

FISHtoFigure Version 1.0.1 User Information

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1 What is FISHtoFigure?

FISHtoFigure is a standalone software tool enabling transcriptomic analysis of QuPath quantified smFISH datasets in a user-defined, RNA target-oriented manner. Capable of multi-mRNA target, multi-dataset analysis, FISHtoFigure facilitates the rapid, streamlined analysis of large datasets comprising many images in an intuitive fashion. Users can partition transcriptomic data in an automated way simply by defining a target or series of targets to analyse, allowing analysis to be conducted for specific cell types and transcriptional profiles.

FISHtoFigure can produce differential transcriptome analysis for quantified smFISH microscopy data, with the development focus being the analysis of RNAscope images (<https://acdbio.com/rnascopemultiplex-fluorescent-v2-assay>). Currently, FISHtoFigure is limited to the analysis of QuPath output files, however, due to the method by which FISHtoFigure carries out analysis, the software is adaptable to other formats of quantified microscope image data.

FISHtoFigure allows users analyse smFISH data for specific combinations of mRNA targets, isolating cells which are positive for a particular target, or multiple targets, across multiple datasets simultaneously in order to quickly analyse specific differences in the transcriptome between datasets that would otherwise be very difficult to parse.

2 FISHtoFigure Usage

2.1 Quantification with QuPath

QuPath (<https://github.com/qupath>) is an open source bioimage analysis platform designed to process large image datasets such as those generated in smFISH experiments. QuPath has built-in functions to identify cell boundaries based on nuclear or membrane stains and identify subcellular fluorescent spots (i.e. those representing mRNA transcripts in smFISH images). ACDBio, the developer of the popular smFISH platform: RNAscope, recommend QuPath for quantification of RNAscope images, however, upto this point downstream analysis has required data be exported from QuPath and analysed using custom analysis pipelines written by the user. FISHtoFigure allows users to conduct these downstream analyses without custom scripts. The FISHtoFigure tool uses QuPath quantified output files as inputs. ACDBio have created a useful document for the analysis of smFISH data in QuPath which can be downloaded here: <https://acdbio.com/qupath-rna-ish-analysis>

2.2 QuPath Output File

QuPath is an image analysis platform capable of quantifying cellular boundaries and subcellular objects (such as the spots representing transcripts in smFISH data). FISHtoFigure was developed to conduct downstream analysis of smFISH data quantified using QuPath. Following quantification of cells and transcripts, QuPath will output a spreadsheet-like file containing image information in the form of a txt file. This file contains rows corresponding to individual cells or subcellular spots, and columns containing specific information about cells or subcellular objects such as centroid coordinates (the x and y coordinates associated with the nucleus of the cell), the number of spots within the cell, and the fluorescent intensity of a transcriptional spot. Due to the volume of information within fluorescence image files, QuPath output files are generally hundreds of columns and thousands of rows long, FISHtoFigure harvests the specific information and conducts differential transcriptome analyses.

2.3 Preprocessing Tool

Many smFISH experiments will comprise numerous images, a simple Graphical User Interface (GUI) driven tool has been created to concatenate the outputs from QuPath for multiple images into a single file which can then be processed in FISHtoFigure. Within this preprocessing tool, the user should enter the full path to the directory containing image data and click "Find" to submit this path, clicking "Run" will then run the program and produce the concatenated file in txt and csv format within the directory.

2.4 FISHtoFigure Software Tool

A GUI has been created for the main FISHtoFigure program, shown annotated in Figure 1. Below, each part of the GUI is described:

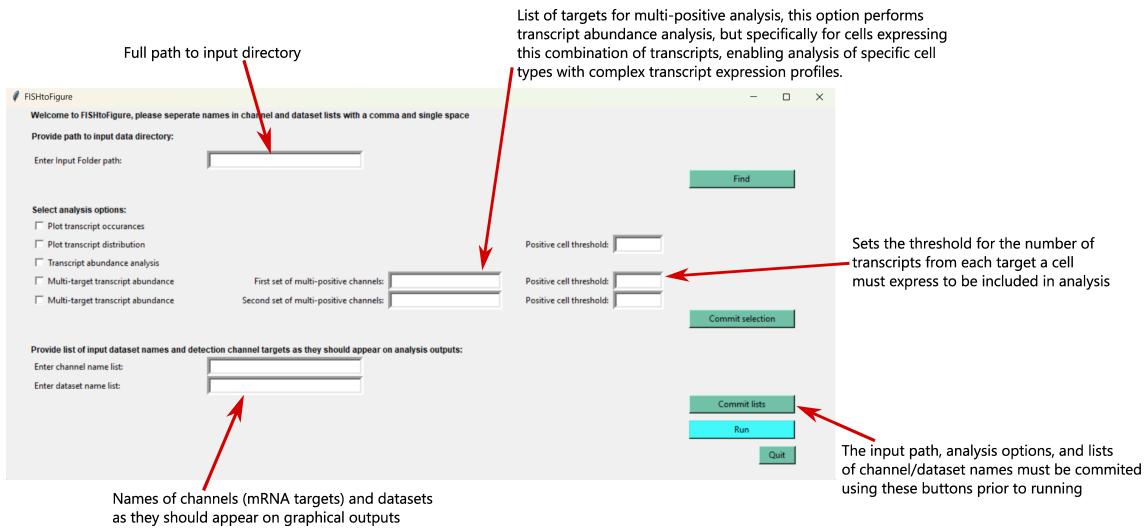


Figure 1: A simple GUI has been created such that users can use FISHtoFigure with minimal interfacing with the underlying Python program.

2.4.1 1: Input Folder Path

Users should enter the full path to the directory containing the files to be analysed. **Note:** This should not be the path to the data files themselves. On Windows 10 and later, the path to a directory can be copied by right-clicking the folder and clicking "Copy as Path".

2.4.2 2: Analysis Options

Users can then define which analysis outputs they desire in the analysis options section, examples of outputs are shown in Figures 4 and 5. If users wish to use the (Multi-positive cell analysis) function of FISHtoFigure requiring the input of channel names, channel names should be input exactly as they appear in the user-defined channel list below separated by a comma and a space. Once selected, analysis options can then be submitted via the "Commit Selection" button.

2.4.3 2b: Positive-cell Threshold

Note: The user doesn't have to set a threshold, if the user is not interested in setting a threshold for analysis this can be left blank, the default value is 1 transcript per target per cell.

In some experiments, mRNA targets may have a high background which may get in the way of analysis. The positive cell threshold allows users to control the number of transcripts of each mRNA target which a cell must express to be included in analysis. This should be an integer.

Note: Due to the way in which intensity is calculated, the threshold for multi-positive analysis is not applied to the intensity. Therefore, if the user specifies a threshold above 1 for multi-positive analysis intensity analysis will not be performed to avoid confusion about what is and isn't thresholded. I hope to change this in a future version so that intensity can be thresholded.

2.4.4 3: Channel and Dataset names

Finally, the user should provide lists of the channels (mRNA targets) contained in their data. The names of targets in the list must be in the order they appear in the QuPath output data, this order is the same as the order they appear in when analysing images in QuPath e.g., Channel 1 in QuPath will be labelled with the first name in the list entered in the GUI. Target channel names within the list should be separated by a comma and a single space. A list containing the names associated with each dataset should be inputted below, even if the user is only analysing

a single dataset. This list will be used for assigning names to each input dataset during analysis and plotting, e.g. "Naive" and "Infected". Dataset names should be inputted such that they align with the order of datasets within the input directory (e.g., "Dataset1.txt" in the input directory will be labelled with the first name in the list entered in the GUI).

3 Input Data

FISHtoFigure is currently capable of working with standard Qupath output text files of images with an arbitrary number of detection channels, however, there are certain limitations on the input data as detailed here.

Input datasets should take the form of a txt file (either a standard QuPath output file or a concatenated file comprising multiple QuPath files created using the preprocessing tool) and all datasets to be analysed should be stored within a single input directory, the path to which can be defined in the GUI, discussed below.

3.1 Naming convention

Input datasets should be named according to the following convention "*****X***.txt**" where ******* represents a variable, non-numerical string with no spaces (eg the dataset/samplename) and **X** represents a numerical value unique to that dataset. Numerical variable **X** must be **1** in the first dataset to be analysed, and subsequent datasets must be labelled in ascending order.

Examples of appropriate input dataset names: Inf1.txt, Naive12.czi.txt, DatasetSection31SampleA.txt

Examples of inappropriate input dataset names: Sample12Section10.txt, Section5.czi, Dataset.txt, Inf1.txt.csv, 12Data.txt, Naive Data 1.txt

3.2 Preprocessing

In the case of datasets comprising multiple images, the Qupath outputs for said images can be concatenated in an automated manner by running the preprocessing tool on the directory containing these datasets. Running the preprocessing tool produces a "Concatenated Dataset" file in both txt and csv format. The txt format file can then be processed by the main FISHtoFigure program. The "Concatenated Dataset" file is saved to the input directory specified in the preprocessing tool GUI.

3.3 Input Format

FISHtoFigure harvests cell and subcellular spot information by searching the input file for rows and columns containing cellular and subcellular information such as cell centroids, subcellular spot intensities, and the number of spots within cells. The flags used to pick out these rows and columns are defined such that a standard Qupath output file will already contain the necessary flagged column and row headings, however, for clarity a description of the flags searched for by FISHtoFigure is given below:

3.4 Rows:

Rows represent either a cell or a single subcellular object (such as a single subcellular spot) within a cell. A row representing a cellular object will be followed by rows representing individual subcellular objects within that cell until all subcellular objects are exhausted at which point the subsequent row will represent a new cell. The information used to determine if a row represents a cell or a subcellular object is contained in the "Name" column.

3.4.1 Name: PathCellObject

Represents a cell object, this row contains information relating to subcellular spot and cluster count for each target channel within this cell. It does not contain information about intensities of subcellular objects within the cell.

3.4.2 Name: Subcellular spot: Channel X object

Represents a single subcellular spot in the target channel X, this contains information about the intensity of each channel, though only the Channel X intensity will be non-zero.

3.4.3 Subcellular cluster: Channel X object

When using QuPath to quantify transcripts, users must set an upper limit on the size of fluorescent spots which represent transcripts. If a fluorescent signal is larger than this limit, QuPath stores this as a "cluster" and estimates the number of spots represented by the cluster based on a combination of the size and fluorescent intensity. These rows contain the same information as for rows representing subcellular spots, but for fluorescent clusters.

3.5 Columns:

Column headers comprise the majority of flags used in the analysis of datasets by FISHtoFigure. These are detailed below:

3.5.1 Centroid X μm

Contains a float value representing the X-axis centroid (Centroid being the centre of the cell nucleus) information for the cell, this is used by FISHtoFigure to produce the scatter plot of cells coloured and sized based on transcriptomic spot information.

3.5.2 Centroid Y μm

Equivalent Y-axis information to the above.

3.5.3 Subcellular: Channel X: Num spots estimated

A float (non-integer) representing the estimated number of spots in the cell, estimate is based on the combination of all spots and clusters in the cell.

3.5.4 Subcellular: Channel X: Num single spots

Contains an integer representing the number of subcellular single spots contained in the current cell. Excludes clusters.

3.5.5 Subcellular: Channel X: Num clusters

An integer number representing the number of clusters within the current cell.

3.5.6 Subcellular cluster: Channel X: Mean channel intensity

Contains a float (non-integer) representing the intensity for Channel X for the current subcellular object.

3.5.7 Multi-dataset analysis

In the case that multiple datasets are to be compared (e.g. comparing a dataset comprising all naive controls with that of infected counterparts) datasets in text file format can simply be placed in the same directory, named following the naming convention laid out above. Datasets will be interpreted and plotted together. Note that when defining the dataset names in the GUI, dataset names should be given in the order that the datasets appear in the input directory.

4 FISHtoFigure Capabilities and Outputs

When running FISHtoFigure, a new directory is created within the input directory specified in the GUI called "F2F Output", this stores all analytical outputs created including figures, datasets associated with figures, and analytical txt format outputs.

An example of the generated "F2F Output" folder output from running all of FISHtoFigure's analysis options with two input datasets is shown in Figure 2.

Name	Status	Date modified	Type	Size
📁 [Cd79a, Il10] Positive Cells	✓	30/07/2022 18:00	File folder	
📁 [Cx3cr1, Il10ra] Positive Cells	✓	30/07/2022 18:00	File folder	
📁 General Transcript Abundance Analytics	✓	30/07/2022 18:00	File folder	
📄 Analysis Ouput	✓	30/07/2022 18:00	Text Document	1,558 KB
xl Infected output	✓	30/07/2022 18:00	Microsoft Excel Co...	2,131 KB
xl Infected Spot Count Occurance Histogram	✓	30/07/2022 18:00	PNG File	269 KB
xl Infected Spot Distribution by Channel	✓	30/07/2022 18:00	PNG File	3,280 KB
xl Naive output	✓	30/07/2022 18:00	Microsoft Excel Co...	573 KB
xl Naive Spot Count Occurance Histogram	✓	30/07/2022 18:00	PNG File	233 KB
xl Naive Spot Distribution by Channel	✓	30/07/2022 18:00	PNG File	1,358 KB

Figure 2: Example of the F2F output directory for two input datasets: "Naive" and "Infected". Here, two multi-channel expression profiles were analysed: "Cd79a, Il10" positive cells and "Cx3cr1, Il10ra" positive cells.

4.1 CSV Data

The initial process of FISHtoFigure consists of the conversion of each of the Qupath output text files in the designated directory into an equivalent csv file. This csv format is required for the rest of the data analysis carried out by FISHtoFigure while additionally being easily interpretable by programs such as excel. The generated csv files are saved to a newly generated sub-directory titled "csv files" within the directory specified by the user in the GUI in case users wish to conduct custom analysis. These generated csv files are named based on the dataset names in the input directory (e.g., "*Dataset name*.csv").

4.2 Non-Zero Cell Data Harvesting

Following input file format conversion, the first task carried out by FISHtoFigure is the creation of a dataframe containing only cells expressing at least one subcellular spot in at least one detection channel, referred to hereafter as non-zero cells. FISHtoFigure reads the input file in a line-by-line

fashion, locates non-zero cells, and writes this cell’s information to a new file. The resulting non-zero cell dataset is written to a csv file titled ”*Input file name* output” and saved to the ”F2F Output” directory within the user specified directory.

```
Cell with centroid: 132.74, 222.84
Cd79a Spots = 0
Cx3cr1 Spots = 2
Il10 Spots = 0
Il10ra Spots = 0
Cd79a Clusters = 0
Cx3cr1 Clusters = 0
Il10 Clusters = 0
Il10ra Clusters = 0
End of Cell

Cell with centroid: 142.9, 222.66
Cd79a Spots = 0
Cx3cr1 Spots = 0
Il10 Spots = 0
Il10ra Spots = 1
Cd79a Clusters = 0
Cx3cr1 Clusters = 0
Il10 Clusters = 0
Il10ra Clusters = 0
End of Cell

Dataset 2 Transcript Expression Analysis
Percentage of cells with non-zero spot count = 17.058311575282854%
Mean spot count in non-zero cells = 0.7439975990396158
Mean cluster count in non-zero cells = 0.3372599039615846
Mean spot count in all cells = 0.12691342855680132
Mean cluster count in all cells = 0.05753084523626683
```

Figure 3: Short section from the pipeline output for an example RNAScope dataset. Following cellular information, holistic analysis is reported for the dataset before automatically beginning the analysis of any subsequent datasets, reported within the same output file.

4.3 Data Harvesting and Analytics as Text File

While carrying out the non-zero cell harvesting process, FISHtoFigure harvests information such as the cell’s position within the input image (centroid) and subcellular spot information for each spot within the cell. To aid with data interpretation, harvested information for each non-zero cell is written to a text file titled ”Analysis Output” in the ”F2F Output” directory, an example of the format for the output cellular data is shown in Figure 3.

Along with non-zero cellular information, the Analysis output file stores the list of files being analysed, input file header information (The flags being used to harvest information and the user-specified channel names associated with each flag), and some holistic analysis for each image dataset such as percentage of cells containing subcellular spots and mean spot count per cell within the image.

4.4 Transcript Distribution

The ”Plot transcript distribution” option will plot centroid data for cells with non-zero subcellular spot counts for a given input dataset, creating a plot analogous to the input image. As such, this option is recommend for use with single image datasets where centroid information is consistent.

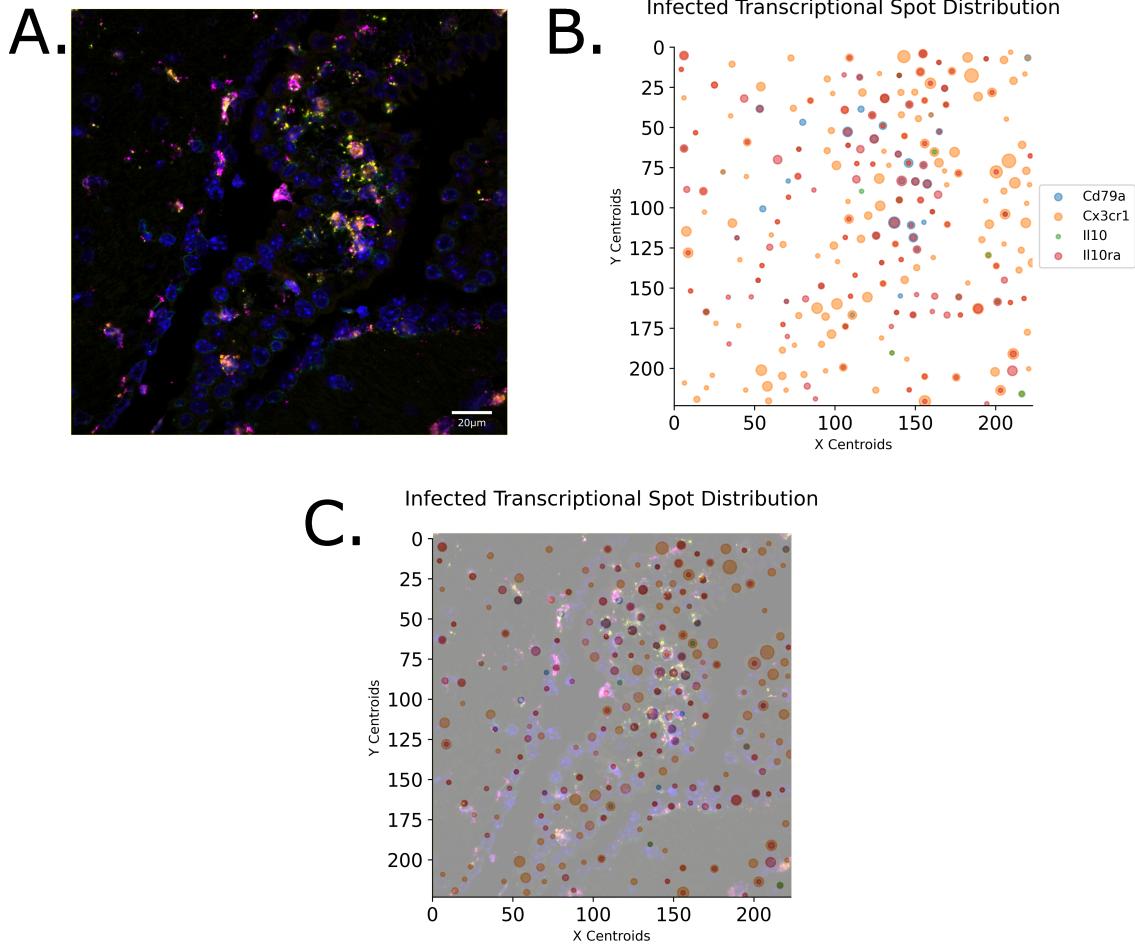


Figure 4: Spatial distribution plotting function example using a dataset comprising a single RNAscope image. A: The fluorescence image acquired via RNAscope, with fluorescent spots representing RNA transcripts. B: Spatial distribution produced by FISHtoFigure. C: An overlay of the RNAscope output image with the FISHtoFigure distribution.

Use with concatenated datasets comprising multiple images will result in overlap since centroid information is recorded per image and spatial relationships between images are not conserved during concatenation. Basically, all images will be plotted on top of one another.

Points within the resulting scatter plot represent individual cells with the colour and size of data points representing mRNA target and subcellular spot count for that target respectively. This function provides not only a highly quantified equivalent of the original input microscopy data, but also a visual output which can be directly compared with the original dataset to validate the harvesting of information from the dataset by FISHtoFigure, as shown in Figure 4.

4.5 Differential Transcriptomic Analysis

Following data harvesting, FISHtoFigure provides the user with a means to conduct transcript expression analysis, this can be conducted for individual images/datasets, or for an arbitrary number of datasets, enabling the comparison of transcript expression between datasets. This analysis produces plots of spot count and intensity by target channel, these plots consist of a box and swarm-plot, where each data point in the swarm plot represents a single cell. In the case of the intensity plot, each data point represents the sum total intensity of all subcellular spots, for a particular mRNA target, within that cell. In the case of the spot count graph, each data point

simply shows the number of subcellular spots for each target in that cell.

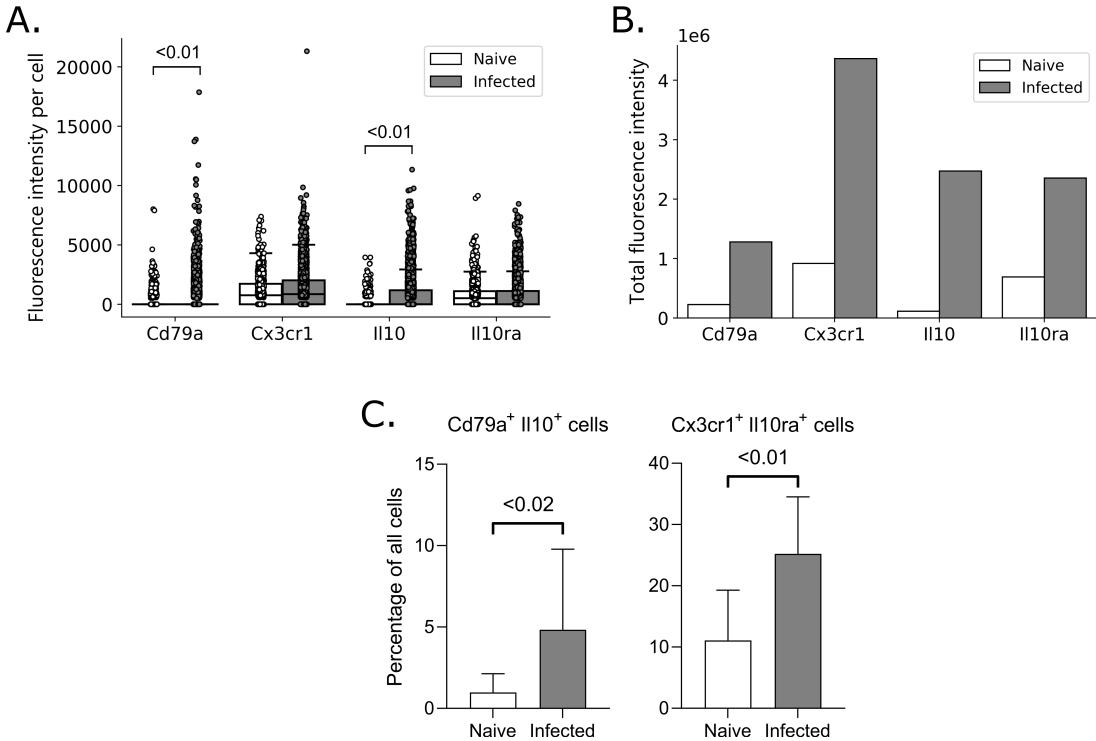


Figure 5: Differential transcript abundance analysis performed using FISHtoFigure for a pair (naive and infected) of concatenated datasets comprising multiple RNAScope images. A: Cell-wise spot count by RNA target channel, each point represents the fluorescent intensity (representative of the number of transcripts) for each mRNA target for a particular cell. B: Total cumulative fluorescent intensity for each mRNA target for all cells in the dataset. C: Multi-target analysis for two distinct cell types, each characterised by a specific pair of mRNA targets ($\text{Cd79a}^+ \text{ II10}^+$ B-cells, and $\text{Cx3cr1}^+ \text{ II10ra}^+$ microglial cells). In addition to graphical outputs, FISHtoFigure outputs data as CSV files for further analysis, here statistics were performed in GraphPad Prism. For further information on these experiments, see the pre-print here: <https://www.biorxiv.org/content/10.1101/2023.06.28.546871v1>.

Additionally, FISHtoFigure produces plots of total subcellular spot number, intensity, and positive cell number for each detection channel as well as the total number of non-zero cells across all channels for each dataset. Examples of the analysis produced using this function are shown in Figure 5.

Along with each generated figure, the dataframe containing the information used to generate that figure is saved as a csv with the same name as the associated figure. This allows the figures generated in FISHtoFigure to be recreated using another graphing software such as GraphPad Prism quickly and easily or for more specific analysis to be conducted on the specific subset of data used to create a particular figure (E.g., Cellular intensities for each target channel for non-zero cells) without need to manual harvest these data from the QuPath output. These datasets are structured to be easily interpretable to ease further analysis, as such, columns are labelled based on the information inputted in the GUI by the user. Each dataset derives it's format from the original QuPath file, as such, each row represents a cell (Other than in the cumulative analyses such as the positive cell number, which comprise only a single row containing the sum totals across all cells for each channel) and each column represents either the spot number or intensity for a particular channel, depending on the dataset.

All figures and associated datasets generated using this function are saved to a newly generated directory titled "General Transcript Abundance Analytics" within the "F2F Output" directory.

An example of a "General Transcript Abundance Analytics" directory generated using a pair of example datasets is shown in Figure 6.

Name	Status	Date modified	Type	Size
Cell-wise Intensity by Channel	✓	30/07/2022 18:00	Microsoft Excel Co...	204 KB
Cell-wise Intensity by Channel	✓	30/07/2022 18:00	PNG File	355 KB
Cell-wise Spot Count by Channel	✓	30/07/2022 18:00	Microsoft Excel Co...	91 KB
Cell-wise Spot Count by Channel	✓	30/07/2022 18:00	PNG File	248 KB
Intensity of Non-Zero Cells by Channel	✓	30/07/2022 18:00	Microsoft Excel Co...	163 KB
Intensity of Non-Zero Cells by Channel	✓	30/07/2022 18:00	PNG File	372 KB
Positive Cell Count (All Channels)	✓	30/07/2022 18:00	Microsoft Excel Co...	1 KB
Positive Cell Count (All Channels)	✓	30/07/2022 18:00	PNG File	160 KB
Positive Cell Count by Channel	✓	30/07/2022 18:00	Microsoft Excel Co...	1 KB
Positive Cell Count by Channel	✓	30/07/2022 18:00	PNG File	162 KB
Total Intensity by Channel	✓	30/07/2022 18:00	Microsoft Excel Co...	1 KB
Total Intensity by Channel	✓	30/07/2022 18:00	PNG File	119 KB
Total Spot Count by Channel	✓	30/07/2022 18:00	Microsoft Excel Co...	1 KB
Total Spot Count by Channel	✓	30/07/2022 18:00	PNG File	163 KB

Figure 6: Example of the generated "General Transcript Abundance Analytics" directory within the "F2F Output" directory. Each figure (saved as a PNG file) has an associated dataset which contains the data required to recreate the figure which is saved as a csv of the same name.

4.6 Custom Transcriptomic Analysis for Multi-Target Expressing Cells

Using the "Multi-target transcript abundance" function, users can define a subset of target channels on which to conduct specific transcriptomic analysis. Users should enter the names of the targets they wish to conduct analysis on as they appear in the user-defined "channel name list" within the GUI, as with the channel and dataset lists, target channel names should be separated by a comma and single space. FISHtoFigure will then search the non-zero cell data harvested from the QuPath file specifically for cells co-expressing all the targets defined by the user in the "First/Second set of multi-positive channels" input section. Cells found to fulfill the specified expression profile are stored to a separate dataframe and differential transcript expression is conducted for this new dataframe as described in the "Differential Transcriptomic Analysis" section above.

As with the "Differential Transcriptomic Analysis" section above, datasets used to generate each figure are saved as csv files with the same name as the associated figure. All figures and associated datasets are saved to a newly generated directory within the "F2F Output" directory. The name of this directory is simply the list of channels selected by the user for this analysis (e.g., "[*Channel 1*, *Channel 2*, *Channel n*] Positive Cells").

Currently, FISHtoFigure facilitates the analysis of two multi-positive cell profiles within the input GUI, allowing for multiple multi-target positive conditions to be explored in parallel.

4.7 Cell Positivity Threshold

If users are using the "Plot transcript distribution" or the "Multi-target transcript abundance" functions, a "Positive cell threshold" can be set. This sets a minimum limit on the number of transcripts a cell must express for it to be deemed positive and thus be included in analysis. This allows for the removal of background expression, for example, if a user wishes to analyse cells which highly express a particular RNA target which is expressed at low levels by all cells, users can set

a threshold such that only highly expressing cells are analysed. This threshold should be input as a single integer, all cells expressing a number of transcripts greater than or equal to the threshold will be included in analysis. If no threshold is specified FISHtoFigure will include all non-zero cells by default.

4.8 Exceptions and Errors

Some common issues which either cause the FISHtoFigure program to crash (errors) or cause some non-crash problem with analysis (exceptions) have been factored into the program design. In these cases FISHtoFigure will produce a txt file briefly describing the issue within the "F2F Output" directory, more detailed explanations of these are given below:

4.8.1 Exception: No cells with multi-positive expression profile

If the user provides a set of mRNA targets for multi-positive analysis but no cells within the dataset express this set of targets, rather than leaving the multi-positive output directory blank this exception is produced. This may occur due to there being no cells with this expression profile at all, however, more commonly this occurs because the user-specified threshold is too high and has removed the cells with this profile from analysis.

4.8.2 Exception: One or more channels with zero intensity

If any of your targets do not display any spots or clusters in your dataset then this channel will have zero intensity. When quantifying data within QuPath, if a target displays no spots or clusters then a column for the intensity of this column is not generated. As such, FISHtoFigure cannot harvest this information (even though it would just be zeros). For this reason, certain calculations relying on intensity data cannot be performed (specifically those relating to total intensity) and are excluded from analysis.

4.8.3 Error: Dataset names

If the user has accidentally given a list of dataset names which is different from the number of txt files within the input directory, this error is produced. Please remember to separate dataset names with a comma and space (", "). If FISHtoFigure is taking a very long time to analyse data then check the specified directory for any error messages.

4.8.4 Error: Channel names

If the user gives a list of target channel names which is different from the number of channels specified in the QuPath output file, this error is produced. When quantifying data within QuPath, each target (fluorescent colour channel) is assigned a specific channel in the output file. Therefore the number of channel names given in the FISHtoFigure GUI should be the same as the number of channels quantified in QuPath (minus the DAPI stain which is used to identify nuclei but not analysed during the subcellular object analysis function in QuPath, see the RNAscope note on using QuPath for subcellular spot detection mentioned above for details on how to perform the QuPath analysis). Please remember to speарате channel names with a comma and single space (", "). If FISHtoFigure is taking a very long time to analyse data then check the specified directory for any error messages.

5 Developer Information

FISHtoFigure is an open source software hosted on GitHub. Though currently FISHtoFigure is designed to process Qupath output files, all partitioning and analysis occurs after data is extracted from the input file and stored as various dataframes. Therefore, adapting the software to interpret

input files from other software such as Fiji or CellProfiler should be achievable by simply changing the flags used to identify information required for analysis. Provided cellular and subcellular information can be quantified in a tabulated format, it is likely FISHtoFigure can be adapted to work with this format.

GitHub: <https://github.com/Calum-Bentley-Abbot/FISHtoFigure.git>

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