*Bioinformatics*, 36(19), 2020, 4972–4974
doi: 10.1093/bioinformatics/btaa635
Advance Access Publication Date: 14 July 2020
Applications Note



Bioimage informatics

# RainbowSTORM: an open-source ImageJ plug-in for spectroscopic single-molecule localization microscopy (sSMLM) data analysis and image reconstruction

**Janel L. Davis[1,†], Brian Soetikno[1,†], Ki-Hee Song[1], Yang Zhang[1], Cheng Sun[2] and Hao F. Zhang[1,*]**

[1]Department of Biomedical Engineering and [2]Department of Mechanical Engineering, Northwestern University, Evanston, IL 60208, USA

*To whom correspondence should be addressed.

†The authors wish it to be known that, in their opinion, the first two authors should be regarded as Joint First Authors.

Associate Editor: Jinbo Xu

Received on April 3, 2020; revised on June 15, 2020; editorial decision on July 5, 2020; accepted on July 7, 2020

## Abstract

**Summary:** Spectroscopic single-molecule localization microscopy (sSMLM) simultaneously captures the spatial locations and full spectra of stochastically emitting fluorescent single molecules. It provides an optical platform to develop new multimolecular and functional imaging capabilities. While several open-source software suites provide subdiffraction localization of fluorescent molecules, software suites for spectroscopic analysis of sSMLM data remain unavailable. RainbowSTORM is an open-source ImageJ/FIJI plug-in for end-to-end spectroscopic analysis and visualization for sSMLM images. RainbowSTORM allows users to calibrate, preview and quantitatively analyze emission spectra acquired using different reported sSMLM system designs and fluorescent labels.

**Availability and implementation:** RainbowSTORM is a java plug-in for ImageJ (https://imagej.net)/FIJI (http://fiji.sc) freely available through: https://github.com/FOIL-NU/RainbowSTORM. RainbowSTORM has been tested with Windows and Mac operating systems and ImageJ/FIJI version 1.52.

**Contact:** hfzhang@northwestern.edu

**Supplementary information:** Supplementary data are available at *Bioinformatics* online.

## 1 Introduction

Single-molecule localization microscopy (SMLM) (Betzig *et al.*, 2006; Rust *et al.*, 2006; Sharonov and Hochstrasser, 2006) overcomes the optical diffraction limit by localizing stochastically emitting fluorescent molecules with high localization precision (typically 10–20 nm). Recently, spectroscopic SMLM (sSMLM) (Bongiovanni *et al.*, 2016; Dong *et al.*, 2016; Zhang *et al.*, 2015), which simultaneously detects the location and full emission spectra of each emission event was reported. Thus far, sSMLM has enabled multicolor imaging (Zhang *et al.*, 2015) and tracking (Huang *et al.*, 2018) of as many as four different fluorescent species using a single excitation source. sSMLM has also led to new functional imaging capabilities through the analysis of variations in the spectra of individual molecules. For example, sSMLM detected the polarity of the environment surrounding dye molecules (Bongiovanni *et al.*, 2016) and enabled the discovery of previously undetected molecular conformations of dyes (Kim *et al.*, 2017). Overall, sSMLM shows great promise to further extend existing SMLM. While a variety of software algorithms and packages are currently available for processing and analyzing traditional SMLM images (Sage *et al.*, 2019), software tools for comprehensive spectroscopic analysis of sSMLM images remain unavailable.

Here, we present RainbowSTORM, an open-source spectroscopic analysis plug-in for ImageJ/FIJI. RainbowSTORM leverages the functionality of the existing SMLM processing tool ThunderSTORM (Ovesny *et al.*, 2014) to attain spatial information while providing crucial spectroscopic tools for system calibration as well as spectral identification and classification. RainbowSTORM uses the spectral centroids (or intensity-weighted spectral means) of each localized stochastic event to define a range of spectral colors and render pseudocolored super-resolution images (Bongiovanni *et al.*, 2016; Dong *et al.*, 2016; Zhang *et al.*, 2015). Multicolor images can be generated by setting different user-defined spectral centroid ranges for channels with predefined colors. We provide a detailed user guide that includes descriptions and workflows for the processes implemented in RainbowSTORM. Derivations for spectroscopic analysis (Song *et al.*, 2018) and flowcharts of the algorithms used in RainbowSTORM are included in the Supplementary Information (SI).

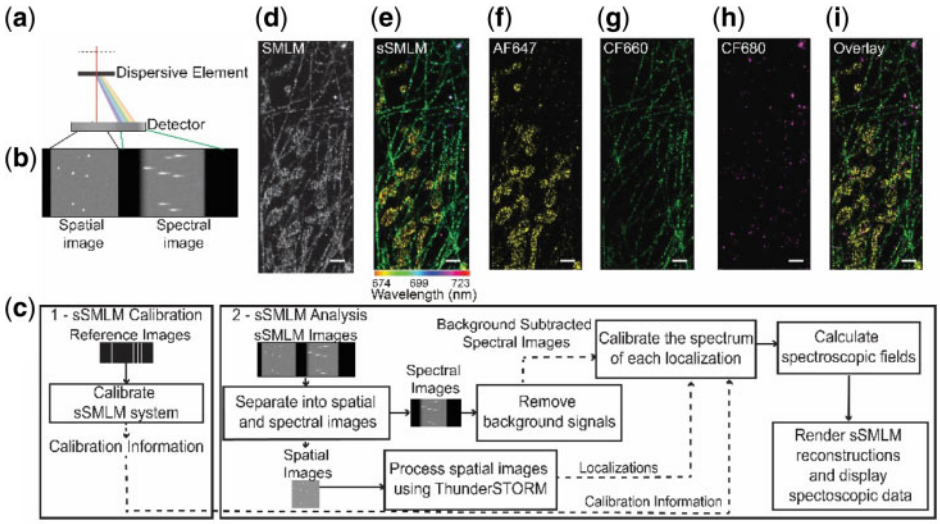


**Fig. 1.** (**a**) Conceptual schematic of a general sSMLM system. (**b**) sSMLM images with the spatial and spectral images captured on different parts of a detector. (**c**) RainbowSTORM workflow showing how the system calibration module interacts with the analysis module, (**d**) SMLM reconstruction and (**e**) Pseudocolored sSMLM reconstruction. Reconstructions of three separate channels showing (**f**) mitochondria labeled with AF647, (**g**) microtubules labeled with CF660, (**h**) peroxisomes labeled with CF680 and (**i**) the overlay of the three channels. Scale bars: 1 $\mu$m

## 2 Features and methods

### 2.1 System calibration

RainbowSTORM calibrates sSMLM images acquired using different systems, where the dispersive element (Fig. 1a) can be either a grating or a prism. While grating-based systems are calibrated by linearly fitting pixel positions to known wavelengths (Dong *et al.*, 2016), prism-based systems are calibrated using second-order (Huang *et al.*, 2018) or third-order (Zhang *et al.*, 2015) polynomial fittings. Calibration in RainbowSTORM can be performed using both calibrated light sources (e.g. calibration lamps or multiple laser lines) and multicolor fluorescent beads.

### 2.2 sSMLM image processing

In addition to providing a flexible calibration tool, RainbowSTORM also includes a sSMLM analysis module for processing sSMLM images (Fig. 1b). The general workflow for RainbowSTORM analysis is outlined in Figure 1c. RainbowSTORM first requires the images to be cropped for spatial and spectral analysis. Next two-dimensional (2D) or three-dimensional (3D) spatial images, captured using the astigmatism method (Huang *et al.*, 2008; Zhang *et al.*, 2015), can be processed using ThunderSTORM. Figure 1d shows the resulting SMLM reconstruction. Next, RainbowSTORM removes background signals from the spectral images and automatically excludes emission events that spatially overlap (see details for both methods in the SI and user guide). RainbowSTORM also enables the preview of the results of spectroscopic analysis using the current processing parameters. Finally, RainbowSTORM identifies the full spectra and calculates the spectroscopic fields for all localizations.

### 2.3 Visualization and post-processing

After processing the spectral images, pseudocolored reconstructions (Fig. 1e) are rendered using the spectral centroids and spatial coordinates of each localization. For 3D sSMLM images, a stack of pseudocolored reconstructions can be rendered, where each frame in the stack is generated using localizations with similar axial positions. The histograms of the calculated spatial and spectral fields can be displayed and used to select subsets of the data for independent visualization. Additionally, localizations with large point spread functions and spectrum widths as well as localizations with low photon counts and precisions in the spatial and spectral domains can be excluded. RainbowSTORM can also apply ThunderSTORM drift-correction files and assess the image quality of sSMLM reconstructions using Fourier Ring Correlation (FRC) analysis (Nieuwenhuizen *et al.*, 2013). The spectral centroid ranges can also be assigned to multiple channels to create multicolor reconstructions using the classification module. For example, Figure 1f–h shows reconstructions of the mitochondria, microtubules and peroxisomes of COS-7 cells, respectively, labeled by Alexa Fluor 647(AF647), CF660 and CF680. Figure 1i shows the overlay of the three images from the selected spectral centroid windows. After post-processing, sSMLM results can be saved. Previously saved sSMLM results can be loaded using the sSMLM import module for further analysis.

## 3 Summary

RainbowSTORM provides a plug-in for performing spectroscopic analysis of 2D and 3D sSMLM images acquired using both grating-based and prism-based sSMLM implementations. RainbowSTORM fills the need for a spectroscopic analysis platform and provides spectral classification methods, spectral and spatial filtering methods, pseudocolored visualization of sSMLM datasets and built-in FRC analysis. Future updates will make RainbowSTORM compatible with a wider range of spatial analysis platforms. Further, additional spectroscopic analysis methods such as spectral unmixing (Davis *et al.*, 2018), machine-learning-based spectral classification (Zhang *et al.*, 2019) and cluster analysis (Bongiovanni *et al.*, 2016; Davis *et al.*, 2020) will be added to RainbowSTORM.

## Acknowledgements

The authors thank Prof. Ke Xu and Dr. Limin Xiang from The University of California, Berkeley for allowing them to test their experimental data using RainbowSTORM.

## Funding

This work was supported by grants from the National Science Foundation [CBET-1706642, EFRI-1830969 and EEC-1530734] and by the National Eye Institute [R01EY026078 and R01EY029121]. J.L.D. was supported by the National Science Foundation Graduate Research Fellowship [DGE-1842165].

K.S. was supported by the Christine Enroth-Cugell and David Cugell Graduate Fellowship in Biomedical Engineering and Visual Neuroscience.

*Conflict of Interest*: none declared.

## References

Betzig,E. *et al.* (2006) Imaging intracellular fluorescent proteins at nanometer resolution. *Science*, **313**, 1642–1645.

Bongiovanni,M.N. *et al.* (2016) Multi-dimensional super-resolution imaging enables surface hydrophobicity mapping. *Nat. Commun.*, **7**13544.

Davis,J.L. *et al.* (2018) Method to identify and minimize artifacts induced by fluorescent impurities in single-molecule localization microscopy. *J. Biomed. Opt.*, **23**, 1–14.

Davis,J.L. *et al.* (2020) Super-resolution imaging of self-assembled nanocarriers using quantitative spectroscopic analysis for cluster extraction. *Langmuir*, **36**, 2291–2299.

Dong,B.Q. *et al.* (2016) Super-resolution spectroscopic microscopy via photon localization. *Nat. Commun.*, **7**12290.

Huang,B. *et al.* (2008) Three-dimensional super-resolution imaging by stochastic optical reconstruction microscopy. *Science*, **319**, 810–813.

Huang,T. *et al.* (2018) Simultaneous multicolor single-molecule tracking with single-laser excitation via spectral imaging. *Biophys. J.*, **114**, 301–310.

Kim,D. *et al.* (2017) Spectrally resolved super-resolution microscopy unveils multipath reaction pathways of single spiropyran molecules. *J. Am. Chem. Soc.*, **139**, 9447–9450.

Nieuwenhuizen,R.P. *et al.* (2013) Measuring image resolution in optical nanoscopy. *Nat. Methods*, **10**, 557–562.

Ovesny,M. *et al.* (2014) ThunderSTORM: a comprehensive ImageJ plug-in for PALM and STORM data analysis and super-resolution imaging. *Bioinformatics*, **30**, 2389–2390.

Rust,M.J. *et al.* (2006) Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods*, **3**, 793–795.

Sage,D. *et al.* (2019) Super-resolution fight club: assessment of 2D and 3D single-molecule localization microscopy software. *Nat. Methods*, **16**, 387–395.

Sharonov,A. and Hochstrasser,R.M. (2006) Wide-field subdiffraction imaging by accumulated binding of diffusing probes. *Proc. Natl. Acad. Sci. USA*, **103**, 18911–18916.

Song,K.H. *et al.* (2018) Theoretical analysis of spectral precision in spectroscopic single-molecule localization microscopy. *Rev. Sci. Instrum.*, **89**, 123703.

Zhang,Z. *et al.* (2015) Ultrahigh-throughput single-molecule spectroscopy and spectrally resolved super-resolution microscopy. *Nat. Methods*, **12**, 935–938.

Zhang,Z. *et al.* (2019) Machine-learning based spectral classification for spectroscopic single-molecule localization microscopy. *Opt. Lett.*, **44**, 5864–5867.