the Expected K values. As a confidence interval option is specified, two additional fields named *LwConfEnv* and *HiConfEnv* will be included in the *Output Table*.

17.1 On the toolbar select the ***Arc Toolbox icon*** then → ***Spatial Statistics Tools*** → ***Analyzing Patterns*** → ***Multi-distance spatial cluster analysis (Ripley's K function)***.

17.2 In the pop-up windows for *Input Feature Class* select ***Export Output #*** for *Output Table* leave the program generated name (***Export Output # MultiDistan)***, for *Compute Confidence Envelope (optional)* choose ***99_PERMUTATIONS***, tick the box ***Display Results Graphically***.

Note

If the image scale and the coordinate system are set as indicated above the tool output will be indicated in *millimeters* but it will correspond to *microns*.
The graph should be exported in JPEG format at a size of 900x510 pixels if used for publication. In the Options window select: Quality 100% and 300 DPI.



Graphic generated by ArcMap as an output of the ***Multi-distance spatial cluster analysis (Ripley's K function) tool***. The observed K value (red line) is larger than the expected K value (blu line) for all distances. Therefore, the distribution is more clustered than a random distribution at the scale of analysis. As the observed K value is larger than the upper confidence envelope value (upper gray dashed line) all along the distance interval computed, spatial clustering for that interval is statistically significant.

Mapping Purkinje neurons clusters

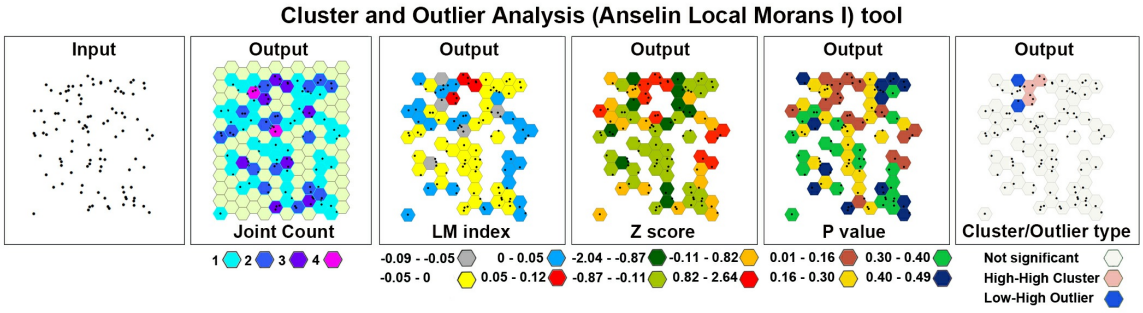
18 After the tool has run, a new layer is added to the map displaying in different colors statistically significant clusters and outliers for a 95 percent confidence level based on the local I index

19 **Cluster and Outlier Analysis (Anselin Local Moran's I)**

The Cluster and Outlier Analysis tool identifies spatial clusters of Purkinje neurons, with high or low values. The tool also identifies spatial outliers. The *Cluster and Outlier Analysis (Anselin Local Moran's I) tool* and the *Optimized Outer Outlier Analysis* tool (see Step 19) calculate a local Moran's I value (LM index the figure), a z-score, a pseudo-p-value, and a COType (Cluster/Outlier

Type) field in the Output Feature Class (map layer) that identifies the cluster with a color code (last panel on the right) for each statistically significant feature. The z-scores and pseudo-p-values represent the statistical significance of the computed index values. A high positive z-score for a feature indicates that the surrounding features have
similar values (either high values or low values). The COType field in the Output Feature Class will be HH for a statistically significant cluster of high values and LL for a statistically significant cluster of low values. A low negative z-score for a feature indicates a statistically significant spatial data
outlier. The COType field (last panel on the right) also identifies statistically significant high and low outliers (HL and LH), indicating if the feature has a high value and is surrounded by features with low values (HL) or if the feature has a low value and is surrounded by features with high values (LH).

The image below shows the results of the Cluster and Outlier Analysis tool applied to a set of 100 randomly generated points (cells) in the 2D space.

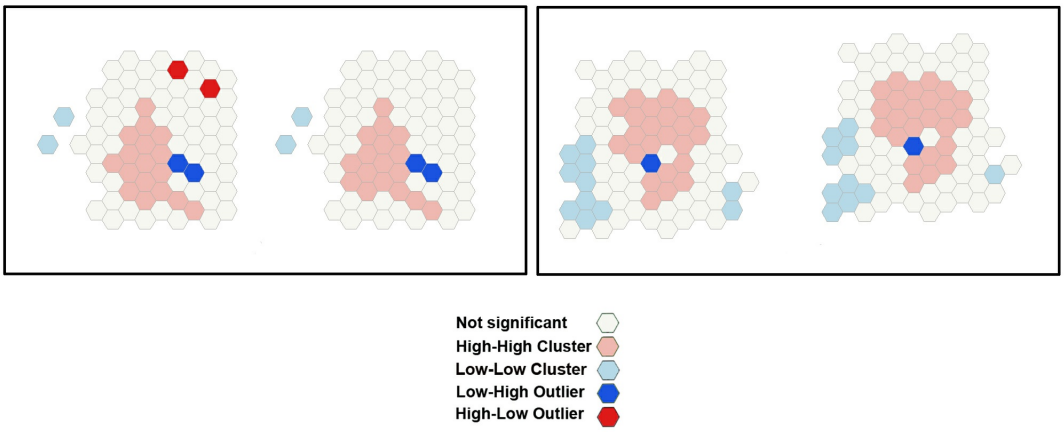


Results of the **Cluster and Outlier Analysis (Anselin Local Moran's I)** tool applied to a set of 100 randomly generated points (i.e. the centers of mass of 100 cells).

19.1 On the toolbar select the ***ArcToolbox*** icon then → ***Spatial Statistics Tools*** → ***Mapping Clusters*** → ***Cluster and Outlier Analysis (Anselin Local Moran's I)***.

19.2 In the pop-up windows for the *Input Feature Class* select ***GenerateTessellation#Spati#***, for the *Input Field* select ***Join-Count***, for *Conceptualization of Spatial Relationship* select ***INVERSE_DISTANCE***, for *Distance Method* select ***EUCLIDEAN DISTANCE***, for *STANDARDIZATION* select ***ROW***. Click ***OK***.

19.3 After the tool has run, a new layer is added to the map displaying in different colors statistically significant clusters and outliers for a 95% confidence level based on the local Moran I index.

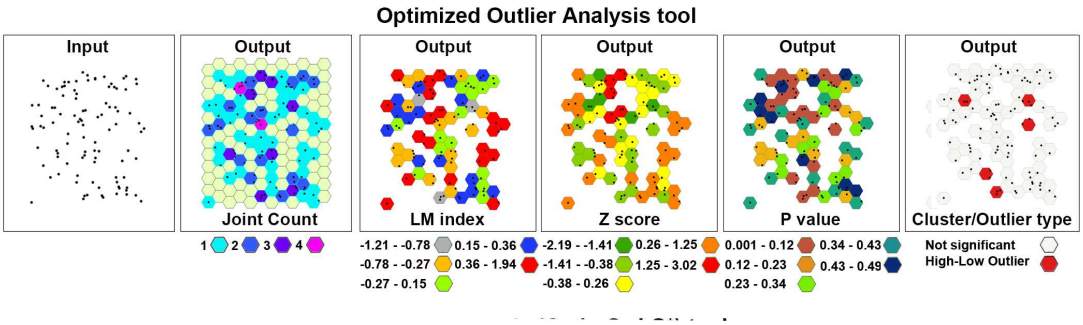


Examples of the output of the **Cluster and Outlier Analysis (Anselin Local Moran's I) tool**. Each rectangle encloses the results of two elaborations on two cerebellar slices with two different conceptualizations of spatial relationship: inverse distance with row correction (left) and fixed distance band (right). When the inverse distance with row correction is applied all cells are considered neighbors of all others. Thus all cells impact or influence all other cells, but the farther away one cell is, the smaller the impact it has. With row standardization spatial weights are standardized; each weight is divided by its row sum, i.e. the sum of the weights of all neighboring cells. With the fixed distance band, an inverse distance conceptualization is also used, and the
software computes a threshold distance value to reduce the number of required computations.

20 Optimized Outlier Analysis

This tool identifies statistically significant spatial clusters of high values (hot spots) and low values (cold spots) as well as high and low outliers. It automatically aggregates incident data, identifies an appropriate scale of analysis, and corrects for both multiple testing and spatial dependence using the False Discovery Rate (FDR) correction method. By the Optimized Outlier Analysis, the COType field will always indicate statistically significant clusters and outliers based on an FDR-corrected
95% confidence level.

The image below shows the results of the Optimized Outlier Analysis tool applied to a set of 100 randomly generated points (cells) in the 2D space.

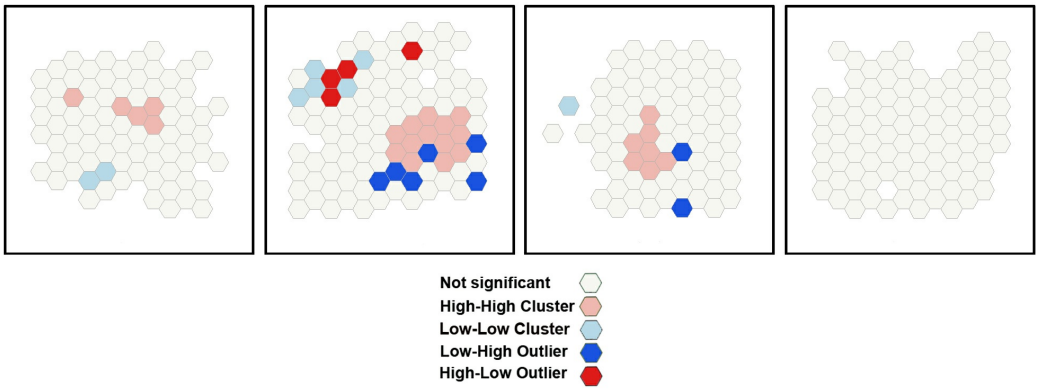


Results of the **Optimized Outlier Analysis (Anselin Local Moran's I)** tool applied to a set of 100 randomly generated points (i.e. the centers of mass of 100 cells).

20.1 On the toolbar select the ***ArcToolbox*** icon then → ***Spatial Statistics Tools*** → ***Mapping Clusters*** → ***Optimized Outlier Analysis***.

20.2 In the pop-up windows for *Input Feature Class* select ***GenerateTessellation#Spati#***, for ***Input Field*** select **Join-Count**; for *Conceptualization of Spatial Relationship* select ***INVERSE_DISTANCE***, for *Distance Method* select ***EUCLIDEAN DISTANCE***, for *STANDARDIZATION* select ***ROW***. Click ***OK***.

20.3 After the tool has run, a new layer is added to the map displaying in different colors statistically significant clusters and outliers based on their z-scores.

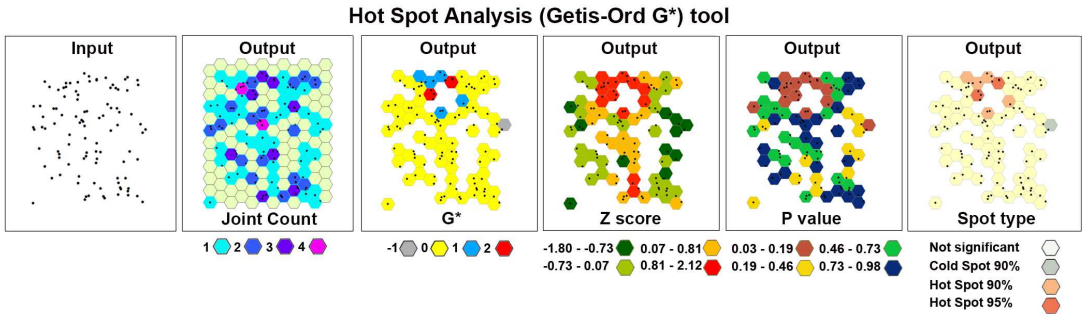


Examples of the output of the **Optimized Cluster and Outlier Analysis (Anselin Local Moran's I)** tool. Each rectangle encloses the results of the elaboration on a cerebellar slice. The parameters of the analysis have been optimized by the software.

21 Hot Spot Analysis (Getis-Ord Gi*)

The Hot Spot Analysis tool calculates the Getis-Ord Gi* statistic of the Purkinje neurons' spatial distribution. The resultant z-scores and p-values show where high or low numbers of Purkinje neurons cluster spatially.

The image below shows the results of the Hot Spot Analysis (Getis-Ord Gi*) tool applied to a set of 100 randomly generated points (cells) in the 2D space.

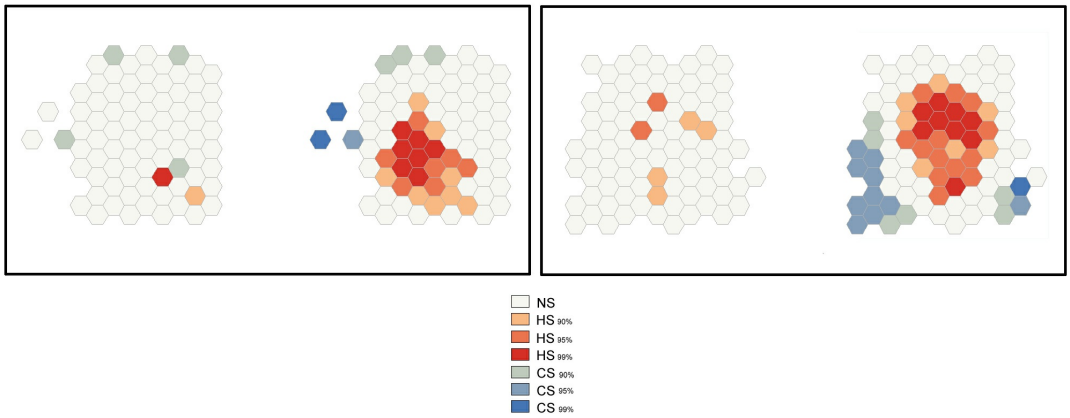


Results of the **Hot Spot Analysis (Getis-Ord Gi*)** tool applied to a set of 100 randomly generated points (i.e. the centers of mass of 100 cells).

21.1 On the toolbar select the ***ArcToolbox*** icon then → ***Spatial Statistics Tools*** → ***Mapping Clusters*** → ***Hot Spot Analysis (Getis-Ord Gi*)***.

21.2 In the pop-up windows for *Input Feature Class* select ***GenerateTessellation#Spati#***, for *Input Field* select ***Join-Count***, for ***Conceptualization of Spatial Relationship*** select ***INVERSE_DISTANCE***, for *Distance Method* select ***EUCLIDEAN DISTANCE***, for *STANDARDIZATION* select ***ROW***. Click ***OK***.

21.3 After the tool has run, a new layer is added to the map (Figure S5) displaying in different colors statistically significant spatial clusters of high values (hot spots) and low values (cold spots) based on their z-scores.

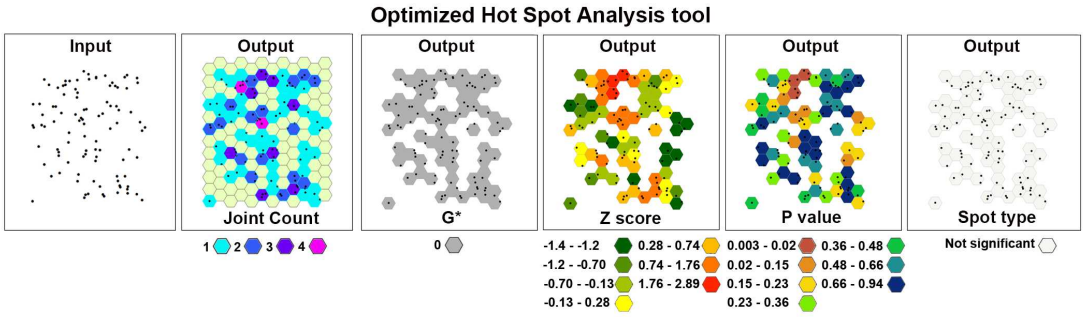


Examples of the output of the **Hot Spot Analysis (Getis-Ord Gi*)** tool. Each rectangle encloses the results of two elaborations on two cerebellar slices with two different conceptualizations of spatial relationship: inverse distance with row correction (left) and fixed distance band (right). When the inverse distance with row correction is applied all cells are considered neighbors of all others. Thus all cells impact or influence all other cells, but the farther away one cell is, the smaller the impact it has. With row standardization spatial weights are standardized; each weight is divided by its row sum, i.e. the sum of the weights of all neighboring cells. With the fixed distance band, an inverse distance conceptualization is also used, and the software computes a threshold distance value to reduce the number of required computations. *Abbreviations:* NS = Not significant; HS = Hot spot; CS = Cold spot. Percent values are confidence intervals.

22 Optimized Hot Spot Analysis

The Optimized Hot Spot Analysis tool calculates the Getis-Ord Gi* statistic of the Purknje neurons' spatial distribution. It evaluates automatically the characteristics of the input feature class to produce optimal results.

The image below shows the results of the Optimized Hot Spot Analysis tool applied to a set of 100 randomly generated points (cells) in the 2D space.



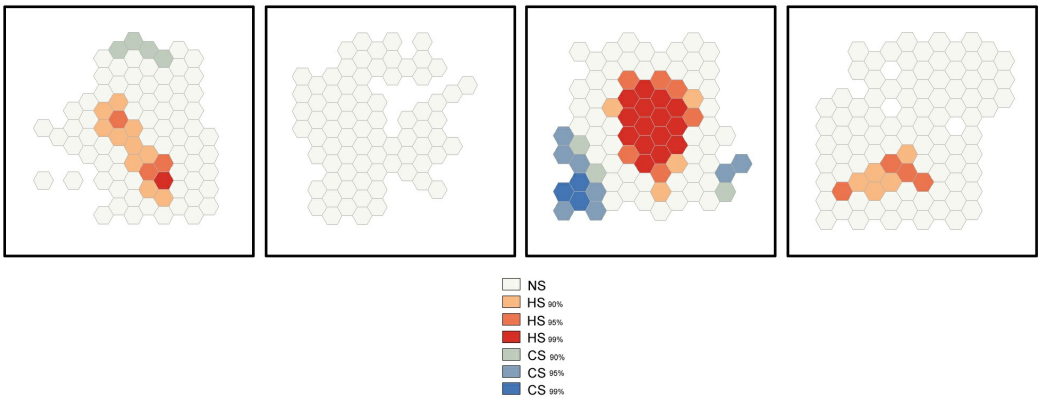
Results of the **Optimized Hot Spot Analysis** tool applied to a set of 100 randomly generated

points (i.e. the centers of mass of 100 cells).

22.1 On the toolbar select the **ArcToolbox icon** then → **Spatial Statistics Tools** → **Mapping Clusters** → **Optimized Hot Spot Analysis**.

22.2 In the pop-up windows for *Input Feature Class* select ***GenerateTessellation#Spati#***, for *Input Field* select ***Join-Count***; for ***Conceptualization of Spatial Relationship*** select ***INVERSE_DISTANCE***, for *Distance Method* select ***EUCLIDEAN DISTANCE***, for *STANDARDIZATION* select ***ROW***. Click ***OK***.

22.3 After the tool has run, a new layer is added to the map displaying in different colors statistically significant spatial clusters of high values (hot spots) and low values (cold spots) based on their z-scores.



Examples of the output of the **Optimized Hot Spot tool**. Each rectangle encloses the results of the elaboration on a cerebellar slice. The parameters of the analysis have been optimized by the software. *Abbreviations:* NS = Not significant; HS = Hot spot; CS = Cold spot. Percent values are confidence intervals.