# SONLab FRET Analysis Tool

User Guide for v2.0.3-build

SONLab Team

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**Abstract**

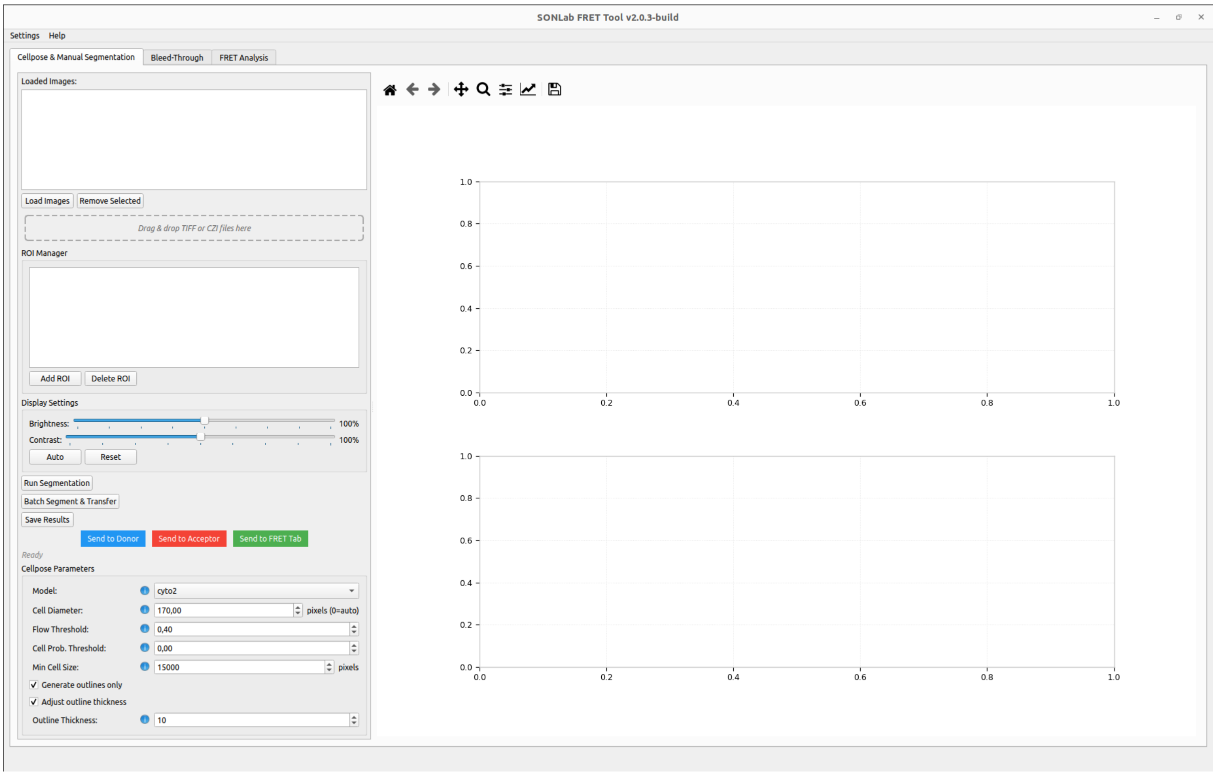
This guide documents the SONLab FRET Analysis Tool, an open-source desktop application for analyzing Fluorescence Resonance Energy Transfer (FRET) microscopy data. The tool combines deep-learning cell segmentation (Cellpose) with standardized pipelines for bleed-through correction and FRET efficiency calculation, enabling reproducible, low-bias analysis of protein–protein interactions. It walks through installation, the user interface, and the three analysis stages—segmentation, bleed-through correction, and FRET calculation—together with the statistics, file formats, and troubleshooting.

# Contents

# 1. Introduction

## 1.1. About the Tool

The SONLab FRET Analysis Tool is organized as a three-stage pipeline, with one tab per stage: **Cellpose & Manual Segmentation**, **Bleed-Through**, and **FRET Analysis**. A typical session moves left to right through these tabs: cells are segmented, the spectral cross-talk is characterized, and the corrected data are used to compute FRET efficiency, statistics, and figures.



Brightness: 100%

Contrast: 100%

Model: cyto2

Cell Diameter: 170,00 pixels (0=auto)

Flow Threshold: 0,40

Cell Prob. Threshold: 0,00

Min Cell Size: 15000 pixels

☑ Generate outlines only

☑ Adjust outline thickness

Outline Thickness: 10

Figure 1: The main window, shown on the Cellpose & Manual Segmentation tab.

## 1.2. Key Features

- **AI-powered segmentation:** Cellpose models with manual polygon refinement.
- **Bleed-through correction:** donor (S1), acceptor (S2), and optional S3/S4 channels with Constant, Linear, or Exponential fitting.
- **Multiple FRET formulas:** FRET/Donor, FRET/Acceptor, Xia *et al.*, Gordon *et al.*, PixFRET, and DFRET.
- **Robust statistics:** assumption-checked significance testing with group comparisons.
- **Rich visualization:** efficiency maps, histograms, box plots, and a Gaussian-mixture distribution analysis.
- **Reproducible outputs:** efficiency maps (TIFF), statistics (CSV), figures (PNG/PDF), and reusable parameter files (JSON).
- **Batch processing** and a customizable interface (theme, font size, compact layout).

### 1.3. System Requirements

- Windows 10/11, macOS 10.15+, or a recent Linux distribution.
- Python 3.10 (required for dependency compatibility).
- Minimum 8 GB RAM (16 GB recommended for large datasets).
- A CUDA-capable GPU accelerates Cellpose segmentation but is not required.
- Screen resolution of 1920×1080 or higher recommended.

## 2. Getting Started

### 2.1. Installation

Two installation methods are provided.

**Installers (recommended).** Platform scripts in the `installers/` directory create a virtual environment, install dependencies, and add a desktop launcher: `install_windows.ps1`, `install_linux.sh`, and `install_mac.sh`. See `installers/README.md` for details.

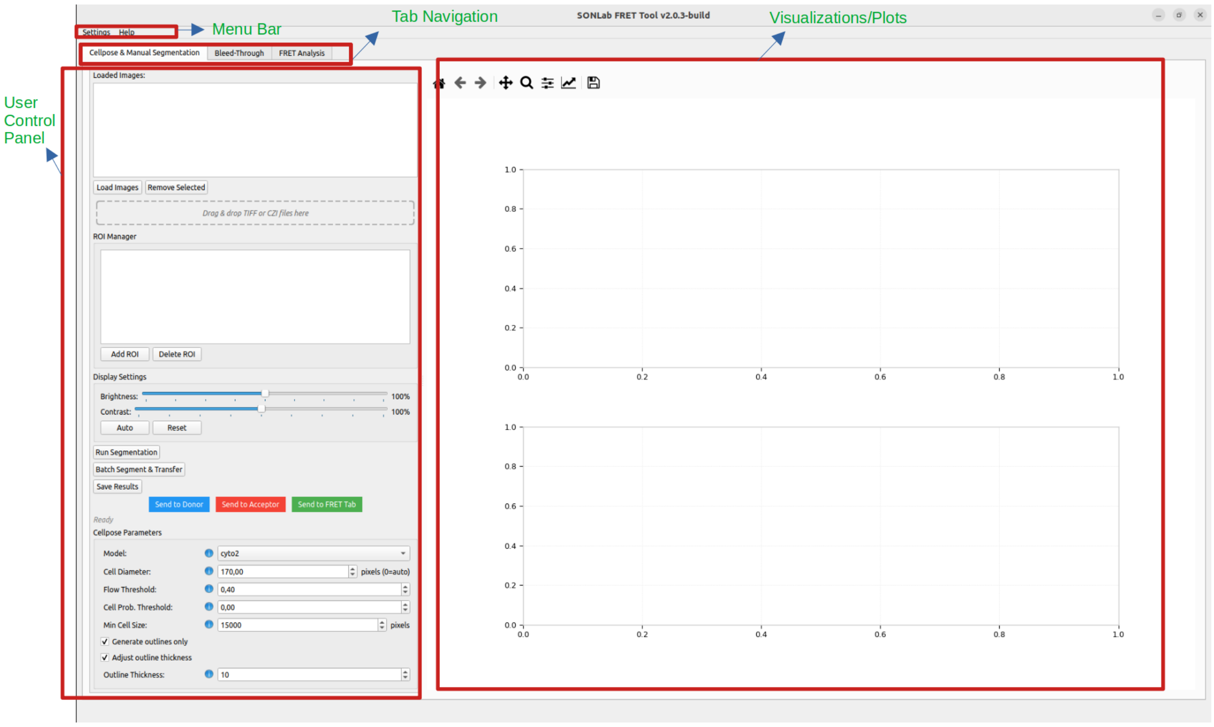
**Manual installation.** For advanced users:

```
git clone https://github.com/sonlab-metu/SONLab-FRET-Tool.git
cd SONLab-FRET-Tool
python3 -m venv venv
source venv/bin/activate          # Windows: .\venv\Scripts\activate
pip install -r installers/requirements.txt
# Install PyTorch for your hardware (CUDA/ROCm/CPU); see the README,
   e.g. CPU:
pip install torch torchvision torchaudio --index-url https://download
   .pytorch.org/whl/cpu
python3 -m GUI.main_gui
```

**Note:** Other Python versions are not supported because of dependency constraints. On Apple Silicon, use the CPU command; PyTorch uses the Metal backend automatically.

### 2.2. User Interface Overview

The window has three regions: the **menu bar** (settings and help), the **tab navigation**, and the **working area** (a left-hand control panel and a visualization area).



Brightness: 100%
Contrast: 100%
Model: cyto2
Cell Diameter: 170,00 pixels (0=auto)
Flow Threshold: 0,40
Cell Prob. Threshold: 0,00
Min Cell Size: 15000 pixels
☑ Generate outlines only
☑ Adjust outline thickness
Outline Thickness: 10

Figure 2: The four regions of the interface: menu bar, tab navigation, user control panel, and visualization area.

## 2.3. Settings and Help

The **Settings** menu offers font-size adjustment, a Light/Dark theme (with a Dark Theme color editor), and a Compact Layout toggle. The **Help** menu provides a guided walk-through, this user guide, and an About dialog.

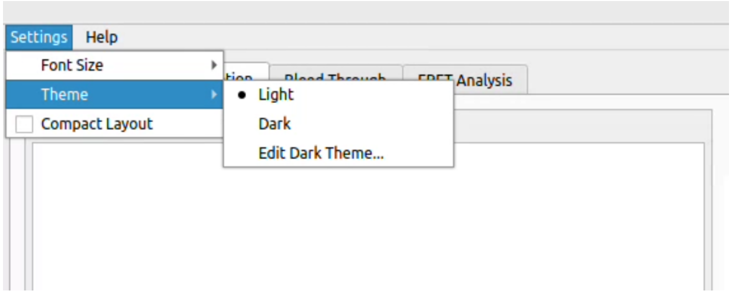
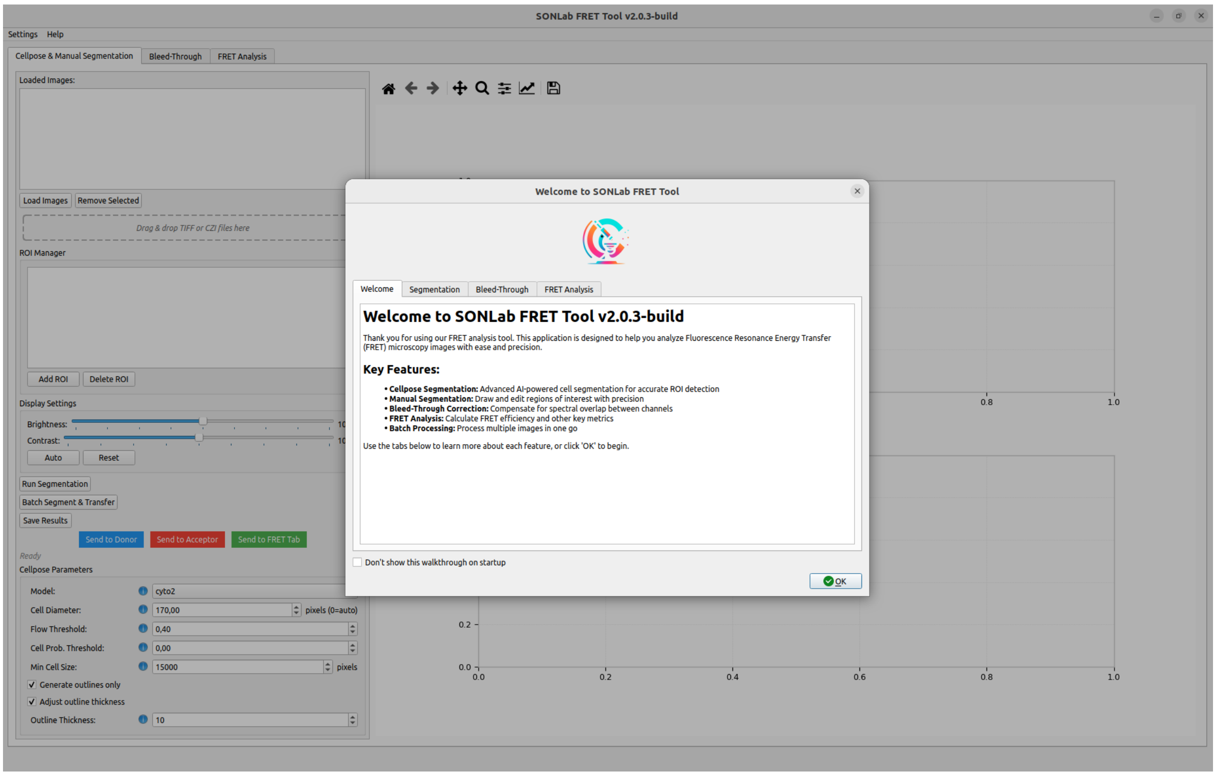


Figure 3: The Settings menu, with the Theme submenu open.

## 2.4. Interface Conventions

- **Info icons** (i) next to controls show a one-line description on hover.
- **Drag & drop** of `.tif`, `.tiff`, and `.czi` files is supported on image lists.
- The **mouse wheel does not change** the value of dropdowns or spin boxes anywhere in the app, preventing accidental edits while scrolling.
- **Pop-out** (↗) buttons open a plot in a larger window; matplotlib toolbars provide zoom, pan, and save.



Model: cyto2

Cell Diameter: 170,00 pixels (0=auto)

Flow Threshold: 0,40

Cell Prob. Threshold: 0,00

Min Cell Size: 15000 pixels

☑ Generate outlines only

☑ Adjust outline thickness

Outline Thickness: 10

**Welcome to SONLab FRET Tool v2.0.3-build**

Thank you for using our FRET analysis tool. This application is designed to help you analyze Fluorescence Resonance Energy Transfer (FRET) microscopy images with ease and precision.

**Key Features:**

- **Cellpose Segmentation:** Advanced AI-powered cell segmentation for accurate ROI detection
- **Manual Segmentation:** Draw and edit regions of interest with precision
- **Bleed-Through Correction:** Compensate for spectral overlap between channels
- **FRET Analysis:** Calculate FRET efficiency and other key metrics
- **Batch Processing:** Process multiple images in one go

Use the tabs below to learn more about each feature, or click 'OK' to begin.

☐ Don't show this walkthrough on startup

Figure 4: The Help → Walk-through dialog introduces each pipeline stage. It can be reopened any time, even after being dismissed on startup.

## 3. Cellpose & Manual Segmentation

### 3.1. Overview

The first tab segments cells using the Cellpose deep-learning models and lets you refine the result by hand with polygon ROIs. Segmentation produces a **label mask**—an image where every pixel of a cell shares the same integer label and background is 0. This mask is stored as the first frame of the saved stack and tells every downstream calculation which pixels belong to which cell.

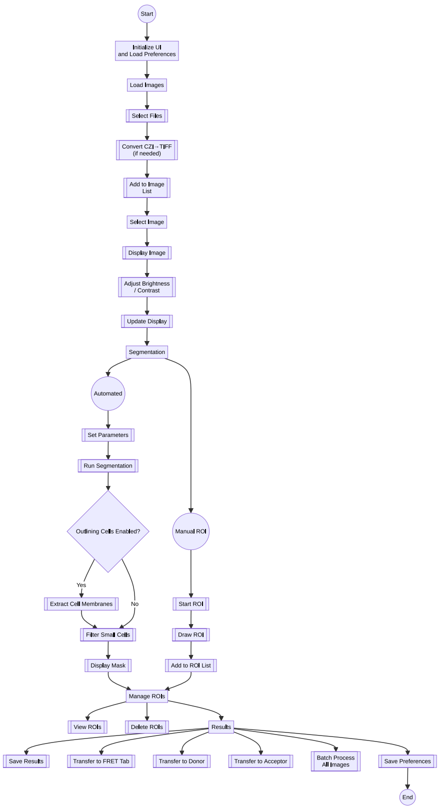
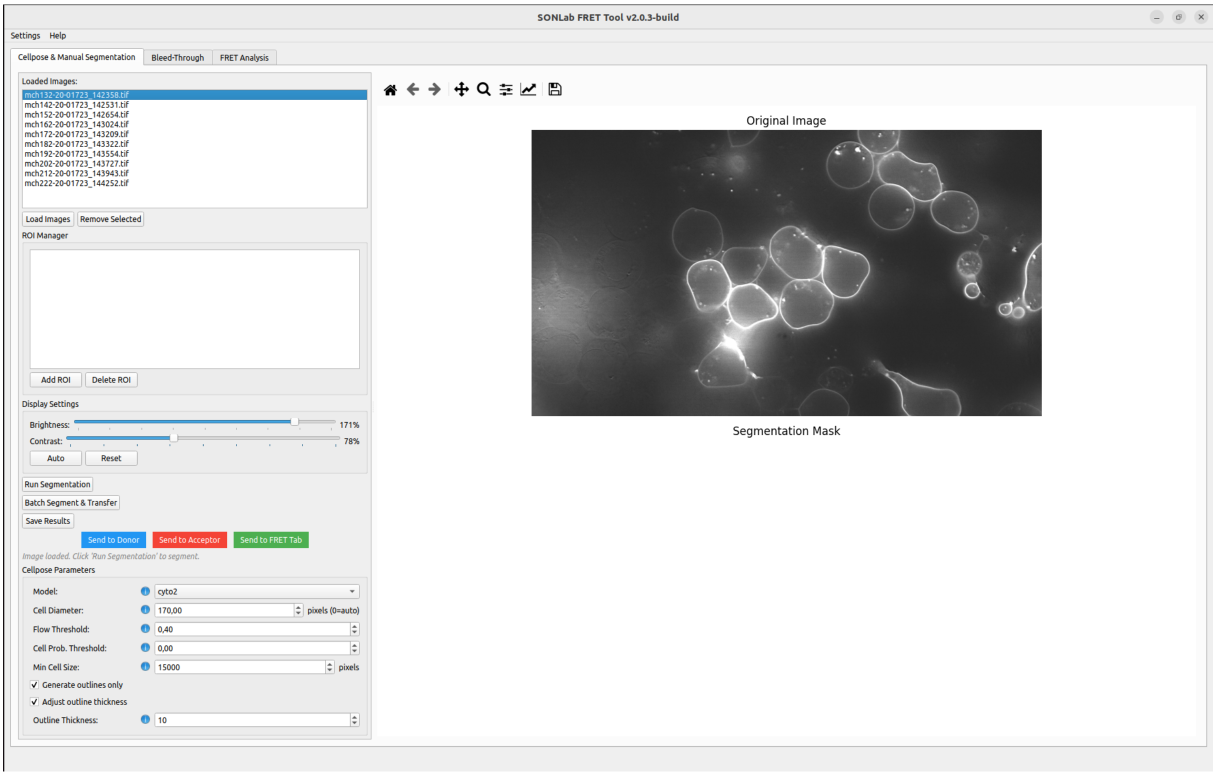


Figure 5: Flow of the segmentation stage, from loading images to transferring the segmented stacks.



Loaded Images:
mch132-20-01723_142358.tif
mch142-20-01723_142531.tif
mch152-20-01723_142654.tif
mch162-20-01723_143024.tif
mch172-20-01723_143209.tif
mch182-20-01723_143322.tif
mch192-20-01723_143554.tif
mch202-20-01723_143727.tif
mch212-20-01723_143943.tif
mch222-20-01723_144252.tif

Brightness: 171%
Contrast: 78%

*Image loaded. Click 'Run Segmentation' to segment.*

Model: cyto2
Cell Diameter: 170,00 pixels (0=auto)
Flow Threshold: 0,40
Cell Prob. Threshold: 0,00
Min Cell Size: 15000 pixels
☑ Generate outlines only
☑ Adjust outline thickness
Outline Thickness: 10

Figure 6: The Segmentation tab: image list and Cellpose parameters on the left; the original image and segmentation-mask area on the right.

## 3.2. Loading Images

Click **Load Images** or drag and drop files onto the drop area. Inputs are multi-frame `.tif`/`.tiff` and Zeiss `.czi`; CZI files are converted automatically to a multi-frame TIFF (FRET, Donor, Acceptor) preserving the original raw intensities. Use **Remove Selected** to drop images; when the list becomes empty the view returns to a blank state.

## 3.3. Cellpose Parameters

Table 1: Cellpose segmentation parameters.

| Parameter | Default | Description |
|---|---|---|
| `Model` | cyto2 | Cellpose model (cyto2, cyto, nuclei, tissuenet, livecell). `cyto2` suits most whole-cell tasks; use `nuclei` for nuclear stains. |
| `Cell Diameter` | 170 px | Approximate cell diameter; set 0 for automatic estimation. The single most impactful parameter. |
| `Flow Threshold` | 0.4 | Maximum flow error per mask. Lower is stricter; higher recovers more cells but may add spurious ones. |
| `Cell Prob. Threshold` | 0.0 | Detection probability cut-off. Lower detects fainter cells (more noise); raise to keep confident detections. |
| `Min Cell Size` | 15000 px | Objects smaller than this are removed. |
| `Generate outlines only` | on | Store outlines instead of filled masks (output prefixed `outline_segmented_` vs. `whole-cell_segmented_`). |
| `Outline thickness` | 10 | Thickness (px) of generated outlines (1–20). |

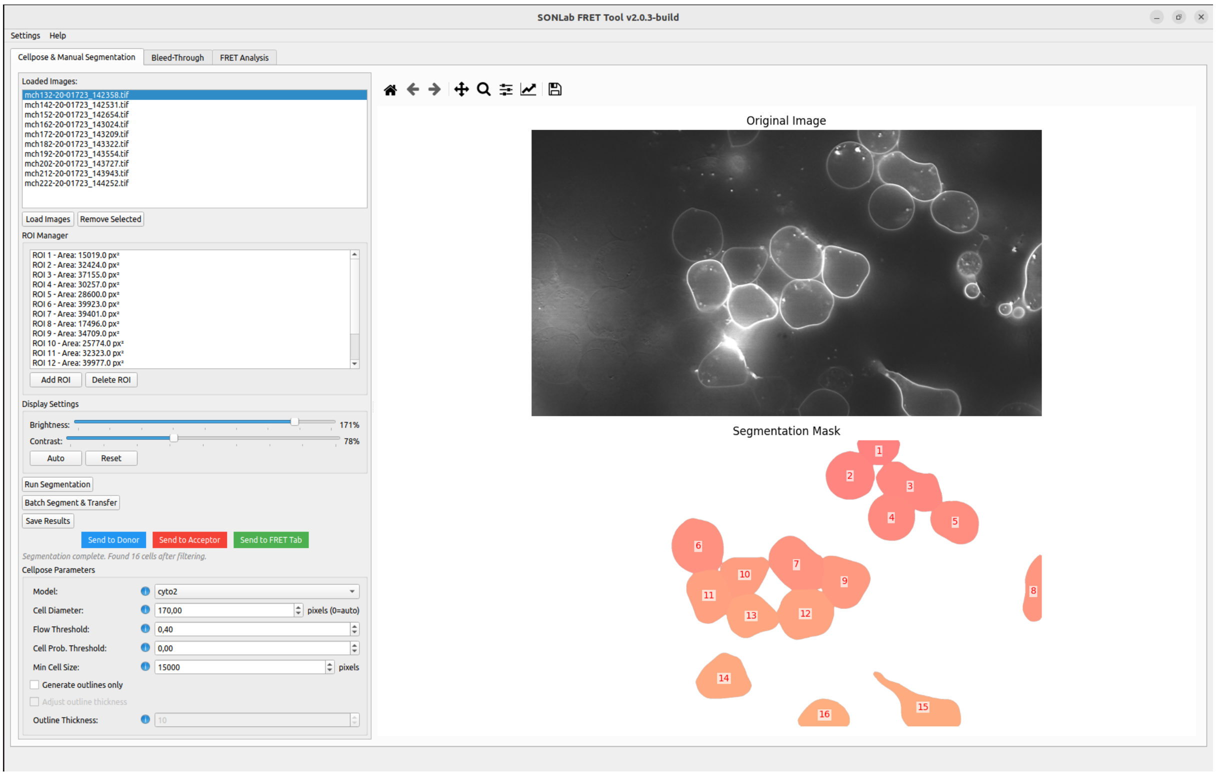
**Note:** If cells are merged, reduce `Cell Diameter` or `Flow Threshold`. If single cells are split, increase the diameter. Remove debris by raising `Min Cell Size`.

## 3.4. Display Settings

The **Brightness** and **Contrast** sliders, **Auto**, and **Reset** affect only the on-screen preview, never the saved data.

## 3.5. Running Segmentation

Click **Run Segmentation** to segment the selected image; the status line reports progress and the number of cells found.



Loaded Images:
mch132-20-01723_142358.tif
mch142-20-01723_142531.tif
mch152-20-01723_142654.tif
mch162-20-01723_143024.tif
mch172-20-01723_143209.tif
mch182-20-01723_143322.tif
mch192-20-01723_143554.tif
mch202-20-01723_143727.tif
mch212-20-01723_143943.tif
mch222-20-01723_144252.tif

ROI 1 - Area: 15019.0 px²
ROI 2 - Area: 32424.0 px²
ROI 3 - Area: 37155.0 px²
ROI 4 - Area: 30257.0 px²
ROI 5 - Area: 28600.0 px²
ROI 6 - Area: 39923.0 px²
ROI 7 - Area: 39401.0 px²
ROI 8 - Area: 17496.0 px²
ROI 9 - Area: 34709.0 px²
ROI 10 - Area: 25774.0 px²
ROI 11 - Area: 32323.0 px²
ROI 12 - Area: 39977.0 px²

Brightness: 171%
Contrast: 78%

*Segmentation complete. Found 16 cells after filtering.*

Model: cyto2
Cell Diameter: 170,00 pixels (0=auto)
Flow Threshold: 0,40
Cell Prob. Threshold: 0,00
Min Cell Size: 15000 pixels
☐ Generate outlines only
☐ Adjust outline thickness
Outline Thickness: 10

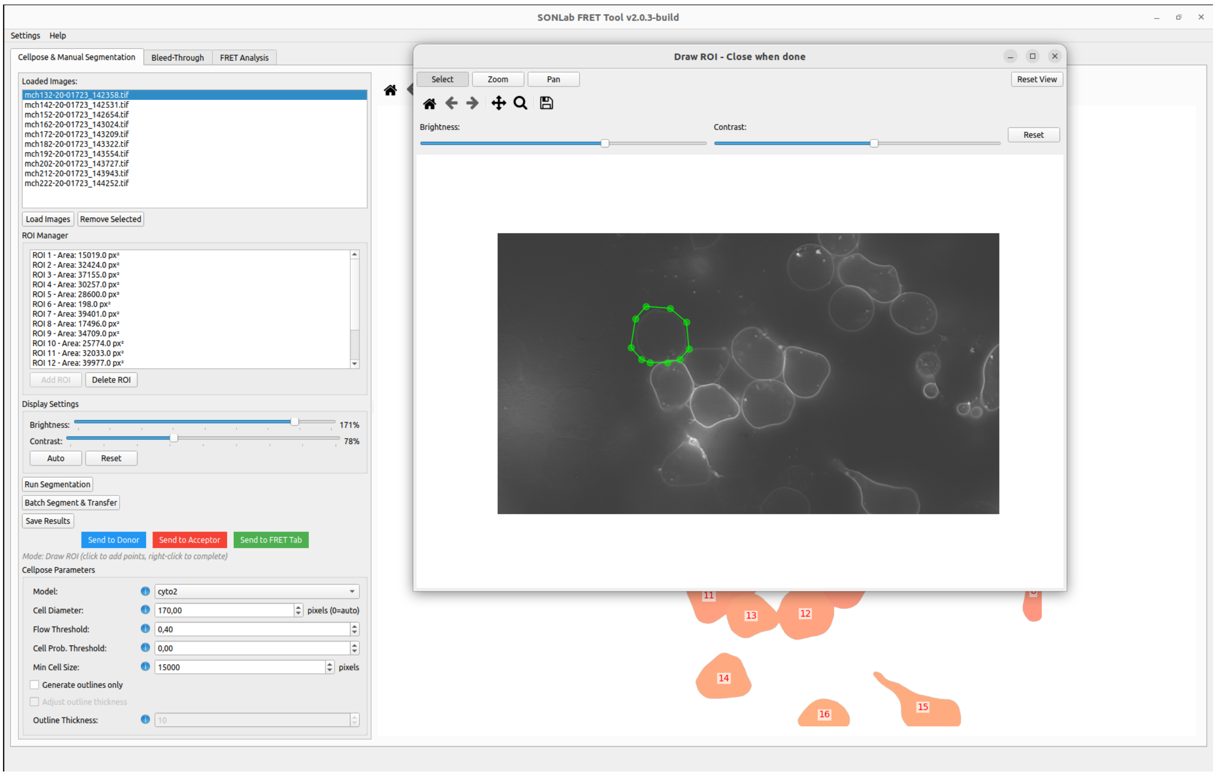
Figure 7: After Run Segmentation: detected cells appear as numbered labels, and the ROI Manager list is populated with one entry per cell.

## 3.6. Manual Refinement (ROI Manager)

Refine the mask using the ROI Manager:

- **Add ROI**: click **Add ROI**, then click on the image to place polygon vertices around a cell and close the polygon. The region gets the next available label.
- **Delete ROI**: select a region in the list and click **Delete ROI**.

Use this to add missed cells, remove wrong regions, or split merged cells.



Loaded Images:
mch132-20-01723_142358.tif
mch142-20-01723_142531.tif
mch152-20-01723_142654.tif
mch162-20-01723_143024.tif
mch172-20-01723_143209.tif
mch182-20-01723_143322.tif
mch192-20-01723_143554.tif
mch202-20-01723_143727.tif
mch212-20-01723_143943.tif
mch222-20-01723_144252.tif

ROI Manager
ROI 1 - Area: 15019.0 px²
ROI 2 - Area: 32424.0 px²
ROI 3 - Area: 37155.0 px²
ROI 4 - Area: 30257.0 px²
ROI 5 - Area: 28600.0 px²
ROI 6 - Area: 198.0 px²
ROI 7 - Area: 39401.0 px²
ROI 8 - Area: 17496.0 px²
ROI 9 - Area: 34709.0 px²
ROI 10 - Area: 25774.0 px²
ROI 11 - Area: 32033.0 px²
ROI 12 - Area: 39977.0 px²

Display Settings
Brightness: 171%
Contrast: 78%

*Mode: Draw ROI (click to add points, right-click to complete)*

Cellpose Parameters
Model: cyto2
Cell Diameter: 170,00 pixels (0=auto)
Flow Threshold: 0,40
Cell Prob. Threshold: 0,00
Min Cell Size: 15000 pixels
☐ Generate outlines only
☐ Adjust outline thickness
Outline Thickness: 10

Figure 8: The ROI drawing window: place polygon vertices around a cell using the Select/Zoom/Pan tools and brightness/contrast controls.

### 3.7. Saving and Transferring

- **Save Results**: save the segmented stack to a `segmented/` folder next to the source image.
- **Send to FRET Tab**: save and add the image to the FRET tab.
- **Send to Donor** / **Send to Acceptor**: send the image to the corresponding Bleed-Through channel.
- **Batch Segment & Transfer**: segment all loaded images and transfer them to the FRET tab, optionally with a group name.

### 3.8. Output Format

Results are saved as a multi-frame TIFF: frame 0 is the label mask, followed by the original image channels (FRET, Donor, Acceptor). Raw intensities are preserved so the stacks are directly usable downstream (see Section 8).

### 3.9. Troubleshooting

- **Cells merged:** lower `Cell Diameter`/`Flow Threshold`. **Cells split:** increase `Cell Diameter`.
- **Debris segmented:** increase `Min Cell Size`. **Faint cells missed:** lower `Cell Prob. Threshold`.
- **Polygon won't complete:** ensure at least 3 vertices and that it is closed.
- **Slow:** use a CUDA-capable GPU, or reduce image/batch size.

# 4. Bleed-Through Correction

## 4.1. Overview

Spectral bleed-through (cross-talk) is the signal that leaks from the donor and acceptor fluorophores into the FRET channel. The Bleed-Through tab estimates the bleed-through coefficients from single-label control images and fits a correction model that the FRET tab applies.

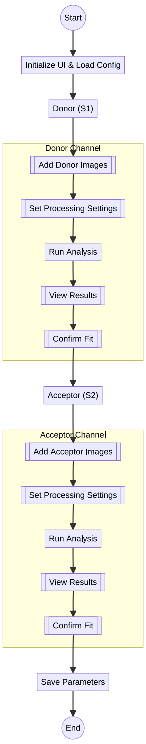
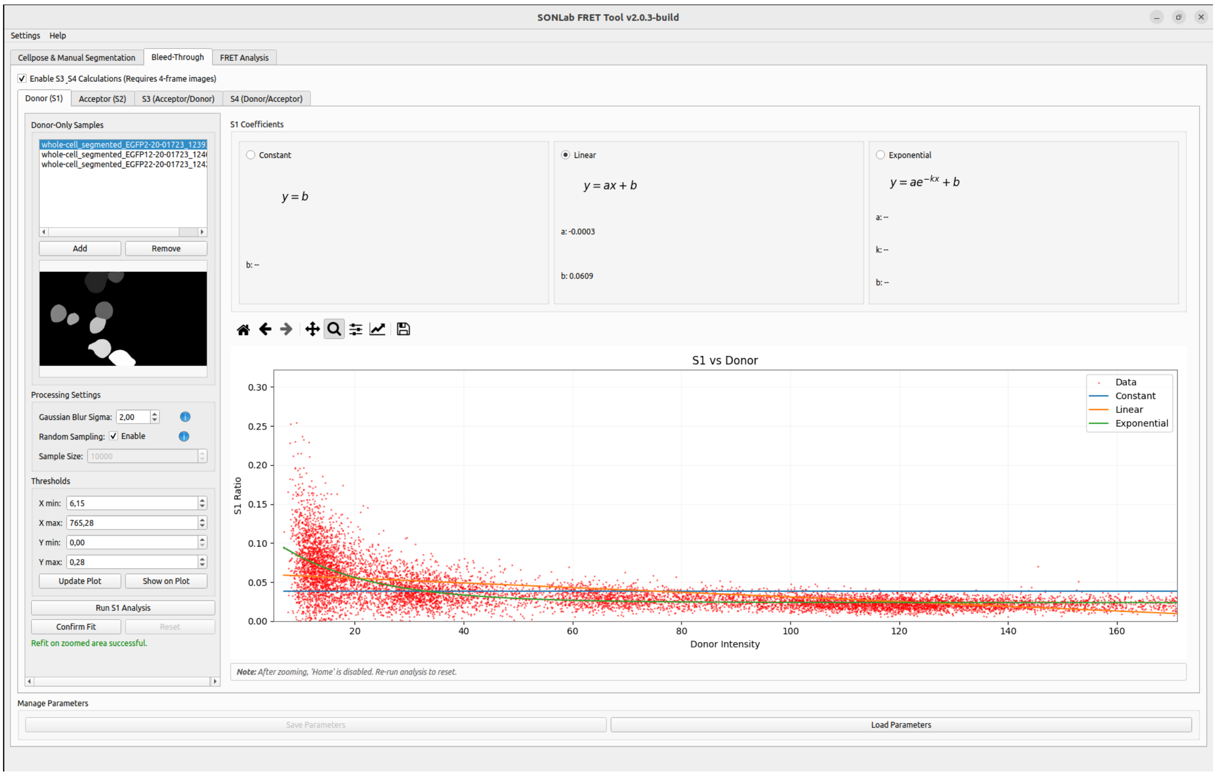


Figure 9: Flow of the bleed-through stage, from loading control images to saving the fitted coefficients.

Table 2: Bleed-through channels.

| Channel | Coefficient | Control sample | Required |
|---|---|---|---|
| Donor (S1) | Donor → FRET | donor-only | Always |
| Acceptor (S2) | Acceptor → FRET | acceptor-only | Always |
| S3 (Acceptor/Donor) | Acceptor cross term | donor-only (4-frame) | Optional |
| S4 (Donor/Acceptor) | Donor cross term | acceptor-only (4-frame) | Optional |

S3 and S4 are enabled with the *Enable S3 & S4 Calculations* checkbox and require four-frame images (mask, FRET, Donor, Acceptor).



☑ Enable S3_S4 Calculations (Requires 4-frame images)

whole-cell_segmented_EGFP2-20-01723_1239
whole-cell_segmented_EGFP12-20-01723_124
whole-cell_segmented_EGFP22-20-01723_124

Gaussian Blur Sigma: 2,00
Random Sampling: ☑ Enable
Sample Size: 10000

X min: 6,15
X max: 765,28
Y min: 0,00
Y max: 0,28

Refit on zoomed area successful.

$y = b$

b: --

$y = ax + b$

a: -0.0003

b: 0.0609

$y = ae^{-kx} + b$

a: --

k: --

b: --

**Note:** *After zooming, 'Home' is disabled. Re-run analysis to reset.*

Figure 10: The Donor (S1) channel: control-image list and processing settings on the left, coefficient panel on top, and the S1-vs-Donor scatter plot with the fitted curves below.

## 4.2. Workflow

1. Select a channel sub-tab (start with Donor (S1)).
2. Add the matching control images.
3. Configure the processing settings and run the analysis.
4. Choose a fitting model and confirm the fit.
5. Repeat for Acceptor (S2) (and S3/S4 if enabled), then save the parameters.

## 4.3. Loading Control Images

Click **Add** (or drag and drop). Use donor-only samples for S1/S3 and acceptor-only samples for S2/S4—typically the segmented stacks produced in the Segmentation tab. A preview of the selected image is shown.

## 4.4. Processing Settings

Table 3: Bleed-through processing settings.

| Setting | Default | Description |
|---|---|---|
| `Gaussian Blur Sigma` | 2.0 | Standard deviation of the smoothing kernel applied before computing ratios. |
| `Random Sampling` | off | Fit on a random subset of pixels (much faster on large data). |
| `Sample Size` | 10000 | Number of pixels sampled when Random Sampling is enabled. |

## 4.5. Running the Analysis and Thresholds

Click **Run Analysis**. A scatter plot of the bleed-through ratio versus intensity is shown with the fitted curve(s), computed inside the segmentation mask. The **Thresholds** panel (X/Y min/max, **Update Plot**, **Show on Plot**) restricts which points are used—useful for excluding saturated or low-signal pixels.

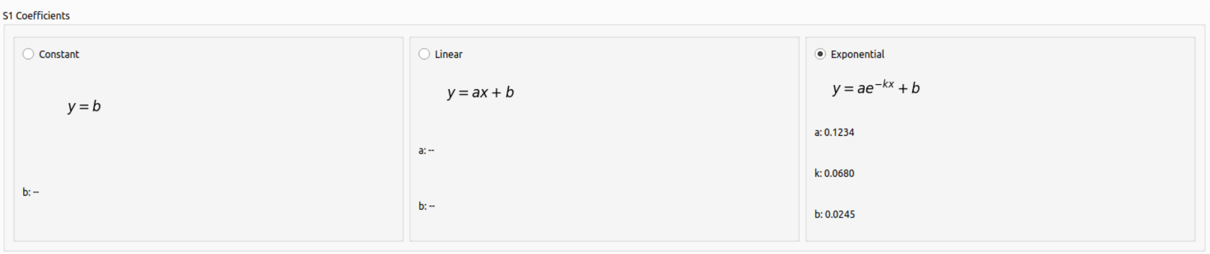
**Note:** After zooming, the toolbar Home button is disabled; re-run the analysis or use the threshold Update Plot to reset the view.

## 4.6. Fitting Models

Table 4: Bleed-through fitting models.

| Model | Equation | When to use |
|---|---|---|
| Constant | $y = b$ | Bleed-through is intensity-independent. |
| Linear | $y = ax + b$ | Bleed-through varies linearly with intensity. |
| Exponential | $y = ae^{-kx} + b$ | Non-linear relationship. The curve decays by default, so a growing relationship yields a negative $k$. |

Select a model with its radio button; the fitted coefficients are displayed. Click **Confirm Fit** to lock the coefficients for the channel, or **Reset** to unlock for a new analysis.



S1 Coefficients

○ Constant

$y = b$

b: --

○ Linear

$y = ax + b$

a: --

b: --

● Exponential

$y = ae^{-kx} + b$

a: 0.1234

k: 0.0680

b: 0.0245

Figure 11: The coefficient panel showing the Constant, Linear, and Exponential models, with Exponential selected and its fitted coefficients shown.

## 4.7. Saving and Loading Parameters

**Save Parameters** writes the model type and coefficients to `bt_params.json` in the default location and **also copies it into each input image directory** (prompting before overwriting). **Load Parameters** restores a saved file; on startup the tool offers to reload the last session's parameters. Loading while an analysis is on screen redraws the plot from the saved coefficients without re-fitting, preserving the confirmed values.

## 4.8. Troubleshooting

- **Poor fit:** try a different model or adjust the Gaussian sigma.
- **Slow:** enable Random Sampling with a smaller sample size.
- **No data points:** confirm the images are single-label controls with a segmentation mask.
- **S3/S4 error:** provide four-frame stacks.

# 5. FRET Analysis

## 5.1. Overview and Workflow

The FRET Analysis tab computes pixel-wise FRET efficiency from segmented, bleed-through-corrected images, organizes images into groups, and produces statistics and figures.

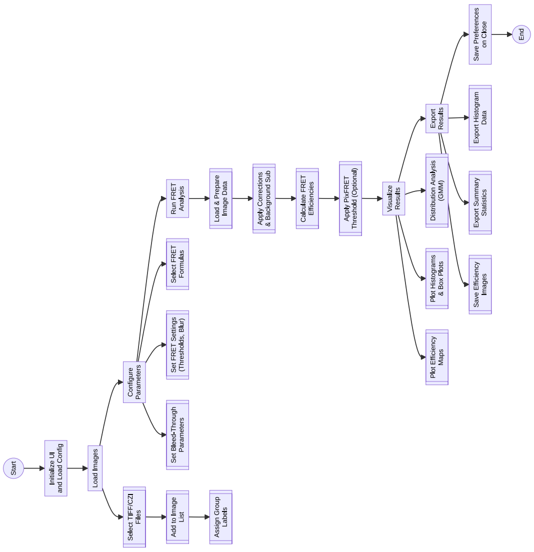
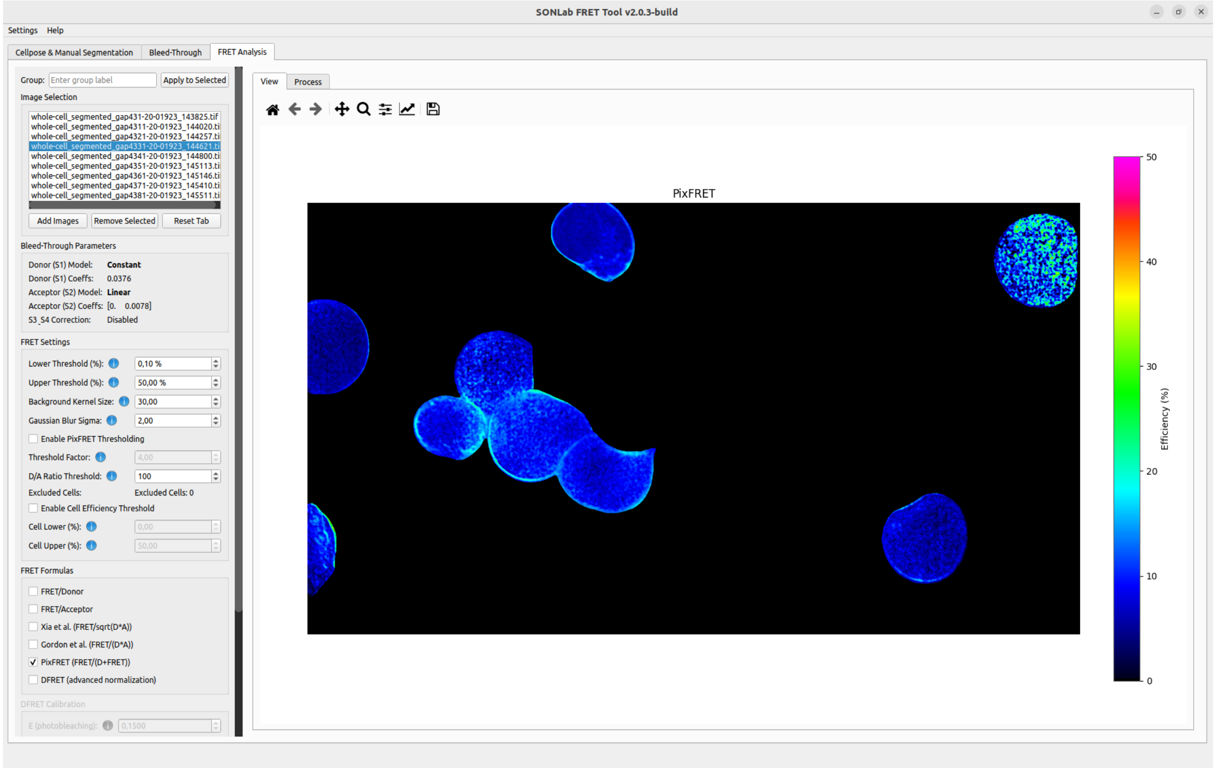


Figure 12: Flow of the FRET stage, from loading segmented images to exporting results.

1. Add segmented images (from the Segmentation tab or disk).
2. Confirm the bleed-through parameters carried over from the Bleed-Through tab.
3. Set thresholds and optional filters; select one or more formulas.

4. (Optional) Calibrate DFRET; assign groups.
5. Run the analysis and review the results.



Group: Enter group label

Image Selection

whole-cell_segmented_gap431-20-01923_143825.tif
whole-cell_segmented_gap4311-20-01923_144020.ti
whole-cell_segmented_gap4321-20-01923_144257.ti
whole-cell_segmented_gap4331-20-01923_144621.ti
whole-cell_segmented_gap4341-20-01923_144800.ti
whole-cell_segmented_gap4351-20-01923_145113.ti
whole-cell_segmented_gap4361-20-01923_145146.ti
whole-cell_segmented_gap4371-20-01923_145410.ti
whole-cell_segmented_gap4381-20-01923_145511.ti

Bleed-Through Parameters

Donor (S1) Model: **Constant**
Donor (S1) Coeffs: 0.0376
Acceptor (S2) Model: **Linear**
Acceptor (S2) Coeffs: [0. 0.0078]
S3_S4 Correction: Disabled

FRET Settings

Lower Threshold (%): 0,10 %
Upper Threshold (%): 50,00 %
Background Kernel Size: 30,00
Gaussian Blur Sigma: 2,00
☐ Enable PixFRET Thresholding
Threshold Factor: 4,00
D/A Ratio Threshold: 100
Excluded Cells: Excluded Cells: 0
☐ Enable Cell Efficiency Threshold
Cell Lower (%): 0,00
Cell Upper (%): 50,00

FRET Formulas

☐ FRET/Donor
☐ FRET/Acceptor
☐ Xia et al. (FRET/sqrt(D*A))
☐ Gordon et al. (FRET/(D*A))
☑ PixFRET (FRET/(D+FRET))
☐ DFRET (advanced normalization)

DFRET Calibration

E (photobleaching): 0,1500

Figure 13: The FRET Analysis tab: image list/grouping, bleed-through parameters, FRET settings, and formula selection on the left; a color-coded efficiency map on the right.

## 5.2. Image Management and Grouping

**Add Images** loads segmented stacks; **Remove Selected** and **Reset Tab** manage the list (clearing the displays when empty). Assign a group name and click **Apply to Selected** to tag images; groups drive the comparative statistics (e.g. control vs. treatment).

## 5.3. Bleed-Through Parameters

The **Bleed-Through Parameters** panel shows the donor and acceptor model and coefficients and the S3/S4 status, populated from the confirmed fits (or a loaded `bt_params.json`) and applied automatically when the analysis runs. If they read *N/A*, confirm and save the fits first.

## 5.4. FRET Settings

Table 5: FRET settings.

| Setting | Default | Description |
|---|---|---|
| `Lower Threshold (%)` | 0 | Minimum efficiency shown on the maps. |
| `Upper Threshold (%)` | 50 | Maximum efficiency shown (values above are clamped in the display). |
| `Background Kernel Size` | 30 | Kernel size for local background subtraction. |
| `Gaussian Blur Sigma` | 2.0 | Optional Gaussian blur; set 0 to disable. |

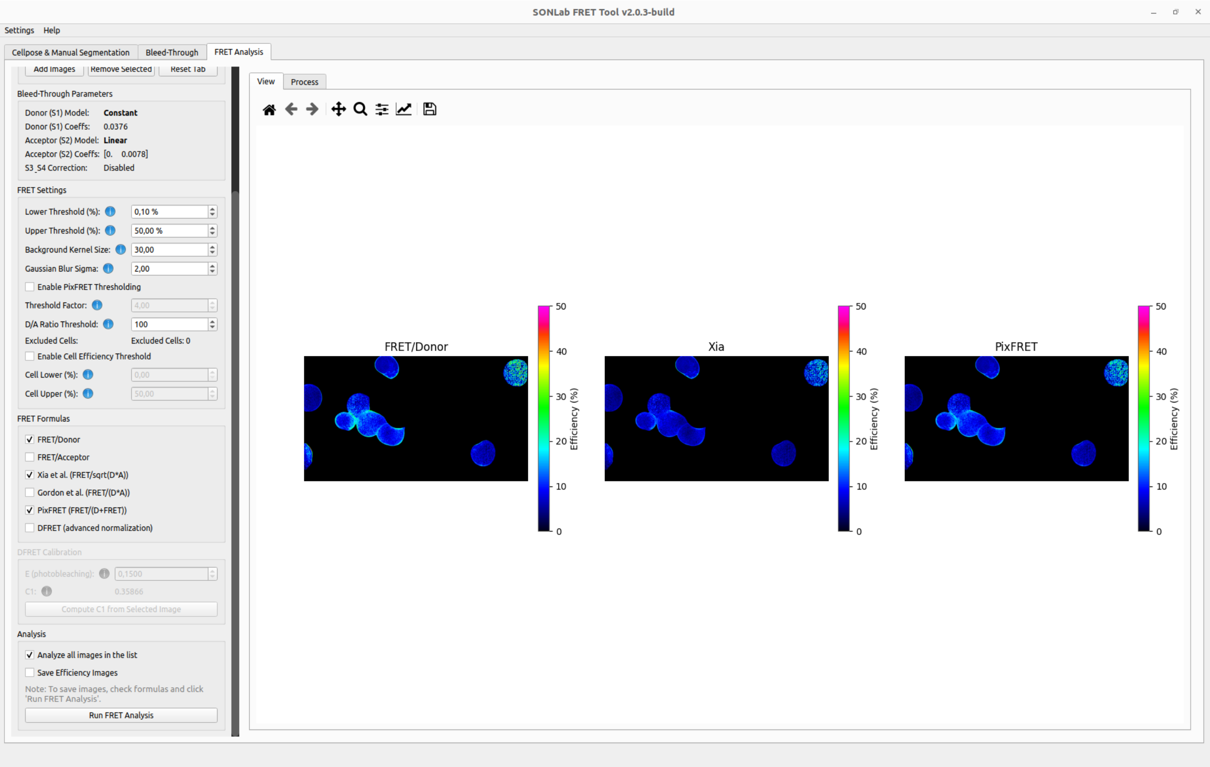
Two optional filters are available: **PixFRET Thresholding** (an intensity-based mask with a `Threshold Factor`) and the **Cell Efficiency Threshold** (`Cell Lower`/`Cell Upper`%) which excludes whole cells whose mean efficiency falls outside a range.

## 5.5. FRET Formulas

Select one or more formulas; each produces its own efficiency map. Here $F$, $D$, and $A$ are the corrected FRET, Donor, and Acceptor intensities.

Table 6: Available FRET efficiency formulas.

| **Formula** | **Expression** | **Notes** |
|---|---|---|
| FRET/Donor | $F/D$ | Basic donor-normalized ratio. |
| FRET/Acceptor | $F/A$ | Acceptor-normalized ratio. |
| Xia *et al.* | $F/\sqrt{D \cdot A}$ | Accounts for both contributions. |
| Gordon *et al.* | $F/(D \cdot A)$ | Alternative dual normalization. |
| PixFRET | $F/(D+F)$ | Common for sensitized-emission FRET. |
| DFRET | advanced | Calibrated, photobleaching-anchored efficiency. |


Bleed-Through Parameters
Donor (S1) Model: **Constant**
Donor (S1) Coeffs: 0.0376
Acceptor (S2) Model: **Linear**
Acceptor (S2) Coeffs: [0. 0.0078]
S3_S4 Correction: Disabled

FRET Settings
Lower Threshold (%): 0,10 %
Upper Threshold (%): 50,00 %
Background Kernel Size: 30,00
Gaussian Blur Sigma: 2,00
☐ Enable PixFRET Thresholding
Threshold Factor: 4,00
D/A Ratio Threshold: 100
Excluded Cells: Excluded Cells: 0
☐ Enable Cell Efficiency Threshold
Cell Lower (%): 0,00
Cell Upper (%): 50,00

FRET Formulas
☑ FRET/Donor
☐ FRET/Acceptor
☑ Xia et al. (FRET/sqrt(D*A))
☐ Gordon et al. (FRET/(D*A))
☑ PixFRET (FRET/(D+FRET))
☐ DFRET (advanced normalization)

DFRET Calibration
E (photobleaching): 0,1500
C1: 0.35866

Analysis
☑ Analyze all images in the list
☐ Save Efficiency Images
Note: To save images, check formulas and click 'Run FRET Analysis'.

Figure 14: Multiple formulas can be computed at once; each selected formula produces its own efficiency map for side-by-side comparison.

## 5.6. DFRET Calibration

DFRET applies an advanced normalization (after Hochreiter *et al.*) anchored to a photobleaching measurement. Enter `E (photobleaching)`—the FRET efficiency (0–1) from a donor–acceptor fusion construct—and either provide `C1` (the donor normalization factor) or click **Compute C1 from Selected Image**. DFRET requires a positive $E$ to proceed.

### 5.7. Running the Analysis

In the **Analysis** panel, tick *Analyze all images in the list* to process every image (otherwise only the selected one), then click **Run FRET Analysis**. The bleed-through correction is applied and efficiency maps, statistics, and plots are produced.

### 5.8. Troubleshooting

- **BT parameters *N/A*:** confirm and save the fits in the Bleed-Through tab.
- **DFRET won't run:** enter a positive $E$ or compute $C1$.
- **Maps empty:** check the thresholds and that frame 0 is a mask.
- **All cells excluded:** loosen or disable the Cell Efficiency Threshold.

## 6. Results and Visualization

After running the analysis, results are shown for the **Current Image** and **All Images**, each with **Summary**, **Histogram & Box Plot**, and **Distribution Analysis** sub-tabs.

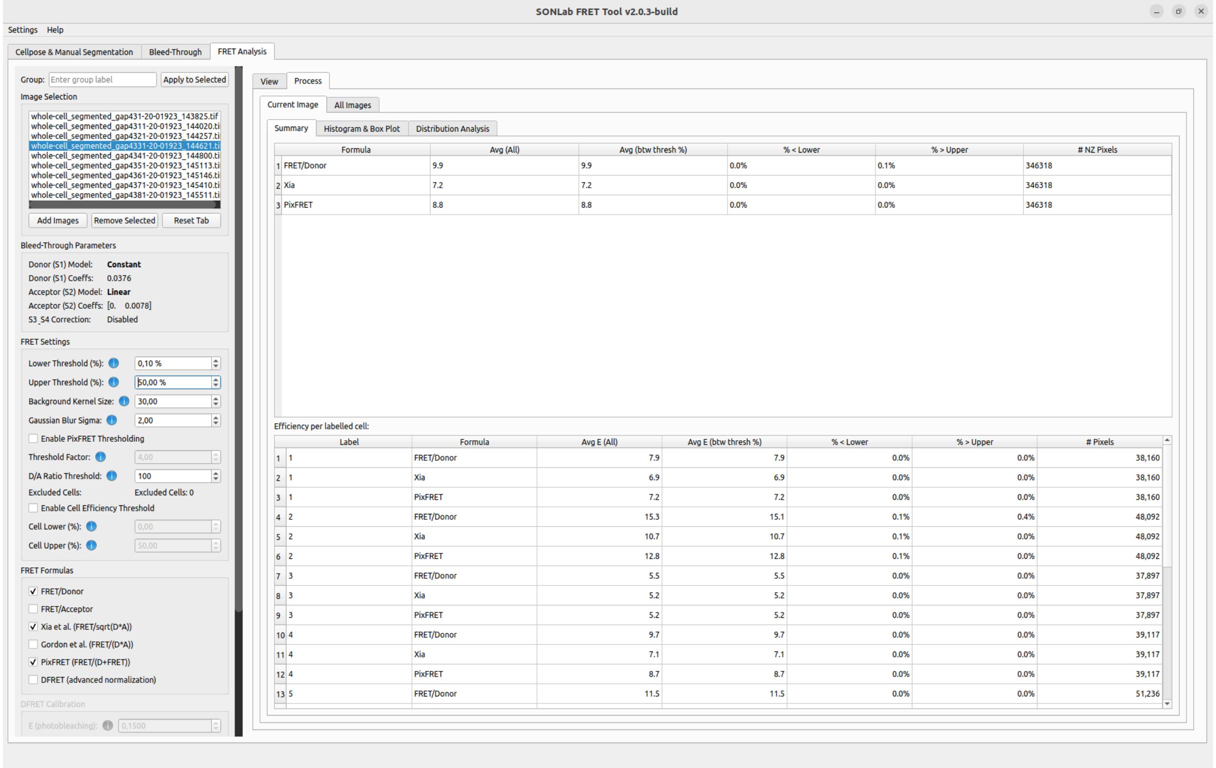
### 6.1. Efficiency Maps

Each formula is rendered as a color-coded efficiency map scaled to the Lower/Upper thresholds (values above the upper threshold are clamped for display). Toolbars provide zoom/pan/save, and the pop-out button opens a larger window.

### 6.2. Statistics Tables

The **Current Image** summary reports, per formula: the `Avg (All)` mean over all non-zero pixels (independent of the display threshold), the `Avg (btw thresh)` mean within the threshold range, the percentages of non-zero pixels below/above the thresholds, and the non-zero pixel count. A per-cell table breaks these down by label.

**Note:** `Avg (All)` and `Avg (btw thresh)` are deliberately distinct: they differ whenever cells contain pixels outside the threshold range, which is also what makes the below/above percentages meaningful.



Group: Enter group label

Image Selection

whole-cell_segmented_gap431-20-01923_143825.tif
whole-cell_segmented_gap4311-20-01923_144020.ti
whole-cell_segmented_gap4321-20-01923_144257.ti
whole-cell_segmented_gap4331-20-01923_144621.ti
whole-cell_segmented_gap4341-20-01923_144800.ti
whole-cell_segmented_gap4351-20-01923_145113.ti
whole-cell_segmented_gap4361-20-01923_145146.ti
whole-cell_segmented_gap4371-20-01923_145410.ti
whole-cell_segmented_gap4381-20-01923_145511.ti

Bleed-Through Parameters

Donor (S1) Model: **Constant**
Donor (S1) Coeffs: 0.0376
Acceptor (S2) Model: **Linear**
Acceptor (S2) Coeffs: [0. 0.0078]
S3_S4 Correction: Disabled

FRET Settings

Lower Threshold (%): 0,10 %
Upper Threshold (%): 50,00 %
Background Kernel Size: 30,00
Gaussian Blur Sigma: 2,00
☐ Enable PixFRET Thresholding
Threshold Factor: 4,00
D/A Ratio Threshold: 100
Excluded Cells: Excluded Cells: 0
☐ Enable Cell Efficiency Threshold
Cell Lower (%): 0,00
Cell Upper (%): 50,00

FRET Formulas

☑ FRET/Donor
☐ FRET/Acceptor
☑ Xia et al. (FRET/sqrt(D*A))
☐ Gordon et al. (FRET/(D*A))
☑ PixFRET (FRET/(D+FRET))
☐ DFRET (advanced normalization)

DFRET Calibration

E (photobleaching): 0,1500

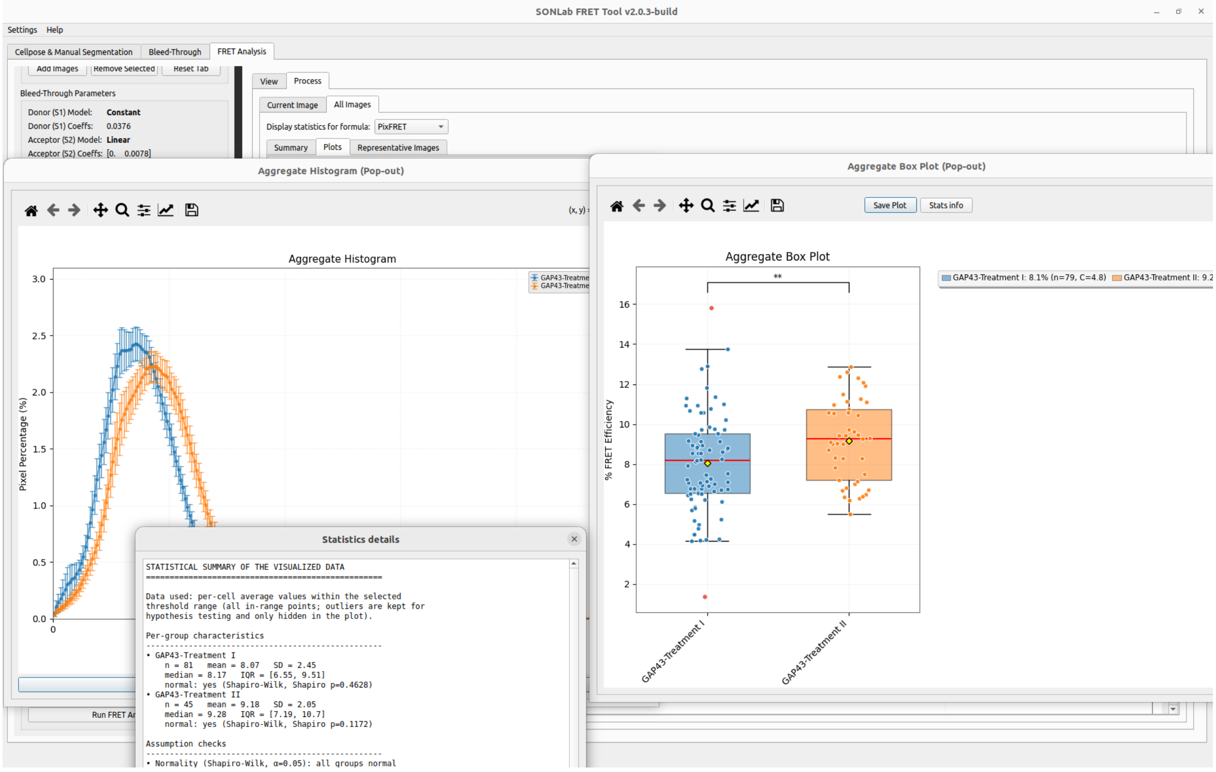
| | Formula | Avg (All) | Avg (btw thresh %) | % < Lower | % > Upper | # NZ Pixels |
|---|---|---|---|---|---|---|
| 1 | FRET/Donor | 9.9 | 9.9 | 0.0% | 0.1% | 346318 |
| 2 | Xia | 7.2 | 7.2 | 0.0% | 0.0% | 346318 |
| 3 | PixFRET | 8.8 | 8.8 | 0.0% | 0.0% | 346318 |

Efficiency per labelled cell:

| | Label | Formula | Avg E (All) | Avg E (btw thresh %) | % < Lower | % > Upper | # Pixels |
|---|---|---|---|---|---|---|---|
| 1 | 1 | FRET/Donor | 7.9 | 7.9 | 0.0% | 0.0% | 38,160 |
| 2 | 1 | Xia | 6.9 | 6.9 | 0.0% | 0.0% | 38,160 |
| 3 | 1 | PixFRET | 7.2 | 7.2 | 0.0% | 0.0% | 38,160 |
| 4 | 2 | FRET/Donor | 15.3 | 15.1 | 0.1% | 0.4% | 48,092 |
| 5 | 2 | Xia | 10.7 | 10.7 | 0.1% | 0.0% | 48,092 |
| 6 | 2 | PixFRET | 12.8 | 12.8 | 0.1% | 0.0% | 48,092 |
| 7 | 3 | FRET/Donor | 5.5 | 5.5 | 0.0% | 0.0% | 37,897 |
| 8 | 3 | Xia | 5.2 | 5.2 | 0.0% | 0.0% | 37,897 |
| 9 | 3 | PixFRET | 5.2 | 5.2 | 0.0% | 0.0% | 37,897 |
| 10 | 4 | FRET/Donor | 9.7 | 9.7 | 0.0% | 0.0% | 39,117 |
| 11 | 4 | Xia | 7.1 | 7.1 | 0.0% | 0.0% | 39,117 |
| 12 | 4 | PixFRET | 8.7 | 8.7 | 0.0% | 0.0% | 39,117 |
| 13 | 5 | FRET/Donor | 11.5 | 11.5 | 0.0% | 0.0% | 51,236 |

Figure 15: Current Image → Summary: the per-formula statistics table (top) and the per-cell breakdown (bottom).

The **All Images** view aggregates every analyzed cell across images and groups, and can be exported to CSV.



Bleed-Through Parameters

Donor (S1) Model: **Constant**

Donor (S1) Coeffs: 0.0376

Acceptor (S2) Model: **Linear**

Acceptor (S2) Coeffs: [0. 0.0078]

Display statistics for formula: PixFRET

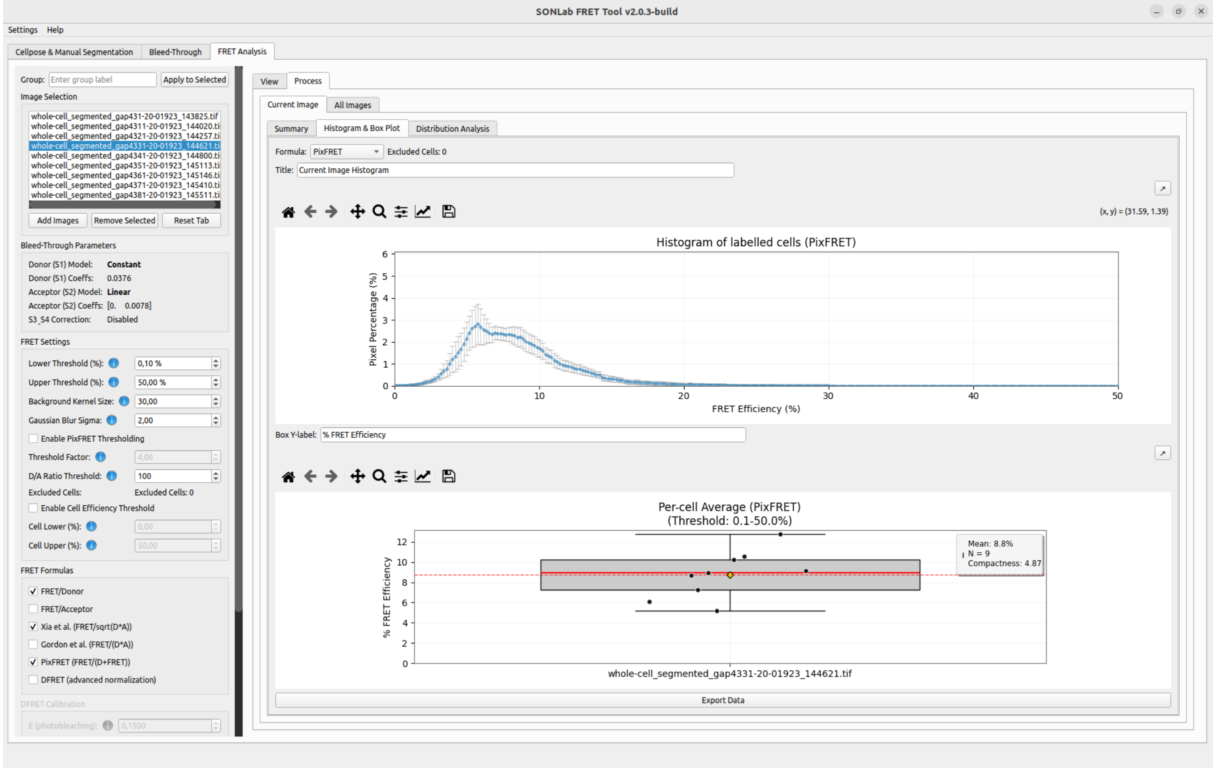
GAP43-Treatment I: 8.1% (n=79, C=4.8) GAP43-Treatment II: 9.2

```
STATISTICAL SUMMARY OF THE VISUALIZED DATA
==================================================

Data used: per-cell average values within the selected
threshold range (all in-range points; outliers are kept for
hypothesis testing and only hidden in the plot).

Per-group characteristics
--------------------------------------------------
• GAP43-Treatment I
    n = 81   mean = 8.07   SD = 2.45
    median = 8.17   IQR = [6.55, 9.51]
    normal: yes (Shapiro-Wilk, Shapiro p=0.4628)
• GAP43-Treatment II
    n = 45   mean = 9.18   SD = 2.05
    median = 9.28   IQR = [7.19, 10.7]
    normal: yes (Shapiro-Wilk, Shapiro p=0.1172)

Assumption checks
--------------------------------------------------
• Normality (Shapiro-Wilk, α=0.05): all groups normal
```

Figure 16: All Images → Plots: the aggregate histogram and box plot (popped out) compare groups across every analyzed image, with a statistics summary.

## 6.3. Histograms and Box Plots

The histogram shows the distribution of efficiency values (the aggregate version averages per-cell histograms with an error band); the box plot shows per-cell averages, with groups side by side in the aggregate view. An **SEM/SD** toggle controls the error representation, titles/labels are editable, and every plot has pop-out and save controls.



Group: Enter group label

whole-cell_segmented_gap431-20-01923_143825.tif
whole-cell_segmented_gap4311-20-01923_144020.ti
whole-cell_segmented_gap4321-20-01923_144257.ti
whole-cell_segmented_gap4331-20-01923_144621.ti
whole-cell_segmented_gap4341-20-01923_144800.ti
whole-cell_segmented_gap4351-20-01923_145113.ti
whole-cell_segmented_gap4361-20-01923_145146.ti
whole-cell_segmented_gap4371-20-01923_145410.ti
whole-cell_segmented_gap4381-20-01923_145511.ti

Donor (S1) Model: **Constant**
Donor (S1) Coeffs: 0.0376
Acceptor (S2) Model: **Linear**
Acceptor (S2) Coeffs: [0.    0.0078]
S3_S4 Correction: Disabled

Lower Threshold (%): 0,10 %
Upper Threshold (%): 50,00 %
Background Kernel Size: 30,00
Gaussian Blur Sigma: 2,00
☐ Enable PixFRET Thresholding
Threshold Factor: 4,00
D/A Ratio Threshold: 100
Excluded Cells: Excluded Cells: 0
☐ Enable Cell Efficiency Threshold
Cell Lower (%): 0,00
Cell Upper (%): 50,00

☑ FRET/Donor
☐ FRET/Acceptor
☑ Xia et al. (FRET/sqrt(D*A))
☐ Gordon et al. (FRET/(D*A))
☑ PixFRET (FRET/(D+FRET))
☐ DFRET (advanced normalization)

E (photobleaching): 0,1500

Formula: PixFRET Excluded Cells: 0
Title: Current Image Histogram
(x, y) = (31.59, 1.39)
Box Y-label: % FRET Efficiency
whole-cell_segmented_gap4331-20-01923_144621.tif

Figure 17: Current Image → Histogram & Box Plot: the efficiency histogram of labelled cells and the per-cell average box plot.

## 6.4. Statistical Significance Testing

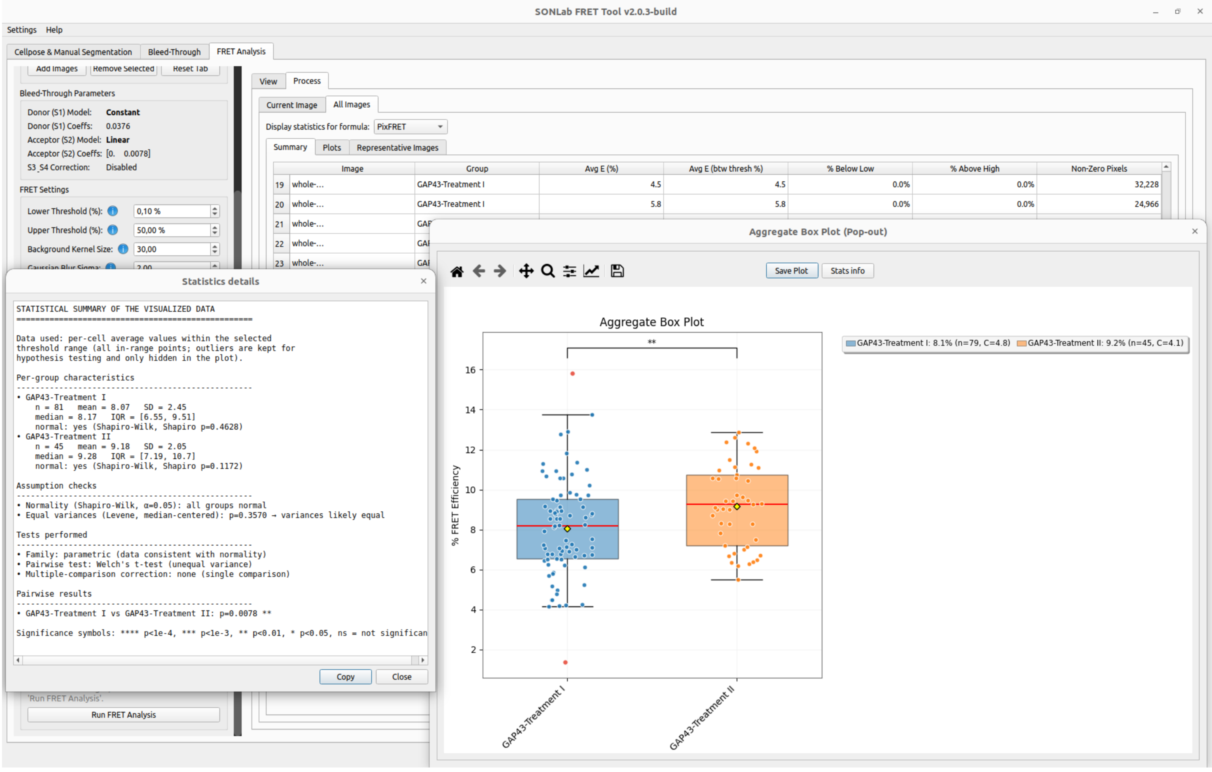
When two or more groups are compared, significance bars are drawn and the test is chosen **from the data** rather than always using the ordinary normality-assuming forms:

- Each group is screened for normality (Shapiro–Wilk).
- **All groups normal** → Welch's *t*-test (pairs) and Welch's ANOVA (omnibus); robust to unequal variances.
- **Any group non-normal** → Mann–Whitney *U* (pairs) and Kruskal–Wallis (omnibus).
- For > 2 groups, post-hoc pairwise comparisons use Holm–Bonferroni correction and are only reported when the omnibus test is significant.

Table 7: Significance symbols.

| Symbol | Meaning |
|---|---|
| `****` | $p < 0.0001$ |
| `***` | $p < 0.001$ |
| `**` | $p < 0.01$ |
| `*` | $p < 0.05$ |
| `ns` | not significant |

The **Stats info** button on a box plot opens a read-only dialog summarizing the per-group descriptive statistics, the normality and equal-variance checks, the chosen test family, the omnibus result, and each pairwise comparison (copyable to the clipboard).



Bleed-Through Parameters

Donor (S1) Model: **Constant**
Donor (S1) Coeffs: 0.0376
Acceptor (S2) Model: **Linear**
Acceptor (S2) Coeffs: [0. 0.0078]
S3_S4 Correction: Disabled

FRET Settings

Lower Threshold (%): 0,10 %
Upper Threshold (%): 50,00 %
Background Kernel Size: 30,00

Display statistics for formula: PixFRET

| | Image | Group | Avg E (%) | Avg E (btw thresh %) | % Below Low | % Above High | Non-Zero Pixels |
|---|---|---|---|---|---|---|---|
| 19 | whole-... | GAP43-Treatment I | 4.5 | 4.5 | 0.0% | 0.0% | 32,228 |
| 20 | whole-... | GAP43-Treatment I | 5.8 | 5.8 | 0.0% | 0.0% | 24,966 |
| 21 | whole-... | | | | | | |
| 22 | whole-... | | | | | | |
| 23 | whole-... | | | | | | |

```
STATISTICAL SUMMARY OF THE VISUALIZED DATA
==================================================

Data used: per-cell average values within the selected
threshold range (all in-range points; outliers are kept for
hypothesis testing and only hidden in the plot).

Per-group characteristics
--------------------------------------------------
• GAP43-Treatment I
    n = 81   mean = 8.07   SD = 2.45
    median = 8.17   IQR = [6.55, 9.51]
    normal: yes (Shapiro-Wilk, Shapiro p=0.4628)
• GAP43-Treatment II
    n = 45   mean = 9.18   SD = 2.05
    median = 9.28   IQR = [7.19, 10.7]
    normal: yes (Shapiro-Wilk, Shapiro p=0.1172)

Assumption checks
--------------------------------------------------
• Normality (Shapiro-Wilk, α=0.05): all groups normal
• Equal variances (Levene, median-centered): p=0.3570 → variances likely equal

Tests performed
--------------------------------------------------
• Family: parametric (data consistent with normality)
• Pairwise test: Welch's t-test (unequal variance)
• Multiple-comparison correction: none (single comparison)

Pairwise results
--------------------------------------------------
• GAP43-Treatment I vs GAP43-Treatment II: p=0.0078 **

Significance symbols: **** p<1e-4, *** p<1e-3, ** p<0.01, * p<0.05, ns = not significan
```

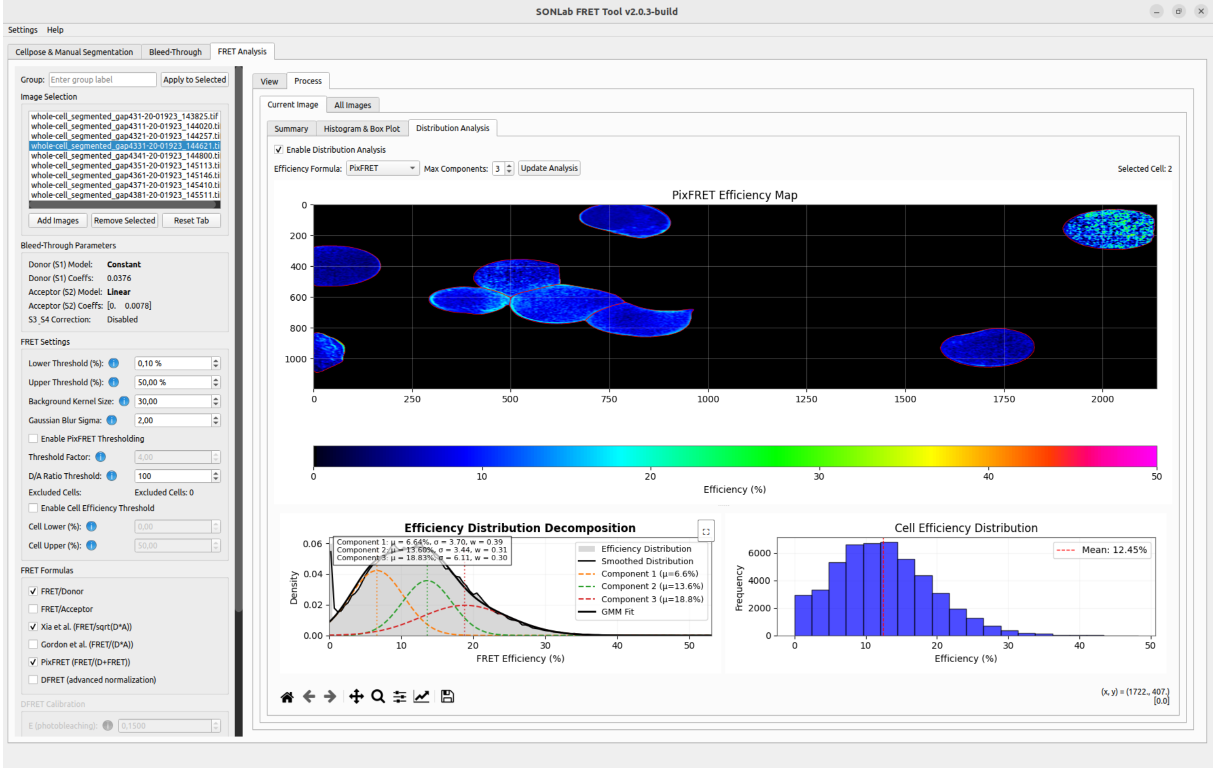
'Run FRET Analysis'.

🟦 GAP43-Treatment I: 8.1% (n=79, C=4.8) 🟧 GAP43-Treatment II: 9.2% (n=45, C=4.1)

Figure 18: The aggregate box plot comparing two groups, with the Statistics details dialog reporting the descriptive statistics, assumption checks, the chosen test, and the pairwise result.

## 6.5. Distribution Analysis

The **Distribution Analysis** sub-tab decomposes a cell's efficiency distribution into Gaussian components, revealing sub-populations. Enable it, choose the efficiency formula and the `Max Components` (1–5), click **Update Analysis**, then click a cell on the map to select it. The panel shows the efficiency map, the Gaussian-mixture decomposition of the selected cell's distribution, and the cell efficiency histogram.



Group: Enter group label

Image Selection

whole-cell_segmented_gap431-20-01923_143825.tif
whole-cell_segmented_gap4311-20-01923_144020.ti
whole-cell_segmented_gap4321-20-01923_144257.ti
whole-cell_segmented_gap4331-20-01923_144621.ti
whole-cell_segmented_gap4341-20-01923_144800.ti
whole-cell_segmented_gap4351-20-01923_145113.ti
whole-cell_segmented_gap4361-20-01923_145146.ti
whole-cell_segmented_gap4371-20-01923_145410.ti
whole-cell_segmented_gap4381-20-01923_145511.ti

Bleed-Through Parameters

Donor (S1) Model: **Constant**
Donor (S1) Coeffs: 0.0376
Acceptor (S2) Model: **Linear**
Acceptor (S2) Coeffs: [0. 0.0078]
S3_S4 Correction: Disabled

FRET Settings

Lower Threshold (%): 0,10 %
Upper Threshold (%): 50,00 %
Background Kernel Size: 30,00
Gaussian Blur Sigma: 2,00
☐ Enable PixFRET Thresholding
Threshold Factor: 4,00
D/A Ratio Threshold: 100
Excluded Cells: Excluded Cells: 0
☐ Enable Cell Efficiency Threshold
Cell Lower (%): 0,00
Cell Upper (%): 50,00

FRET Formulas

☑ FRET/Donor
☐ FRET/Acceptor
☑ Xia et al. (FRET/sqrt(D*A))
☐ Gordon et al. (FRET/(D*A))
☑ PixFRET (FRET/(D+FRET))
☐ DFRET (advanced normalization)

DFRET Calibration

E (photobleaching): 0,1500

☑ Enable Distribution Analysis

Efficiency Formula: PixFRET Max Components: 3 Selected Cell: 2

Component 1: μ = 6.64%, σ = 3.70, w = 0.39
Component 2: μ = 13.60%, σ = 3.44, w = 0.31
Component 3: μ = 18.83%, σ = 6.11, w = 0.30

Figure 19: Distribution Analysis: the efficiency map (top), the Gaussian-mixture decomposition (bottom left), and the cell efficiency distribution (bottom right).

### 6.6. Representative Images and Export

**Find Rep Image** selects the image that best represents a group. Outputs: efficiency maps (TIFF, when saving is enabled), aggregate statistics (CSV), and figures (PNG/PDF) via each plot's Save button.

## 7. Workflows and Data Flow

### 7.1. Recommended Workflow

1. **Segment** your images; refine ROIs; send controls to the bleed-through channels and experimental images to FRET.
2. **Characterize bleed-through** for each channel, confirm the fits, and save the parameters.
3. **Compute FRET**: verify the coefficients, choose formulas and thresholds, assign groups, run, and export.

### 7.2. Data Flow Between Tabs

Table 8: How data moves between tabs.

| Data | Produced in | Used in | Mechanism |
|---|---|---|---|
| Segmented stacks | Segmentation | BT, FRET | Send buttons, batch transfer, or files |
| BT coefficients | Bleed-Through | FRET | Confirmed fits; `bt_params.json` |
| Groups | FRET / batch | FRET stats | Group tags |
| Maps & statistics | FRET | external | TIFF / CSV / figures |

The label mask travels with the image as frame 0 of every segmented stack, so each tab knows which pixels belong to which cell, and raw channel intensities are preserved through saving.

### 7.3. Performance Tips

- A CUDA-capable GPU greatly accelerates Cellpose segmentation.
- Enable Random Sampling for large bleed-through datasets.
- Use Batch Segment & Transfer for many images at once.

## 8. File Formats

### 8.1. Input Images

Multi-frame `.tif`/`.tiff` and Zeiss `.czi`. CZI files are converted on load to a multi-frame TIFF (FRET, Donor, Acceptor) preserving raw intensities; a fourth channel is included when present for S3/S4.

### 8.2. Segmented Stacks

Saved to a `segmented/` folder, prefixed `outline_segmented_` or `whole-cell_segmented_`.

Table 9: Segmented stack frame layout.

| Frame | Content |
|---|---|
| 0 | Segmentation label mask (0 = background, 1..N = cell labels) |
| 1 | FRET channel |
| 2 | Donor channel |
| 3 | Acceptor channel (present for 4-frame data) |

### 8.3. Parameters and Outputs

`bt_params.json` stores the per-channel model type, coefficients, and processing settings (written to the default location and copied into each input directory). FRET efficiency maps are saved as 32-bit float TIFFs (`FRET_Analysis_Results/<name>_<formula>_efficiency.tif`) storing percentage values; statistics export to CSV and figures to PNG/PDF.

**Note:** The label mask must be frame 0 of any stack fed to the Bleed-Through or FRET tabs. Three-frame stacks suffice for S1/S2; four-frame stacks are required for S3/S4.

# 9. Troubleshooting and FAQ

## 9.1. Common Issues

- **Python not found / import errors:** use Python 3.10, activate the virtual environment, and reinstall the requirements (and PyTorch for your hardware).
- **GPU not detected:** verify the CUDA/ROCm version matches the installed PyTorch; the tool still runs on CPU.
- **Stale plot after removing images:** removing the last image clears the views; otherwise re-select an image or use Reset Tab.
- **Mouse wheel won't change a field:** this is intentional—click and type, or use the arrows.
- **Out of memory:** process fewer images, enable sampling, and close other applications.
- **Unexpected efficiency values:** verify the frame order (mask, FRET, Donor, Acceptor).

## 9.2. FAQ

**Which images go into the Bleed-Through tab?** Single-label controls: donor-only for S1/S3 and acceptor-only for S2/S4.

**Do I have to segment before FRET?** Yes—the mask (frame 0) tells the calculation which pixels belong to which cell.

**Can I reuse a bleed-through model?** Yes; save and load `bt_params.json` (a copy is also stored next to your input images).

**Where are results saved?** Efficiency maps to `FRET_Analysis_Results/`, statistics to CSV, and figures via each plot's Save button.

# 10. Contact and Support

- Issues and discussions: the project's GitHub repository, `https://github.com/sonlab-metu/SONLab-FRET-Tool`
- Email: sonlab@metu.edu.tr

*If you use the tool in your research, please cite: Nursoy, A. Z., Cevheroğlu, O., & Son, Ç. D. Automated FRET Analysis for Enhanced Characterization of Protein–Protein Interactions. Microscopy Research and Technique. https://doi.org/10.1002/jemt.70147*