Basic workflow for dual-color detection and co-localization

Here we list the main steps to perform an mRNA detection for two colors and calculating spot-colocalization with **FISH-quant**[1] (The functionality is available in v3). For more details on the individual steps, we refer to the FISH-quant manual.

Co-localization between the two channels is calculated as a linear assignment problem (LAP) solved with the Hungarian algorithm. We use the Matlab function `hungarianlinker`[2] and `munkres`[3] for this purpose.

Image names have to have a common part, and a unique part which is indicative of the channel. In this example we assume that the channel indicator is *_color1* and *_color2*. Images could be called *smFISH_pos1_color1.tif* and *smFISH_pos1_color1.tif*

1. Create folders to store data (images, outlines, results). We recommend defined separate folders for outlines and results for the two colors.

2. Open main interface **FQ**

    a. Define folders

    b. Define experimental parameters

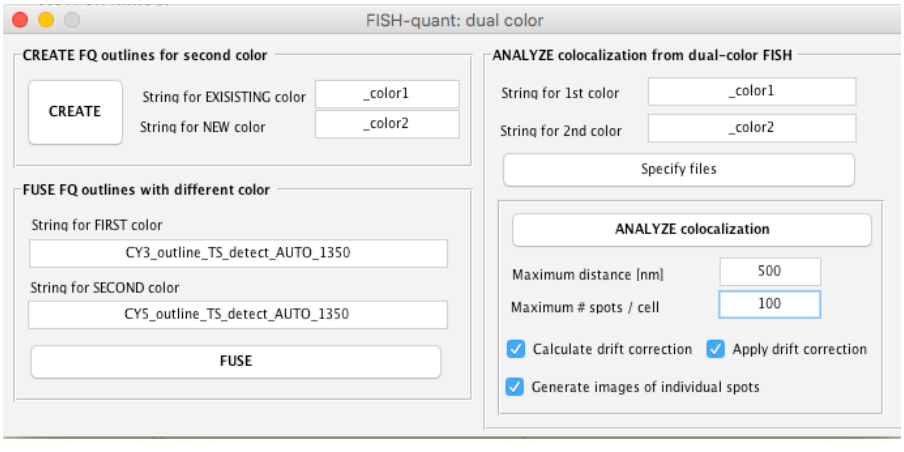
3. Define all outlines for first color

4. Use Outlines from first color to generate outlines for second color. Use dedicated tool **FQ_DualColor.** The relevant part is in the upper left of the interface

    a. You have to specify which part of the file-names has to be replaced to go from one color to another. For instance, *_color1* by *_color2.*

    b. Press on **Create**. You can specify all outlines that you want to use to create the new outlines in the second color. The new outlines will be saved in subfolder of the original outlines

    Note: the script will replace the name of the image within the outline file as well. The wavelength will be the same – this doesn't matter since the processing is pretty much independent of this.



5. Perform **spot detection** for for first color
    a. Find best detection/fitting settings
        - Filtering
        - Detection thresholds
        - Thresholds after fitting

b. Perform batch-detection for all cells in all images. Save *Results for each images,* which will be needed for the co-localization analysis.

   Note: for really dense samples, the fitting might pose problems since a spot could be affected by its neighbors. We therefore provide the option to fit spots not at all, or only in cells with fewer mRNAs than the user defined number threshold. This is available from the button **Restrict # of fitted spots** in the main FQ interface.

6. Repeat detection for **second color** as detailed above for first color.

7. Perform **co-localization analysis**. This is done in the right part of the **FQ_DualColor** interface

   a. You have to specify again the two strings for the first and second color.

   b. Then click on **Specify files.** Here you have to specify multiple files/folders needed for the analysis. No processing is performed
      - All result FILES of the FIRST color that you want to analyze
      - FOLDER where result files of second color are stored.
      - Folder with images of first color
      - Folder with images of second color

   c. You can then determine co-localization by pressing on **Analyze colocalization.** You can also explore how the data behaves by changing the various parameters. The various result files described below will be generated

      - *Maximum distance*
        This is the maximum allowed distance for two spots to be considered co-localized. At the end of the analysis, a plot will be shown with the number of co-localized spots as a function of the distance just up to the defined thresholds

      - *Maximum number of spots/cell*
        Cells with more spots than this threshold will not be included in the analysis

      - *Calculate drift correction*
        When enabled, this option will calculate the average drift in each direction from all spots that are co-localized. You have to analyze the data again to apply it. This is a simple drift, no rotational correction will be considered. Also, this correction might not be good enough for large chromatic aberration (especially towards the border of the image).

      - *Apply drift correction*
        Apply the drift correction in the new analysis run as a correction where it will be subtracted from the spot positions in the second channel. Results will be saved in a new folder such that results before and after drift correction can be compared.

      - *Generate images of individual spots*
        When enabled, an image of each spot will be generated and saved in a folder **+** if t has a co-localization, and folder called **-** if it doesn't have a co-localization. This helps to judge how well the analysis works in general.

        ATTENTION. This can take a long time if you have many spots. We recommend using this option only for a selected number of cells/images.

   d. Different result files will be in the folder with the results of the spot detection of channel 1.

      - *A* text file specifying for each cell how many co-localized spots have been found.
      - *A plot with the number of detected spots as a function of distance*
      - *(If enabled) a plot with the drift in each direction*
      - *(If enabled) plots of localized and not localized spots*

References

1. Mueller, F. *et al.* FISH-quant: automatic counting of transcripts in 3D FISH images. *Nat. Methods* **10,** 277–278 (2013).
2. Hungarian based particle linking - File Exchange - MATLAB Central. at <http://fr.mathworks.com/matlabcentral/fileexchange/33968-hungarian-based-particle-linking>
3. Hungarian Algorithm for Linear Assignment Problems (V2.3) - File Exchange - MATLAB Central. at <http://fr.mathworks.com/matlabcentral/fileexchange/20652-hungarian-algorithm-for-linear-assignment-problems--v2-3->