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Conventional BODIPY conjugates for live-cell super-resolution microscopy and single-molecule tracking

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Cover Letter

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To:
Benjamin Werth
Senior Editor, JOVE

Dear Benjamin, October 28th, 2019

We hereby would like to submit our manuscript entitled *"Conventional BODIPY conjugates for live-cell super-resolution microscopy and single-molecule tracking"* as a manuscript for publication in JoVE. As per our previous communication, you identified this work as an interesting topic for a JoVE article and a video protocol. In this work we describe, optimize and employ a protocol about our recent and surprising finding that the myriad of versatile BODIPY conjugates available for conventional fluorescence microscopy can be used for multi-color Single-Molecule Localization Microscopy (SMLM) in living cells. Besides being one of the most widely used fluorophores, BODIPYs exhibit other advantages over existing labeling strategies for SMLM. We demonstrate the versatility of this protocol by presenting insights into how the dynamics and the nanoscopic spatial distribution of fatty acids and lipid droplets in yeast are regulated by the metabolic state of cells. We also demonstrate the use of this novel labeling protocol in living mammalian cells. Since SMLM techniques are increasingly contributing to discoveries across different disciplines, we believe that our manuscript would be suitable and a helpful resource for the broad reader/viewership of JoVE.

SMLM is based on localizing single fluorophores. It is therefore critical to separate the fluorescence of densely packed molecules in time in order to avoid their spatial overlap. Here we optimize a protocol for our recently published (https://www.nature.com/articles/s41467-019-11384-6) and novel labeling strategy for SMLM: the harnessing of red-shifted ground state dimers, which transiently form through bi-molecular encounters of conventional BODIPY conjugates. While BODIPY dyes have been previously shown in ensemble spectroscopy experiments to form red-shifted ground state dimers, our paper was the first report to demonstrate the detection of their bright fluorescence on a single molecule level. We optimize and demonstrate the versatility of this approach for SMLM by resolving and quantifying the spatial distribution and dynamics of single fatty acids and lipid droplets in living cells with two colors. The obtained super-resolution images exhibit a dramatic change in the localization of fatty acids into puncta along the plasma membrane upon fasting, suggestion a spatial protection mechanism against lipotoxicity. Likewise, the mobility of fatty acids shows a strong transition from free diffusion to confined immobilization in these puncta upon fasting.

While a multitude of different labeling strategies have been developed for SMLM including photoswitchable BODIPY probes, we are certain that our work will be of high impact and usefulness to the field due to the **novel mechanism and extreme simplicity** of this approach: no chemical modification, special imaging buffers, cell fixation, genetic tagging, (toxic) photoswitching, washing etc. is necessary. Just adding any of the commercially available BODIPY-conjugates in any color to the live cell culture and exciting transient ground state dimers at a red-shifted wavelength will do the trick. All data in this manuscript is original but similar to data recorded in our recent publication.

We suggest the following reviewers who are experts in the field:

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Please let me know if you have further questions.

Sincerely,

Elias M. Puchner

TITLE:

Conventional BODIPY Conjugates for Live-Cell Super-Resolution Microscopy and Single-Molecule Tracking

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Fluorescence microscopy, Super-resolution microscopy, single molecule tracking, single-molecule localization microscopy, BODIPY, ground-state dimers, yeast, mammalian cells

SUMMARY:

Conventional BODIPY conjugates can be used for live-cell single-molecule localization microscopy (SMLM) through exploitation of their transiently forming, red-shifted ground state dimers. We present an optimized SMLM protocol to track and resolve subcellular neutral lipids and fatty acids in living mammalian and yeast cells at the nanoscopic length scale.

ABSTRACT:

Single molecule localization microscopy (SMLM) techniques overcome the optical diffraction limit of conventional fluorescence microscopy and can resolve intracellular structures and the dynamics of biomolecules with ~20 nm precision. A prerequisite for SMLM are fluorophores that transition from a dark to a fluorescent state in order to avoid spatio-temporal overlap of their point spread functions in each of the thousands of data acquisition frames. BODIPYs are well-established dyes with numerous conjugates used in conventional microscopy. The transient formation of red-shifted BODIPY ground-state dimers (D_{II}) results in bright single molecule emission enabling single molecule localization microscopy (SMLM). Here we present a simple but versatile protocol for SMLM with conventional BODIPY conjugates in living yeast and mammalian cells. This procedure can be used to acquire super-resolution images and to track single BODIPY-D_{II} states to extract spatio-temporal information of BODIPY conjugates. We apply this procedure to resolve lipid droplets (LDs), fatty acids, and lysosomes in living yeast and mammalian cells at the nanoscopic length scale. Furthermore, we demonstrate the multi-color imaging capability with BODIPY dyes when used in conjunction with other fluorescence probes. Our representative

results show the differential spatial distribution and mobility of BODIPY-fatty acids and neutral lipids in yeast under fed and fasted conditions. This optimized protocol for SMLM can be used with hundreds of commercially available BODIPY conjugates and is a useful resource to study biological processes at the nanoscale far beyond the applications of this work.

INTRODUCTION:

Single-molecule localization microscopy (SMLM) techniques such as stochastic optical reconstruction microscopy (STORM) and photo-activated localization microscopy (PALM) have emerged as methods for generating super-resolution images with information beyond Abbe's optical diffraction limit[1,2] and for tracking the dynamics of single biomolecules[3,4]. One of the requirements for probes compatible with SMLM is the ability to control the number of active fluorophores at any time to avoid spatial overlap of their point spread functions (PSF). In each of the thousands of data acquisition frames, the location of each fluorescent fluorophores is then determined with ~20 nm precision by fitting its corresponding point-spread function. Traditionally, the on-off blinking of fluorophores has been controlled through stochastic photoswitching[1,2,5] or chemically induced intrinsic blinking[6]. Other approaches include the induced activation of fluorogens upon transient binding to a fluorogen-activating protein[7,8] and the programmable binding-unbinding of labeled DNA oligomers in total internal reflection fluorescence (TIRF) or light sheet excitation[9]. Recently, we reported a novel and versatile labeling strategy for SMLM[10] in which previously reported red-shifted dimeric (D_{II}) states of conventional boron di-pyromethane (BODIPY) conjugates[11–13] are transiently forming and become specifically excited and detected with red-shifted wavelengths.

BODIPYs are widely used dyes with hundreds of variants that specifically label sub-cellular compartments and biomolecules[14–16]. Because of their ease of use and applicability in living cells, BODIPY variants are commercially available for conventional fluorescence microscopy. Here, we describe a detailed and optimized protocol on how the hundreds of commercially available BODIPY conjugates can be used for live-cell SMLM. By tuning the concentration of BODIPY monomers and by optimizing the excitation laser powers, imaging and data analysis parameters, high-quality super-resolution images and single molecule tracking data is obtained in living cells. When used at low concentration (25−100 nM), BODIPY conjugates can be simultaneously used for SMLM in the red-shifted channel and for correlative conventional fluoresce microscopy in the conventional emission channel. The obtained single molecule data can be analyzed to quantify the spatial organization of immobile structures and to extract the diffusive states of molecules in living cells[17]. The availability of BODIPY probes in both green and red forms allows for multi-color imaging when used in the right combination with other compatible fluorophores.

In this report, we provide an optimized protocol for acquiring and analyzing live-cell SMLM data using BODIPY-C_{12}, BODIPY (493/503), BODIPY-C_{12} red and lysotracker-green in multiple colors. We resolve fatty acids and neutral lipids in living yeast and mammalian cells with ~30 nm resolution. We further demonstrate that yeast cells differently regulate the spatial distribution of externally added fatty acids depending on their metabolic state. We find that added BODIPY-fatty acids (FA) localize to the endoplasmic reticulum (ER) and lipid droplets (LDs) under fed conditions whereas BODIPY-FAs form non-LD clusters in the plasma membrane upon fasting. We

further extend the application of this technique to image lysosomes and LDs in living mammalian cells. Our optimized protocol for SMLM using conventional BODIPY conjugates can be a useful resource to study biological processes at the nanoscale with the myriad available BODIPY conjugates.

PROTOCOL:

NOTE: For yeast cloning and endogenous tagging please refer to our recent publication[10].

1. Preparation of yeast cell samples for imaging

1.1. Prepare a liquid overnight culture of a w303 yeast strain. Using a sterile wooden stick, spot a small amount of yeast cells from an agar plate containing yeast extract–peptone–dextrose into a culture tube with ~2 mL of synthetic complete dextrose (SCD) medium. Incubate the tube overnight in a shaking incubator at 270 rpm and 30 °C.

1.2. Perform a 1:50 morning dilution of the cells in SCD. Continue to culture the diluted cells for 4 h at 30 °C in a 270 rpm shaking incubator, allowing the cells to grow in exponential phase and to reach an optical density (OD) of ~0.6.

NOTE: The procedure can vary here depending on which metabolic state is being studied. BODIPY conjugates do not require cells in the exponential growth phase. However, be cognizant of autofluorescence from dead cells during the stationary phase, as it can cause a background signal too strong to analyze single BODIPY-D_{II} emitters.

1.3. For studying fasting cells, grow the yeast culture for 2 days without exchange of media.

1.4. At ~30 min prior to plating the cells, incubate a chambered coverglass with 80 µL of 0.8 mg/mL sterile Concanavalin A (ConA) in deionized H_2O at room temperature. After 30 min, wash the coverglass three times with deionized H_2O.

1.5. At ~30 min before imaging, pipette the cells on the chambered coverglass, with the correct volume of fresh SCD to achieve an optical density of ~0.12 (typically 60 µL yeast culture at OD ~0.6 in 240 µL SCD). Let the cells settle and adhere to the ConA surface for 30 min.

1.6. Add the desired BODIPY conjugate directly to the chambered coverglass at a final concentration of ~100 nM.

NOTE: A BODIPY concentration optimization experiment may be required depending on BODIPYs local density in a particular cellular compartment.

2. Preparation of mammalian cells for SMLM imaging

2.1. Maintain the mammalian U2OS cells in non-fluorescent DMEM with 10% fetal bovine serum,

4 mM glutamine, 1 mM sodium pyruvate and 1% penicillin-streptomycin antibiotics in a T25 flask.

NOTE: Cells can also be maintained in DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics, however, the medium needs to be exchanged before imaging with a non-autofluorescent solution.

2.2. Split the cells at 70–80% confluency 1:5 in a single well of an 8-well plate. Culture the cells in the 8-well plate for 12 to 24 h before imaging.

2.3. Add BODIPY-C_{12}, LysoTracker Green or any other BODIPY conjugate at a final concentration of 100 nM (stock solutions in dimethyl sulfoxide [DMSO]) 10 min prior to imaging. This time can vary based on the desired experiment.

NOTE: Imaging can be performed at ambient temperature (23 °C) using live cell imaging solution. However, imaging at 37 °C and 5% CO_2 with non-fluorescent DMEM mixed with 10% fetal bovine serum, 4 mM glutamine, 1 mM sodium pyruvate and 1% penicillin-streptomycin antibiotics is preferred to keep cells closer to physiological conditions and to make biological conclusions.

3. Equipment preparation

3.1. Mount the appropriate filter sets in the emission path based on the emission color of BODIPY being used.

NOTE: A quad-band dichroic mirror (zt/405/488/561/640rdc) first separates the excitation from the emission light. The green emission (525 nm) and red emission (595 nm) are then split by a dichroic long-pass beam splitter (T562lpxr) followed by band-pass filters ET525/50 in the green channel and ET 595/50 in the red channel. The two channels are then projected to different areas of the same camera chip. Similarly, the red emission (595 nm) and the far-red emission are first split by a dichroic long-pass beam splitter (FF652-Di01) followed by band-pass filters ET 610/75 in the red and FF731/137 in the far-red channel.

3.2. Turn on the microscope, microscope stage, lasers (488 nm, 561 nm) and camera. Here an inverted microscope with a perfect focus system and an EMCCD camera cooled to -68 °C are used.

3.3. Add a drop of immersion oil on the microscope objective.

3.4. Open the Hal4000 software (see the **Table of Materials**) that controls the LED light for bright field imaging, laser powers, laser shutters and camera settings for imaging. Set the EMCCD gain to 30 and the camera temperature to -68 °C. Prepare the camera and corresponding software to record movies at 20 Hz.

NOTE: This technique applies to any wide-field microscope capable of photo-activated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM)

imaging. Corresponding software may vary.

3.5 Turn on the microscope stage heater and set it to a temperature of 37 °C and to a CO_2 level of 5%. Adjust the objective correction collar accordingly.

3.6. Mount the sample on the microscope stage and focus until the focusing system engages. Move the stage using the stage controller until healthy cells appear in the field of view.

NOTE: For imaging with yeast cells at room temperature, there is no need to turn on the heater of CO_2 control.

3.7. Turn on the appropriate lasers for the excitation of monomers as well as dimers. For BODIPY green or LysoTracker green, we use a 561 nm laser to excite D_{II} states for SMLM and a 488 nm laser to excite monomers for conventional fluorescence.

NOTE: For BODIPY red, use a 640 nm laser to excite D_{II} states for SMLM and a 561 nm laser to excite the monomers for conventional fluorescence. For BODIPY red, adjust the 561 nm and 640 nm laser powers to visualize bulk fluorescence in the red channel and single molecule bursts in the far-red channel. The typical power for 561 nm is ~0.06 W/cm^2 and ~5 kW/cm^2 for 640 nm. For BODIPY green, expect to also see conventional fluorescence in the green emission channel under 561 nm excitation. For BODIPY red, expect to see conventional fluorescence in the red channel under 640 nm excitation. This signal arises from anti-Stokes emission, which becomes useful for monomer/dimer co-localization images with continuous laser excitation.

4. Data acquisition

4.1. Load laser shutter sequences for the excitation of monomers as well as dimers.

NOTE: We typically use nine single molecule excitation frames at 561 nm excitation followed by one conventional excitation frame at 488 nm excitation. This offers a brighter conventional fluorescence signal and avoids leaking of another 488 nm excitable fluorophore such as green fluorescent protein (GFP) into the red single-molecule detection channel in multi-color imaging applications. Alternatively, turn on the 561 nm laser continuously and rely on the anti-Stokes emission for conventional images in the shorter wavelength channel.

4.2. Tune the laser powers such that single molecule fluorescence bursts are detected in the red-shifted emission channel under 561 nm excitation, and conventional fluorescence appears in the green emission channel with 488 nm excitation. Typical laser powers of the 561 nm laser will be around 0.8–1 kW/cm^2 for SMLM, and 0.035–0.07 W/cm^2 for the 488 nm laser in the conventional fluorescence imaging mode.

4.3. Choose a destination folder for movies and record 5,000–20,000 acquisition frames to collect enough localizations for reconstructing super-resolution images.

4.4. Move to different fields of view and repeat the steps above to collect data from more cells.

5. Data analysis and single-molecule tracking

5.1. Load the movie into a SMLM analysis software.

NOTE: Any software[18] can be used. We use INSIGHT (see the **Table of Materials**) and cross-validate the results using the ThunderSTORM[19] plugin for imageJ (Fiji).

5.2. Visually screen the movie and adjust contrast settings such that single-molecule fluorescent blinking is visible. If needed restrict the region or the frame range for SMLM data analysis if parts of the sample are continuously fluorescing.

5.3. Set single molecule identification parameters for fitting with 2D Gaussian PSFs (ROI: 7 x 7 pixels with pixel size 160 nm, width 260–650 nm, height > 50 photons). Visually screen through some example frames to check the identification parameters and reliably detect the distinct single molecule fluorescent bursts (see **Figure 1C**).

NOTE: Certain identification parameters such as height and width can be slightly adjusted to optimize the recognition of visually perceived single molecule fluorescence signals.

5.4. Perform SMLM image analysis with the optimized identification parameters and then render each single molecule as a 2D Gaussian whose width is weighted by the inverse square root of the detected number of photons.

5.5. Assess the quality of the data. Use restricted frame ranges to observe single molecule distributions at more specific instances in time. This can account for organelle movement during data acquisition.

5.6. To further analyze the spatial distribution and dynamics of the molecule distributions, export the obtained molecule list containing the coordinates, frames of appearance, photons, widths and heights of the fitted localizations. Import the molecule list in custom written analysis procedures.

5.7. For obtaining spatial information of the single molecule distribution, calculate the radial distribution function ρ(r), which represents the density of localizations as a function of the radial distance[20]. To obtain ρ(r), calculate unique pair-wise distances of all localizations, construct the histogram with bins centered at r_i with height $H(r_i)$ and with a width dr and divide by $2\pi r_i*dr$; ($\rho(r_i) = H(r_i)/\pi(r_i+dr)^2-r_i^2$). The radial distribution function can then be used to quantify and compare the degree of clustering as well as the characteristic size of clusters.

5.8. To obtain dynamic information about the diffusion of molecules, link localizations that appear for example within 3 pixels (0.48 µm) in consecutive data acquisition frames to create single molecule traces.

NOTE: The linking distance will depend on the diffusion of molecules and the density of localizations. The maximum linking distance can be estimated by analyzing the density of localizations in each frame[21,22]. The average density was determined to be 0.043 localizations per μm^2; thus a 0.48 µm radius was within a low enough density to ensure that different molecules were not linked together.

5.9. Average the displacements for different lag times Δt from multiple traces lasting at least three lag times to create a mean squared displacement (MSD) vs. Δt plot. Fit the MSD vs. Δt curve with the equation $MSD = 4D\Delta t + \sigma^2$ to calculate the average diffusion coefficient D[3].

REPRESENTATIVE RESULTS:

Here, we present an optimized sample preparation, data acquisition and analysis procedure for SMLM using BODIPY conjugates based on the protocol from the above (**Figure 1A**). To demonstrate an example of the workflow for acquiring and analyzing SMLM data, we employ BODIPY (493/503) in yeast to resolve LDs below the optical diffraction limit (**Figure 1B–F**). Examples of the different multi-color imaging modes of BODIPY in conjunction with other probes such as GFP, mEos2 are shown in **Figure 2**. We manipulate the metabolic state in yeast by growing them in same media for ~48 h and show that BODIPY-C12 forms immobile non-LD clusters in cell periphery upon fasting in contrast to their incorporation into LDs under fed conditions (**Figure 3**). To further extend the SMLM capability of BODIPY conjugates to mammalian cells, we image BODIPY-C12 and LysoTracker-green in live U2OS cells (**Figure 4**).

FIGURE LEGENDS:

Figure 1: Optimization of SMLM data acquisition and analysis using BODIPY dyes. (**A**) Workflow for optimizing single molecule fluorescence signals and post-processing of the SMLM data from BODIPY conjugates. (**B**) LED image (left), conventional fluorescence image (middle, excitation: 488 nm, emission: 525 nm) and anti-Stokes image (right, excitation: 561 nm, emission: 525 nm) of yeast cells stained with the LD marker BODIPY (493/503). (**C**) Single SMLM frames showing singe BODIPY D_{II} emitters (excitation: 561 nm, emission: 595 nm) at too low density (left), optimal density (middle) and too high density (right). (**D**) Optimization of SMLM analysis parameters. With a too high photon number threshold, the software misses valid single molecule signals (left), detects all molecules with an optimal photon threshold (middle) and detects false localizations with too low photon thresholds (right). (**E**) SMLM image of BODIPY (493/503) resolves the size of LDs (left, zoom) with a mean diameter of 125 nm. (**F**) Single molecule tracking reveals confined diffusion of BODIPY (493/503) inside LDs (left). Traces are used to compute the MSD vs. time curve, which exhibits sub-diffusive behavior inside LDs (right). Scale bar = 1 µm, zoom = 100 nm.

Figure 2: Multi-color SMLM imaging using BODIPY conjugates in living cells. (**A**) Conventional image of BODIPY-C12 under 488 nm excitation (left). Corresponding SMLM image using D_{II} states of BODIPY-C12 under 561 nm excitation (middle) and zoom (right) revealing BODIPY-C12 in emerging LDs. (**B**) Conventional fluorescence image of the ER labeled with Sec63-GFP under 488 nm excitation (left). Simultaneously recorded conventional fluorescence image of BODIPY-C12

red with 561 nm excitation (middle) and SMLM image using 640 nm excitation (right). (**C**) Sequential two-color SMLM imaging of Sec63-mEos2 and BODIPY-C12 green D_{II} states. First, mEos2 is imaged with high 405 nm photo-activation and 561 nm excitation (left) followed by long data acquisition without 405 nm activation (middle). Scale bar = 1 μm.

Figure 3: Differential fatty acid distribution upon fasting in yeast cells. (**A**) Schematic describing different metabolic states (fed and fasted condition) based on the duration of growth in the SCD medium. B) Conventional fluorescence images (top) show that BODIPY-C12 red co-localizes with BODIPY (493/503) under fed conditions indicating incorporation into LDs. The SMLM image (lower, left) shows dense BODIPY-C12 puncta in LDs and single molecule traces (lower, right) exhibit diffusion along cellular membranes. (**C**) Under fasted condition, BODIPY-C12 forms puncta in the cell periphery that do not co-localize with LDs (upper: left, middle, lower left). The SMLM image resolves the puncta and confined traces of BODIPY-C12 red (lower, right). (**D**) The radial distribution function (left) shows higher clustering of BODIPY-FAs upon fasting. The mean-square displacement vs. time plot of single molecule tracking (right) confirms immobilization of BODIPY-C12 upon fasting. Scale bar = 1 μm.

Figure 4: Imaging of BODIPY dyes in live mammalian U2OS cells. (**A**) Conventional fluorescence image (left) of BODIPY-C12 at 488 nm excitation. The corresponding SMLM image using D_{II} states (right) at 561 nm excitation shows the nanoscopic distribution of D_{II} states in U2OS cell. The insets show magnifications of lipid droplets (scale bar = 500 nm). (**B**) Conventional image of lysosomes in U2OS cells using LysoTracker green at 488 nm excitation (left). The corresponding SMLM image of immobile lysosomes (right, scale bar = 5 μm) at 561 nm excitation. Inset: SMLM image of an optically diffraction limited lysosome (scale bar 100 nm). The BODIPY-C12 images were recorded in live cell imaging solution at 23 °C. The images of lysosomes using LysoTracker green were recorded in non-fluorescent DMEM with 10% fetal bovine serum, 4 mM glutamine, 1 mM sodium pyruvate and 1% penicillin-streptomycin antibiotics at 37 °C.

DISCUSSION:

In this protocol, we demonstrated how conventional BODIPY conjugates can be used to obtain SMLM images with an order of magnitude improvement in spatial resolution. This method is based on exploiting previously reported, red-shifted D_{II} states of conventional BODIPY dyes, which transiently form through bi-molecular encounters. These states can be specifically excited and detected with red-shifted wavelengths and are sparse and short-lived enough for SMLM. By tuning the concentration of BODIPY monomers along with laser parameters, an optimal density of localizations and signal-to-noise can be achieved. We resolved the intracellular distribution and mobility of fatty acid analogs and neutral lipids with ~30 nm resolution (theoretical Thompson's formula) in living yeast cells under fed and fasted conditions. We also found that ~40% of BODIPY D_{II} states stay on for two or more data acquisition frames at 20 Hz, enabling single-molecule tracking to quantify mobility under different conditions. Our results show the differential localization and mobility of BODIPY-FAs upon fasting and suggest a protection mechanism against lipotoxicity. Our ability to track single BODIPY molecules and to resolve the size of LDs and BODIPY-FAs puncta below the optical diffraction limit under different metabolic states is only possible with the developed SMLM capability of conventional BODIPY conjugates.

The exact molecular mechanisms and pathways involved in the spatial regulation of the fatty acid distribution and uptake are the subject of our future studies. Furthermore, we extended the SMLM capability of conventional BODIPY conjugates to living mammalian cells by resolving BODIPY-FAs and lysosomes in U2OS cells.

Using D_{II} states of conventional BODIPY conjugates for SMLM has advantages over other probes since hundreds of different BODIPY variants are commercially available that label specific molecules or cellular compartments in living cells. The sample preparation is as easy as adding the dye at low (~100 nM) concentrations before imaging without any washing. In contrast to other PALM/STORM probes that bleach over time, BODIPY monomers are unaffected by the excitation of their D_{II} states and therefore provide an almost never depleting source for single molecule signals in long term imaging. Since D_{II} states arise due to spontaneous bi-molecular encounters, SMLM using D_{II} states requires no externally added buffer to induce blinking[23]. Similarly, there is no need for high-energy photo-activation as required for newly synthesized photo-activatable BODIPY versions[24] or SMLM of some conventional BODIPY dyes[21], which could be detrimental for cell health during long term imaging[25,26]. Moreover, SMLM with D_{II} states creates an inherent background suppression of non-specifically interacting probes because of the quadratic dependence of D_{II} states on the monomer concentration. Therefore, a higher contrast is achieved in SMLM images compared to traditional probes whose monomeric signal is detected.

BODIPYs exhibit a faint anti-Stokes fluorescence that enables the excitation of monomers and dimers with a single laser at high excitation power. On the one hand, this property can be exploited for simultaneous conventional fluorescence and SMLM imaging to track and resolve moving structures. On the other hand, it makes it harder to combine BODIPY D_{II} states with other probes for multi-color imaging as the BODIPY signal occupies two emission channels. However, multi-color imaging is possible when probes are carefully chosen as shown in **Figure 2B** with Sec63-GFP and BODIPY-C12 red. Similarly, sequential two-color SMLM is possible with other photo-activatable probes like mEos2 as shown in **Figure 2C**. Other possible combinations for two-color SMLM include the use of green BODIPY conjugates and a 640 nm excitable dyes such as JF646 bound to the halo tag[27].

In summary, we have presented an optimized protocol for SMLM using conventional BODIPY dyes to investigate the spatio-temporal distribution of fatty acids, neutral lipids and lysosomes the nanoscopic length scale in living yeast and mammalian cells. With minor modifications, this protocol can be equally applicable for SMLM with hundreds of other BODIPY conjugates across different cell types.

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DISCLOSURES:
The authors declare no competing interests.

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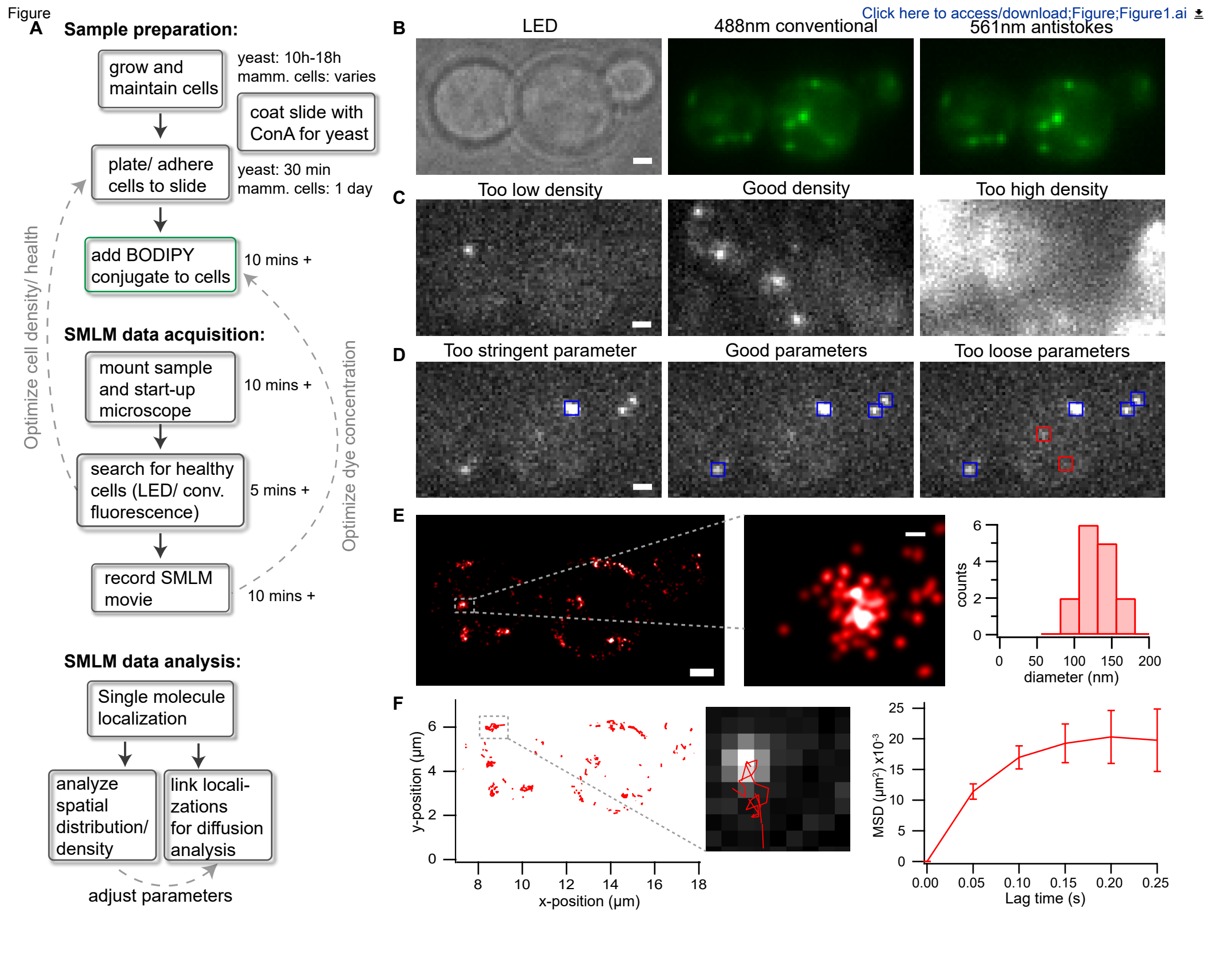
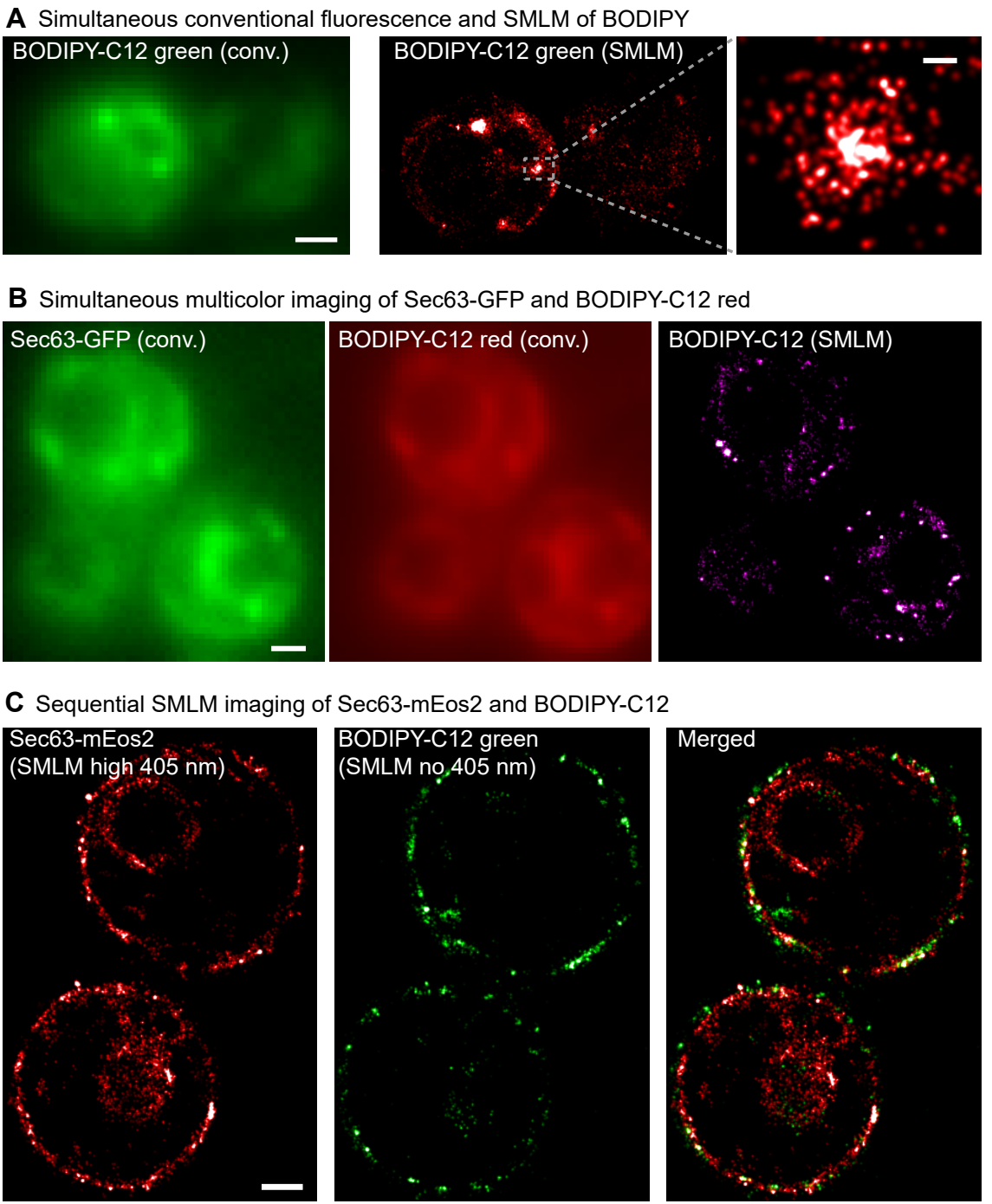
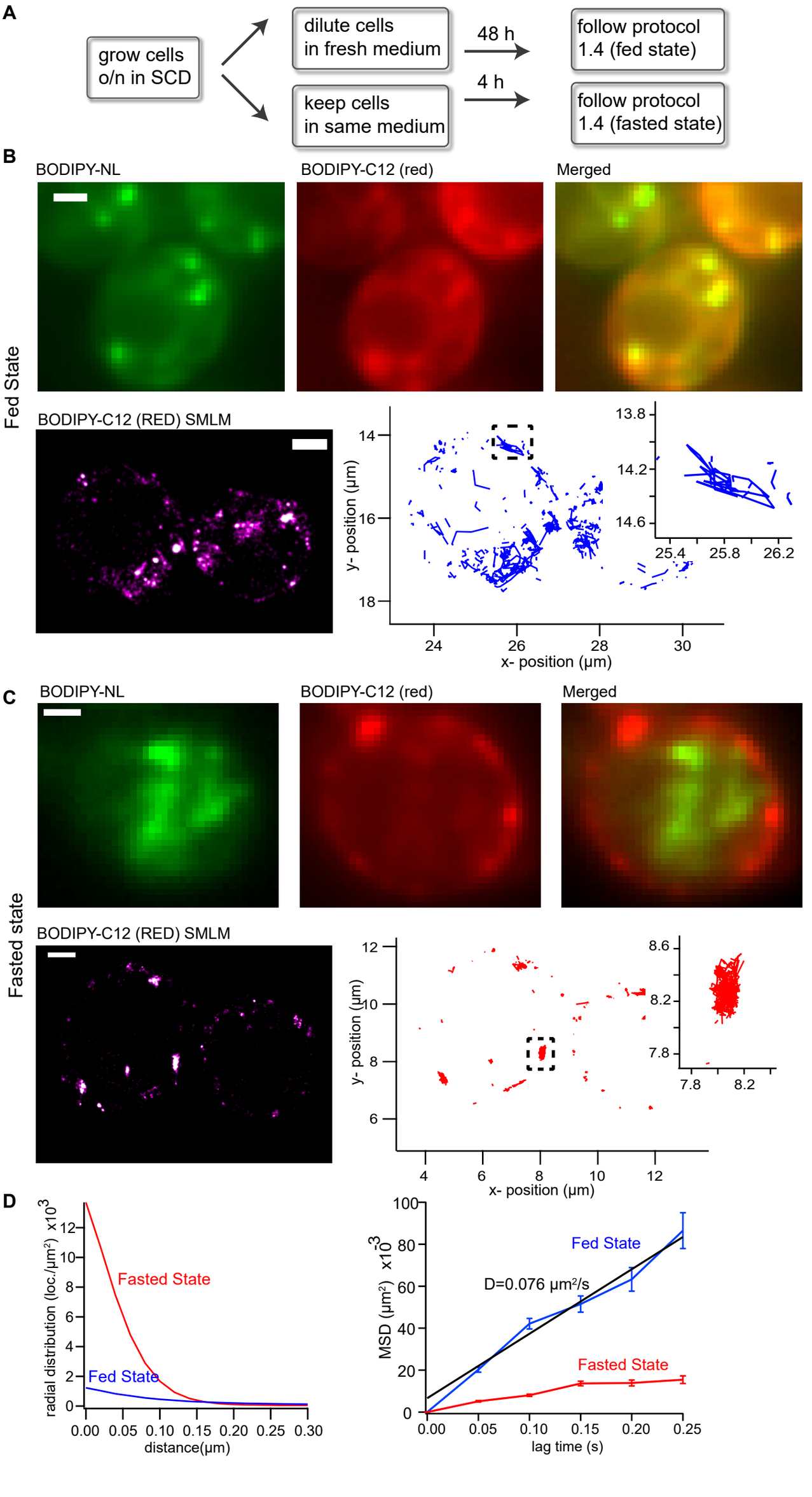
Figure







Figure

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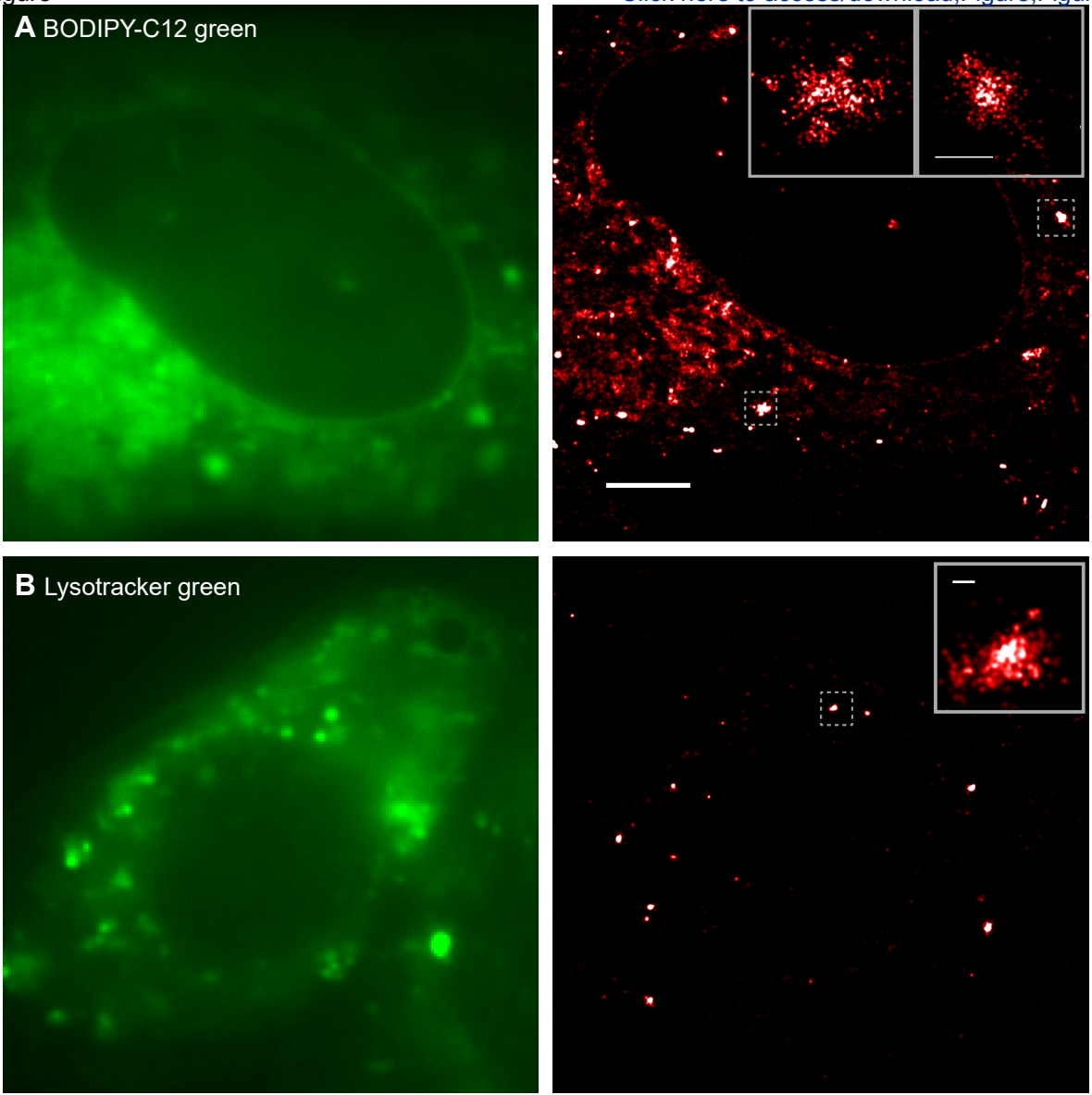

Name of Material/ Equipment	Company	Catalog Number
BODIPY C12	ThermoFisher	D3822
BODIPY C12 Red	ThermoFisher	D3835
BODIPY(493/503)	ThermoFisher	D3922
Concanavalin A	Sigma-Aldrich	C2010
Drop-out Mix Complete w/o nitrogen base	US Biological	D9515
Dextrose	Sigma-Aldrich	G7021
Eight Well	Cellvis	C8-1.58-N
Eight Well, Lb-Tek II	Sigma-Aldrich	
ET525/50	Chroma	
ET595/50	Chroma	
ET610/75	Chroma	
Fetal Bovine Serum (FBS)	Gibco	26140079
FF652	Semrock	
FF731/137	Semrock	
FluoroBrite DMEM	ThermoFisher	A1896701
Hal4000	Zhuang Lab, Harvard University	
Ixon89Ultra DU-897U	Andor	
Laser 405, 488, 561, 640 nm	CW-OBIS	
Insight3	Zhuang Lab, Harvard University	
L-Glutamine	Gibco	25030-081
live-cell imaging solution	ThermoFisher	A14291DJ
Lysotracker Green	ThermoFisher	L7526
Mammalian ATCC U2OS cells (Manassas, VA)	Dr. Jochen Mueller (University of Minnesota)	
Nikon-CFI Apo 100 1.49 N.A	Nikon	
Penicillin streptomycin	Gibco	15140-122
Sodium Pyruvate	Gibco	11360-070
T562lpxr	Chroma	
Trypsin-EDTA	Gibco	15400-054
W303 MATa strain	Horizon-Dharmacon	YSC1058
Yeast Nitrogen Base	Sigma-Aldrich	Y1250

zt405/488/561/640rdc

Chroma

Comments/Description
Green fatty acid analog
Red fatty acid analog
Neutral lipid marker
Cell immobilization on glass surface
Amino acids for SCD
Carbon source for SCD
Chambered Coverglasses
Chambered Coverglasses
Bandpass filter
Bandpass filter
Bandpass filter
Serum
Beam splitter
Bandpass filter
Cell culture medium
Data acquisition software
EMCCD camera for photon detection
Lasers for excitation
Single molecule localization software
Amino acid required for cell culture
Imaging buffer
Bodipy based lysosome marker
Oil immersion objective
Antibiotics
Supplement for cell culture
Beam splitter
Dissociation of adherent cell
Parental yeast strain
Nitrogen base without amino-acids

Quadband dichroic mirror

Dear editorial team, dear reviewers,

We are thankful for the positive assessment and the useful comments that helped us improve our manuscript. Below we provide a point-by-point response to editorial comments and comments from the peer reviewers that explain the changes we made to the revised manuscript.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We proofread our revised manuscript and hope there are no more typos.

• Protocol Language:

1) Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Example not in imperative voice: 2.5, 3.1.

We changed this in the revised manuscript.

2) Limit steps to no more than 4 sentences (2-3 actions) each.

• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:

1) 2.6: Is imaging to be conducted at this step, or are you using this as a segue into the next section? If imaging is being performed here, more details are required regarding the equipment and settings.

Imaging is not yet performed in this step. Hence we have removed this in the revised manuscript.

2) 4.1: This is more of a discussion than an instruction, please clearly describe the steps to be followed. Short discussions (1-2 sentences can be a note following an instructional step.

We clarified this in the revised version.

3) Section 5: mention explicit details regarding software actions.

We made those changes in the revised manuscript.

4) 5.1: Please specifically describe the actions using a single software.

5) 5.3-5.8: Unclear what is being done and how. If you wish to film this, software screenshots will need to be supplied.

We think it is best to film up to the image reconstruction. We like to remove this part for filming.

6) 5.4: How is the quality assessed?

This means assessing the quality of localizations to avoid detecting false localizations and autofluoresence due to cell death etc.

• Protocol Highlight: Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

We have highlighted the parts for filming.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

4) Notes cannot be filmed and should be excluded from highlighting.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We believe we have discussed the above aspects in our manuscript.

• Figures: Please remove the embedded figures from the manuscript. Figure legends, however, should remain within the manuscript text, directly below the Representative Results text.

We corrected this in the revised manuscript.

• References: Please spell out journal names.

We corrected this in the revised manuscript.

• Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are FluoroBrite, Nikon Ti-E, Matlab, Igor pro, Insight, etc

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

We have made sure to omit commercial sounding language as much as possible.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All figures are original.

Reviewer #1:
Manuscript Summary:
The authors describe using BODIPY dyes for single-molecule imaging/tracking. The article is clearly written and steps are easy to follow.

Major Concerns:
None. I think the article could be published. Minor issues the authors might want to address (if they agree that these suggestions would improve the paper) are listed below.

We thank the reviewer for the positive assessment and the helpful comments that helped us improve our manuscript.

Minor Concerns:
1. Since "single-molecule tracking" is in the title, I think it might help to have some more details regarding this. How long (in time or frames) might one expect to be able to track a BODIPY molecule?

Yes, we agree with the reviewer. Based on the on-time distribution, ~ 40 % of localizations (n=6569 localizations) appeared in two or more frames at 20 Hz, which we now mention in the manuscript.

2. Step 2.6 seems out of place. I think it should be listed under "4. Data Acquisition".

Yes, we now list it under “Data Acquisition”.

3. It wasn't immediately clear to me how the two-color imaging was being done. Perhaps add more detail -- are the two color channels split and sent to the same camera? two cameras used?

Thank you for pointing this out. The emission colors were first split and then send to different areas of the same camera chip. We clarified this in the manuscript.

4. Are there reasons for imaging at 20 Hz rather than some other speed? I imagine this will depend on how long the molecules emit. Do the authors know how long the dye molecules remain "on"?

We typically use 20 Hz for live cell PALM with mEos2 to achieve a high signal to noise ration. We chose 20 Hz for BODIPY to have a better comparison for photon counts with mEos2. Based on the average photon counts (977 per localization) and on time distribution (~60% for one acquisition frame), higher imaging speeds should be possible.

5. In some single-particle tracking applications, one can allow a particle/molecule to be missing from one frame but still link that molecule in the surrounding frames. Would that be okay to do with BODIPY?

We agree with the reviewer and for some mEos2 tracking application we indeed allow for a fluorophore to e.g. be dark in one frame and link it to the next frame. However, since BODIPY D|| states arise from transient bi-molecular encounters of dense monomers, it will be unlikely to detect/difficult to link the fluorescence from same two monomers once they have disassociated. So we think it is better not to link molecules once they are off.

6. Line 195 reads "... The later will offer a brighter conventional signal." I'm not sure I understand why exactly. Could the authors explain this?

Thank you for pointing out this confusion. BODIPYs also exhibit anti-stokes fluorescence that comes from the excitation of the fraction of monomers in higher vibrations states that are much less in number compared to stokes fluorescence that comes from monomers in lower vibrational state. Hence, 488 nm excitation of monomers (Stokes fluorescence) leads to brighter signal compared to 561 nm excitation (Anti-Stokes fluorescence). We clarified this in the revised manuscript.

Reviewer #2:
Manuscript Summary:

The manuscript submitted by Adhikari et al., "Conventional BODIPY conjugates for live-cell super-resolution microscopy and single-molecule tracking," serves as a detailed method for the paper "Single-molecule localization microscopy and tracking with red-shifted states of conventional BODIPY conjugates in living cells" published on Nature Communications in 2019. The authors used transient red-shifted BODIPY ground-state dimers to localize lipid droplets, fatty acids, and lysosomes in living yeast. Further, they resolved fatty acids and neutral lipids with ~30 nm resolution using BODIPY-C12 red in combination with lysotracker-green. The work also demonstrates two color localization of Sec63 molecules and fatty acids in single cells using BODIPY-C12 green and mEos2 under different metabolic states. Overall, this is a very interesting work that provides applications in other biological processes in living cells. This manuscript is suitable for JoVE publication with minor revisions.

Major Concerns:
None

We thank the reviewer for the positive assessment and the helpful comments that helped us improve our manuscript.

Minor Concerns:
1. On page 2 line 109 and line 113, the authors did not specify the incubation temperature of ConA. Are these steps performed at room temperature?

Yes, these steps are at room temperature and we added the incubation time and temperature in the revised manuscript.

2. On page 2 line 131, is the 8-well plate glass-bottom? What is the vendor?

Yes, it is a glass-bottom. We used the 8-well from LabTek (Sigma-Aldrich) and Cellvis. This information is provided in the separate materials sheet that JoVE requires.

3. On page 3 line 150, the quad-band dichroic mirror is missing 488 band.

We thank the reviewer for catching this. We corrected this in the revised manuscript.

4. On page 3 line 166, which microscope stage heater/incubator was used? The vendor information should be included.

We included this information in the separate materials sheet.

5. On page 5 line 229 and 244, the authors should provide the equations used to fit curves. What is the equation used to determine radial distribution function p(r)? Which equation was chosen to determine the average diffusion coefficient? In Fig 1F, the MSD reached a plateau at long lag time. Was a confined diffusion equation used to determine the average diffusion coefficient?

Thank you for pointing this. We used MSD=4DΔt + 2σ^2 to determine diffusion coefficient (D) from MSD curves. For ρ(r), we computed density of localizations at a distance r_i as $\rho(r_i)= H(r_i)/(\pi((r_i+dr)^2-r_i^2)$ by making histogram $H(r_i)$ with bin width dr from counts of pairwise distances of unique pairs. This information is now included in the revised manuscript. In Fig 1F, we did not fit any equation to calculate the diffusion co-efficient. We just made a qualitative statement on the sub-diffusive nature of BODIPY (493/503) inside LDs.

6. Which method was used to estimate the 30 nm resolution?

We used theoretical resolution estimation using Thompson's formula and indicate this in the revised manuscript.