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## EASI-FISH Spot Counting in Drosophila Brain Using RS-FISH

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

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## Abstract

This protocol describes how to utilize the Radial Symmetry-FISH (RS-FISH) software for fluorescent spot detection and counting (Bahry, E., Breimann, L., Zouinkhi, M. et al., 2022) to analyze images of the adult *Drosophila* brain labeled by Expansion-Assisted Iterative Fluorescence *In Situ* Hybridization (EASI-FISH) (Wang, Y., Eddison, M., Fleishman, G. et al., 2021). In expansion microscopy, tissues are embedded and expanded isotropically in a swellable hydrogel to achieve a higher spatial resolution (Chen, F., Tillberg, P.W. & Boyden, E.S., 2015). EASI-FISH combines this method with RNA fluorescence *in situ* hybridization, utilizing the hybridization chain reaction (HCR). In images taken by light sheet or confocal microscopy, individual transcripts are detected as bright spots. These spots are sufficiently separated from each other after 2x or 3x expansion of the brains to perform spot counting, unless the gene is question is too highly expressed. To use this protocol, RS-FISH should be installed as a Fiji plugin according to the instructions detailed on the [RS-FISH GitHub repository](#). The cell body masks were produced in Amira using the techniques described in the "[Using Amira to generate cell body masks in fluorescent light sheet microscopy images of EASI-FISH samples](#)" protocol.

## Attachments



EASI-

FISH\_Spot\_Count...

195KB

## Image Cropping and Channel Selection

- 1 Crop all relevant images so that spot counting only occurs in the area around the cell body.
- 1.1 Open the "shrink 2" mask, the "shrink 5" mask, and the original CZI image file by
  - In the **Bio-Formats Import Options** window that appears when loading the CZI file, ensure that **View stack with** is set to "Hyperstack" and **Stack order** is set to "XYCZT"
  - Click **OK**

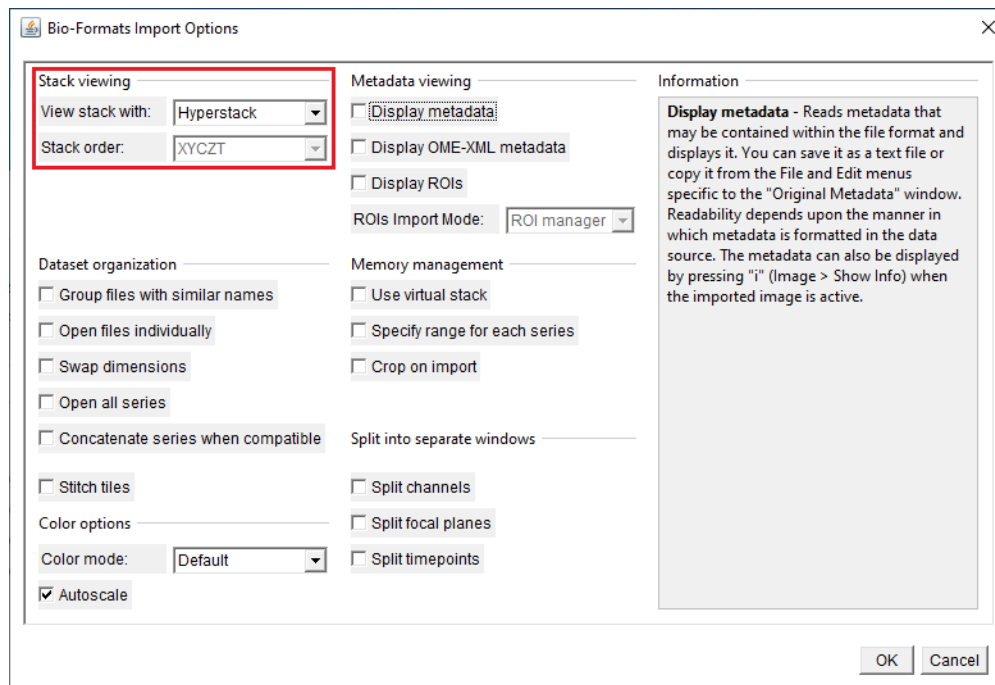


Fig 1.1 Bio-Formats Import Options window

- 1.2 Run **Plugins → Macros → Record...** to bring up a **Recorder** window that lists every action done in Fiji
  - Use the macro recorder to take note of parameters for future reference
- 1.3 Select the "shrink 2" mask window and run **Image → Stacks → Z Project...**
  - In the **ZProjection** window that appears, make sure that the **Projection type** is set to "Max Intensity"
  - Save the resulting maximum intensity projection (MIP) as a TIF file by running **File → Save As → Tiff...**
- 1.4 Click on the MIP window and select a rectangular area around the cell body using the **Rectangle** tool on the Fiji toolbar

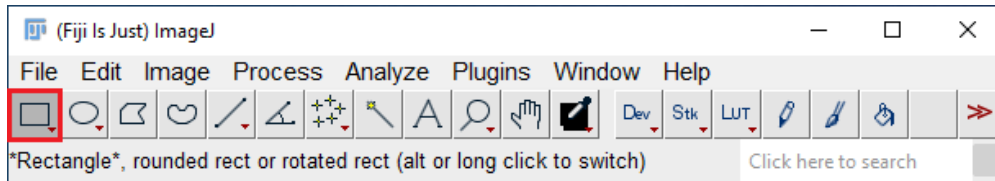


Fig1.4 Rectangle Tool

- Leave a margin around the cell body of approximately 50 pixels on all sides
- Save the dimensions of the rectangle by copying them from the **Recorder** window

1.5 Select the original "shrink 2" mask file window and run **Edit → Selection → Restore Selection**

- Once the rectangle selection appears on the image run **Image → Crop**
- Save the output as a TIF file and name it "...mask\_cropped"  
(e.g., "Gel1\_Brain2\_L\_SS76094\_2x\_shrink2\_mask\_cropped.tif")
- Repeat the process for the "shrink 5" mask

1.6 Select the original CZI image file and run **Edit → Selection → Restore Selection**

- Once the rectangle selection appears on the image run **Image → Crop**
- Save the output as a TIF file and name it "...image\_cropped"  
(e.g., "Gel1\_Brain2\_L\_SS76094\_2x\_image\_cropped.tif")
- Keep the cropped image file open and close all other windows

2 Isolate and edit the channels with RNA expression that will be used for spot counting.

2.1 Select the cropped image window and run **Image → Color → Split Channels**

- Close the windows for channels 1 & 4
- Save channels 2 & 3 as separate TIF files  
(e.g., "C2-Gel1\_Brain2\_L\_SS76094\_2x.tif" and "C3-Gel1\_Brain2\_L\_SS76094\_2x.tif")

2.2 Select the channel 2 window and adjust the settings to make the spots more visible

- Increase the size of the image by hitting the "up" arrow key (↑) on the keyboard until the spots in the image are an adequate size
- Click the **LUT** button on the Fiji toolbar and select "Grays"

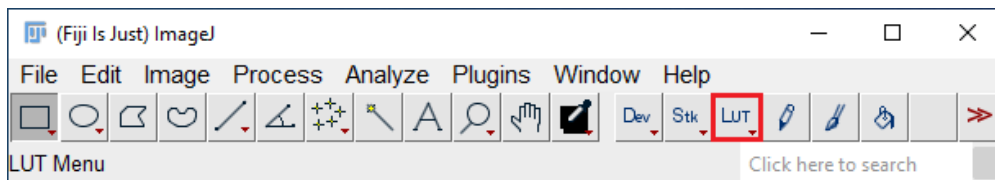


Fig 2.2a LUT menu

- Run **Process → Subtract Background...** and set the **Rolling ball radius** to 50.0 pixels. Click **OK** to apply the background subtraction to the entire stack

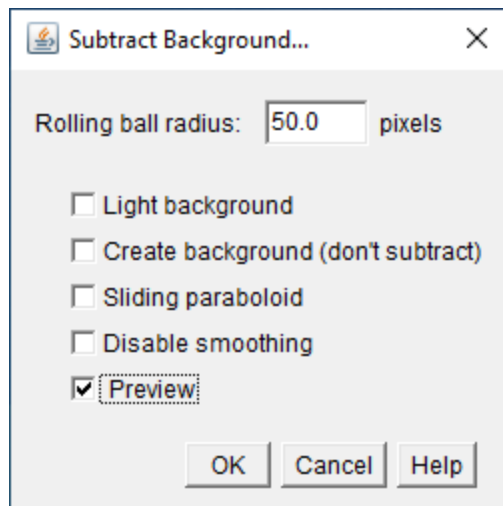


Fig 2.2b Subtract Background window

- If the brightness of the image is not optimal run **Image → Adjust → Brightness/Contrast... → Reset** (do NOT click **Apply**). Scroll through the entire stack to make sure that none of the signal is oversaturated
- Save the processed image as a TIF file and name it "...bg\_subtract" (e.g., "C2-Gel1\_Brain2\_L\_SS76094\_2x\_bg\_subtract.tif")
- The "...bg\_subtract" image is what will be used for all subsequent steps

## Calculate the Anisotropy Coefficient

- 3 In order to accurately detect 3D locations, it is important that the spots being counted are relatively spherical. The anisotropy coefficient is the factor by which the image needs to be adjusted to achieve more spherical spots for the RS-FISH process.
- 3.1 Select the channel 2 window and run **Plugins → RS-FISH → Tools → Calculate Anisotropy Coefficient**
  - In the **Anisotropy** window that appears, select the correct name from the **Image** dropdown list and make sure the **Detection Mode** is set to "Gauss Fit"
  - Click **OK**
- 3.2 Move the rectangle that appears in the image to a location where it either surrounds a cell body or where there are multiple isolated spots that can be counted
  - Copy the dimensions of the rectangle from the **Recorder** window and save them for later use
- 3.3 Spots that will be used to calculate the coefficient will be outlined in red in the image window. Adjust the following parameters in the **Adjust difference-of-gaussian values**

window so that as many spots as possible are included, with particular attention paid to spots that are well isolated from the rest

- Adjust the **Sigma** up or down to increase or decrease the size of the radius of the red outline circle
- Adjust the **Threshold** up or down to increase or decrease the number of spots included
- Click **Done**

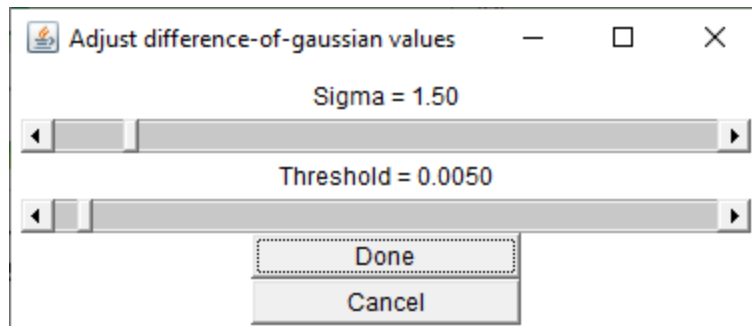


Fig3.3 Adjust difference-of-gaussian values window

#### Note

Start with the default settings (**Sigma** = 1.50 and **Threshold** = 0.0050) and see what the result looks like. If the result isn't optimal, simply redo the process and adjust the parameters as needed

### 3.4 When the calculation has finished, the anisotropy coefficient will appear in a **Log** window

- The anisotropy coefficient should be close to 1.0 (unless the spots greatly deviate from something close to circular)

#### Note

The normal range for the anisotropy coefficient is usually between 0.8 and 1.2

### 3.5 To check the anisotropy coefficient, apply it to a resliced image

- Select the channel 2 window and run **Image** → **Stacks** → **Reslice [/]...**
- In the **Reslice** window that appears, make sure that **Avoid interpolation** is checked and click **OK**

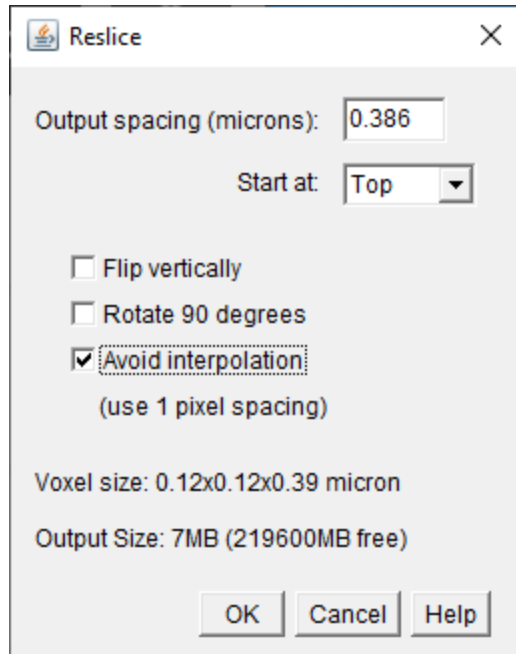


Fig 3.5a Reslice window

- Now select the resliced image and Run **Image → Scale...**
- In the **Scale** window that appears, enter the calculated anisotropy coefficient as the **Y Scale** value and click **OK**

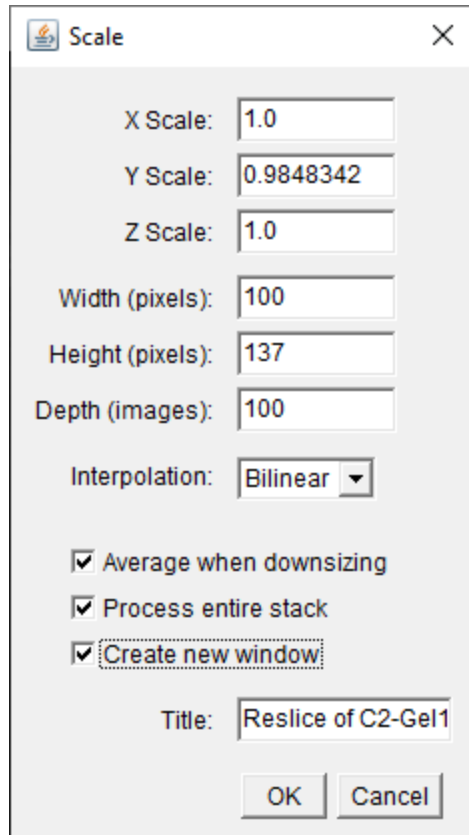


Fig 3.5b Scale window

- Compare the original reslice to the scaled reslice. Find a Z-section where spots in the original reslice appear either elongated or flattened and see if the same spots in the scaled reslice are more circular. The spots of RNA expression in the EASI-FISH images are usually already round, so the effect may be minimal
- Close both reslice images

3.6 If the anisotropy coefficient looks correct, take note of the value by copying the "Anisotropy coefficient" line from the **Log** window

## Run RS-FISH

- 4 Use RS-FISH to determine the total number of individual spots of RNA expression in the image.
- 4.1 Select the channel 2 window and run **Plugins → RS-FISH → RS-FISH**
  - In the **Radial Symmetry (2d/3d)** window that appears, select the correct name from the **Image** dropdown and make sure that the **Mode** set to "Interactive"
  - The **Anisotropy coefficient** line should show the coefficient that was just calculated (rounded to 4 decimal places)



- In the **Computation** section, **Robust fitting** should be set to "RANSAC," the boxes next to **Compute min/max intensity from image** and **Use anisotropy coefficient for DoG** should be checked, and **Spot intensity** should be set to "Linear Interpolation"
- Under **Visualization**, the box next to **Add detections to ROI-Manager** should NOT be checked
- Click **OK**

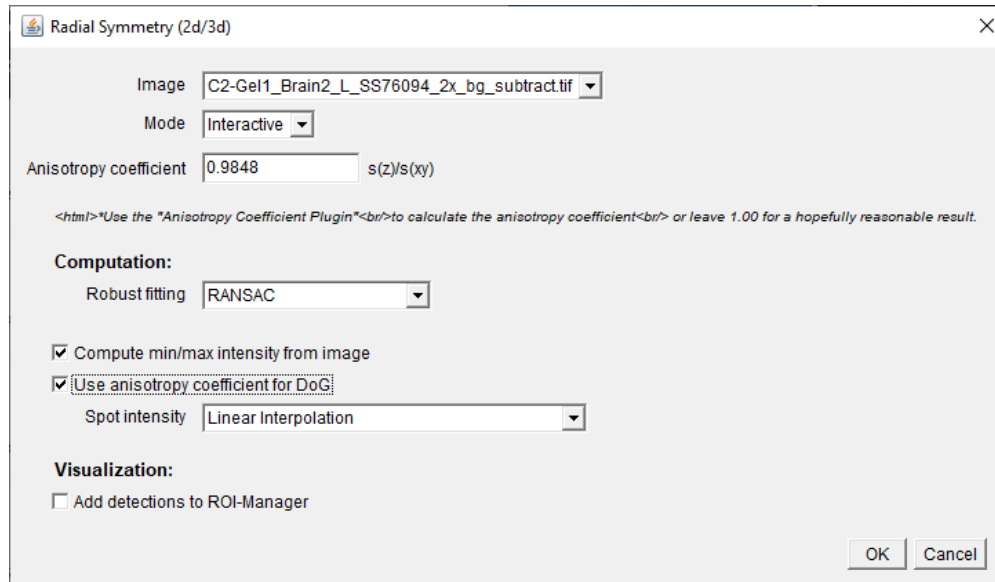


Fig 4.1 Radial Symmetry (2d/3d) window

- 4.2 Detected spots will now be outlined with a red circle in the image window. A blue cross inside the circle indicates that the RANSAC algorithm used outlier removal to detect the spot
  - Click inside the rectangle in the image window to make sure that it is in the same position that was used to calculate the anisotropy coefficient
- 4.3 Another window called **RANSAC preview** will also appear, which displays the currently detected spots. The preview will change as the parameters are adjusted
- 4.4 Adjust the following parameters in the **Adjust difference-of-gaussian values** to include as many spots as possible that are not part of the noise or the background. Be sure to scroll through all Z-sections in the stack to ensure that the parameters provide the desired results for the entire image
  - As before, the **Sigma** value will determine the size of the area for detection. When setting this value, keep in mind that the red outline circles should be a bit larger than the actual spots
  - As before, the **Threshold** value will determine the limit for spot detection. If the image contains more noise or if there are "halos" around the spots that are also being

detected, set this value higher to compensate (the normal range is anywhere from 0.00500 to 0.00800)

#### Note

Just like when calculating the anisotropy coefficient, start with the default settings (**Sigma** = 1.50 and **Threshold** = 0.00500) and see what the result looks like. If the result isn't optimal, simply redo the process and adjust the parameters as needed

- 4.5 For this project, the parameters in the **Adjust RANSAC values** window are being left at their default values
- The **Support region radius** (which selects the radius for each spot where gradients are used for radial symmetry) should be set to 3
  - The **Inlier ratio** (the ratio of the gradients that are part of the current spot) should be set to 0.10
  - The **Max error** (the maximum error allowed for fitting) should be set to 1.5000
  - **Local background subtraction** should be set to "No background subtraction"

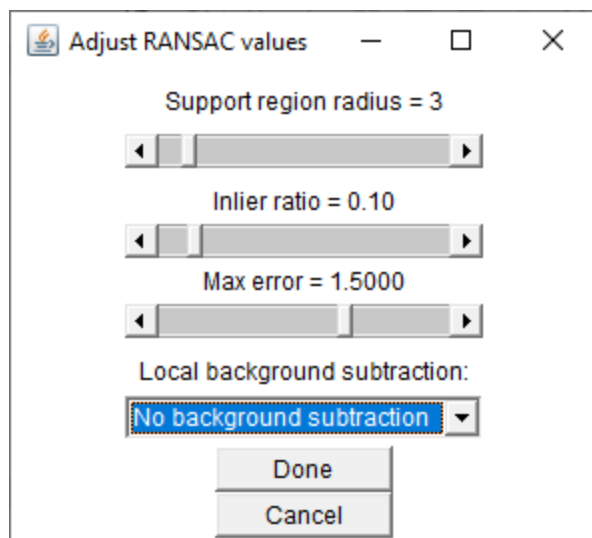


Fig 4.5 Adjust RANSAC values window

- 4.6 Once the parameters have been set, take note of them before clicking **Done** in the **Adjust RANSAC values** window
- Once the process has finished, the image window will show the selected spots in red and a new **Intensity distribution** histogram window will appear
  - Take a screenshot of the histogram window and save it as a PNG labeled "...original\_c#" (e.g., "SS76094\_L\_original\_c2.PNG")

- 4.7 Adjust the minimum intensity threshold by clicking at different points along the histogram
- The histogram window can be expanded to make it easier to select a certain value
  - Scroll through the image stack as the threshold is adjusted to make sure only true spots are being detected
  - Carefully check areas that either have a large amount of noise or that have “halos” around the spots to make sure they are not included
  - Consider not only the shape of the histogram and where the peak lies, but also the intensity value at which the peak occurs. If the peak of the histogram is at a low intensity ( $\leq 200$ ) be sure to set the threshold point beyond it

#### Note

In most cases, spots with an intensity below 200 are not considered to be actual signal and should not be included. For Gad1 RNA expression, the threshold should generally be set around 300-500, but this can vary depending on the image

- 4.8 If the parameters need to be adjusted based on the detections seen in the image window, close the **Intensity distribution** window and run **Plugins → RS-FISH → RS-FISH** again until the optimal settings are reached
- Keep track of all parameter values as they are adjusted
  - Move the rectangle in the image window to the same position that was used to calculate the anisotropy coefficient
  - Overwrite the “original\_c#” PNG with a new screenshot each time that RS-FISH process is repeated
- 4.9 Once the parameters have been finalized and the intensity threshold has been set, take another screenshot of the histogram window and save it as a PNG labeled “...final\_c#” (e.g., “SS76094\_L\_final\_c2.PNG”)
- 4.10 To complete the process, select the **Intensity distribution** window and click **OK - press to proceed to final results**
- Copy the “Final #detections (before intensity check),” the “intensityThreshold,” and the “Spots found” lines from the **Log** window for future reference
  - The histogram window will be replaced by a window called **smFISH localizations** that lists the coordinates of all detected spots. Run **File → Save As** from this window (not from the main Fiji toolbar) and edit the file name so it includes the cell body ID and the channel number. Be sure to manually change the file type to CSV (e.g., “SS76094\_L\_smFISH localizations\_c2.csv”)
- 5 Repeat the entire process for channel 3, starting from step 2.2 under “Image Cropping and Channel Selection.”

 [go to step #2.2](#)



- 6 If there is more than one cell body in the image, the parameters for both the anisotropy coefficient calculation and for the RS-FISH process can be used as starting points for a new cell since the image acquisition settings should be relatively consistent.

## Mask Filtering

- 7 The mask filtering process narrows down the total number of spots to only those that are present within the boundaries of the mask.
  - 7.1 Create a new folder named "Results" within the spot counting folder
  - 7.2 Run **Plugins → RS-FISH → Tools → Mask Filtering**
    - In the **Pick files to include** window that appears, drag the CSV file into the **Inputs File/Folder** line
    - Drag the cropped mask file into the **Mask File** line
    - Drag the "Results" folder into the **Output Folder** line (this will avoid possible overwrite of existing files)
    - The name of the selected CSV should appear at the bottom of the window in the ***selected files*** section. If it doesn't, change the **exclude files smaller than (KB)** to "0"
    - Click **OK**

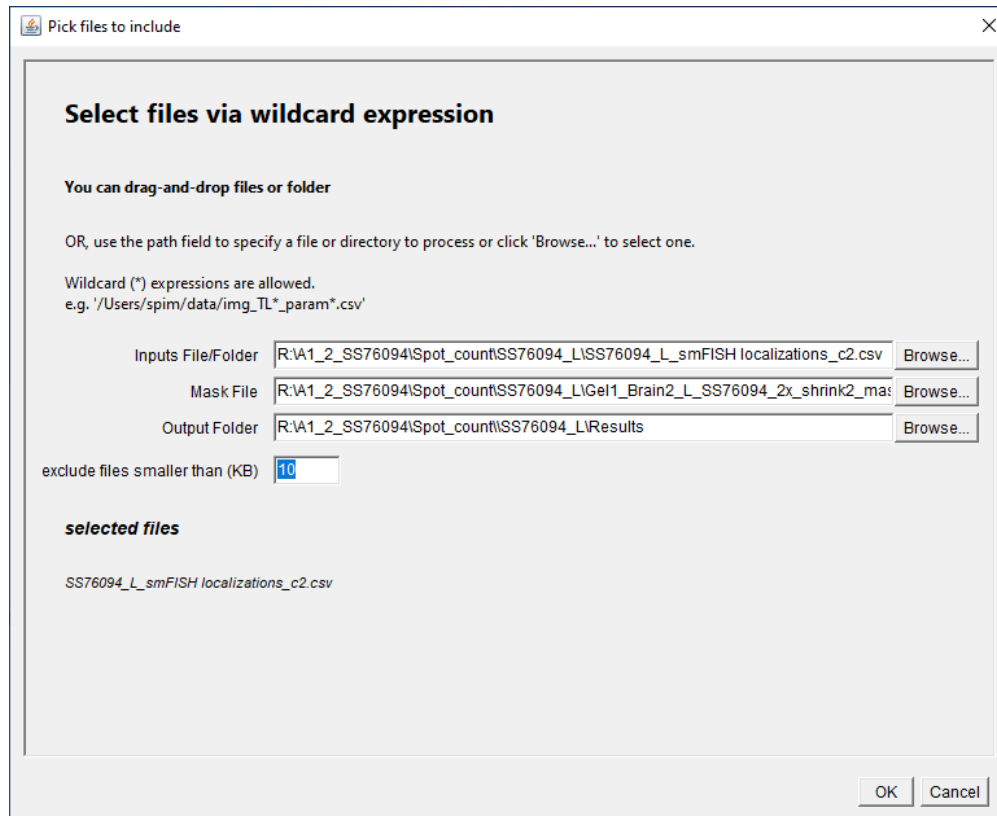


Fig7.2 Pick files to include window

- 7.3 A dialogue window will show the number of files filtered, and a new CSV file will be written to the selected output directory
  - Edit the name of the filtered CSV file to include the shrink size of the mask (e.g., "SS76094\_L\_shrink2\_smFISH localizations\_c2\_filtered.csv")
- 7.4 Move the filtered CSV out of the "Results" folder and place it in the parent spot counting folder. This will allow the "Results" folder to be used repeatedly
- 7.5 Repeat the filtering process until the initial results for channels 2 & 3 have been filtered for both the "shrink 2" and the "shrink 5" masks

## Checking Results in Big Data Viewer

- 8 Big Data Viewer can be used to visualize the spots of RNA expression detected within the mask by highlighting them in the original channel image.
- 8.1 Open the "...bg\_subtract" image for the desired channel in Fiji and run **Plugins → RS-FISH → Tools → Show Detections (BigDataViewer)**

- 8.2 A window called **Display overlaid points with BDV** should appear
  - For **Options**, select “Display a currently open image and load points from CSV” from the dropdown
  - Leave the **Sigma (points)** slider at 1.00
  - Click **OK**
- 8.3 In the **Select open image and CSV(s)** window, make sure the correct image name is displayed, and drag the filtered CSV file for the corresponding channel (at either “shrink 2” or “shrink 5”) into the **Select CSV** line
- 8.4 Once the **BigDataViewer** window is open, hover over the right side of the window and click on the blue arrow that appears to view the sidebar
  - Under **Sources**, click on the image name above “detections”
  - Use the now active slider at the bottom of the **Sources** section to lower the maximum threshold by dragging the circle on the right side towards the left. The image should become visible when the maximum is lowered down to around 1000 or 3000

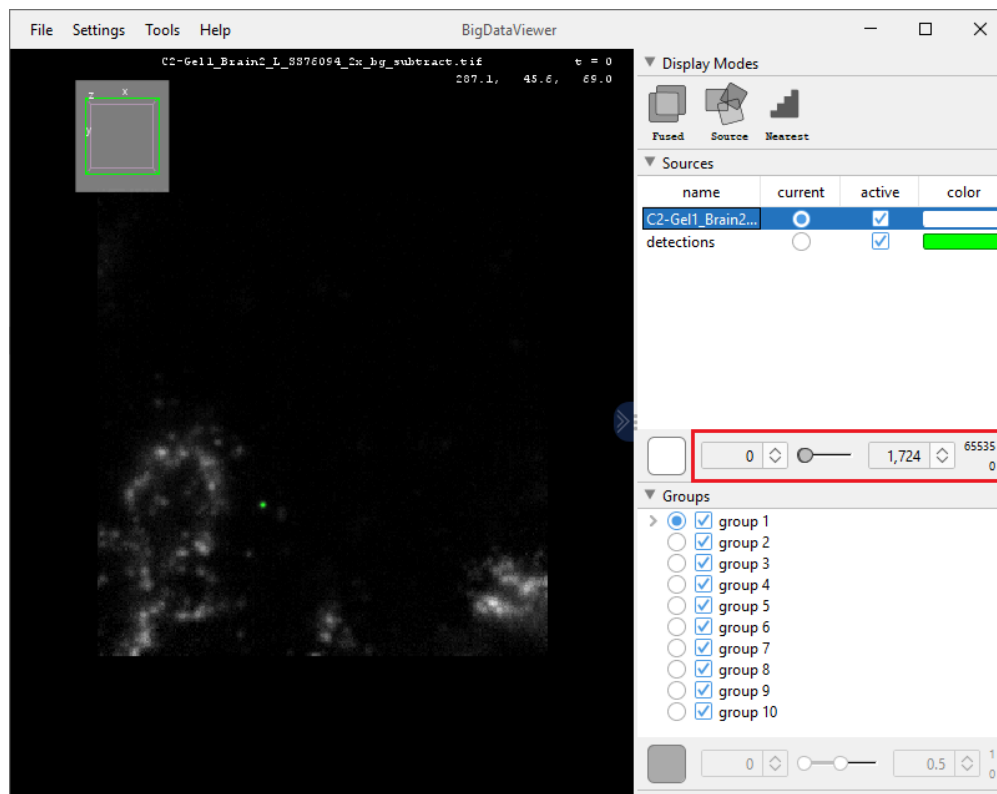


Fig8.4 BigDataViewer window

- 8.5 Scroll through the image to find the green spots that were detected within the mask and make sure they look correct
  - This method is helpful when determining if expression from neighboring cells has been included in the final spot count



## Protocol references

Bahry, E., Breimann, L., Zouinkhi, M. *et al.* RS-FISH: precise, interactive, fast, and scalable FISH spot detection. *Nat Methods* **19**, 1563–1567 (2022). <https://doi.org/10.1038/s41592-022-01669-y>

Chen, F., Tillberg, P.W. & Boyden, E.S. Expansion microscopy. *Science* **347** (6221), 543–548, <https://doi.org/10.1126/science.1260088>, (2015).

Wang, Y., Eddison, M., Fleishman, G. *et al.* EASI-FISH for thick tissues defines lateral hypothalamus spatio-molecular organization. *Cell* **184** (26), 6361–6377 (2021) <https://doi.org/10.1016/j.cell.2021.11.024>

GitHub repository: <https://github.com/PreibischLab/RS-FISH>

Park, G., Weigel, A., Petruncio, A. *et al.* Using Amira to generate cell body masks in fluorescent light sheet microscopy images of EASI-FISH samples. **protocols.io** (2023) [dx.doi.org/10.17504/protocols.io.rm7vzb18rvx1/v1](https://dx.doi.org/10.17504/protocols.io.rm7vzb18rvx1/v1)