ThunderFISH

Easy ImageJ pipeline for increased throughput of RNA-FISH experiments

v.1.0 Manual

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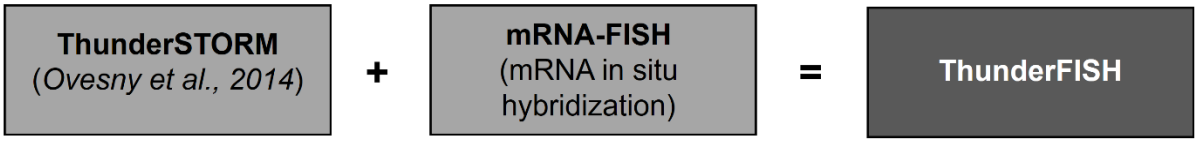
1. Introduction

What is ThunderFISH?

It's a simple protocol that allows counting single molecule transcripts in single cells using mRNA-FISH labelling and clever data processing. Originally developed for mouse embryonic stem cells with lowly expressed genes but works too for other cell lines.

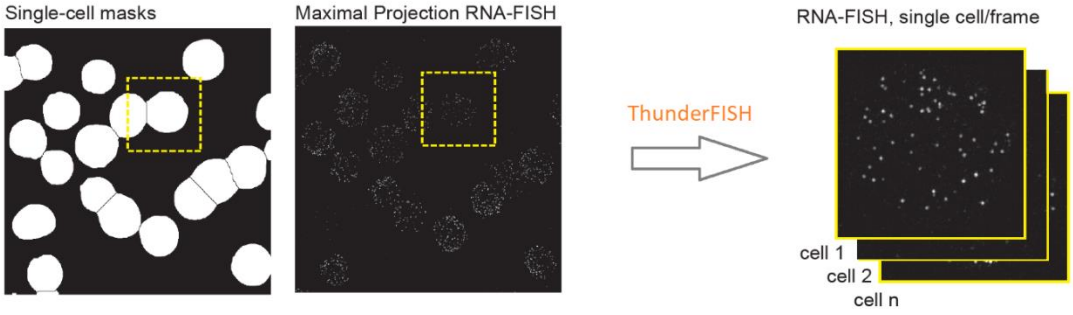


Why ThunderFISH?

While excellent alternatives like FISH-QUANT (*Mueller et al., 2013*) exist and provide great precision in 3D spot quantification, ThunderFISH is worthwhile due to its great **speed** and **throughput**. In our hands ThunderFISH allows acquisition and analysis of up to 10,000 cells in one day using a standard fluorescence wide-field microscope!

How does it work?

ThunderFISH is a method that comprises i) easy **sample preparation protocol** combined with ii) usage of ThunderFISH ImageJ/FiJi **script** to pre-process RNA-FISH microscopy images of full fields of view to make them compatible with ImageJ plugin *ThunderSTORM* (*Ovesny et al., 2014*) that in turn allows counting of diffraction limited spots with great speed - in minutes you'll obtain your transcript counts per cell as a .csv file. **Just follow this manual!**



What is it dedicated for?

This protocol and data analysis pipeline is designed to analyse standard RNA-FISH image data in mouse embryonic stem (mES) cells for genes with medium to low expression levels, i.e. when <50 transcripts per cell occur on average (see *Dobrinic et al., 2020* for the original publication) – this number has been obtained for mES cells and depends on cell volume; larger cell types will allow counting more transcripts. It is also meant to help capture rare cells.

2. Get ready to start with ThunderFISH

Download Imagej/FiJi:

The latest version of Fiji/ImageJ is available here: https://imagej.net/Fiji/Downloads

We tested the ThunderFISH scripts using: ImageJ 1.52p, Java 1.8.0_172 (64-bit), Win 10

Imagej/Fiji packages required:

ThunderSTORM – Imagej/Fiji plugin for reconstruction of single molecule localization microscopy data such as STORM and PALM (*Ovesny et al., 2014*).

For information and installation follow the official guidelines:

https://zitmen.github.io/thunderstorm/

Restart your Fiji. If the installation was successful, you should be able to find ThunderSTORM under ImageJ/Plugins/ThunderSTORM in ImageJ drop-down list.

Download ThunderFISH script:

https://github.com/aleks-szczure/ThunderFISH

Equipment:

Regular wide-field microscope with a set of fluorescence filters, motorized stage that allows acquisition of 3D images, and an EMCCD or sCMOS camera connected to a computer. ThunderFISH was tested on Windows 7 and Windows 10 machines.

Other important information:

For comparison with other methods of spot counting see our paper Dobrinic et al., 2020

3.1. ThunderFISH sample preparation

1) Plate cells on a 10 cm plastic plate and culture until 80% confluence is reached (approx. 30M cells per plate).
2) Wash the **adherent** cells once with 1x PBS, aspirate it and add 1.5 ml 0.5% trypsin. Incubate for 3 minutes at 37°C. Wait until majority of the cells detaches.
3) Add 3.5 ml of PBS and resuspend thoroughly the cells. NOTE: it is important to avoid any cell-clumps and have a single cell suspension, hence pipette the solution of cells multiple times this applies to all subsequent steps too.
4) For **suspension cultures** begin here. Collect the cells to a 15 ml centrifugation tube and centrifuge for 3 min at 300 g.
5) Aspirate the solution leaving out approx. 200ul of the solution with the cell pellet. Use 1 ml-pipette and resuspend the cell pellet. Add 2 ml of 3.7% paraformaldehyde for 10 min to fix the cells. NOTE: insufficient cell resuspension will lead to fixation of the cells to each other forming cell clumps.
6) Centrifuge for 3 min at 300 g, aspirate formaldehyde solution and replace it with 2 ml of ice-cold 70% ethanol. Resuspend the pellet and incubate the cells in a fridge for at least 30 min. The cells in 15 ml tube can be stored in such conditions up to 10 days. NOTE: as an alternative to ethanol we recommend 0.5% TX-100/PBS solution.
7) Centrifuge for 3 min at 300 g, discard ethanol solution by inverting the tube to the sink. Resuspend the pellet in 2 ml of 2 x SSC, 10% (v/v) formamide solution.
8) Centrifuge for 3 min at 300 g. Using 1ml pipette aspirate thoroughly any liquid. Add 200ul of the staining solution containing 2 x SSC, 10%(v/v) formamide, and 20% (w/v) dextran sulfate containing 1 µl of standard RNA-FISH probe set. Carefully resuspend the pellet. NOTE: the concentration of FISH-probes should be optimized for each probe-set.
9) Incubate the tube with staining reaction at 37°C overnight. NOTE: minimum staining time required is 4 h.
10) Add 2 ml of 2 x SSC, 10% (v/v) formamide solution and centrifuge for 3 min at 300 g. Discard solution and replace it with 2 ml of 2 x SSC, 10%(v/v) formamide solution containing appropriate DAPI concentration. Incubate for at least 15 min at 37°C. NOTE: If no DAPI staining is required in your experiment repeat this step to wash out residual probe.
11) Centrifuge for 3 min at 300 g and replace the solution with 2 ml of 2 x SSC with appropriate Agglutinin-Alexa488 concentration for 5 min at room temperature (read provider's manual for more details).
12) Fill up the tube with PBS and centrifuge for 3 min at 300 g. Aspirate supernatant with 1 ml pipette leaving approximately 100 µl, resuspend the cell pellet (see **Fig. 1A** for details).
13) Apply approx. 10 µl Vectashield H-1000 mounting medium on a glass microscopy slide. Add 5 µl of the cell solution, pipette multiple times the droplet on a glass slide to avoid cell clumps and evenly distribute the cells in a solution of mounting medium.
14) Apply a 0.17 mm-thick microscope coverslip from the top and allow the solution of cells expand evenly underneath (**Fig. 1B**) and add a tissue to collect an excess of mounting media. Press very hard from the top using your pointing finger multiple times, for as long as an excess of liquid appears on a tissue. This will result in a single cell monolayer (**Fig. 1C**). NOTE: this step is crucial for successful microscopy experiment where cells are not allowed to overlap with each other in 3D. This would compromise further analysis and reliable cell separation.
15) Seal the coverslip with nail-polish. Your sample is ready for imaging!

ADVICE: Too many cells in a field of view may lead to errors due to cells merging during image tresholding by ThunderFISH pre-processing script. In contrast, few cells will yield little information and will necessitate prolonged microscopy measurements and larger data that will be processed longer. Therefore, we advise always preparing 2 – 3 slides per sample using varying volume of the cell suspension.

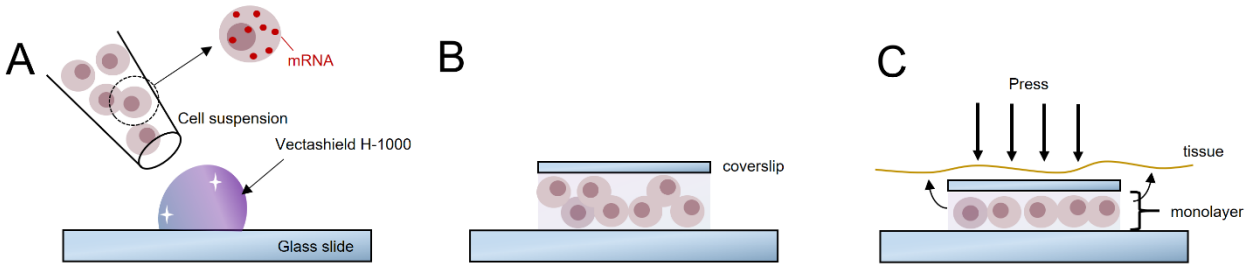


Figure 1. Microscopy sample preparation for ThunderFISH, individual steps depicted. **A**) Labelled cell suspension is mixed with mounting medium on a glass slide. **B**) Microscopy coverslip is applied from the top leading to even distribution of the solution but not cell monolayer. **C**) A paper tissue is applied from the top and the coverslip is pressed down (arrows). This allows the cells to be easily separated from each other as they are now in 2 dimensions instead 3 and all have round, globular shapes – image tresholding from now should be piece-a-cake!

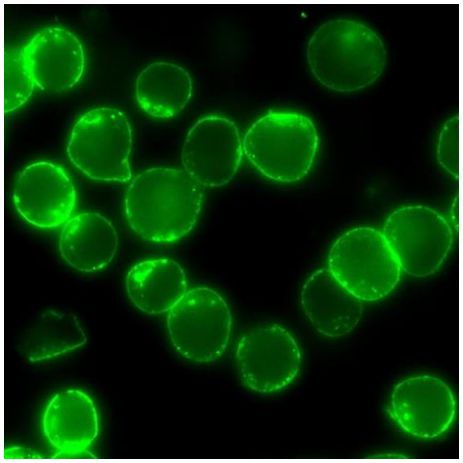


Figure 2. Optimal mouse embryonic stem cell density in a single field of view for ThunderFISH processing. Green colour represents Agglutinin-staining of the cell membrane. Maximal projection of 30 images taken every 0.35 µm. Note that thanks to this sample preparation mES colonies are broken down to single cells that can be easily separated from each other. Most of the cells are very round and have circularity > 0.8 - hence will be taken into account by ThunderFISH. Cells touching the image boarders are going to be discarded from image analysis.

3.2. Microscopy

When you have already optimized the cell concentration in your sample you are ready to carry out some imaging.

In our experiments we acquire usually 45 - 60 separate fields of view in 3D per sample. This on regular allows us analysis of 500 – 1200 cells which is enough to precisely describe transcript distribution among cells for vast majority of genes. Here are some basic parameters of our setup and settings used to give you first impression where to start:

Camera: 1200 x 1200 px^2 (the larger the better), pixel size corresponds to 96 nm in the sample, 600 ms exposure time. CAUTION: we strictly recommend to stick to such or similar camera pixel size. Much larger pixel size will lead to under-sampling of the diffraction limited spots corresponding to transcripts. This may lead to their merging due to compromised systems resolution. In principle we discourage from using camera pixel size > 130 nm per pixel. Note that all ThunderFISH parameters are optimized for appropriate sampling using correct sampling! If you are not sure what pixel size does your imaging system require please use SVI Nyquist Calculator for most precise estimation: https://svi.nl/NyquistCalculator

Objective: oil-immersed 60x, PLAN APO, 1.4 NA, with additional 2x lens. Objectives with lower magnification in most of the cases are not advisable.

Excitation: 550 nm diode at maximum intensity, Quasar570-labelled probes (Stellaris) that we are using barely bleach in such conditions. Cy3 probes in our hands bleached more readily but provided sufficient signal. Quasar670 (Stellaris) performs well but from our experience probe concentration requires a careful optimisation as too high concentration seems to result in self-quenching.

Imaging settings: 30 images every 0.35 µm are acquired for each of the fields of view Depending on the experiment different other channels are included as well such as DAPI etc. Channels are acquired sequentially to speed up acquisition. ADVICE: the closer the sampling rate in Z to Nyquist sampling, the higher the quality of the data in ThunderFISH. This is due to multiple acquisition of signal from the same transcripts. We strongly discourage using Z-step exceeding 0.4 µm as in our experience it affects the data quality in spite of significant acceleration of the imaging process.

3.3. ThunderFISH analysis step by step

3.3.1 Images

After performing 3D-imaging of your samples prepared in the previous section we recommend exporting the data to .tiff format with channels split (see **Fig. 3**, Left A – C for examples of desired data arrangement). Let's assume you carried out imaging in a similar fashion to us, namely, acquiring 3 channels using n = 30 Z-slices every 0.35 µm and exported it as a .tiff file. As the result of loading it to ImageJ your stack will have 3 x 30 = 90 slices; First 30 corresponding to DAPI, next 30 corresponding to GFP, and last 30 representing RNA-FISH channel (See **Fig. 3**, Left, A).

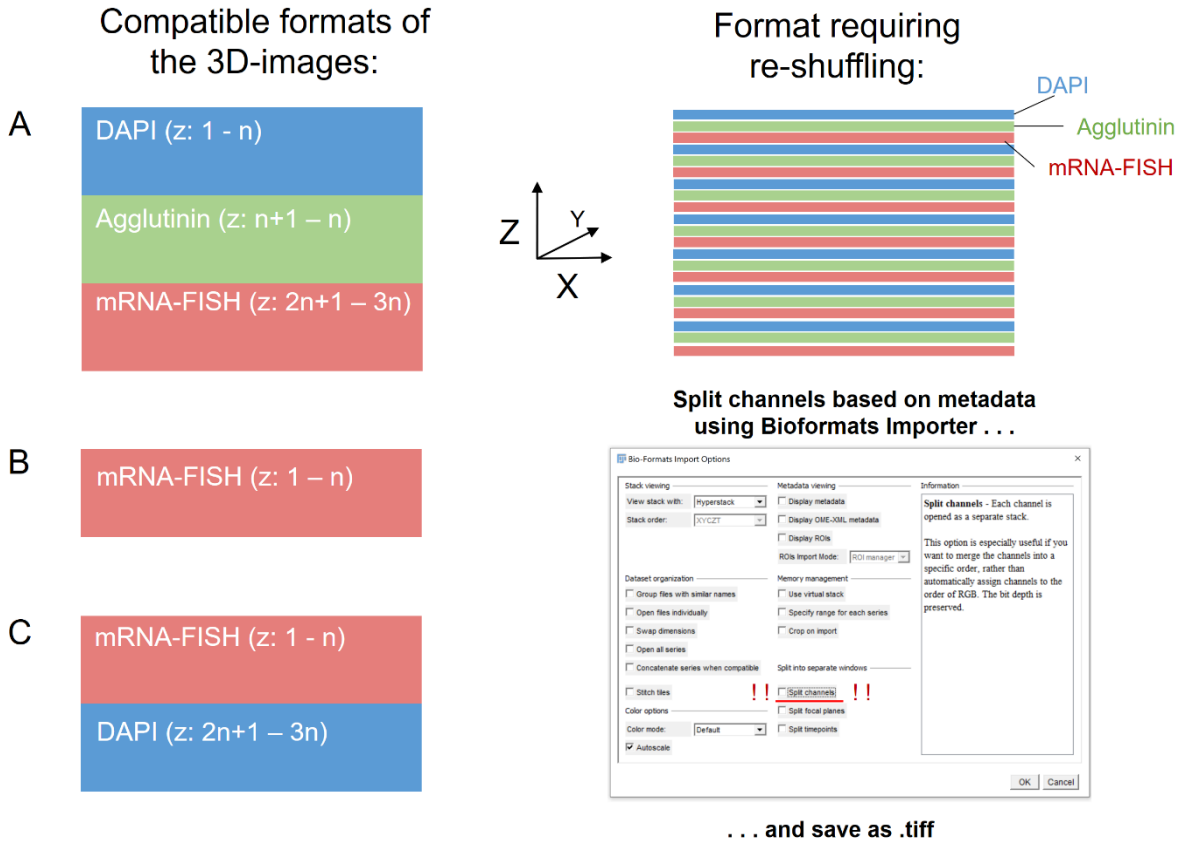
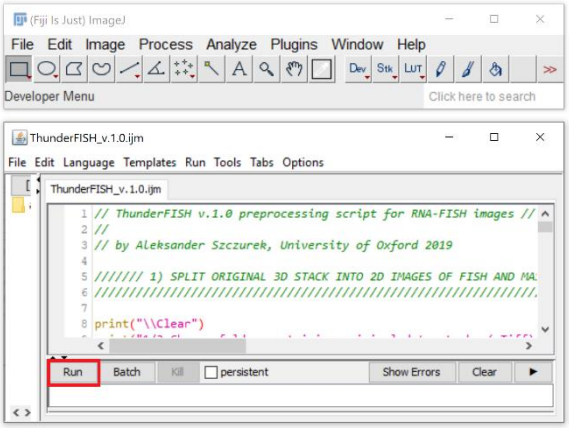


Figure 3. Formatting 3D image data to make it compatible with ThunderFISH. Left) Examples of data arrangement (A - C) compatible with ThunderFISH pre-processing scripts. n Z-slices is arranged in following order: 1_DAPI, 2_DAPI, 3_DAPI, 1_GFP, 2_GFP, 3_GFP, 1_FISH, 2_FISH, 3_FISH etc. Number of channels does not matter as long as all channels are arranged separately (arranged as channels, not as Z-slices). Right) Example of 3D-data stack that won't be recognised by ThunderFISH scripts. Such data needs to be imported to ImageJ using Bioformats Importer; channels need to be split and saved as .tiff files that can be easily processed by ThunderFISH (Save such 'reschuffled' stacks using ImageJ > File > save as > Tiff).

3.3.2 Running the ThunderFISH scripts on the test data

To begin with ThunderFISH data processing follow paragraphs A to E of the instruction below:

A) Download the whole GitHub folder (https://github.com/aleks-szczure/ThunderFISH) on your personal computer and drag-and-drop `ThunderFISH_v1.0.ijm` to ImageJ. The code



will appear before your eyes (**Fig. 4**):

Figure 4. ThunderFISH code window. You can freely modify the code here and save it (File > Save). However, if you want to run the code using default settings simply press “Run” button.

Brief synopsis: This code takes your 3D microscopic images in .tiff format stored in one input directory of your choice, performs 2D projection of your images, appropriate background subtraction, and produces images of i) mask and ii) RNA-FISH in separate directories. Subsequently, mask images are broken down to single cell mask images that are used to produce single cell RNA-FISH images that are automatically merged into a separate stack compatible with ThunderSTORM (Ovesny et al., 2014). You get all that done with one click (“Run” button)!

B) Download examples of our raw 3D-images provided in the GitHub folder (l i n k DROPBOX?) and prepare your directory. We recommend organising it in a following manner (**Fig. 5**):

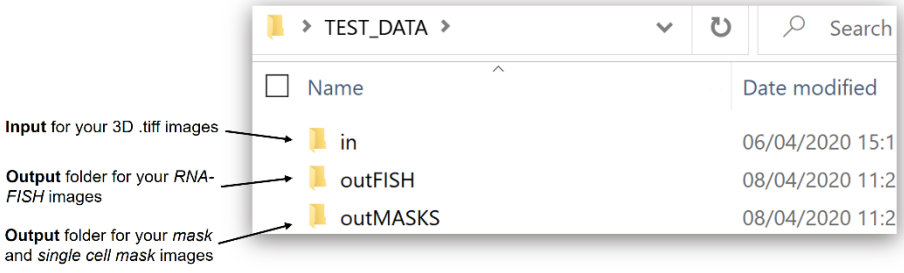


Figure 5. Prepare your directory where ThunderFISH will be operating. Three folders are required: One input folder where only your 3D .tiff images will be stored (either examples of our data or yours), and two output folders where RNA-FISH and Masks will be saved. The exact names of subdirectories do not matter, however, we recommend following this naming pattern to avoid confusion.

NOTE: Please avoid dots (".") in your folders' names.

C) After you downloaded the exemplary data, extract all three .tiff images it contains to the "`in`" folder. Run ThunderFISH using default settings by pressing "Run" button indicated with red square in **Fig. 4**. Communicate window will pop up and prompt you to indicate all three subfolders one by one. Press "OK" every time and indicate all three directories in a following order:

1) 'yourfolder/in',

2) 'yourfolder/outFISH',

3) 'yourfolder/outMASKS'.

After couple of minutes ThunderFISH will display a communicate that the first part has been successfully accomplished and will await user's response. You will be now able to check on results of thresholding: Go to yourfolder/outMASKS directory and explore it. You will find there masks of full fields of view (`_Ave_MASK_C0002` extension) and single cell masks (`_Ave_MASK_C0002_MASK` extension). Review the single cell masks in explorer window (**Fig. 6**). Delete masks representing e.g. merged cells or cells that have been overly cut as a result of whatershedding. NOTE: the number of such cells at this stage depends strongly on Circularity parameter range used in the code (default 0.75 – 1.0 setting ensures very few such cases at the expense of potential cell loss, to modify that parameter see **Table 1** for more details). Cells touching the image boarder are automatically discarded as they could lead to transcript undercounting.

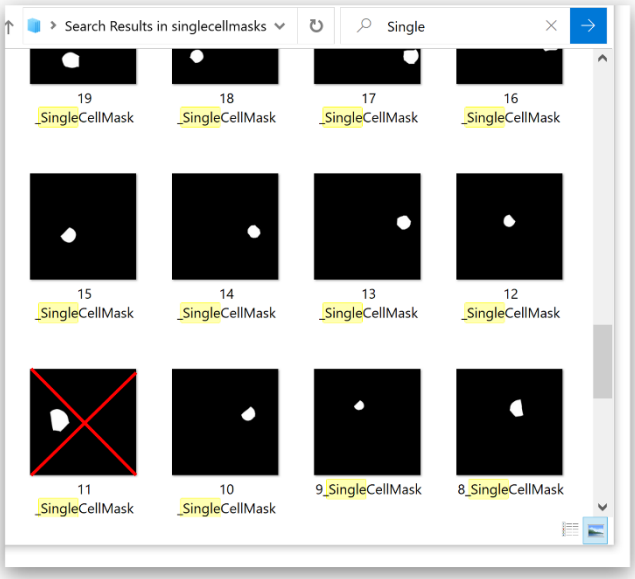


Figure 6. Discard manually any inappropriate single cell masks that have been left out: review the single cell masks in 'yourfolder/outMASKS/masks/' e.g. by searching for a phrase 'Single' (Microsoft Windows).

D) After you discarded inappropriate single cell masks (`_Ave_MASK_C0002_MASK` extension) from 'yourfolder/outMASKS/masks' directory hit 'OK' button. Now ThunderFISH will process RNA-FISH images and single cell masks that you accepted, and will convert them into a single-cell data-stack compatible with ThunderSTORM spot counting ImageJ plugin. The data-stack will be saved to 'yourfolder/outFISH' as well as will be displayed in ImageJ after ThunderFISH is finished. Each frame of this data stack corresponds to a single cell RNA-FISH image (2D maximal projection) with subtracted background (number of frames of this stack corresponds to the total number of cells, **Fig. 7B**, red circle). Single mRNA signals should be visible. Lack of spots in an individual frame corresponds to a cell that does not express that particular gene at the moment of fixation. Review the directories by dragging and dropping resulting images to ImageJ.

E) Analyse number of transcripts per cell of the stack (keep it open in ImageJ!) using ThunderSTORM plugin. Go to *ImageJ* > *Plugins* > **ThunderSTORM** > *Camera setup*, and change camera pixel size to the one that you used (in our case it is 96 nm). Now go to ImageJ > *Plugins* > *ThunderSTORM* > *Run analysis* (**Fig. 7A**), leave most of the settings as default settings except *Peak intensity threshold*. We recommend trying out values from the range of `5` to `10*std(Wave.F1)`. See **Fig. 7A** for an example of parameters used.

In a moment a ThunderSTORM results spreadsheet should appear in front of your eyes as well transcripts detected will be highlighted with red crosses appearing now in your single-cell stack (**Fig. 7B**) – browse it to confirm that spots were detected correctly. Results window displays a list of spots identified across the entire data set: each row corresponds to one spot and its parameters, among them are such of no interest to us as *sigma* or *intensity*, and such that will be crucial for our analysis like *frame* – this parameter in case of our data corresponds to a cell number; rows with the same *frame* number correspond to the same cell. Hence, go to 'ThunderSTORM: results' and press 'Plot histogram'. In order to produce data with transcripts per cell change the parameter to *frame* and number of bins to your total number of cells (**Fig. 7C**).

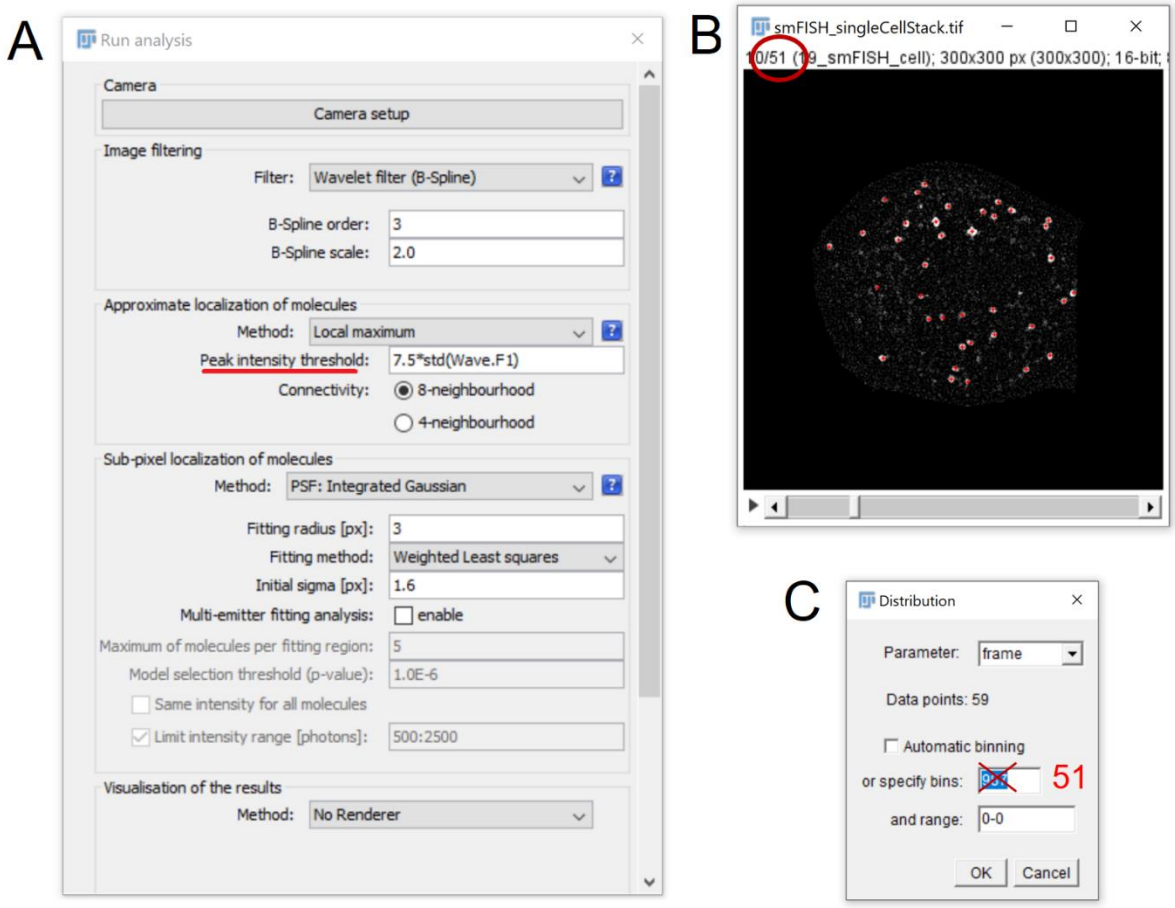


Figure 7. Spot counting of the single-cell RNA-FISH data-stack prepared by ThunderFISH. Go to Image > Plugins > ThunderSTORM > Run analysis. **A**) Example of parameters used to run the analysis. Adjust peak intensity threshold. We do not recommend using values from outside a range 5 – 10. **B**) Example of a single cell image with highlighted RNA-FISH spots detected. It's visible that some have been lost during the analysis and hence it might be crucial to repeat this analysis with lower *Peak intensity threshold*. **C**) Plot the distribution of number of transcripts per cell by pressing 'Plot histogram' button, then changing the parameter to *frame* and specifying number of cells instead of bin number. Press 'OK' to obtain the distribution and export the numbers.

New window appeared before your eyes called 'frame Distribution'. Press 'List' button to display number of transcripts per cell. In order to save it as a .csv file go to File > Save as and specify your name with .csv extension, for example "yourname.csv". Open this file in Microscoft Excel to plot the distribution. Measure mean number of transcripts per cell, and transcription variability as coefficient of variation (see **Fig. 8**).

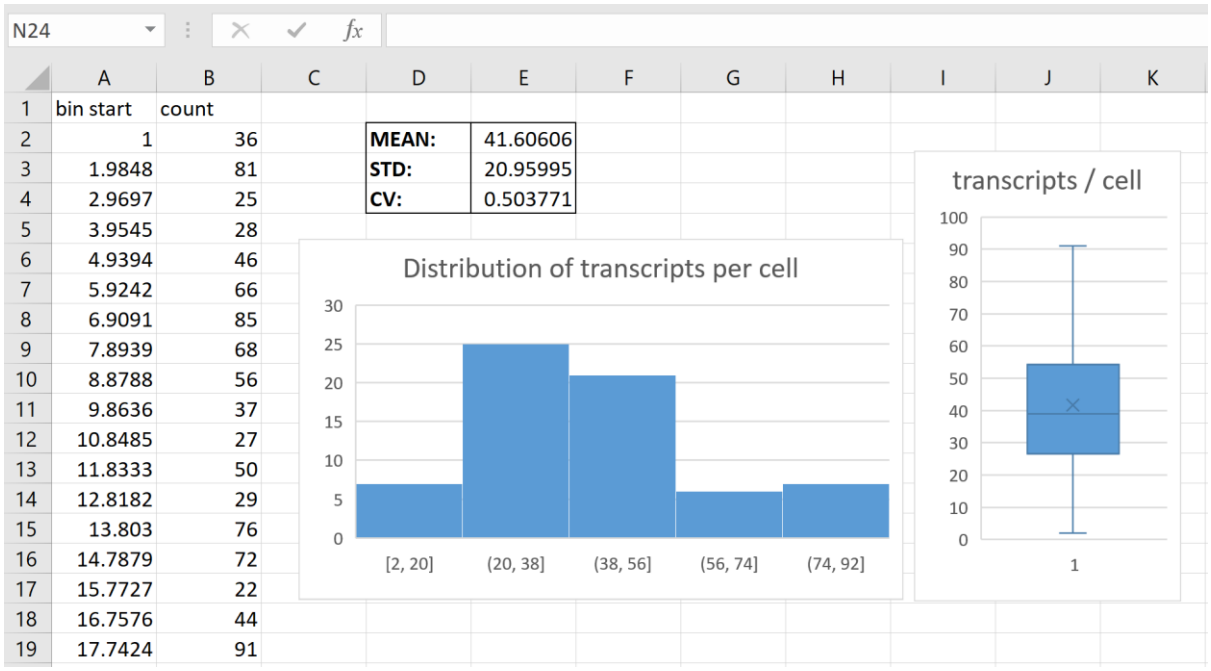


Figure 8. Example of the transcript per cell distribution obtained for the exemplary ThunderFISH data included in this manual. Confirm that you obtained similar values!

3.3.2 Running the ThunderFISH scripts on your data

After you've tried with our exemplary data you are finally ready to run your own data through the ThunderFISH pipeline! But before you begin you need to make sure that the script reads properly your data and uses optimal parameters. See **Table 1** below for detailed explanation what you **need** and can change in specific lines of code to get it to work with your data. For your convenience each such element is also marked in the code with a following comment '`//@user`' – use CTRL+F to find such comment and identify parts of code where parameters are.

Table 1. Interpretation of parameters used in the code. Feel free to change them!

Parameter	Code line	Meaning

Dividing the stack to separate channels (*obligatory*)	30 - 38	1) This is the only parameter that you **must adjust to your data** (number of Z-stacks and channels may differ in your acquisitions from ours). Default settings assume that each data stack contains 3 channels (DAPI (1-30), GFP (31-60), and FISH (61-90)), 30 images each. Hence following command is used:
		`run("Make Substack...", " slices=61-90");`
		
		This command is compatible with the test data so take a careful look at it in ImageJ before running ThunderFISH!
		
		Example: If your data contains, say, only 25 Z-stacks with DAPI (1-25) and FISH (26-50) channels then change this command to:
		`run("Make Substack...", " slices=26-50");`
		
		2) If you wish to threshold cells not based on FISH background signal (default, whole cells) but e.g. on DAPI signal because you are interested only in nuclear RNA change the command in line 36:
		`run("Make Substack...", " slices=61-90");`
		to:
		`run("Make Substack...", " slices=1-30");`

Background subtraction radius FISH	33	Default = 2, we recommend using value 2 or 3 for images with ~100 nm camera pixel size: if your images have a different pixel size *P* we recommend changing median filter radius accordingly: *X* = 2*(96/*P*), where *X* correspond to the filter radius.
Background subtraction radius MASK	41	Default = 150, removes extracellular background and facilitates image thresholding. Crucial in creating accurate mask. Default value assumes data having camera pixel size.
Median filter radius	98	Default = 5, blurs small image features of size <5 pixels in an image used for thresholding the cells. We recommend running the script with default value and comparing original image with `_Ave_MASK_C0002` extension with resulting image with `_Ave_MASK_C0002_MASK` extension. The default value of 5 has been tested for images with 96 nm

		pixel size: if your images have a different pixel size *P* we recommend changing median filter radius accordingly *X* = 5*(96/*P*), where *X* correspond to the filter radius.
Other thresholding parameters	100 - 107	Default = *Huang*, for most of the cases default settings are sufficient, however, if you're not happy with your thresholding results you can freely change this part of code. Among many other thresholding settings are: Mean, Otsu, Moments, Minimum, Yen and many more.
Single cell mask Size and Circularity	140	Size, default is larger than 6000 px^2. It ensures discarding smallest objects that do not correspond to the cells. Circularity, default is larger than 0.75. It's quite a stringent value, if you feel as if you're losing many cells from your analysis you can decrease it to 0.6. We do not recommend decreasing it below that value.
Single cell mask intensity multiplier	205	Default = 10. Single cell masks are binary images representing 255 pixel intensity for the cells and 0 intensity for the extracellular background (rest). The mask is processed: 255 a.u. x 10 = 2550 a.u. If your microscopy images are much brighter than our test data, this parameter can be further increased.
Size of the final stack for Thunder-STORM	227	Default = 350 x 350 px^2. Depends on your cell size and camera pixel size. Lower values can be used to save space but may cropp parts of the cells. It is further cropped to its final dimensions in line 308.

Organise your data in folders in a similar manner as outlined for our exemplary data.

After you've change obligatory parameters responsible for appropriate loading of your data, and perhaps others that you wished to adjust too (**Table 1**), it's time to press 'Run' button and allow ThunderFISH to analyse your data! Good luck!

References

Ovesny

Mueller

Dobrinic

*Schindelin, J.; Arganda-Carreras, I. & Frise, E. et al. (2012), "Fiji: an open-source platform for biological-image analysis", Nature methods **9(7)**: 676-682, PMID 22743772, doi:10.1038/nmeth.2019* (on Google Scholar).