**Batch Analysis Pipeline for 4-Channel TIFF Images**

**Overview**

This MATLAB pipeline performs **batch analysis of four-channel TIFF images** (e.g., STED or confocal datasets).

For each selected image, the script generates a comprehensive set of **visualization figures** and **quantitative overlap coefficients**, and exports all numerical results into a single CSV file for downstream statistical analysis.

The pipeline is fully automated, requires **no manual ROI selection**, and is designed to ensure **reproducibility and consistency across large datasets**.

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**Input Requirements**

- Multi-page TIFF files with **at least four image planes**
- Channel assignment:
  - **Channel 1**: Red
  - **Channel 2**: Green
  - **Channel 3**: Blue
  - **Channel 4**: Gray (displayed as intensity added to RGB channels)

All images are assumed to be aligned and of identical dimensions across channels.

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**How to Run the Analysis**

1. Open MATLAB and add the script directory to the MATLAB path.
2. Run the main batch function:
3. batch_analyze_4ch_tiffs2
4. A file selection dialog will appear. Select **one or multiple 4-channel TIFF files**.
5. The script will process each file sequentially and report progress in the MATLAB Command Window.

Each image is analyzed independently; if one file fails, the remaining files will continue to be processed.

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**Output Summary**

For each TIFF file, the following outputs are generated:

**Figures (saved as .fig files)**

- **Fig1–Fig3**: Combined into a single main figure
- **Fig4**: Pairwise channel overlap analysis
- **Fig5**: Triple-channel overlap analysis

Figures are saved in the same directory as the input TIFF file, using the original filename as a prefix.

**Numerical Results**

- All overlap coefficients are aggregated into a table
- A timestamped CSV file is saved in the same directory
- The results table is also assigned to the MATLAB base workspace as:
- batch_overlap_results

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**Figure Descriptions**

**Fig1 — Original Normalized Images**

Each channel is independently normalized to the range $[0, 1]$ using linear scaling. Merged RGB images are generated to visualize pairwise and multi-channel combinations.

**Purpose**

- To inspect raw intensity distributions
- To provide a reference view independent of contrast enhancement or thresholding

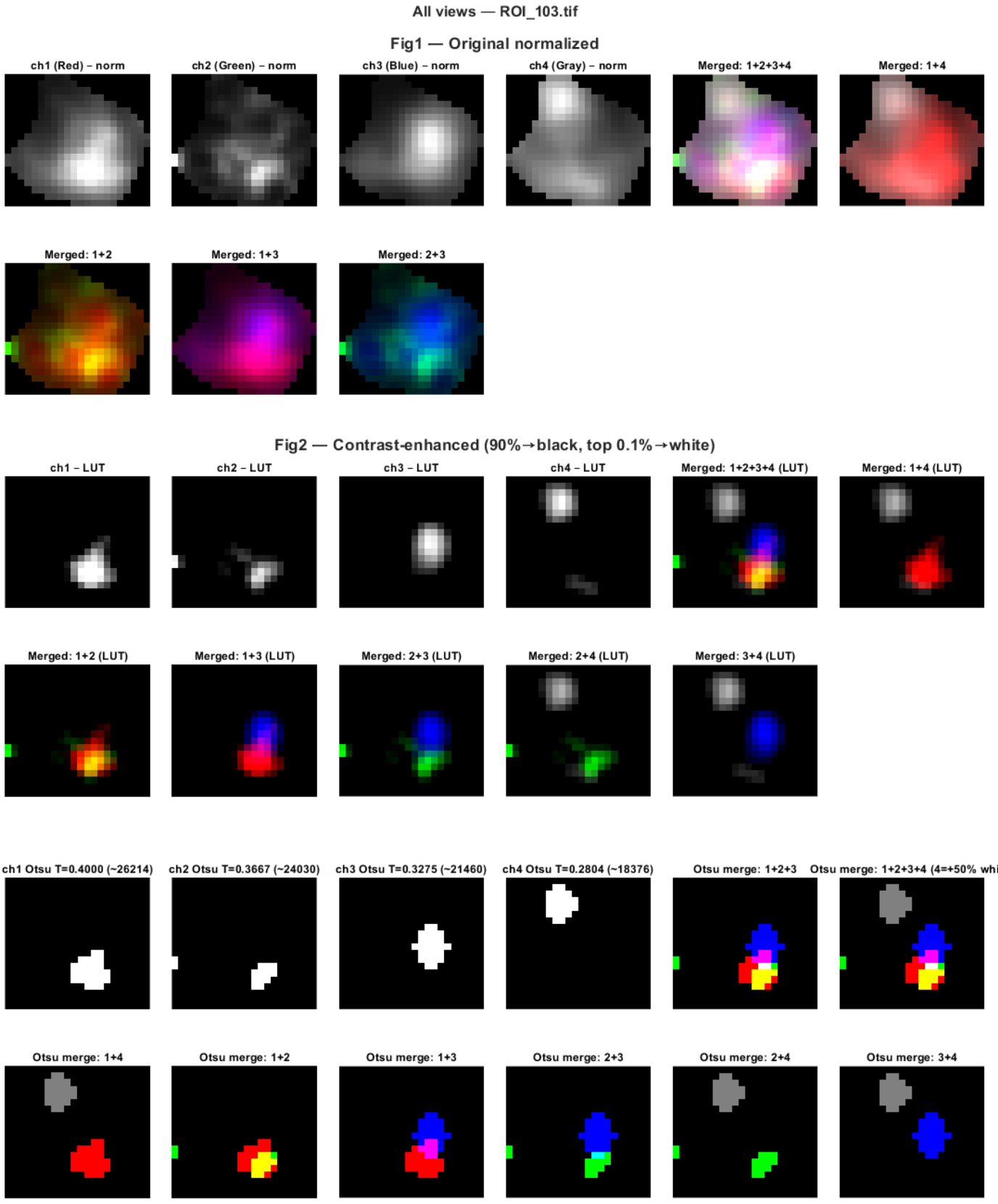


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**Fig2 — Contrast-Enhanced (LUT-Applied) Images**

A percentile-based LUT is applied to each channel:

- Intensities below the **90th percentile** are mapped to black
- Intensities at or above the **99.9th percentile** are mapped to white
- Intermediate values are linearly scaled

The original 16-bit dynamic range is preserved during processing before display.

**Purpose**

- To enhance weak structures
- To approximate manual LUT adjustments commonly performed in ImageJ/Fiji
- To standardize contrast across datasets

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**Fig3 — Otsu Thresholded Binary Images**

Otsu's method is applied to the LUT-enhanced images to generate binary masks for each channel. Merged pseudocolor images illustrate spatial overlap of thresholded signals.

The fourth (gray) channel is displayed as a white intensity overlay.

**Purpose**

- To visualize automatic thresholding results
- To compare intensity-based and structure-based representations
- To assess the upper bound of detectable overlap

[Figure 3 placeholder]

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**Fig4 — Pairwise Overlap Analysis**

All six pairwise channel combinations are analyzed:

- ch1–ch2
- ch1–ch3
- ch2–ch3
- ch1–ch4
- ch2–ch4
- ch3–ch4

For each pair, a row of panels displays:

1. Merged LUT-enhanced image
2. Normalized channel A
3. Normalized channel B
4. Pixel-wise overlap map with overlap coefficient

**Purpose**

- To visualize spatial overlap
- To quantify pairwise colocalization using a consistent definition

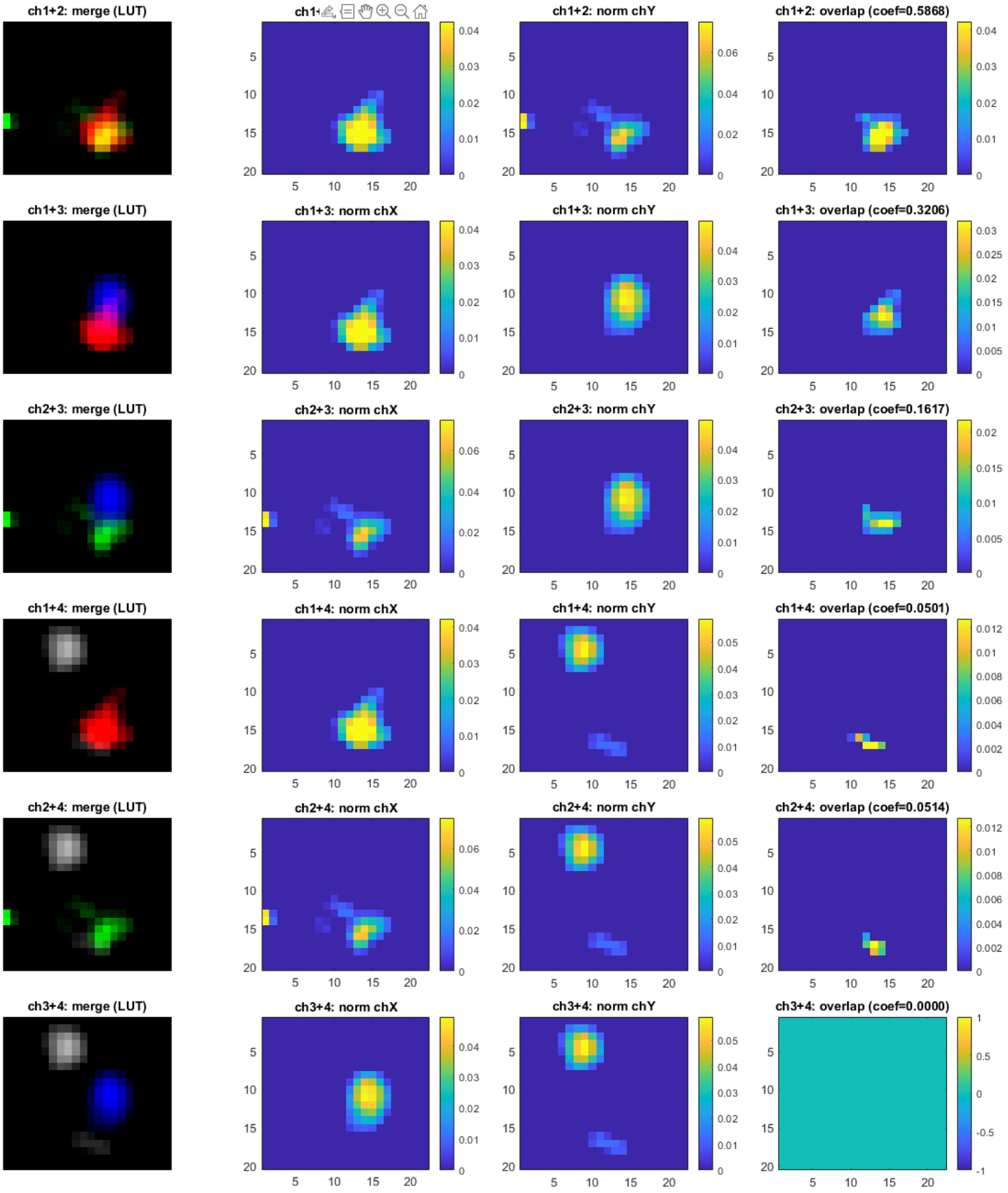



**Fig5 — Triple-Channel Overlap Analysis**

All four triple-channel combinations are analyzed:

- ch1–ch2–ch3

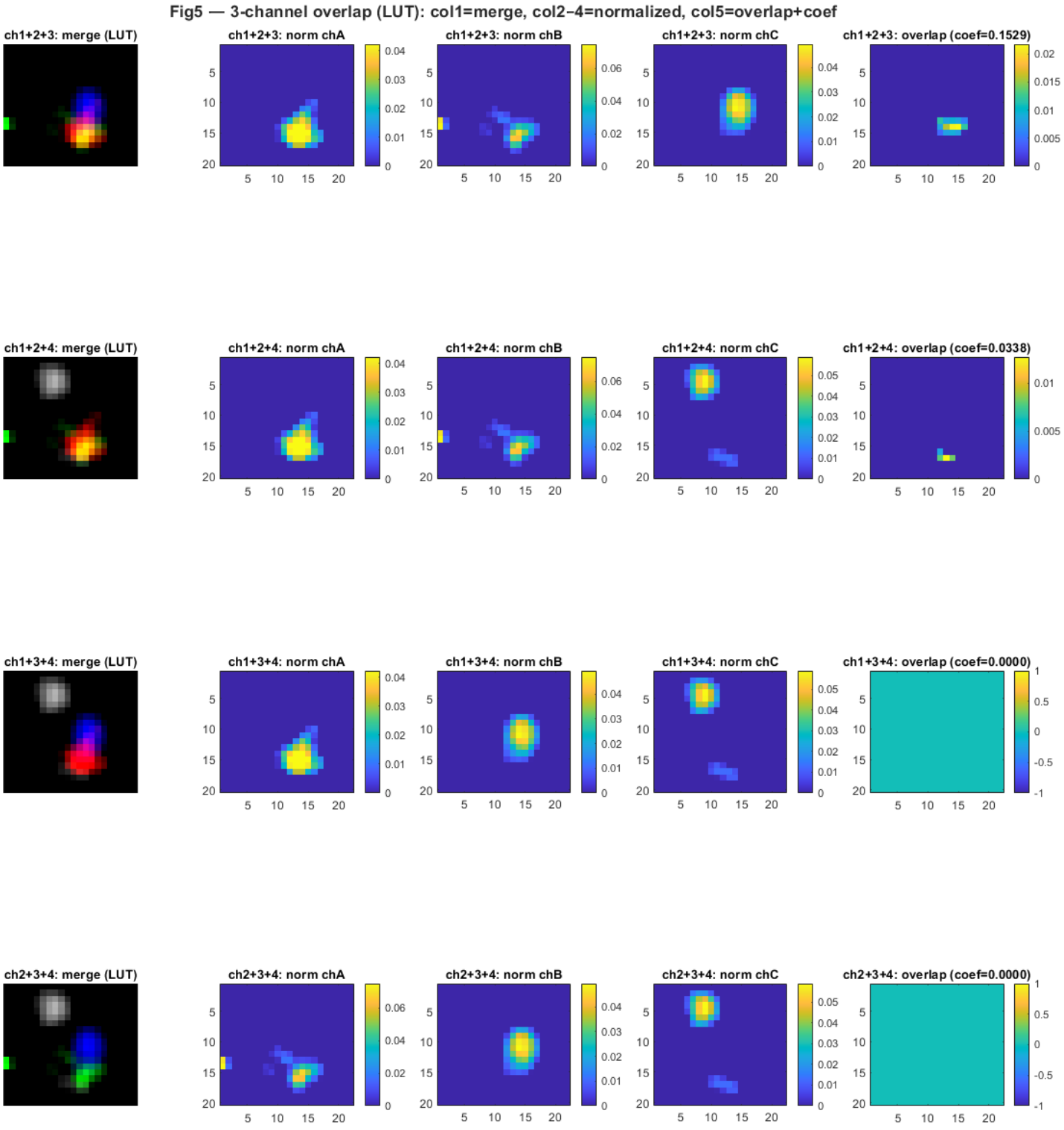
- ch1–ch2–ch4
- ch1–ch3–ch4
- ch2–ch3–ch4

Overlap is computed as the **pixel-wise minimum intensity across three normalized channels**.
Each row displays:

1. Merged LUT-enhanced image
   2–4. Normalized individual channels
2. Triple-overlap map with overlap coefficient

**Purpose**

- To quantify higher-order colocalization
- To identify regions where three molecular components co-exist

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**Definition of Overlap Coefficients**

For each channel $X$, intensities are normalized such that:

$$\sum X = 1$$

**Pairwise overlap**

$$\text{overlap}(X, Y) = \sum \min(X, Y)$$

**Triple overlap**

$$\text{overlap}(X, Y, Z) = \sum \min(X, Y, Z)$$

This definition captures **intensity-weighted spatial co-occurrence**, analogous to Manders-type metrics but extended to multi-channel comparisons.

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**CSV Output Format**

Each row corresponds to a single TIFF file. Columns include:

- coef12, coef13, coef23, coef14, coef24, coef34
- coef123, coef124, coef134, coef234

Missing values are filled with NaN to ensure consistent table structure.

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**Notes on Reproducibility**

- No manual thresholding or ROI selection is used
- All processing steps are deterministic
- Visualization and quantification are derived from the same underlying data
- The pipeline is suitable for batch-level statistical comparisons across experimental conditions

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**Typical Use Cases**

- STED or super-resolution colocalization analysis
- Multi-protein synaptic organization studies
- High-throughput comparison of molecular overlap across genotypes or treatments