Method for code (Microglia\_Analysis\_CY5.m):  
  
Image analysis was performed using MATLAB R2021b (Mathworks) to count microglia labeled with the fluorescent marker CY5 in images of rat spinal cord slices. The code reads in overlay images showing CY5-labeled microglia, performs background subtraction and thresholding to identify microglia somata, and counts the number of microglia in each image.

Key parameters used in the analysis include:

* resolution = 0.75488 μm/pixel (image resolution)
* r = 35 μm (radius for selecting microglia somata)
* threshold\_percentile = 99.0 (percentile threshold for CY5 intensity)
* cell\_size = 100 pixels (expected soma size)

The overlay images are read in and background fluorescence is removed using a 100x100 pixel median filter. Thresholding is then performed on the CY5 channel at the 99th percentile intensity to select microglia somata. Contiguous groups of pixels above this intensity threshold with an area of at least 100 pixels (corresponding to an approximate soma diameter of 35 μm) are counted as individual microglia. The x,y coordinates and a numeric label are overlaid on the image to show the identified cells. Finally, the total count of microglia somata for each image is printed and the processed image is saved with "\_binarycount" appended to the filename. This automated analysis provides an efficient means to quantify microglia numbers in spinal cord sections.

The key image processing steps are performed in the selectcells.m function, which applies the thresholds and size criteria to the CY5 channel input image to identify clusters of pixels corresponding to cell somata. This function is called by the main script to identify microglia somata and enable cell counting.

Code explanation part-by-part:

1.Set up

* Add necessary MATLAB pathways
* Define root data folder and folder with overlay images
* Get listing of overlay image files
* Set parameters:

1. resolution = image resolution in μm/pixel
2. r = radius for selecting cells
3. threshold\_percentile = intensity threshold percentile
4. cell\_size = expected cell soma size
5. Loop through each overlay image file

2a. Preprocessing

- Read in overlay image

- Background subtraction using median filter on each channel

2b. Thresholding

- Calculate intensity threshold based on set percentile on CY5 channel

- Apply threshold to select somata using selectcells.m function

- Function applies size criteria to get cell objects

2c. Analysis

- Overlay x,y coordinates and labels on identified cells

- Print total cell count

2d. Output

- Save processed image with cell ID overlay

End main loop