**Uncovering dynamic transcriptional regulation of methanogenesis via** **single-cell imaging of archaeal gene expression**

**1. Introduction**

This code package is designed to analyze confocal image stacks of microbial cells labeled using smHCR-FISH. In this document, we provide the minimum information needed to use the code.

The code requires pre-installation of MATLAB (2023a) with the image analysis, curve fitting, optimization, and parallel processing toolboxes. In addition, ImageJ (v1.54j) needs to be installed for cell segmentation. To run the code smoothly, we highly recommend a 64-bit computer system with a multi-core processor and large (≥30 GB) RAM. To install the package, simply copy the folder “smHCR-FISH” to your computer, and add “smHCR-FISH/MATLAB” to the MATLAB search path.

**2. Image conversion and pre-analysis processing**

Before running the code, download ‘bioformats\_package.jar’ from the website <https://www.openmicroscopy.org/bio-formats/downloads/>, and put this file in the subfolder “MATLAB”. Then go to the folder “smHCR-FISH/Confocal”, and create a subfolder (e.g. “Exp1”) to store your confocal image files. Sample image files are available at http://gofile.me/4yuzx/zbFlbhcpr/LMO-1 FISH data. Next, in the folder “smHCR-FISH”, find the Excel file “Duallist.xls”. Open the file, and add the information regarding your data using the following format:

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Folder name** | **Leave Blank** | **Channel index** | **Tiling method** | **Channel name** | **Running index** |
| Confocal/Exp1/ |  | [1,1,2,3,4] | [1,1] | {'DAPI','A488','TMR','A647'} | T |

After saving the Excel file, run the following scripts in MATLAB:

1. To convert the confocal CZI files into TIF images, run “cziconvert2('Duallist.xls');”
2. To register images from different hybridization round, run “align\_stacknew ('Duallist.xls');”. For images that cannot be registered by the code, run “manual\_align” for manual registration.
3. To create a results folder and save basic information for further analysis, run “Dualprocess0;”

Once done, you will have a subfolder called “stacks” inside your data folder (e.g. “Exp1”) containing all TIFF files, and another subfolder “Results” for future analysis results.

**3. Cell segmentation**

1. For automatic segmentation, using the ‘StarDist’ Plugin in ImageJ, and manual refinement of the ROI result, create a subfolder “RoiSet”, and save the manual refinement results in the “RoiSet”.
2. To create a segmentation masks, run the MATLAB “mask\_rec”.

All segmentation results are stored in the subfolder “masks” inside your data folder.

**4. smFISH spot analysis**

To identify smFISH spots and quantify their intensities, run the following scripts:

1. For automatic spot quantification, run the MATLAB GUI “stack\_RNA”. Choosing “Analysis types” (“foci”) in the GUI.
2. To extract the typical intensity of a single mRNA, run “hist\_fit\_RNA\_stage;”.
3. To double-check and improve the identification of mRNAs, run the MATLAB GUI “stack\_RNA\_check”.

Results of mRNAs are stored in the subfolders “Histogram” inside your data folder.

**5. Analysis of transcriptional regulation**

1. After completing steps 1-4, run “foci\_mask” to count signal spots within a single cell.
2. To fit the mRNA distribution to a two-state model, use MATLAB function

“TwoState\_FSP\_NSX\_st\_twocopy\_P0\_simu”.

**6. Analysis of Cellular Gene Expression in Relation to Iron mineral**

1) Create a subfolder (e.g. “fe\_position”) to store bright field images with iron mineral. Using the ‘StarDist’ Plugin in ImageJ to identify the location of the iron mineral, and save the results in this subfolder.

2) To create the iron mineral segmentation masks, run the MATLAB “fe\_mask”.

3) To register iron mineral images from different hybridization rounds, run “fe\_mask\_align” for manual registration.

4) To compare and analyze the gene expression between cells on the iron mineral and those outside of it, run “fe\_mask\_free\_adhere”.

**7. Others**

1. To count cell numbers, run “cell\_num;”
2. To quantify the ribosome levels, run “Mean\_16s”.
3. To count division cells, run “spline\_axis\_zf”. For manual check, run “modal\_check”.