AURA: Automated Universal RNAscope Analysis for high-throughput applications

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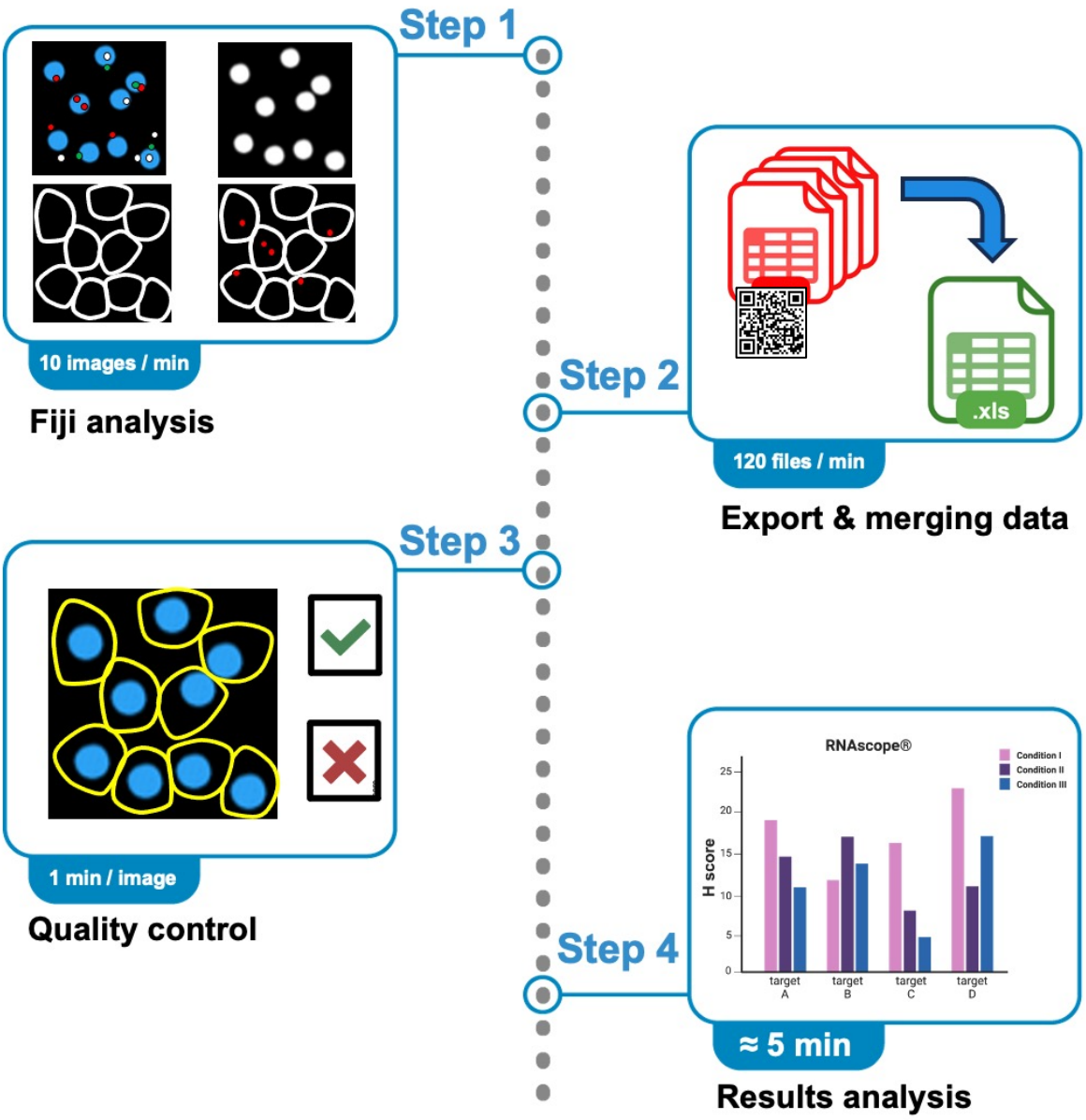
Summary

Fluorescence-based *in-situ* hybridization methods such as RNAscope technology enable highly sensitive and specific visualization of individual RNA molecules (identified as fluorescent specks) within intact cells, enabling precise spatial and cellular localization of RNA transcripts. However, manual analysis of images is time-consuming. We developed a novel, open-source, user-friendly tool to facilitate high-throughput applications for specks-like staining. The protocol seamlessly integrates automated processes, offering a universal solution for efficient transcriptomic analysis, such as, but not limited to, RNAscope in various biological contexts.

Graphical abstract



Before you begin

Perform RNAscope or equivalent technology and imaging

Timing: 2-3 days

1. Perform your RNAscope [1] analysis according to the manufacturer's instructions.

Note: *The protocol has been tested for the RNAscope multiplex V2 assay, but it applies to any equivalent analysis involving intracellular fluorescent and chromogenic specks-like staining, extending its utility beyond in situ transcriptomics, and appealing to a wide scientific community.*

2. Acquire images by confocal microscopy.

Note*: We recommend acquiring at least 10 images per sample to ensure enough high-quality, well-segmented images for analysis. An average of 150 high-quality nuclei per image is recommended.*

3. Download the Fiji software at https://imagej.net/software/fiji/#downloads within a Linux, MacOS, or Windows operative system.
4. If your acquired images are not in .TIFF format, convert them using Fiji.

Install plugins and macro in Fiji

Timing: 5 minutes

1. Download AURA macro and plugins:
   a) To download the AURA macro go to https://github.com/FlorianBrnrd/aura-data-processing, open the "AURA" folder, and open the AURA_macro_v1.1.iJm, click on the download command (Figure 1A, red square).
   b) To download the plugins go to https://mcib3d.frama.io/3d-suite-imagej/#download, click on the "Download Bundle link", and download the 3D ImageJ Suite.
2. Install the plugins and the macro:
   a. Open the Fiji folder: for MacOS use the secondary click on the Fiji application and select the Show Package Contents option (Figure 1B, red circle), for Windows users select the Fiji app folder. Proceed with steps c) and d) for both operative systems.
   c) Drag the three plugins marked with a red square in the downloaded folder "3D ImageJ Suite" (Figure 1C, red square) into the "plugins" folder (Figure 1D, blue circle).

d) Drag the downloaded AURA macro (AURA_macro_v1.ijm) into the "toolsets" folder (Figure 1D, purple circle) contained in the "macros folder" (Figure 1D, red circle).

Figure 1. Install the AURA plugins and macro. (**A**) Screenshot of the AURA macro code, the red square indicates the download command. (**B**) Screenshot of the secondary click on the Fiji app for MacOs. (**C**) Content of the "3D ImageJ Suite" folder, the red square indicates the three plugins to be installed. (**D**) Screenshot of the Fiji folder content. Plugins are dragged into the "plugins" folder (blue circle), and the AURA macro file is dragged into the "toolset" folder (purple circle) contained within the "macros" folder (red circle).

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
RNAscope™ Multiplex Fluorescent Reagent Kit v2	Bio-techne	Cat# 323100
Custom RNAscope™ target probe(s)	Bio-techne	
RNAscope™ 4-plex LS Multiplex Negative Control Probe	Bio-techne	Cat# 321838
RNAscope™ 3-plex LS Multiplex Negative Control Probe	Bio-techne	Cat# 320878

RNAscope™ LS 2.5 4-plex Positive Control Probe- Hs	Bio-techne	Cat# 321808
RNAscope™ 3-plex Positive Control Probe- Ms	Bio-techne	Cat# 320881
Software and algorithms		
ImageJ/Fiji	reference[2,3]	https://imagej.net/software/fiji/#downloads
3D ImageJ Suite Plugins	reference[4]	https://mcib3d.frama.io/3d-suite-imagej/#download
AURA macro	This work	https://github.com/FlorianBrnrd/aura-data-processing
LibreOffice	The Document Foundation	https://www.libreoffice.org/
Other		
Leica SP8 Laser Confocal Microscope or equivalent	Leica Microsystems	https://www.leica-microsystems.com/products/confocal-microscopes/p/leica-tcs-sp8/

***Note**: The custom RNA probe refers to the target RNA of interest.*

Step-by-step method details

Fiji macro analysis

Timing: 1 min per 10 pictures

This step includes nucleus segmentation and quantification of the RNA signal visible as specks (herein defined as dots) in the images.

1. **Launch the AURA macro:**
   - **a)** Click the red double arrow in the Fiji toolbar (Figure 2A) and select the AURA macro from the drop-down menu.
   - **b)** The AURA macro shortcut will appear in the Fiji menu toolbar (Figure 2B).

c) Click on the AURA macro shortcut button (Figure 2B).

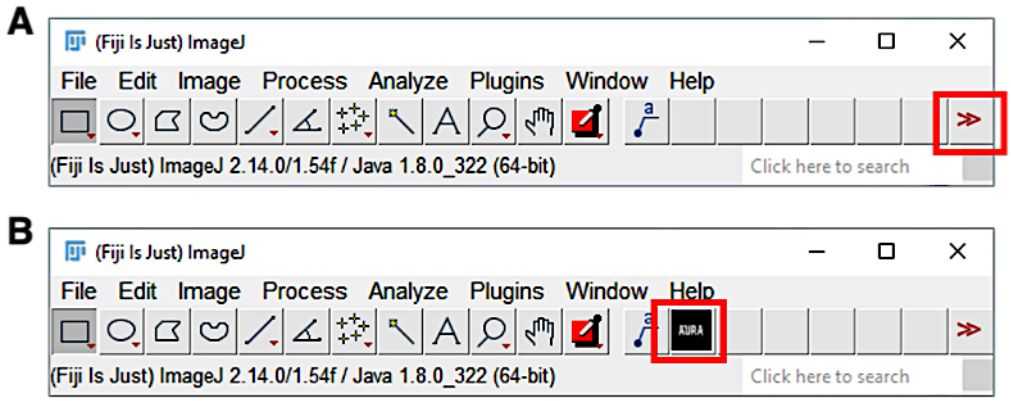


Figure 2. Fiji interface. (A) Selection of the AURA macro from the dropdown menu (red square). **(B)** AURA macro shortcut (red square).

2. **Selection of Image folder:** the macro begins by prompting the user to select the folder containing the images to be analyzed. The final results (step 6) will also be saved in the selected folder.

   a) Select the folder of the image series to open.

Notes: *Images must be in .TIFF format.*

3. **User input for analysis settings**: A dialog box prompts users to provide settings such as expansion around nuclei, the number of channels and their names, and the minimum dot size tolerance for the analysis (Figure 3).

   a) Choose the maximum expansion area around nuclei (Figure 3A), which is used to identify individual cells and quantify the number of dots within a cell (Figure 4A and B).

   Note: *The default pre-set value is 0,1µm, but the expansion depends on the cell size and tissue type. We recommend testing several expansion values on a few images to find the correct value for the segmentation of your samples (Figure 4A). Staining with surface markers may help to define the suitable expansion.*

   b) Choose the number of channels, including the channel used for nucleus staining that you want to analyze (minimum 2 channels and maximum 15 channels) (Figure 3A).

   c) Based on your negative control picture choose the minimum dot size tolerance for the analysis (Figure 3A).

Note: *This procedure is required if the negative control has a few dots smaller than the positive control. These background dots can be used as reference to choose the size tolerance. The default pre-set value is 0,1µm (Figure 4B). If no background is detected in the negative control the threshold for size dot can be changed to 0, and all dots in the test sample will be segmented and counted.*

d) Name your channel based on the specific marker that you used (Figure 3B).

Notes: *Do not use special characters: % $! & / \ : ; « » : % & # @.*

e) Choose the channel used for nucleus staining amongst all channels (Figure 3C).

Note: *The settings will be saved at the end of the analysis in the filename "Analysis_Settings.txt".*

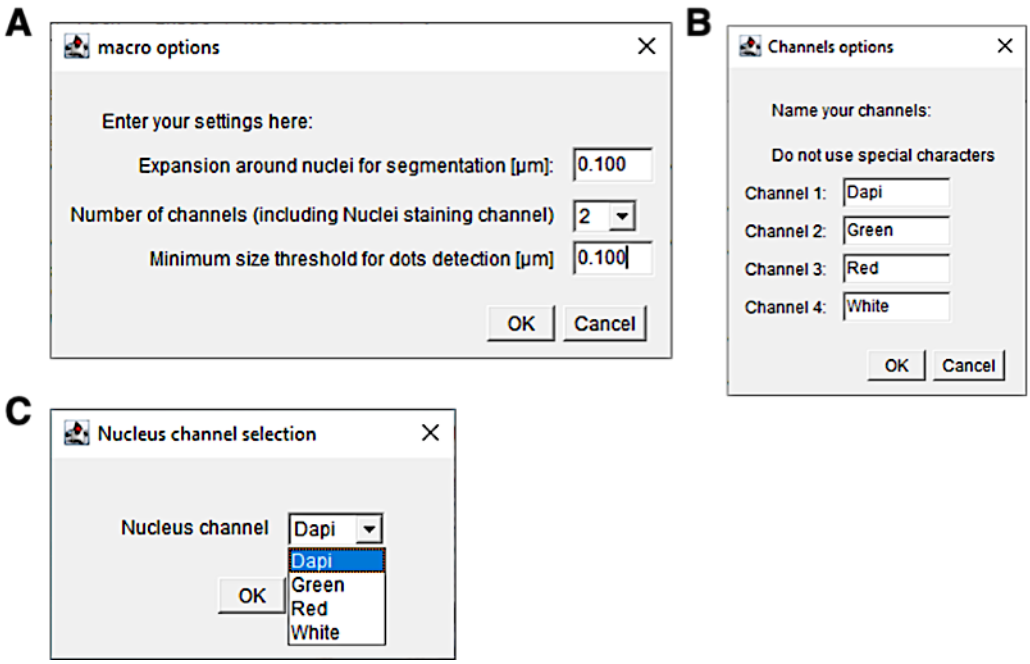


Figure 3. Macro options dialog used to configure settings for image analysis. (A) Macro Options Dialog. This dialog box allows users to set parameters for segmentation and dot analysis. Expansion around nuclei for segmentation [µm]: specify the expansion radius around the nuclei for segmentation, set to 0.100 µm (default setting), and can be changed on the dialogue if required. Number of channels (including DAPI): indicates the total number of channels used in the analysis, including the nuclei staining channel, minimum 2 and maximum 15. Minimum RNAscope dot size [µm]: defines the minimum size threshold for RNAscope dots to be considered in the analysis, set to 0.100 µm (default setting), and can be changed on the dialogue if required. **(B)** Channels Options Dialog. Users can label each channel used in the analysis. **(C)** Nucleus Channel Selection Dialog. The user selects the channel used for nucleus identification inside a dropdown menu.

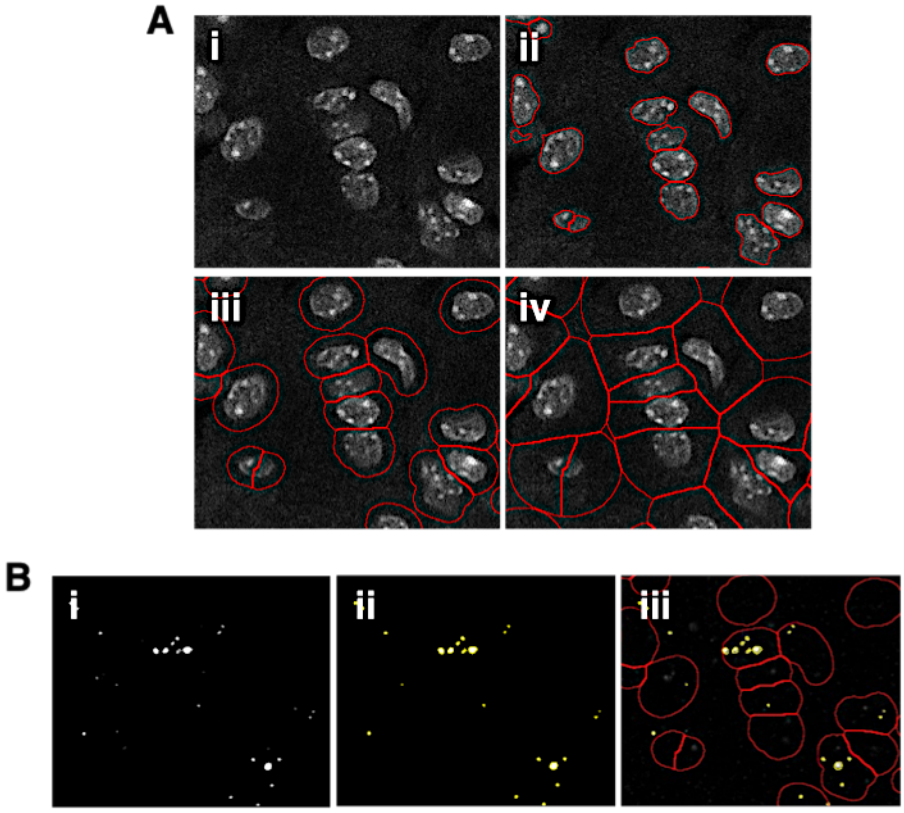
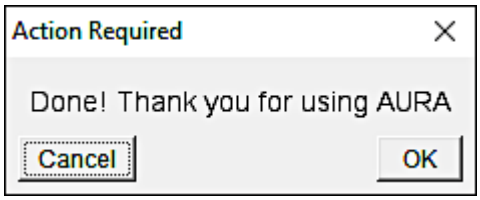


Figure 4. Segmentation results. (A) Example of nuclei segmentation process and cell area identification: i) nuclei staining; ii) nuclei segmentation; iii) results of segmentation with an expansion around nuclei of maximum 2 µm; iv) results of segmentation with an expansion around nuclei of maximum 7 µm. **(B)** Example of dots segmentation process and dot analysis: i) RNAscope fluorescent dots; ii) Dots segmentation highlighted in yellow; iii) Dots counting per cell based on the defined the cell area.

4. End of the macro processing

This message will appear when the analysis has been completed, click on “OK"



5. **Facultative step: customize the AURA macro code**

If the segmentation is satisfactory proceed directly to step 6.

If the segmentation of the samples is not suitable for your image, the macro code can be modified. Critical code lines for the nuclei and dots segmentation are reported below. This step requires Fiji coding expertise.

a) **Parameters for nucleus segmentation**

```
//////////////////// Nucleus Segmentation ////////////////////////////////////
selectImage(NucleusChannel);
run("Duplicate...", "title=nuclei_QC"); //For Quality control
selectImage(NucleusChannel);
run("Enhance Contrast", "saturated=0.35");
run("Apply LUT");
run("Mean...", "radius=2");
run("Gaussian Blur...", "sigma=2");
setAutoThreshold("Otsu dark");
run("Convert to Mask");
run("Open");
run("Watershed");
//////////////////////////////////////////////////////////////////////////////
```

b) Parameters for defining cell area

```
//////////////////// Voronoi-Based Cell Segmentation ////////////////////////
run("Duplicate...", "title=voronoi");
run("Voronoi");
setThreshold(1, 255);
setOption("BlackBackground", false);
run("Convert to Mask");
selectImage(NucleusChannel);
run("3D Watershed Voronoi", "radius_max=cell_size");
run("8-bit");
run("Invert");
imageCalculator("OR create", "voronoi","VoronoiZones");
selectImage("Result of voronoi");
run("Invert");
run("Select None");
run("Analyze Particles...", "show=Masks exclude");
run("Create Selection");
roiManager("add");
selectImage("Result of voronoi");
run("Select None");
run("Analyze Particles...", "show=Masks exclude add");

        roiManager("Select", 0);
        roiManager("Rename", "AllNuclei");
nNuclei=roiManager("count");
for (i = 1; i < nNuclei; i++) {
        roiManager("Select", i);
        roiManager("Rename", "cell_"+i);
        }
//////////////////////////////////////////////////////////////////////////////
```

c) Parameters for dots segmentation

```
//////////////////// Dot Segmentation Function ////////////////////////////////////
function ApplyDotSegmentation() {
 run("Enhance Contrast", "saturated=0.4");
 run("Gaussian Blur...", "sigma=0.5");
 setAutoThreshold("Yen");
   run("Convert to Mask");
   run("Erode");
   run("Dilate");
   run("Invert");
   run("Watershed");
}
//////////////////////////////////////////////////////////////////////////////////
```

d) **Parameter for dot analysis.** This step is repeated automatically for each channel you identified in Step 3.

```
//////////////////// Dot Analysis ////////////////////////////////////////////
for (i = 0; i < ChannelsTotal && i < 15; i++) {
   if (channelNames[i] != NucleusChannel) {
      selectImage(channelNames[i]);
      run("Duplicate...", "title=analyze_" + channelNames[i]);

      ApplyDotSegmentation();

      selectImage("analyze_" + channelNames[i]);
      run("Analyze Particles...", "size=" + dot_size + "-Infinity show=Masks");
      rename(channelNames[i] + "_mask");
      run("Duplicate...", "title=" + channelNames[i] + "_1");

      nNuclei = roiManager("count");
      for (k = 1; k < nNuclei; k++) {
         selectWindow(channelNames[i] + "_" + k);
         roiManager("Select", k);
         run("Analyze Particles...", "summarize");
         rename(channelNames[i] + "_" + (k+1));
      }
Table.deleteColumn("%Area");
Table.deleteColumn("Average Size");
Table.deleteColumn("Total Area");
saveAs("Results", Results_Folder_Path+replace(ori, ".tif",
"_"+channelNames[i]+".csv"));
run("Close");
//////////////////////////////////////////////////////////////////////////////
```

6. Macro-output

a) Inside your Images folder, three sub-folders will be generated (Figure 5):

- Quality_Control
- Results

- Regions of interest (ROIs)

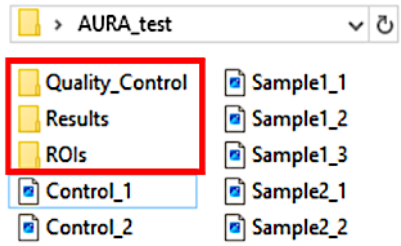


Figure 5. Representative image of the three subfolders generated by AURA, marked with a red square.

The Quality control folder contains already processed quality control pictures for each image (Figure 6A), including:

- nuclei segmentation, marked in red on the nuclei channel image to assess segmentation quality (Figure 6Bi).
- counted dots, marked in yellow and showed in association with the nuclei segmentation (in red) for each channel (Figure 6Bii).

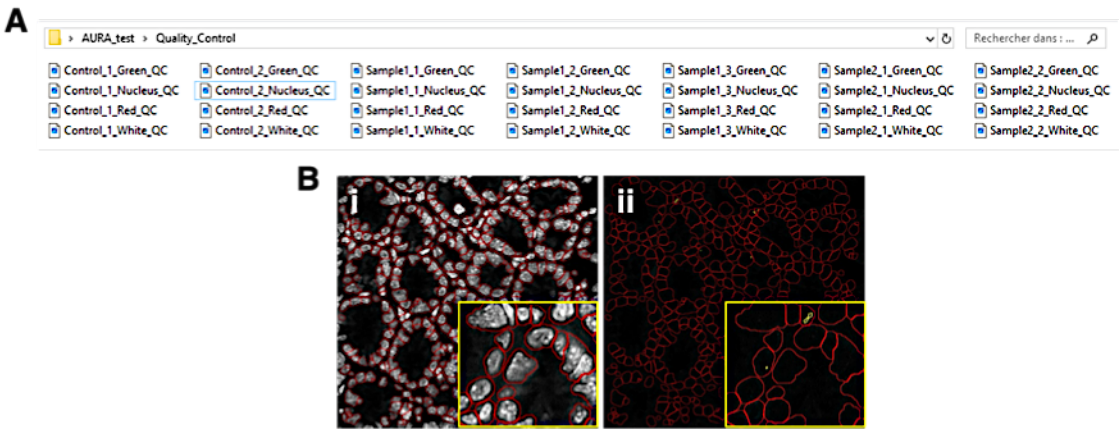


Figure 6 Quality control folder. (**A**) The subfolder contains individual quality control pictures for each image and analyzed channel (here Green, Nucleus, Red and White), saved as .TIFF format. Files are named according to the sample identifiers, followed by the "channel_name_QC" (e.g., "Control_1_Green_QC", "Sample1_1_Nucleus_QC"). (**B**) Representative example of the quality control image showing i) nuclei segmentation, displayed in red; (ii) Dots detection quality control. Dots are displayed in yellow with nuclei segmented in red.

The Results folder (Figure 7A) contains:

- .CSV files generated for each channel per analyzed image

- A single .TXT file that summarizes the analysis settings selected in Step 3.

The ROIs folder (Figure 7B) contains the raw data of the regions of interest generated by the macro, including the nuclei labelling. Visualisation of the raw data is done by dragging the RoiSet file (first) and the corresponding .TIFF image file for the selected channel (second) into the Fiji interface (Figure 7C). The ROI Manager drop-down menu will open (Figure 7D left panel). To display the segmentation, select “Show all” and “labels”. The image with the chosen ROI will open (Figure 7D, right panel).

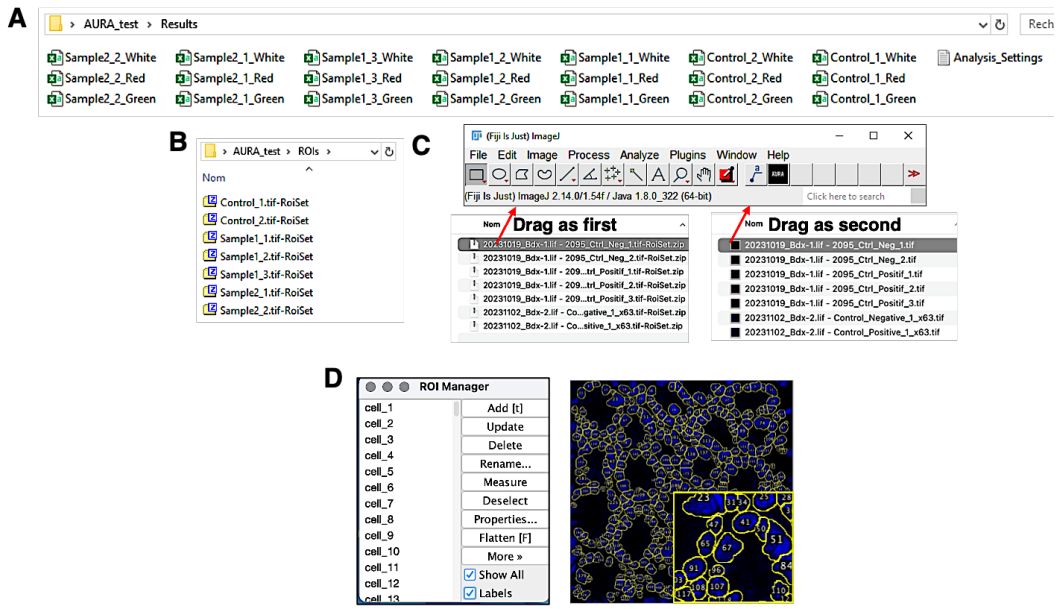


Figure 7. (A) The Results Folder contains the result data for each image across the different channels, saved in .CSV format. Files are named according to the image identifiers, followed by the channel name (e.g., "Sample2_1_White", "Control_1_Red"). **(B)** The ROIs Folder contains sets of regions of interest (ROIs) used in the analysis, saved as .TIFF format. Files are named according to the image identifiers (here Sample and Control), followed by "RoiSet" to indicate ROI data (e.g., "Control_1.tif-RoiSet", "Sample1_3.tif-RoiSet"). **(C)** Open the ROI manager in Fiji. **(D)** ROI manager drop-down menu (left panel) and representative ROIset displayed on nuclei channel (right panel). Each nucleus has an assigned number which will appear in the result file as slice # (e.g. slice 1 = Nucleus #1).

Export & merge data

Timing: 120 files/min

This step includes merging all .CSV files generated by the AURA macro in one .XLSX file. This step is performed with the web app written in *Python* named "Aura Data Processing" and available at: https://aura-data-processing.streamlit.app/.

7. Set parameters for data processing

a) Provide an experiment name

i) Without whitespace

b) Choose your type of analysis

c) Upload your AURA data

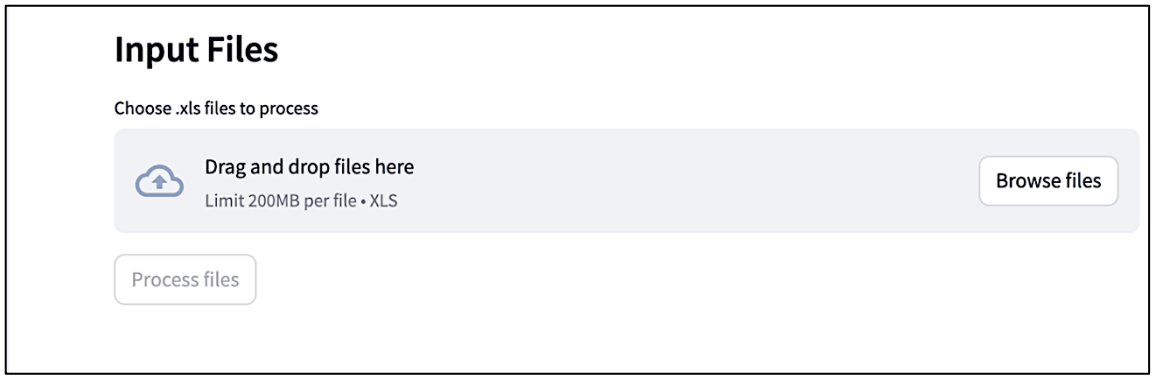
i) Choose the input format

- .CSV files or .ZIP folder

d) Upload your files accordingly

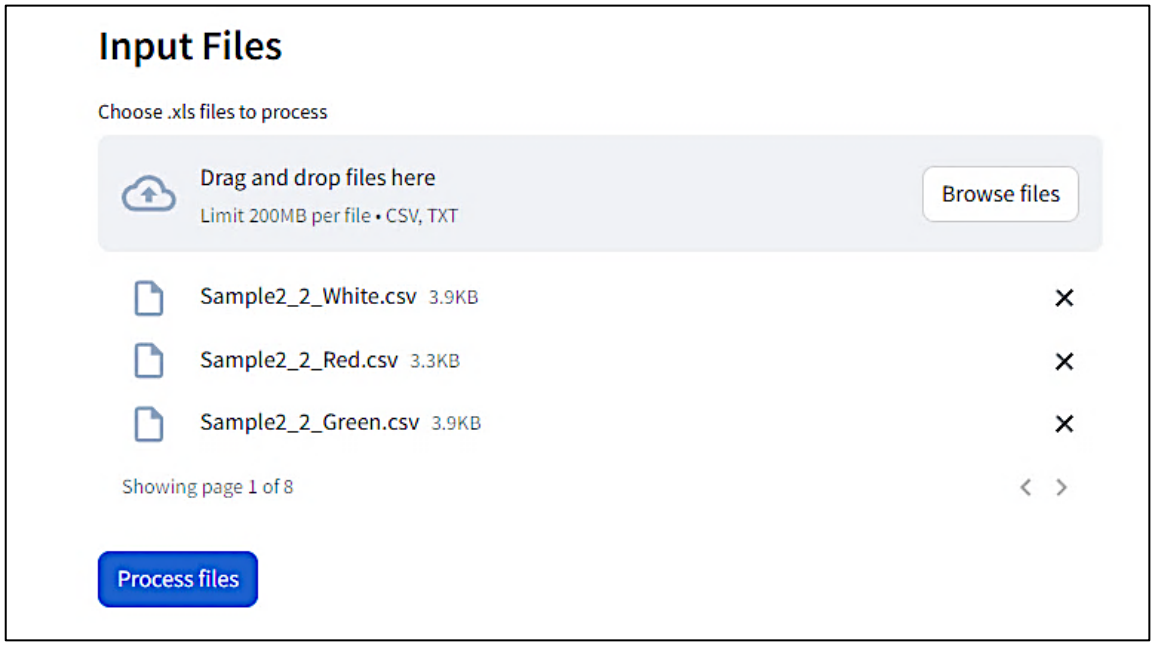
i) Drag & drop files onto the dedicated area or select files through your file manager (Browse files button)

Note: *Upload all the results .CSV files* ***along with the Analysis_Settings.txt*** *file generated by the AURA macro.*

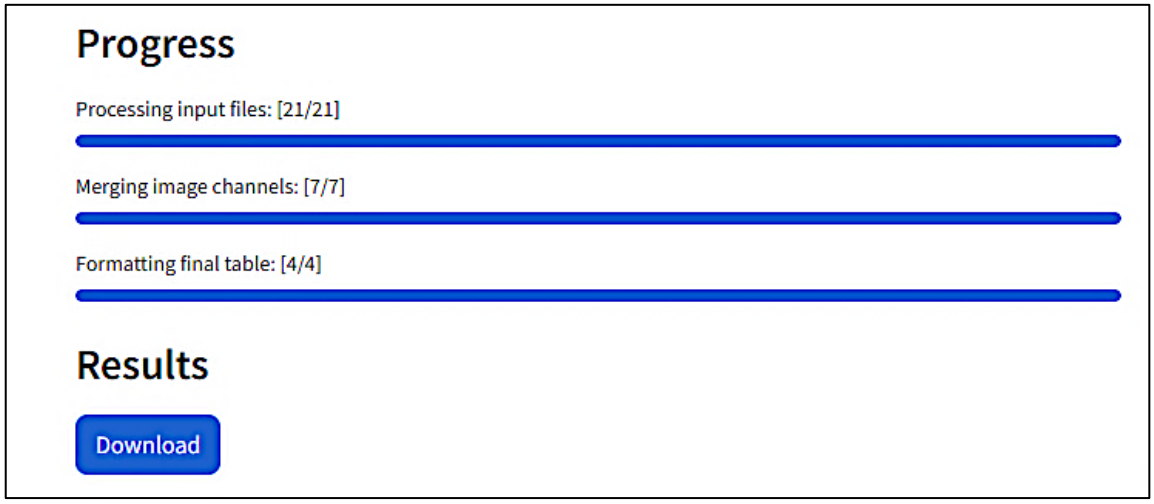


e) Click on "Process files" to run the script

***Note**: the button "Process files" becomes clickable only after uploading your files and providing an experiment name.*



f) Follow script progression



8. **Download the results file in .XLSX format**
   a) Click on "Download" to get your results in a single .XLSX file

Quality control

Timing: 1 min by picture

The quality control picture with nuclei overlaid on the original image is used to inspect the segmentation quality. These images are saved in the Quality_control subfolder (step 6, "Step-by-Step methods detail" section and Figure 6) and should be stored for findability and accessibility [5].

9. **Segmentation quality control**
   a) Perform a visual quality control on all the analyzed images on the nuclei and dots segmentation:
      i) All nuclei segmentation pictures are saved as "sample_name**"_Nucleus_QC**
      ii) All dots segmentation pictures are saved as "sample_name"**_marker_name_QC**

Result analysis

10. RNAscope analysis

RNAscope data can be analyzed in various ways:

a) By the percentage of positive cells.

b) By quantifying the average number of dots or transcripts per cell.

c) By the H-scoring [6]. The H-score is a metric derived from RNAscope image analysis to visualize dynamic levels of a particular RNA expression within a cell population. The H-score is obtained by categorizing cells into bins based on their expression level, indicated by the number of detectable dots within a cell as follows:

- Bin 0 indicates no detectable staining.
- Bin 1 includes cells with 1 to 3 dots.
- Bin 2 comprises cells with 4 to 9 dots.
- Bin 3 includes cells with 10 to 15 dots.
- Bin 4 represents cells with more than 15 dots.

To measure the H-score, each bin is weighted by multiplying the percentage of cells within a bin category by the bin number. The sum of these weighted values yields the final H-score. The formula is expressed as follows:

$$\textbf{H-score} = \sum_{\substack{\textbf{Bin} \\ \textbf{0} \rightarrow \textbf{4}}} \textbf{(Percentage of cells per bin x Bin number)}$$

d) By the RNA probes co-localization based on the percentage of cells displaying one or more dots for different targets.

11. Results output:

a) Excel sheet for each sample (Figure 8A), containing the following information:

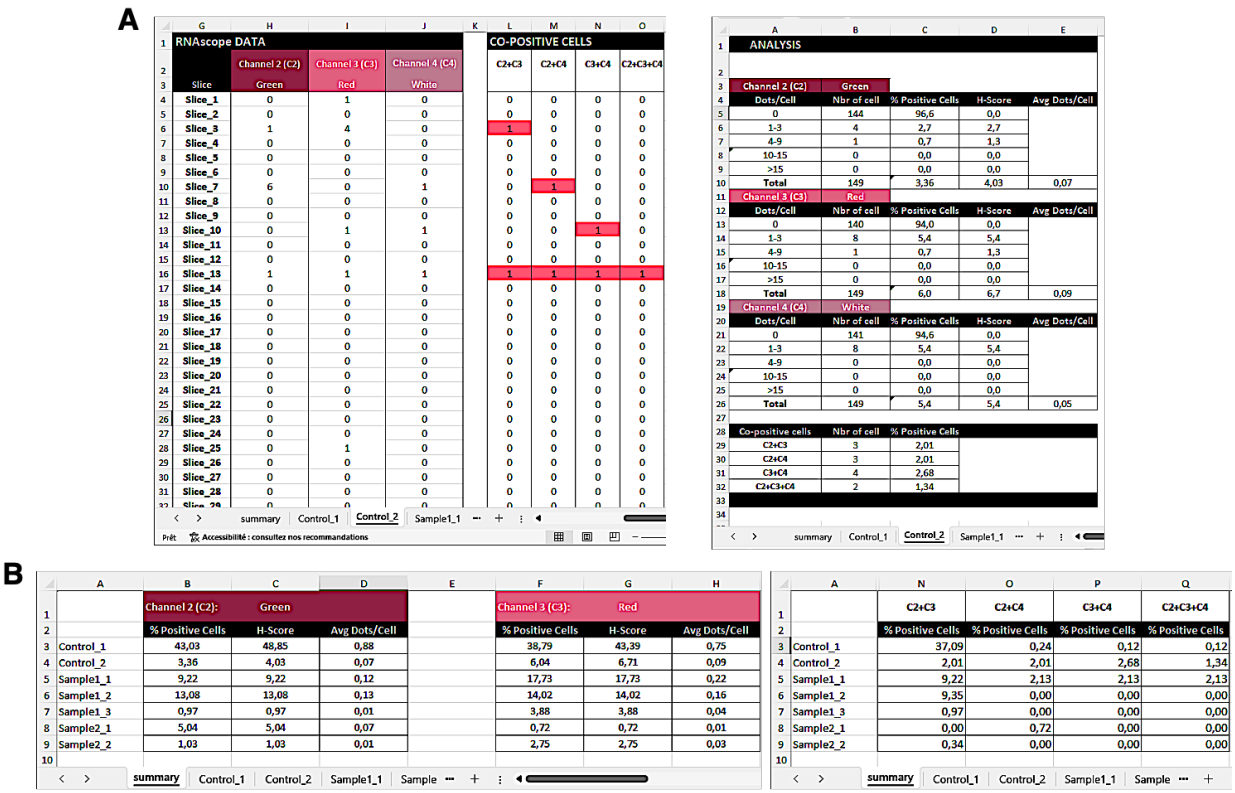
i) Number of dots for each cell and for each channel analyzed, and for double or multiple positive cells (herein defined as co-positive cells).

ii) RNAscope analysis table with:

- Number of cells per each RNAscope bin for each channel
- Percentage positive cells per each RNAscope bin for each channel
- H-score per channel
- Average number of dots per cell per channel
- Number and percentage of co-positive cells

b) Excel sheet containing a consolidated summary of the results for all the samples (Figure 8B) containing the following information:

- Percentage of positive cells per channel
- H score per channel
- Average number of dots per cell per channel
- Percentage of co-positive cells



A

RNAscope DATA

Slice	Channel 2 (C2) Green	Channel 3 (C3) Red	Channel 4 (C4) White	CO-POSITIVE CELLS C2+C3	C2+C4	C3+C4	C2+C3+C4
Slice_1	0	1	0	0	0	0	0
Slice_2	0	0	0	0	0	0	0
Slice_3	1	4	0	1	0	0	0
Slice_4	0	0	0	0	0	0	0
Slice_5	0	0	0	0	0	0	0
Slice_6	0	0	0	0	0	0	0
Slice_7	6	0	1	0	1	0	0
Slice_8	0	0	0	0	0	0	0
Slice_9	0	0	0	0	0	0	0
Slice_10	0	1	1	0	0	1	0
Slice_11	0	0	0	0	0	0	0
Slice_12	0	0	0	0	0	0	0
Slice_13	1	1	1	1	1	1	1
Slice_14	0	0	0	0	0	0	0
Slice_15	0	0	0	0	0	0	0
Slice_16	0	0	0	0	0	0	0
Slice_17	0	0	0	0	0	0	0
Slice_18	0	0	0	0	0	0	0
Slice_19	0	0	0	0	0	0	0
Slice_20	0	0	0	0	0	0	0
Slice_21	0	0	0	0	0	0	0
Slice_22	0	0	0	0	0	0	0
Slice_23	0	0	0	0	0	0	0
Slice_24	0	0	0	0	0	0	0
Slice_25	0	1	0	0	0	0	0
Slice_26	0	0	0	0	0	0	0
Slice_27	0	0	0	0	0	0	0
Slice_28	0	0	0	0	0	0	0

ANALYSIS

Channel 2 (C2) Green				
Dots/Cell	Nbr of cell	% Positive Cells	H-Score	Avg Dots/Cell
0	144	96,6	0,0	
1-3	4	2,7	2,7	
4-9	1	0,7	1,3	
10-15	0	0,0	0,0	
>15	0	0,0	0,0	
Total	149	3,36	4,03	0,07
Channel 3 (C3) Red				
Dots/Cell	Nbr of cell	% Positive Cells	H-Score	Avg Dots/Cell
0	140	94,0	0,0	
1-3	8	5,4	5,4	
4-9	1	0,7	1,3	
10-15	0	0,0	0,0	
>15	0	0,0	0,0	
Total	149	6,0	6,7	0,09
Channel 4 (C4) White				
Dots/Cell	Nbr of cell	% Positive Cells	H-Score	Avg Dots/Cell
0	141	94,6	0,0	
1-3	8	5,4	5,4	
4-9	0	0,0	0,0	
10-15	0	0,0	0,0	
>15	0	0,0	0,0	
Total	149	5,4	5,4	0,05

Co-positive cells	Nbr of cell	% Positive Cells
C2+C3	3	2,01
C2+C4	3	2,01
C3+C4	4	2,68
C2+C3+C4	2	1,34

B

	Channel 2 (C2): Green			Channel 3 (C3): Red		
	% Positive Cells	H-Score	Avg Dots/Cell	% Positive Cells	H-Score	Avg Dots/Cell
Control_1	43,03	48,85	0,88	38,79	43,39	0,75
Control_2	3,36	4,03	0,07	6,04	6,71	0,09
Sample1_1	9,22	9,22	0,12	17,73	17,73	0,22
Sample1_2	13,08	13,08	0,13	14,02	14,02	0,16
Sample1_3	0,97	0,97	0,01	3,88	3,88	0,04
Sample2_1	5,04	5,04	0,07	0,72	0,72	0,01
Sample2_2	1,03	1,03	0,01	2,75	2,75	0,03

	C2+C3	C2+C4	C3+C4	C2+C3+C4
	% Positive Cells	% Positive Cells	% Positive Cells	% Positive Cells
Control_1	37,09	0,24	0,12	0,12
Control_2	2,01	2,01	2,68	1,34
Sample1_1	9,22	2,13	2,13	2,13
Sample1_2	9,35	0,00	0,00	0,00
Sample1_3	0,97	0,00	0,00	0,00
Sample2_1	0,00	0,72	0,00	0,00
Sample2_2	0,34	0,00	0,00	0,00

Figure 8. Example output summary data files. (A) Summary for each sample. Left panel: representative .XLSX sheet containing information for the number of dots per nucleus in each channel (slice 1 = nucleus 1) and the number of dots per nucleus for co-positive cells in the three analyzed channels in one selected sample (Control_2). Co-positive cells are highlighted in colour. Right panel. Representative .XLSX sheet reporting the percentage of cells in each of the RNAscope bin categories (% positive cells), the H-score, and the average number of dots per cell (Avg Dots/Cell) per each acquired channel (here Green, Red, and White), as well as the number and percentage of co-positive cells in one selected sample (Control_2). **(B)** Consolidate summary for all samples. Left panel: summary .XLSX sheet presenting the percentage of cells with dots, the H-score, the average number of dots per cell for each sample analysed in the single channels. Right panel: summary .XLSX sheet presenting the percentage co-positive cells in the three channels analyzed.

Expected outcomes

This protocol offers a simple, open-source, low-cost, easy-to-use macro/script to analyze large amounts of RNAscope microscopy data. The output includes segmented nuclei and dots identification, which are saved as separate files. The script merges separated .CSV files in one unique .XLSX file with a pre-analysis of the data for each sample and a final analysis summary for all the samples. Saved quality control images allow for visual validation of the segmentation

accuracy. Furthermore, this method permits cell-by-cell control and will enable data storage and management according to the findability and accessibility guidelines [5].

Quantification and statistical analysis

The quantification of the percentage of single or multiple positive cells is directly provided in the RNAscope result output Excel data sheet. Statistical analyses can be performed using Prism 7 and Graph Pad Software, or any statistical analysis software, applying the appropriate statistical methods depending on the experimental set-up, e.g. comparing two or multiple experimental groups.

Limitations

Nucleus staining and imaging quality:

Nucleus staining and image acquisition quality are crucial for accurate analysis. Good DAPI staining and image acquisition should exhibit low background noise and minimal overlap between nuclei. Poor-quality staining and image capturing can lead to incomplete segmentation and unreliable quantification. Figure 9 provides examples of poor and good-quality DAPI staining and imaging, with the corresponding nuclei segmentation results.

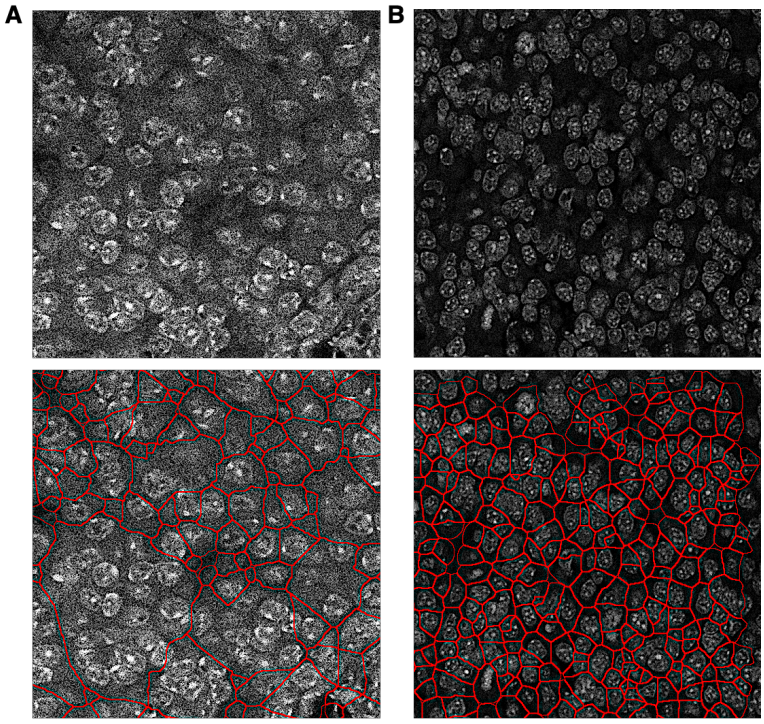


Figure 9. Examples of DAPI staining image files. Representative micrographs showing a bad quality **(A)** and good quality picture **(B)**. The lower panel shows the results of the AURA nuclei segmentation.

Analyzing only one channel:

Currently, the AURA macro does not support the analysis of only one of the acquired channels; therefore, a complete analysis of all the channels chosen in step 3a will be automatically performed.

Troubleshooting

Problem 1:

Error message when starting the AURA macro.

Potential solution

This may occur if all the plugins are not installed or if special characters were used when naming your channels

- Ensure that all the plugins (downloaded in step 3 "Before you begin" section) are installed.
- Avoid using special characters in channels' names.

Problem 2:

Using a zero value for nuclei expansion results in poor segmentation (step 3a, "Step-by-Step methods" detail section).

Potential solution

The macro considers a value of 0 as infinite expansion; hence the default value is set at 0.1µm.

Problem 3:

Inaccurate nuclei segmentation.

Potential solution

Inaccurate segmentation can result from poor DAPI staining or image quality.

- Make sure to exclude any images from your analysis if they are unusable. It is recommended to take multiple pictures (10 per sample) to ensure a sufficient number of high-quality pictures for the analysis.
- Adjust the settings inside the macro script (code at lines 22-35) to improve segmentation accuracy (step 5, "Step-by-Step methods detail" section).
- Perform empirical analysis by manually counting the nuclei while retaining the dots segmentation.
- Retake pictures of your sample, ensuring better nuclei staining and nuclei acquisition.

Problem 4:

Dots are not counted by the AURA macro.

Potential solution

This can happen if the threshold set for dot detection is too high.

- Reduce the threshold for dot detection to ensure more dots are counted (step 3c, "Step-by-Step methods detail" section).
- Adjust the relevant macro code at lines 40-51 to fine-tune dot detection parameters (step 5, "Step-by-Step methods detail" section).

Problem 5:

Remembering analysis parameters (after step 3).

Potential solution

Failure to recall parameters may render the process of replicating or reviewing the analysis more complex. Refer to the file named Setting_Analysis.txt in the Results folder, where settings parameters are stored.

Problem 6:

Error message when connecting to the web app (step 7, "Step-by-Step methods detail" section).

Potential solution

The web app will automatically enter sleep mode after several days of inactivity. Click the refresh button and wait a few minutes for the app to start.

Problem 7:

AURA Data Processing *Python* App does not process files (step 8, "Step-by-Step methods detail" section).

Potential solution

- Ensure that you have uploaded the Setting_Analysis.txt file, which distinguishes files with similar names.
- Try uploading your results folders as a .ZIP file to ensure that all necessary files are included.

Problem 8:

- Issues on the summary page when opened with Microsoft Excel (step 10, "Step-by-Step methods detail" section). The problem occurs if your data file names are longer than 31 characters, which is not supported in Microsoft Excel.

Potential solution

- Open your file with LibreOffice Calc, which does not have this limitation.

Problem 9:

Using the AURA macro for chromogenic RNAscope pictures.

Potential solution

This step requires color separation to separate the markers into different color channels in Fiji, which is done as follows:.

- Images need to be in the RGB format: in the Fiji menu navigate to Image > Type and scroll down to select RGB color.
- In the Fiji menu navigate to Image > Color and scroll down to select Color Deconvolution.
- From the Color Deconvolution dropdown menu select "From ROI" (scroll down if it is not visible among the first choices).
- Use the rectangle tool to select examples of nuclei, probes, and background stains.
- Run the color deconvolution to generate separate images for each channel. Save images in .TIFF format in a folder and proceed with the AURA protocol.

Resource availability

Lead contact:

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Jean Descarpentrie (jean.descarpentrie@umu.se).

Technical contact:

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Wilfried Souleyreau (wilfried.souleyreau@u-bordeaux.fr).

Data and code availability:

The AURA macro is available at https://github.com/FlorianBrnrd/aura-data-processing.

The web app "AURA data processing", which allows users to merge .CSV files into a unique .XLSX file, can be found at https://aura-data-processing.streamlit.app/.

The script for the web app is available at https://github.com/FlorianBrnrd/aura-data-processing.

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Author contributions

Conceptualization, O.C.B.M., W.S.; methodology, J.D., W.S.; app design, F.B.; writing, J.D., W.S., TF; funding acquisition and supervision, LB, TM, I.S.P., T.F.

Declaration of interests

The authors declare no competing interests.

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