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# Quantitative Assessment of $\alpha$ -synuclein Inclusions in Multiple System Atrophy

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**We use this protocol and it's working**

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## Abstract

Morphological differences in the  $\alpha$ -synuclein inclusions of Multiple System Atrophy (MSA) brains were observed, specifically in the putamen and the cerebellar white matter. This protocol was established for the purpose of quantifying features of glial cytoplasmic inclusions (GCIs). These features include: the size of GCIs, density of all  $\alpha$ -synuclein pathology (i.e., neuronal, neuritic, and glial), density of GCIs only, and the number of GCIs. The 5G4 antibody was used in the study as it revealed a wider spectrum of pathology but the protocol can also be used to explore quantitative features of GCIs using different anti- $\alpha$ -synuclein antibodies. We emphasize the importance of utilizing open-access tools and aim to promote accessibility and cost-effective image analysis for quantifying features of GCIs in MSA using immuno-stained whole slide images.



## Materials

Tissue sections immuno-stained with anti- $\alpha$ -synuclein antibody

Digital Tissue Scanner

Whole Slide Image Viewer

Photoshop (licensed)/Paint.NET (free)

Image J

Microsoft Excel

## Digital Setup and Scanning

- 1 To indicate the area for scanning, snapshot the sections using a Microscope Slide Scanner (i.e., TissueSnap<sup>TM</sup>), and place ROIs on the stained tissue area using the corresponding scanner software (i.e., HuronTechnologies).

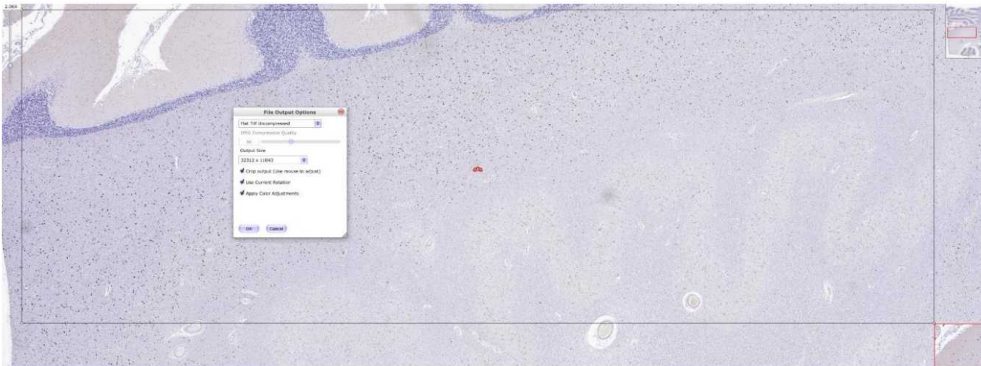


- 2 Set maximum magnification to 40X for higher resolution and more accurate measurement of inclusion size.
- 3 Scan the sections (i.e., TissueScope LE120). Keep the maximum magnification consistent across all your images.

## Selecting Subregion of Interest

- 4 Using the corresponding image viewer (i.e., HuronViewer), set image to 2.06X magnification and snip the region of interest.

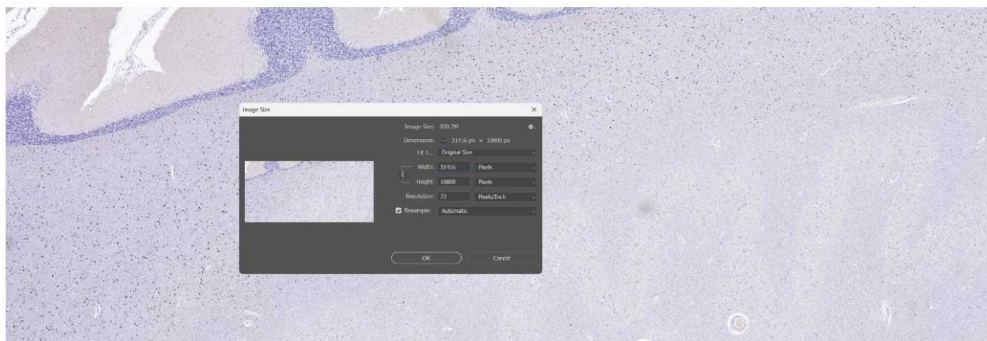
The output size was 32312 × 11043 px for our study. Keep the output size relatively consistent across all your images.



## Image Processing

- 5 Using Adobe Photoshop, crop each image to the same size, including the region of interest in the frame.

The cropped size was 31416 × 10800 px for our study as this was the maximum dimension that can be used for all the raw images collected from step 2. Keep the image size consistent across all your images.

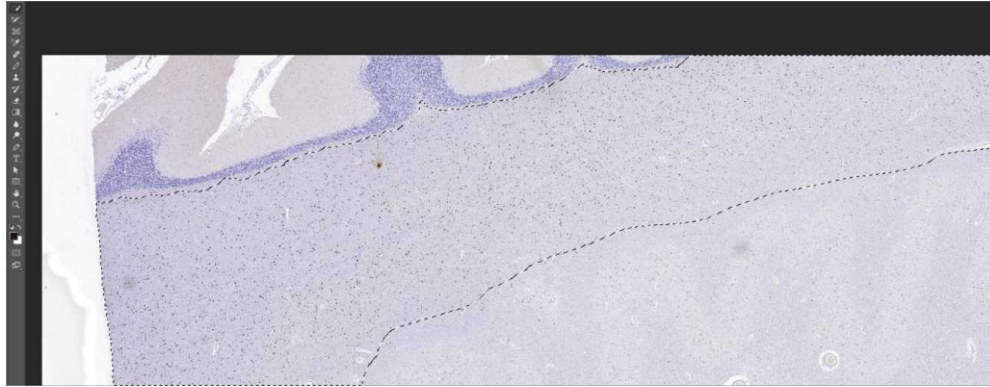


- 6 Save this as your "pre-processed" copy.

## Cropping Region of Interest

- 7 Using Adobe Photoshop, select the area of interest (i.e., putamen from the basal ganglia sections or the cerebellum WM from the cerebellum sections) with the Quick Selection Tool.

Regions were selected with the (+) tool and mis-selected regions were de-selected with the (-) tool. Alternatively, the eraser tool can be used to remove areas that are not of interest.

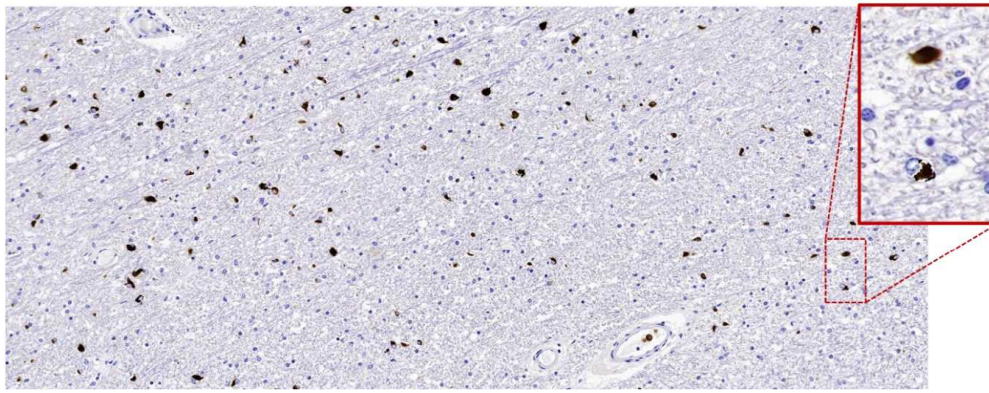


- 8 Copy the selection by pressing Ctrl+C and paste onto a new canvas of the same size and setting by pressing Ctrl+V. The new canvas should be the same size (i.e., 31416 × 10800 px), and have a resolution of 300 pixels/inch, in 8-bit color mode, with a white background.
- 9 Flatten image: Layer > Flatten Image, and save. This is your "region" copy.

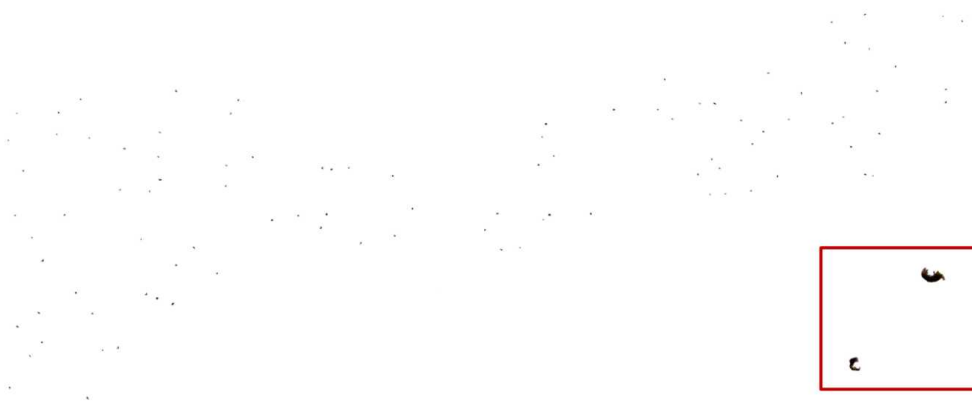
## Setting Up Size Threshold

- 10 Using the "region" copy of your image, zoom to 100% view and use the Magic Wand Tool with the Shift-key pressed, to select 100 random GCIs with visible nucleus.

For images that has less than 100 inclusions, choose as close to 100 as possible.



- 11 Zoom out time to time to ensure equal collection of inclusions across the whole region. If you made an accidental selection, press Ctrl+Z.
- 12 Optically dissect these selected inclusions by pressing Ctrl+C and paste onto a new canvas of the same size and setting as your previous image by pressing Ctrl+V.

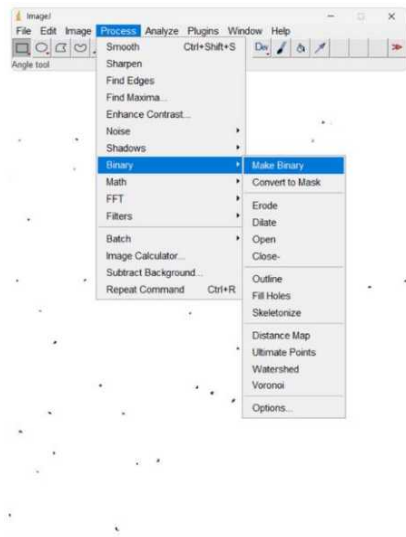


- 13 Adjust the brightness/contrast: Image > Adjust > Brightness/Contrast > Brightness: 150 and Contrast: 100 > OK.
- 14 Flatten Image and save as "threshold" copy.

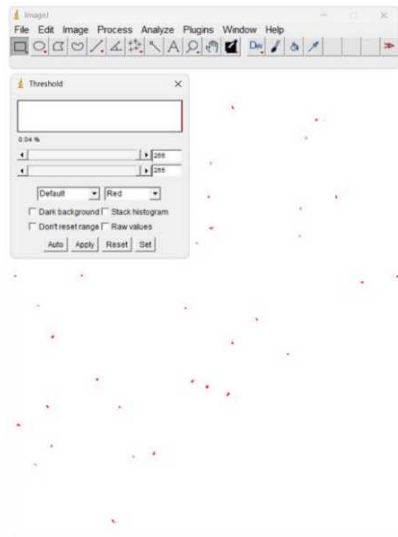
## Obtaining Size Threshold Values

- 15 Using Image J, open your "threshold" image and process the image into binary: Process > Binary > Make Binary

This step is to make sure that the program can measure the size of inclusions with better accuracy and will eliminate any background noise for the final measurement.

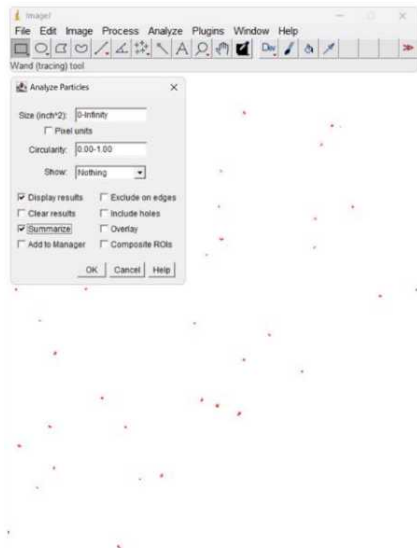


- 16 Adjust the threshold to measure only the black pixels: Image > Adjust > Threshold > Min: 255 and Max: 255



- 17 Before you measure the inclusions, make sure that any default scale is removed: Analyze > Set Scale... > Click to Remove Scale > OK.
- 18 Measure the size of the inclusions: Analyze > Analyze Particles > Size: 0-Infinity > Display Results > OK

This will generate 100 numbers in  $\text{px}^2$  which can be exported to an XML file.



19 File > Save As... > Save

20 Using Microsoft Excel, sort the measurements from smallest to largest. Record the minimum and maximum size in  $\text{px}^2$ .

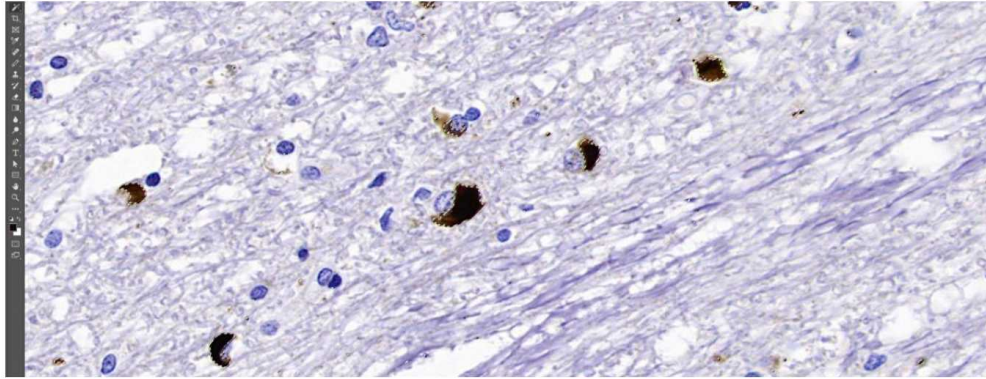
## Capturing All Inclusions in Regions of Interest

21 Using Adobe Photoshop, open the "region" copy and zoom to 100%.

22 Select all the inclusions: Select > Color Range > Use Color Picker Tool to Pick the Color of the Inclusion > Fuzziness: 175 > Ok.

We used the color picker to adjust the intensity of color needed to eliminate selecting the background and only the inclusions.

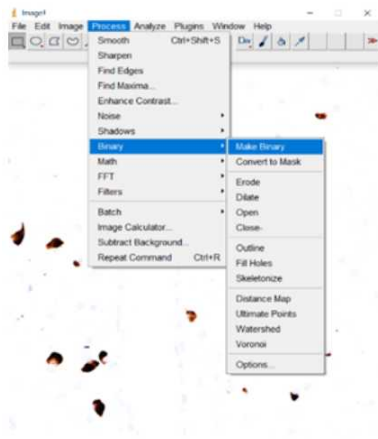
Fuzziness was set to 175 for all images to avoid selection of the background. Keep the picked color and fuzziness consistent across all your images.



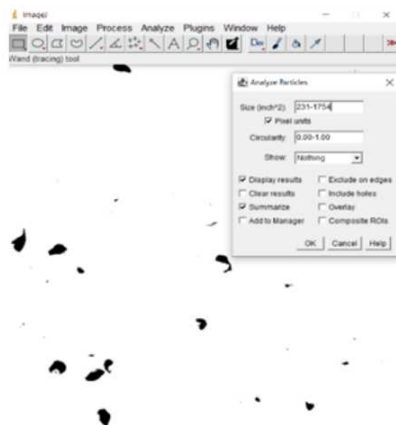
- 23 Copy the selected inclusions by pressing Ctrl+C and paste onto a new canvas of the same size and settings by pressing Ctrl+V.
- 24 Adjust the brightness/contrast: Image > Adjust > Brightness/Contrast > Brightness: 150 and Contrast: 100 > OK.
- 25 Flatten the image and save. This is your "total inclusion" image.

## Measuring Inclusion Size

- 26 Open the "total inclusion" image on Image J and process the image into binary: Process > Binary > Make Binary.



- 27 Adjust the threshold using the minimum and maximum inclusion sizes measured: Analyze > Analyze Particles > Size: min-max (px<sup>2</sup>), Circularity: 0.00-1.00, Show: Nothing > Display Results > OK

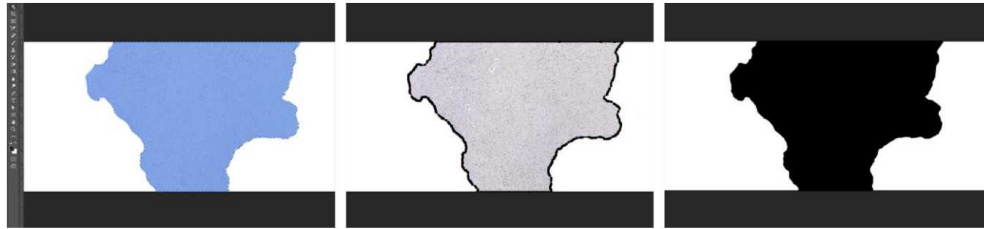


## Creating Shape of the Total Area of Tissue

- 28 Using Adobe Photoshop, open the "region" copy of your image of the region of interest (i.e., the putamen or cerebellar white matter).

- 29 Select the Object Selection Tool and click on your tissue.

When you hover over the tissue, the tissue should be highlighted in blue. If it is not highlighted, you can manually outline the tissue with the mouse and the selection will snap to shape of the tissue.



- 29.1 Click on the Paths tab on the bottom right corner.
- 29.2 Click on the "make pathwork from selection" button.  
Edit > Define custom shape > name your shape (i.e., case number-putamen).
- 29.3 Click on the "fill path with foreground color" button (make sure you select the color black by dragging the circle to the bottom left corner of the palette prior to clicking on this button).
- 30 Image > Mode > Grayscale.
- 31 Save this as your "total area" copy.

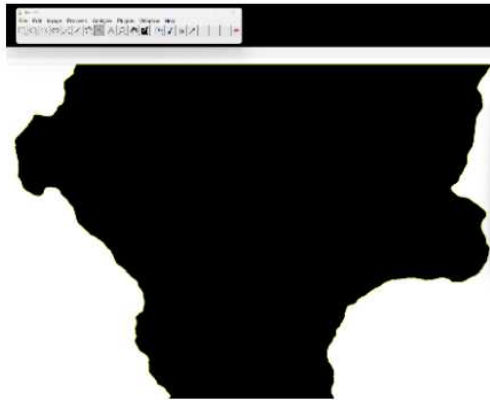
## Measuring Total Area of Tissue

- 32 Using Image J, open your "total area" image and process the image into binary: Process > Binary > Make Binary.

Some images might change the black shape into white when the image is converted to binary.

If so, go to Edit > Invert.

- 33 Using the Wand (tracing) Tool, click on the tissue shape (the tissue should be outlined in yellow) and measure the total area of tissue: Analyze > Measure.



- 34 Record this value.

### Measuring the Total Area of $\alpha$ -Synuclein Burden (px<sup>2</sup>)

- 35 Using Image J, open the "total inclusion" image and process the image into binary:  
Process >  
Binary > Make Binary.
- 36 Measure the total area of  $\alpha$ -synuclein burden: Analyze > Analyze Particles > Summarize > Ok.
- 37 Record the value under Total Area in the Summary window.

### Calculating the Density of All $\alpha$ -Synuclein Pathology (% area)

- 38 Using the recorded values for the total area of tissue, divide the total area of all  $\alpha$ -synuclein immunoreactivity with the total area of tissue to get a decimal number. Multiply by 100 to obtain a percentage.

$$\% \text{ area} = \frac{\text{Total Area of All } \alpha\text{Syn Immunoreactivity (px}^2\text{)}}{\text{Total Area of Tissue (px}^2\text{)}} \times 100$$

## Measuring and Calculating the Density of GCIs (% area)

- 39 Using Image J, open the "total inclusion" image and process the image into binary:  
Process >  
Binary > Make Binary.
- 40 Measure using threshold values obtained from steps 15-20: Analyze > Analyze Particles  
> Size: min-max (px<sup>2</sup>), Circularity: 0.00-1.00, Show: Nothing > Display Results > OK.
- 41 Record the value under Total Area in the Summary window.
- Alternatively, this value can be recorded from section "Measuring Inclusion Size".
- 42 Using the recorded values for the total area of tissue, divide the total area of GCIs with the total  
area of tissue to get a decimal number. Multiply by 100 to obtain a percentage.

$$\% \text{ area} = \frac{\text{Total Area of GCIs (px}^2\text{)}}{\text{Total Area of Tissue (px}^2\text{)}} \times 100$$

## Measuring and Calculating the Number of GCIs (GCIs/mm<sup>2</sup>)

- 43 Using Image J, open the "total inclusion" image and process the image into binary:  
Process > Binary > Make Binary.
- 44 Measure using threshold values obtained from steps 15-20: Analyze > Analyze Particles  
> Size: min-max (px<sup>2</sup>), Circularity: 0.00-1.00, Show: Nothing > Display Results > OK.
- 45 Record the value under Count in the Summary window.
- Alternatively, this value can be recorded from section "Measuring Inclusion Size".



- 46 Using the recorded values for the total area of tissue, first convert the total area of tissue in  $\mu\text{m}^2$  to  $\text{mm}^2$ .
- 47 Divide the number of GCIs by total area of tissue ( $\text{mm}^2$ ) and round to the nearest whole number.

$$\# \text{ of GCIs} = \frac{\text{Total \# of GCIs}}{\text{Total Area of Tissue (mm}^2\text{)}}$$

## Protocol references

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