SONLab FRET Analysis Tool

User Guide for v1.1a

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Abstract

This guide provides detailed documentation for the SONLab FRET Analysis Tool v1.1a, a comprehensive solution for analyzing Fluorescence Resonance Energy Transfer (FRET) data. The tool offers both manual and automated segmentation capabilities, bleed-through correction, and advanced FRET calculation features.

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1. Introduction

1.1. About SONLab FRET Analysis Tool

The SONLab FRET Analysis Tool is a powerful application designed for analyzing Fluorescence Resonance Energy Transfer (FRET) data. It provides a user-friendly interface for processing and analyzing microscopy images to study molecular interactions and dynamics.

1.2. Key Features

- Manual and automated region of interest (ROI) selection
- Advanced image segmentation algorithms
- Bleed-through correction for the FRET channel. For donor and acceptor, there will be a update soon.
- Comprehensive FRET calculation and visualization
- Statistical analysis and data export
- Support for multi-frame TIFF images

1.3. System Requirements

- Windows 10/11, macOS 10.15+, or Linux with Python 3.8+
- Minimum 8GB RAM (16GB recommended for large datasets)
- 2GB free disk space
- Screen resolution: 1920x1080 or higher recommended

2. Getting Started

2.1. Installation

Since it is a standalone program, there is need for installation. Simply run the executable that is compatible with your operating system (OS).

2.2. User Interface Overview

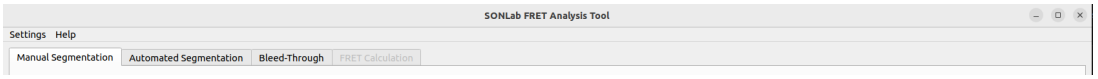

Figure 1: Main interface of the SONLab FRET Analysis Tool.

The application interface is organized into the following main sections:

- **Menu Bar**: Access to file operations, settings, and help
- **Tab Navigation**: Switch between different analysis modules
- **Preview Panels**: View original and processed images
- **Parameter Controls**: Adjust analysis parameters
- **Status Bar**: Display current status and progress (not in this early alpha version but will be in the later beta version.)

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3. Automated Segmentation

3.1. Overview

The Automated Segmentation tab provides tools to automatically detect and segment regions of interest (ROIs) in your FRET images.

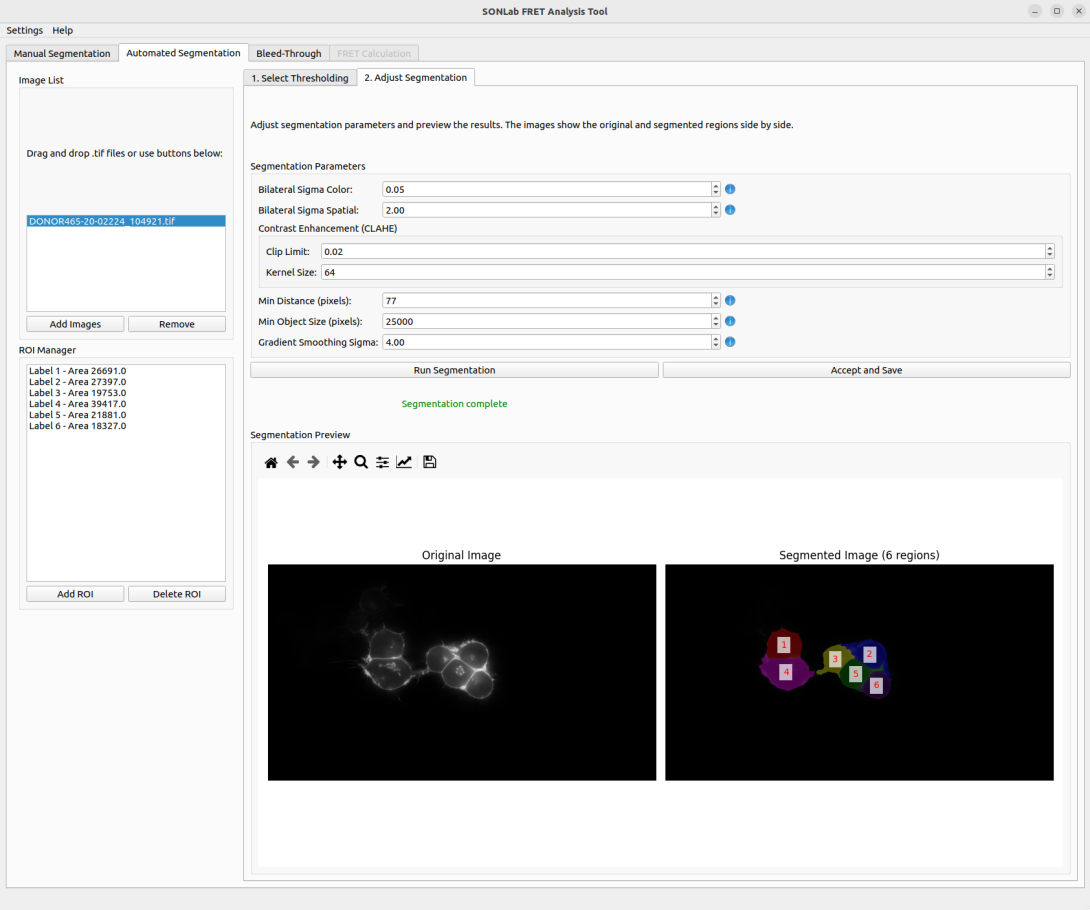

Figure 2: Automated Segmentation tab with thresholding and parameter adjustment panels.

This tab is divided into two main sections: Thresholding Method Selection and Segmentation Parameter Adjustment.

3.2. Workflow

1. Load your TIFF image(s)
2. Select a thresholding method from the first tab that fits your image the best
3. Adjust segmentation parameters in the second tab
4. Preview the results
5. Save the segmentation

3.3. Thresholding Methods

The tool offers the following thresholding algorithms to separate foreground from background:

- **Otsu's Method**: Automatically determines the optimal threshold by minimizing intra-class variance
- **Li's Minimum Cross Entropy**: Minimizes cross-entropy between original and thresholded image

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- **Mean**: Uses the mean of pixel values as threshold
- **Isodata**: Iterative threshold selection using the isodata algorithm
- **Yen's Method**: Maximizes the sum of entropies of the foreground and background
- **Triangle**: Uses the triangle method for thresholding

3.4. Segmentation Parameters

Table 1: Segmentation Parameters

Parameter	Description
`Bilateral Sigma Color` *Range:* 0.01-1.0 *Default:* 0.05	Controls the standard deviation for color/tonal similarity in bilateral filtering. Larger values allow more distant colors to be averaged.
`Bilateral Sigma Spatial` *Range:* 0.1-10.0 *Default:* 2.0	Controls the spatial extent of the bilateral filter. Larger values smooth over larger regions.
`Clip Limit` *Range:* 0.001-0.5 *Default:* 0.02	Limits the contrast enhancement in CLAHE. Higher values increase local contrast.
`Kernel Size` *Range:* 8-256 *Default:* 64	Size of the kernel used for CLAHE. Affects the locality of contrast enhancement.
`Min Distance` *Range:* 1-200 *Default:* 77	Minimum distance between cell centers for watershed segmentation. Increase for larger, more spread-out cells.
`Min Object Size` *Range:* 100-100000 *Default:* 30000	Minimum size of objects to keep after segmentation. Smaller objects will be removed.
`Gaussian Sigma` *Range:* 0.1-10.0 *Default:* 4.0	Standard deviation for Gaussian blur applied before thresholding. Helps reduce noise.

3.5. Usage Instructions

1. **Load Images**: Click the *Add Images* button or drag and drop TIFF files into the interface.
2. **Select Thresholding Method**: In the first tab, preview different thresholding methods and select the one that best separates your cells from the background.
3. **Adjust Parameters**: In the second tab, fine-tune the segmentation parameters based on

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your image characteristics.

4. **Preview**: The interface shows the original image and segmentation results side by side.
5. **Save Results**: Once satisfied with the segmentation, save the results.

3.6. Manual ROI Manipulation

The Automated Segmentation tab includes tools for manual refinement of segmentation results:

3.6.1 ROI Manager

The ROI Manager panel (left side) provides tools to manage regions of interest:

- **Add ROI**: Draw a custom polygon ROI on the image
  1. Click the *Add ROI* button
  2. Click on the image to place vertices of the polygon
  3. Complete the polygon to finish drawing
  4. The new ROI will be assigned the next available label number
- **Delete ROI**: Remove the selected ROI
  1. Select an ROI from the list
  2. Click the *Delete ROI* button
- **ROI List**: Shows all detected and manually added ROIs with their label numbers and areas

3.6.2 Refining Segmentation

After automated segmentation, you can:

- Add missing cells by drawing new ROIs
- Remove incorrectly segmented areas by deleting their ROIs
- Combine multiple ROIs by deleting and redrawing them
- Split merged cells by deleting the ROI and creating separate ROIs for each cell

3.7. Troubleshooting

- **Over-segmentation**: Increase the `Min Distance` parameter or decrease the `Clip Limit`.
- **Under-segmentation**: Decrease the `Min Object Size` or try a different thresholding method.
- **Poor edge detection**: Adjust the `Bilateral Sigma Color` and `Bilateral Sigma Spatial` parameters.
- **Noisy segmentation**: Increase the `Gaussian Sigma` to reduce noise.
- **ROI drawing issues**: Ensure the polygon is closed (right-click or press Enter to complete)
- **ROI not visible**: Check that the ROI is complete (at least 3 points)

3.8. Output

The segmentation results are saved as a multi-frame TIFF file with the segmentation mask as the first frame, followed by the original image frames.

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4. Manual Segmentation

4.1. Overview

The Manual Segmentation tab allows you to manually draw and manage Regions of Interest (ROIs) on your FRET microscopy images.

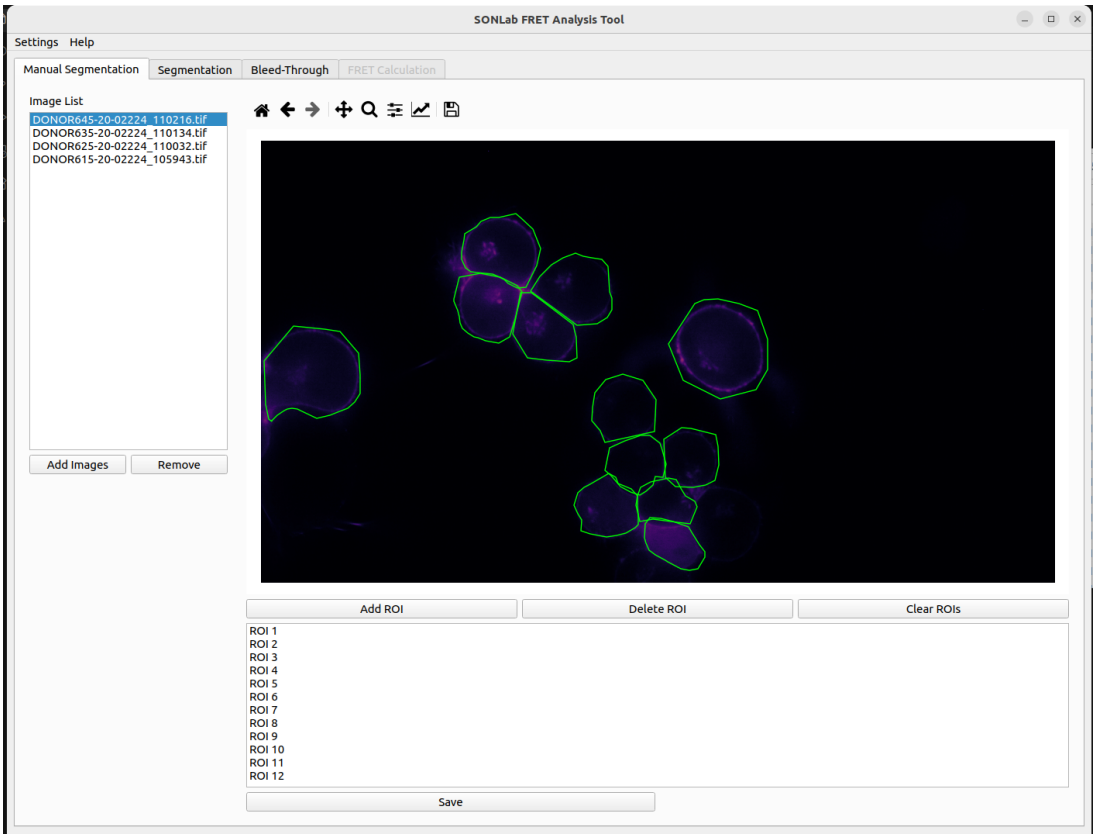

Figure 3: Manual Segmentation tab showing polygon ROI drawing tools and ROI list.

This is particularly useful when automated segmentation methods don't provide satisfactory results or when precise control over ROI selection is required.

4.2. Workflow

1. Load your TIFF image stack (FRET channel will be used for drawing)
2. Add ROIs by drawing polygons on the image
3. Edit or delete ROIs as needed
4. Save the segmentation results

4.3. Key Features

- **Interactive ROI Drawing**: Draw custom polygon ROIs with point-and-click precision
- **ROI Management**: Add, delete, or clear ROIs as needed
- **Multiple Image Support**: Work with multiple images in a single session
- **Drag and Drop**: Easily load images by dragging and dropping TIFF files
- **Visual Feedback**: Real-time visualization of ROIs on the image

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4.4. Usage Instructions

4.4.1 Loading Images

You can load images in three ways:

1. Click the *Add Images* button and select TIFF files
2. Drag and drop TIFF files directly onto the application window
3. Use the file browser to navigate and select images

4.4.2 Working with ROIs

- **Adding ROIs**: Click the *Add ROI* button, then click to place vertices. Enclose your selection to complete the polygon.
- **Selecting ROIs**: Click on an ROI in the list to select it
- **Deleting ROIs**: Select an ROI and click *Delete ROI*, or right-click in the list
- **Clearing All ROIs**: Click *Clear ROIs* to remove all ROIs from the current image

4.5. Handling Overlapping ROIs

When ROIs overlap:

- The application automatically handles overlapping regions
- Pixels in overlapping regions are assigned to the most recently created ROI
- To avoid ambiguity, it's recommended to minimize overlap between ROIs

4.5.1 Saving Results

1. Click the *Save* button
2. The results will be saved as a multi-frame TIFF file in a `segmented` subfolder
3. The output file will be named `segmented_{original_name}.tif`

4.6. Output Format

The saved segmentation file contains the following frames:

1. Frame 1: Label mask (ROIs are numbered starting from 1)
2. Frame 2: Original FRET channel
3. Frame 3: Original donor channel
4. Frame 4: Original acceptor channel

4.7. Troubleshooting

- **ROI not visible**: Ensure the ROI is complete (at least 3 points)
- **Image not loading**: Check that the file is a valid TIFF format
- **ROI drawing issues**: Try zooming in for more precise point placement
- **Save failed**: Verify you have write permissions in the target directory

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5. Bleed-Through Calculation

5.1. Overview

The Bleed-Through tab is used to calculate and correct for spectral bleed-through (BT) between donor-FRET and acceptor-FRET channels in confocal microscopy.

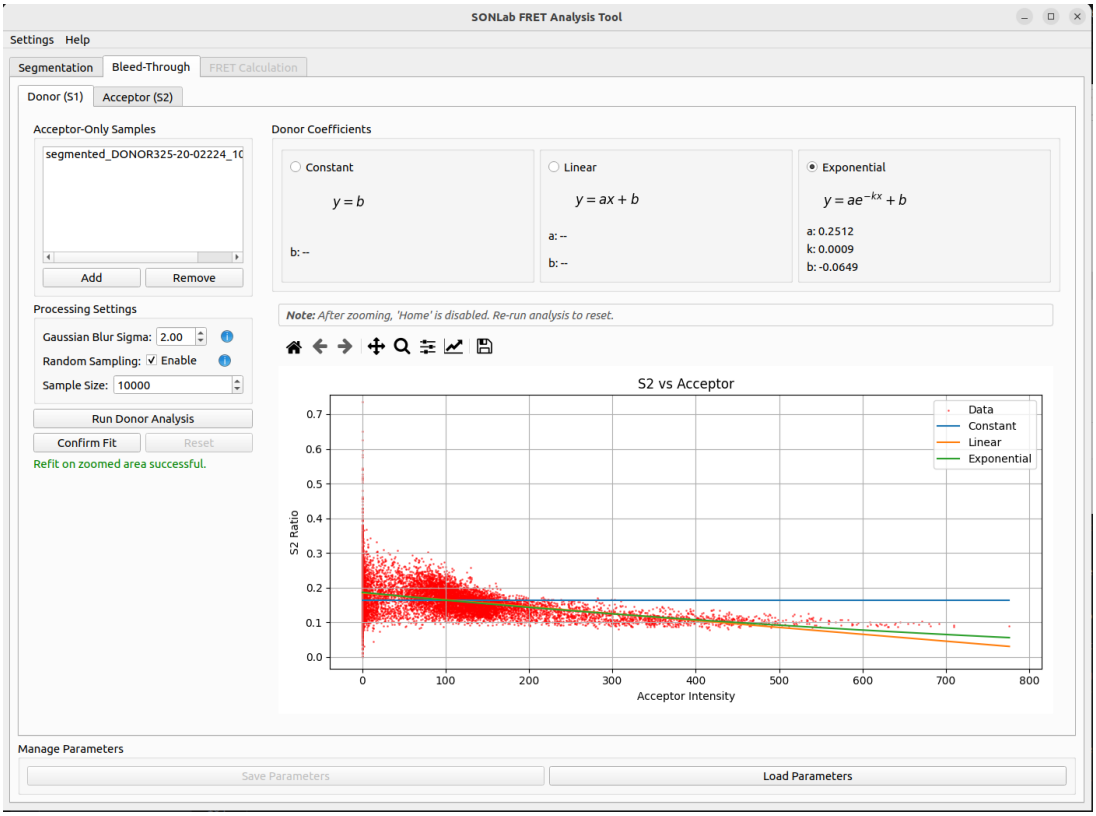

Figure 4: Bleed-Through Calculation tab illustrating donor and acceptor bleed-through analysis.

This is crucial for accurate FRET efficiency calculations. The tab includes two sub-tabs: Donor BT, into FRET channel, (S1) and Acceptor BT, into FRET channel, (S2) for an accurate FRET analyses.

5.2. Workflow

1. Load sample images for both donor-only and acceptor-only samples
2. Configure processing settings for each channel
3. Run the analysis to calculate bleed-through coefficients
4. Review and confirm the fit results
5. Save the parameters for use in FRET calculations

5.3. Key Features

- **Dual-Channel Analysis**: Separate tabs for donor and acceptor channel processing
- **Multiple Fitting Models**: Supports Constant, Linear, and Exponential fitting models
- **Interactive Plotting**: Visualize data points and fitting results
- **Parameter Management**: Save and load analysis parameters
- **Sampling Options**: Process full images or use random sampling for large datasets

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5.4. Usage Instructions

5.4.1 Loading Sample Images

1. Select either the Donor (S1) or Acceptor (S2) tab
2. Click the *Add* button to select sample images
3. Alternatively, drag and drop image files directly onto the tab
4. Ensure you have both donor-only and acceptor-only samples loaded

5.4.2 Configuring Processing Settings

For each channel, configure the following parameters:

- **Gaussian Blur Sigma**: Controls the amount of smoothing applied (default: 2.0)
- **Random Sampling**: Enable to process a subset of pixels for faster analysis
  - **Sample Size**: Number of pixels to sample (default: 10,000)

5.4.3 Running the Analysis

1. Click the *Run Analysis* button for the respective channel
2. The system will process the images and display scatter plots with fitting results
3. Review the fit quality visually and check the calculated coefficients
4. If not satisfied with the fit, you can focus on a specific area by using the zooming tool
5. If needed, adjust the fitting model or parameters and re-run the analysis
6. Once satisfied with the fit, click *Confirm Fit* button to save and use the parameters in the FRET analysis

5.4.4 Selecting the Fitting Model

Three fitting models are available:

1. **Constant**: Simple offset ($y = b$)
2. **Linear**: Linear relationship ($y = ax + b$)
3. **Exponential**: Exponential relationship ($y = ae^{-kx} + b$) *defined as decaying by default, which means when you have a growing curve you will have a negative k

5.4.5 Saving and Loading Parameters

- After confirming both donor and acceptor fits, click *Save Parameters*, if you want to use the same coefficients later.
- Parameters are saved to `bt_params.json`
- Use *Load Parameters* to restore previous settings
- The system will automatically detect and offer to load the last session's parameters

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5.5. Output and Results

For each channel, the following information is provided:

- Scatter plot of the data points with the selected fit
- Calculated coefficients for each fitting model
- Interactive plot controls for zooming and panning
- Status messages and fit quality indicators

5.6. Troubleshooting

- **Poor Fit**: Try a different fitting model or adjust the Gaussian sigma
- **Slow Performance**: Enable random sampling with a smaller sample size
- **No Data Points**: Verify that input images are in the correct format and channel
- **Fit Not Updating**: Click the home button in the plot toolbar to reset the view or re-run the analysis

6. FRET Calculation

6.1. Overview

The FRET Calculation tab provides tools for calculating and analyzing FRET efficiency based on the corrected data.

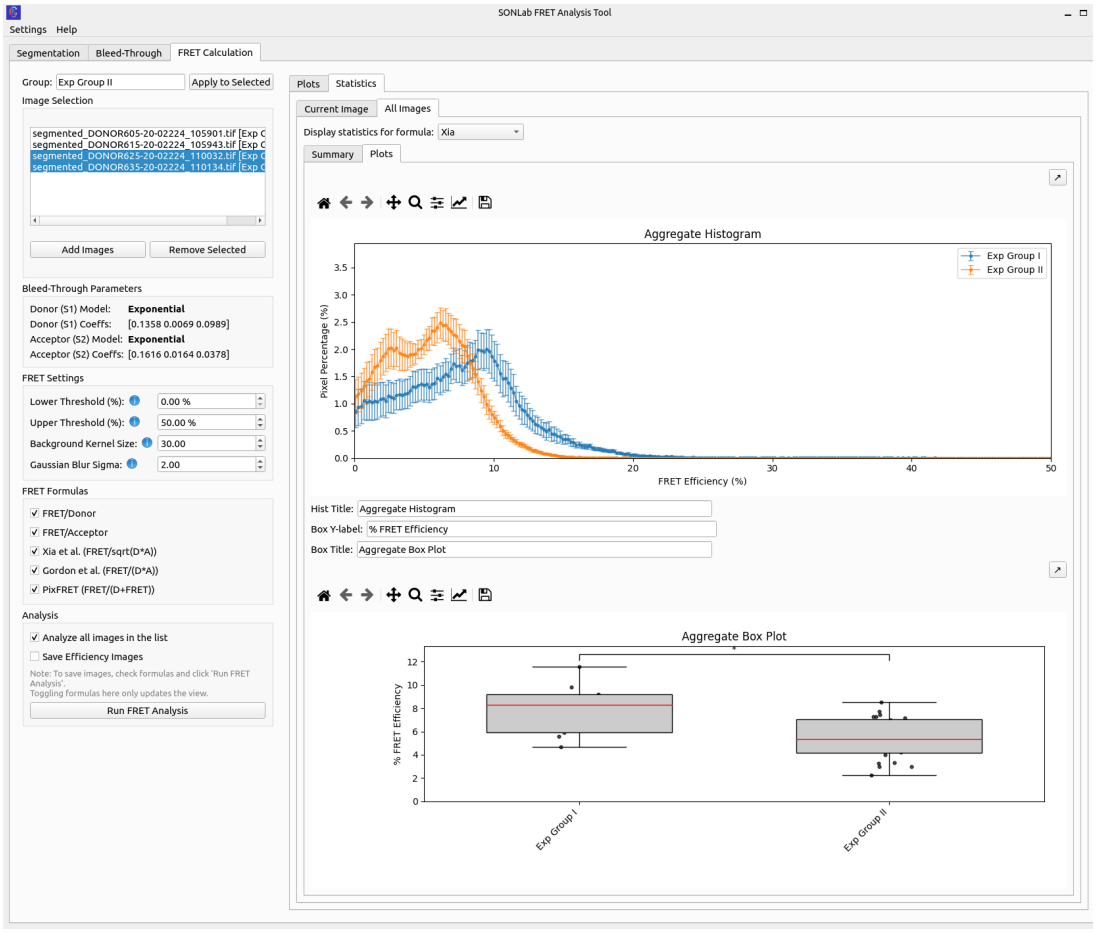

Figure 5: FRET Calculation tab with efficiency map and statistical plots.

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6.2. Workflow

1. Load corrected images (after bleed-through analysis)
2. Select FRET efficiency formula(s)
3. Configure analysis parameters
4. Run the analysis
5. Review and export results

6.3. Image Management

- **Add Images**: Load one or more TIFF/CZI files containing corrected FRET data
- **Grouping**: Assign images to groups for comparative analysis
- **Image List**: View and manage loaded images with their group assignments

6.4. FRET Settings

- **Lower Threshold (%)**: Minimum FRET efficiency value to display (default: 0%)
- **Upper Threshold (%)**: Maximum FRET efficiency value to display (default: 50%)
- **Background Kernel Size**: Size of the kernel for local background subtraction (default: 30)
- **Gaussian Blur Sigma**: Sigma value for optional Gaussian blur (default: 2.0, set to 0 to disable)

6.5. FRET Formulas

Multiple FRET efficiency formulas are available:

- **FRET/Donor**: Basic ratio of FRET to Donor intensity
- **FRET/Acceptor**: Ratio of FRET to Acceptor intensity
- **Xia et al.**: FRET/sqrt(D*R) - accounts for both donor and acceptor contributions
- **Gordon et al.**: FRET/(D*R) - alternative normalization method
- **PixFRET**: FRET/(D+FRET) - commonly used for sensitized emission FRET

6.6. Analysis Options

- **Analyze All Images**: Process all loaded images in sequence
- **Save Efficiency Images**: Export calculated efficiency maps as TIFF files
- **Run FRET Analysis**: Execute the analysis with current settings

6.7. Group Functionality

- The group functionality allows you to organize your data into logical groups
- Create groups based on experimental conditions or treatments
- Compare FRET efficiency between different groups
- The application will maintain the grouping throughout the analysis

6.8. Results Visualization

6.8.1 Current Image

Summary

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- Displays the currently selected image with efficiency maps
- Shows statistics for each enabled FRET formula
- Includes per-label statistics for segmented regions

Histogram & Box Plot

- Interactive histogram of efficiency values
- Box plot showing distribution of per-cell averages
- Customizable axis labels and titles
- Export options for both plots

6.8.2 All Images

Summary

- Aggregated statistics across all analyzed images
- Group comparisons with statistical significance
- Export to CSV for further analysis

Plots

- Aggregate histogram of all labeled cells
- Grouped box plots with statistical comparisons
- Customizable visualization parameters
- Export options for publication-quality figures

6.9. Output Files

When saving is enabled, the following files are generated:

- Efficiency maps for each formula (TIFF format)
- Summary statistics (CSV)
- Histogram data (CSV)
- Plots (PNG/PDF)

7. Troubleshooting

7.1. Common Issues

- **Images not loading**: Check file permissions and format compatibility
- **Segmentation errors**: Adjust the segmentation parameters
- **Performance issues**: Close other applications and reduce number of loaded images if necessary
- **Unexpected results**: Contact with the developer team

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8. Overall Application Logic and Interactions

This section describes how the different components of the SONLab FRET Analysis Tool work together to process and analyze FRET microscopy data.

8.1. Application Workflow

The typical workflow follows these steps:

1. **Image Loading and Preprocessing**
   - Load raw TIFF or CZI images containing FRET, Donor, and Acceptor channels
   - Optionally apply preprocessing steps like background subtraction and Gaussian blur
2. **Segmentation**
   - Use either Manual or Automated Segmentation to define Regions of Interest (ROIs)
   - ROIs are stored and can be modified or deleted as needed
   - Segmented regions are used for all subsequent analysis
3. **Bleed-Through Correction**
   - Characterize and correct for spectral bleed-through between channels
   - Apply correction coefficients to all images
   - Verify correction quality using the provided visualization tools
4. **FRET Calculation**
   - Select and apply one or more FRET efficiency formulas
   - Analyze results using the interactive visualization tools
   - Export data and figures for further analysis or publication

8.2. Data Flow Between Tabs

- **Image Data**: Once loaded, images are available within the tab only
- **ROIs**: Defined in the Segmentation tabs and used in FRET calculations
- **Correction Parameters**: Bleed-through corrections are automatically applied to all relevant analyses
- **Analysis Results**: Can be exported and shared between different sessions

8.3. Performance Considerations

- Processing large images or many ROIs may require significant system resources
- Use the preview features to test parameters on a subset of data
- Save your work frequently, especially after major processing steps
- For batch processing, consider using the command-line interface if available

9. Contact and Support

For technical support or to report issues, please contact:

- Email: zubeyir.nursoy@metu.edu.tr
- Website: `https://www.sonlab.org` – coming soon

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