Supplementary Software 1 METAMORPH

Imaging processing and quantification:

1. Open file.

2. For image containing far red channel >Edit> Duplicate plane (all plane for analysis)
3. For RGB image >Display>Colour Separate

4. Process> Arithmetic
    - Source image 1: All image/plane from previous step
    - Source 2: Constant value
    - Result section – Image: Add
    - Bit depth: 16 bit
    - Operation: Add
    - Constant value: 0 gray levels
    - New image file will be generated (Add-1, Add-2 …)

5. Apps> Multi wavelength cell scoring
    - Number of wavelengths: 4 (depending on the image containing how many channels, channel-1 (Add-1) always for All nuclei)
    - Display result image: Segmentation (Check)
    - Select source image according the channel arrangement (Add-1, Add-2 …)
    - Stain area: Select nucleus, cytoplasm or both
    - Scoring parameter:
        - Min width: *refer to each figure setting* µm
        - Max width: *refer to each figure setting* µm
        - Intensity above local background: *refer to each figure setting* graylevels

6. Configure Data Log (Parameter configuration)
    - Image name
    - Cell: Assigned Label#
    - Cell: Total Area
    - Cell: Positive W2
    - Cell: W2 Stained Area
    - Cell: W2 Stained Integr Intensity
    - Cell: W2 Stained Average Intensity
    - Cell: W2 Nucleus Integr Intensity (*Optional*)
    - Cell: W2 Nucleus Average Intensity (*Optional*)
    - Cell: W2 Cytoplasm Integr Intensity (*Optional*)
    - Cell: W2 Cytoplasm Average Intensity (*Optional*)

    Same parameter configuration for other wavelengths (i.e., W3, W4)

Log column titles (check)

7. For tissue sample images, IbaI signals are used to identify microglia.

- Example, IbaI is represented by wavelength 2 (W2), under the Cell: Positive W2 column, “0” value will be filtered, and the remaining column with “1” value represent microglia (“0” = W2 (IbaI) – negative cells; “1” = W2 (IbaI) – positive cells).

Puncta quantification:

1. Open image file.

2. Save files as Meta Single/Multiple Plane TIFF.

3. Edit> Duplicate > all plane

4. Process> Morphology Filters

- Source image: select puncta plane
- Result image: Top Hat
- Operation: Extract features > Top hat
- Filter shape: Circle
- Diameter 10 pixels
- Reconstruction (check)

5. Process> Arithmetic

- Source image 1: Select nucleus frame
- Source image 2: Constant value
- Result > Image > Add
- Bit depth: 16 bit
- Operation: Add
- Constant values: 0

6. Repeat step 5 on Top Hat generated puncta plane in Step 4.

7. Repeat step 5 on positive marker plane.

8. Apps> Cell scoring

- W1 Source image: Nuclear image generated from Step 5 (i.e., Add)
    - Min width: 6
    - Max width: 12
    - Intensity above local background: 8 gray levels
- W2 Source image: Positive marker image generated from Step 5 (i.e., Add-3)
    - Stained area: Cytoplasm and nucleus
    - Min width: 5
    - Max width: 40
    - Intensity above local background: 20 gray levels
- Display result image: Segmentation (check)

9. Process> Binary operations

- Source image: Segmentation generated in Step 8
- Result image: Binary
- Operation >Binarize

- Parameters: 1 – 3

10. Process> Arithmetic

- Source image 1: puncta plane generated in Step 5 (i.e., Add-2)
- Source image 2: Binary generated at Step 9
- Result image: AND
- Bit depth: 16 bit
- Operation: Logical AND

11. Apps> Granularity

- Granule image: AND generated from Step 10
    - Display result image: Granules (check)
    - Min width: 0.8
    - Max width: 1.3
    - Intensity above local background: 100
- Nuclear image: nuclear plane generated from Step 5 (i.e., Add)
    - Min width: 6
    - Max width: 12
    - Intensity above local background: 5

12. Configure Data Log (Cells)

- Image Name
- Cell: Assigned Label #
- Cell: Granule Count
- Cell: Granule Total Area
- Cell: Granule Integrated Intensity
- Cell: Granule Average Intensity

Colocalization quantification:

1. Open file.

2. For image containing far red channel >Edit> Duplicate plane (all plane for analysis)
3. For RGB image >Display>Colour Separate

4. Process> Arithmetic
    - Source image 1: All image/plane from previous step
    - Source 2: Constant value
    - Result section – Image: Add
    - Bit depth: 16 bit
    - Operation: Add
    - Constant value: 0 gray levels

5. New image file will be generated (Add-1, Add-2 …)

6. Process> Morphology Filters
    - Source image: select planes to measure
    - Result image: Top Hat
    - Operation: Extract features > Top hat
    - Filter shape: Circle
    - Diameter: For LAMP1 > 5 pixels > Use reconstruction (check)
      Gal3 > 5 pixels > Use reconstruction (check)
    - Reconstruction (check)

7. Measure> Threshold Image
    - Inclusive LAMP1: 15-255
    - Inclusive Gal3: 20-255

8. Apps>Colocalization

Quantification Parameters:

(*Unless stated otherwise, W1, W2, W3 and W4 indicates blue, green, red and far red fluorescence signal, respectively.*)

Fig. 2D

Scoring parameter:

	W1	**W2**	**W3**	**W4**
Min width	5	3	3	-
Max width	15	100	100	-
Intensity above local background	5	11	25	-
Minimum stained area	-	10	10	-

Fig. 2F

	W1	**W2**	**W3**	**W4**
Min width	5	5	-	5
Max width	24	24	-	24
Intensity above local background	40	4	-	4
Minimum stained area	-	40	-	40

Fig. 2J

	W1	**W2**	**W3**	**W4**
Min width	4	4	2	-
Max width	30	30	30	-
Intensity above local background	15	20	90	-
Minimum stained area	-	5	5	-

Fig. 3A

	W1	**W2**	**W3**	**W4**
Min width	5	5	5	5
Max width	20	20	20	20
Intensity above local background	50	20	20	20
Minimum stained area	-	100	100	100

Fig. 4A

	W1	W2	W3	W4
Min width	5	5	5	5
Max width	20	20	20	20
Intensity above local background	50	20	20	20
Minimum stained area	-	100	100	100

Fig. 6B

Cell scoring

	W1	W2	W3	W4
Min width	6	5	-	-
Max width	12	40	-	-
Intensity above local background	8	20	-	-
Stained area	-	Cytoplasm and nucleus	-	-

Granularity

	Granule image	Nuclear image	-	-
Min width	0.8	6	-	-
Max width	1.3	12	-	-
Intensity above local background	100	5	-	-

Fig. 6C

	W1	W2	W3	W4
Min width	5	5	3	-
Max width	15	50	50	-
Intensity above local background	40	5	5	-
Minimum stained area	-	10	10	-

Fig. 6D

	W1	W2	W3	W4

Min width	6	5	3	3
Max width	15	50	50	50
Intensity above local background	40	5	8	8
Minimum stained area	-	10	10	10

Fig. 6E

	W1	**W2 (red)**	**W3**	**W4**
Min width	5	2	-	-
Max width	20	20	-	-
Intensity above local background	50	15	-	-
Minimum stained area	-	5	-	-

Fig. 7A

	W1	**W2**	**W3**	**W4**
Min width	5.5	1	1	1
Max width	12	20	20	20
Intensity above local background	16	40	40	90
Minimum stained area	-	30	30	30

Fig. 7B Supplementary Fig. 7

	W1	**W2**	**W3**	**W4**
Min width	5.5	1	1	1
Max width	12	20	20	20
Intensity above local background	16	40	40	90
Minimum stained area	-	30	30	30

Fig. 8A

	W1	**W2**	**W3**	**W4**
Min width	5.5	1	1	1
Max width	15	20	20	20
Intensity above local background	10	40	40	90

Minimum stained area	-	30	30	30

Fig. 9D

	W1	W2	W3	W4
Min width	5	1	5	-
Max width	10	7	10	-
Intensity above local background	5	30	5	-
Minimum stained area	-	5	30	-

Fig. 9D - 2

Cell scoring

	W1	W2	W3	W4
Min width	4	5	-	-
Max width	6	6	-	-
Intensity above local background	5	18	-	-
Stained area	-	Nucleus	-	-

Granularity

	Granule image	Nuclear image	-	-
Min width	1.5	5	-	-
Max width	7	6	-	-
Intensity above local background	70/20	18	-	-

Fig. 9G

	W1	W2	W3	W4
Min width	5	5	5	-
Max width	8	50	15	-
Intensity above local background	5	10	18	-
Minimum stained area	-	50	50	-

Fig. 9G - 2

	W1	W2	W3	W4
Min width	5	3	3	
Max width	8	40	40	
Intensity above local background	5	5	15	
Minimum stained area	-	50	50	

Supplementary Fig. 3A

	W1	W2	W3	W4
Min width	4	10	7	-
Max width	8	30	40	-
Intensity above local background	20	6	5	-
Minimum stained area	-	10	10	-

Supplementary Fig. 4

	W1	W2	W3	W4
Min width	6	3	3	-
Max width	30	20	20	-
Intensity above local background	20	15	20	-
Minimum stained area	-	3	1	-

Supplementary Fig. 6A

	W1	W2	W3	W4
Min width	5	10	10	-
Max width	10	40	40	-
Intensity above local background	5	7	7	-
Minimum stained area	-	10	10	-