Simultaneous spatiotemporal computational super-resolution and multi-parametric fluorescence microscopy

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Method Article

Abstract

We describe here a protocol for performing simultaneous spatiotemporal computational super-resolution and multi-parametric fluorescence microscopy. Our approach does not need specialized instrumentation, utilizes a GPU for faster data evaluation, and produces mutually consistent structure and dynamics data.

Introduction

We describe a protocol to perform simultaneous multi-parametric analysis using fast and sensitive cameras (EMCCD and sCMOS) and a combination of super-resolution and spectroscopy techniques – super resolution radial fluctuation (SRRF)/super-resolution optical fluctuation imaging (SOFI)/deconvolution, fluorescence correlation spectroscopy (FCS) analysis, number and brightness analysis (N&B), and FCS diffusion law analysis.

SRRF [1] (with its roots in SOFI [2]) analyzes the fluctuations in radiality stacks to obtain super resolved images. One could also apply deconvolution algorithms to the raw image stack [3]. Imaging fluorescence correlation spectroscopy (Imaging FCS) [4][5] analyzes the fluctuations in fluorescence intensity in a pixel to estimate the diffusion coefficient (D) and the number of particles in that pixel (N). The relationship between observation area and diffusion coefficient is determined in FCS diffusion law analysis to understand the mode of diffusion [6]. The concentration and brightness at each pixel is estimated from the mean and variance of intensity in a pixel in N&B analysis [7][8].

We introduce a GPU-supported, camera-based approach to achieve high spatiotemporal resolution from a single data set and perform multiple analyses on it to extract maximum information.

Reagents

1. For the point spread function (PSF) calibration of the system, a sample exhibiting free diffusion is needed. We recommend DOPC (2-dioleoyl-sn-glycero-3-phosphocholine; Avanti Polar Lipids, Alabama, USA) bilayer containing a fluorescent label. Examples of fluorescent labels include:

• Lipilight 488 (Idylle, Paris, France) for 488 nm excitation.

• Rhodamine PE (14:0 Liss Rhod PE, Avanti Polar Lipids, Alabama, USA) for 561 nm excitation.

2. Experimental sample (e.g. live cell preparations).

Equipment

1. Total Internal Reflection Fluorescence (TIRF) Microscope (Olympus, Japan):

• IX83 inverted epi-fluorescence microscope

• Motorized cellTIRF-4Line IX3-MITICO illumination combiner

• Laser system of different wavelengths.

2. Dual-emission image splitter (OptoSplit II; Cairn Research, Faversham, UK) (*if dual-channel measurements are required*).

3. Detector:

• Electron multiplying charge-coupled device (EMCCD; iXon^{EM}+ 860, 24 µm pixel size, 128×128 pixels, Andor, Oxford Instruments, UK),

or

• Scientific complementary metal oxide semiconductor (sCMOS; Sona 4.2B-11, 11 µm pixel size, 2048×2048 pixels, Andor, Oxford Instruments, UK).

• Camera control software (Andor Solis, Andor, Oxford Instruments, UK).

4. High numerical aperture (NA) microscope objective (100×, Apo N, NA 1.49 oil-immersion; Olympus, Japan).

• For the iXon^{EM}+ 860 EMCCD and Sona 4.2B-11 sCMOS, this translates to a 240 nm and 110 nm image pixel size, respectively.

5. Magnification changer slider (1×, 1.6× and 2×, IX3-cAS, Olympus, Japan).

• With the extra 2× magnification, the image pixel size for the iXon^{EM}+ 860 EMCCD and Sona 4.2B-11 sCMOS is 120 nm and 65 nm, respectively.

6. On-stage temperature and CO_2 incubator (Chamlide TC, Live Cell Instrument, South Korea).

7. Appropriate emission dichroic mirror and optical filters.

8. Image processing software

a. Fiji-ImageJ

• For Imaging FCS, FCS diffusion law and N&B - Imaging FCS (ImFCS) 1.52 plugin included in ImageJ update site. Also can be downloaded here. The ImFCS 1.52 manual is available here.

• For SOFI and SRRF - NanoJ-SRRF plugin (version 1.14Stable1) included in ImageJ update site.

b. For deconvolution - Huygens Professional version 20.04 (Scientific Volume Imaging, The Netherlands)

9. An NVIDIA graphics processing unit (GPU) for faster analyses.

Procedure

A. Data acquisition

1. Using the appropriate microscopy setup and camera.

• Use the magnification slider if higher magnification is required.

• Use the dual-emission image splitter if dual channel measurements are to be done.

2. Excite the sample with suitable laser powers and record a stack of images at high temporal resolution. As an example, we use the following settings for our measurements:

• Laser powers of 100-300 µW for 488 nm excitation and 0.1-1 mW for 561 nm excitation when using an EMCCD with EM gain of 300. If sCMOS is used as detector, higher laser powers in mW range may be necessary.

3. Record at 1,000 frames per second (fps) for bilayer measurements and 500 fps for cell membrane measurements. If sufficient signal-to-noise ratio (SNR) is achievable, faster recording rates can be used.

• In Andor Solis, for the EMCCD, the kinetic mode of image acquisition was used and the 'baseline clamp' was always used to minimize the baseline fluctuation. The camera was operated using 10 MHz pixel readout speed. Maximum analog-to-digital gain was set to 4.7, and 0.45 µs vertical shift speed was used. The EM gain used was 300.

• Similarly, for the sCMOS, the pixel readout rate used was 200 MHz in an overlap readout mode. The rolling electronic shuttering mode and 12-bit dynamic range was used.

4. Save the image stack as a 16-bit tiff image stack since that is the input for the ImFCS plugin in ImageJ.

B. Data pre-processing

To maintain a good SNR, post-acquisition spatial or temporal binning might be necessary. We suggest the following based on our experience:

a. *Imaging FCS* - 1×1 binning or 2×2 binning depending on SNR. For EMCCD, 1×1 binning usually works at 240 nm image pixel size. If higher magnification or sCMOS is used, 2×2 binning might be required.

b. *FCS diffusion law* - The plot can usually be generated and fit with spatial binning of 1×1 to 5×5. But at low SNR, the smaller bins have large errors and higher spatial binnings might be required.

c. *N&B analysis* - Time bin to at least 10 ms per frame, otherwise the brightness calibration might underestimate the fluorescence probability of the fluorescent protein. We use a higher time binning of 20 ms to get better differentiation of the background and the cell for intensity thresholding,

d. *Computational super-resolution microscopy* - We observed optimal results for SOFI/SRRF after time binning to 200 ms per frame. For deconvolution, temporally bin all the frames to obtain a single time-averaged image.

C. Calibration of point spread function (PSF) for Imaging FCS

1. To calibrate the PSF for the experimental setup, load the bilayer measurement file into the ImFCS plugin in ImageJ.

2. Set the appropriate values for following parameters in the plugin: frame time, correlator (p, q), pixel size, NA, and λ_1.

3. The program will compute and displayed a plot of the diffusion coefficient values for various combinations of PSF and binning values.

4. Refer to the ImFCS manual for more details.

D. Data analyses

Refer to the ImFCS, NanoJ-SRRF and Huygens manuals for more details.

a. *FCS and FCS diffusion law*

1. Load the image stacks in ImFCS 1.52 plugin.

2. Set the appropriate values for following parameters in the plugin: frame time, correlator (p, q), pixel size, NA, λ_1 and bleach correction (refer to ImFCS manual for details).

3. Calculate and fit the ACFs with a suitable diffusion model. To exclude outliers or non-converged fits, threshold the data using the available filters (e.g. *N* or *D* filter).

4. In the "Diff. Law" tab, the diffusion law plot can be generated and fit with a straight line for square binning of desired range.

b. *N&B*

1. Temporally bin the image stack in ImageJ using the **Image → Transform → Bin** command sequence. Set the desired X, Y and Z shrink factor and choose the **Sum** bin method.

2. Load the temporally-binned image stacks in ImFCS 1.52 plugin.

3. Set the appropriate values for following parameters in the plugin: frame time, bleach correction and intensity filter (refer to ImFCS manual for details). The intensity filter excludes the background pixels and include only the pixels containing the cell.

4. Load a dark image (image taken with the camera shutter closed) with the same spatiotemporal dimensions as the measurement image for background correction in the "Bgr NUM" tab.

5. In the "N&B" tab, select "G1" in the "NB mode".

6. Press the "N&B" button in "N&B analysis" to generate the N&B maps.

c. *Computational super-resolution microscopy*

• *SOFI/SRRF*

1. Temporally bin the image stacks as described in the previous N&B section. The **Average** bin method is recommended to avoid pixel count saturation.

2. In the NanoJ-SRRF plugin choose the "SRRF analysis" option. Set the desired values for ring radius, radiality magnification, axes in ring, display_mode, and temporal analysis mode.

3. Click "OK" and the SRRF images will be generated.

4. For SOFI images, follow the same steps as above but set the ring radius, radiality magnification, and axes in ring to the minimum allowed values.

• *Deconvolution*

1. Temporally bin the image stacks to generate single time-averaged images.

2. Load the images into Huygens Professional.

3. Set the microscope type to confocal.

4. Set the X, Y and Z sampling intervals to the ideal Nyquist value.

5. Set the optical parameters according to the microscopy system used: NA, refractive indices of lens immersion oil and embedding medium, objective quality.

6. Set the channel parameters: backprojected pinhole = maximum allowed value, excitation wavelength, emission wavelength, excitation fill factor = 2.

7. The theoretical PSF will be automatically generated. The background value of the image will also be automatically estimated with the emission mode set to lowest and the area radius set to the default 0.7 microns.

8. Use a suitable deconvolution algorithm. e.g. Classic Maximum Likelihood Estimation (CMLE).

9. The deconvolved image will be generated.

Troubleshooting

Time Taken

A typical data set is acquired at 2 ms exposure time for 50,000 frames and requires 100 seconds of measurement.

If using a GPU, the multi-parametric data analyses can be completed in ~5 minutes.

Anticipated Results

Using this protocol, one can generate diffusion coefficient maps, brightness maps, FCS diffusion law plots and computationally super-resolved images from a single data set.

Performing multiple analyses as described above and retaining only those pixels that are validated by all the different techniques produces mutually consistent structure and dynamics data.

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