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Conducting Multiple Imaging Modes with One Fluorescence Microscope

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TITLE:

Conducting Multiple Imaging Modes with One Fluorescence Microscope

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SHORT ABSTRACT:

Here we present a practical guide of building an integrated microscopy system, which merges conventional epi-fluorescent imaging, single-molecule detection-based super-resolution imaging, and multi-color single-molecule detection, including single-molecule fluorescence resonance energy transfer imaging, into one set-up in a cost-efficient way.

LONG ABSTRACT:

Fluorescence microscopy is a powerful tool to detect biological molecules *in situ* and monitor their dynamics and interactions in real-time. In addition to conventional epi-fluorescence microscopy, various imaging techniques have been developed to achieve specific experimental goals. Some of the widely used techniques include single-molecule fluorescence resonance energy transfer (smFRET), which can report conformational changes and molecular interactions with angstrom resolution, and single-molecule detection-based super-resolution (SR) imaging, which can enhance the spatial resolution approximately ten- to twentyfold compared to diffraction-limited microscopy. Here we present a customer-designed integrated system, which merges multiple imaging methods in one microscope, including conventional epi-fluorescent imaging, single-molecule detection-based SR imaging, and multi-color single-molecule detection, including smFRET imaging. Different imaging methods can be achieved easily and reproducibly by switching optical elements. This set-up is easy to adopt by any research laboratory in biological

sciences with a need for routine and diverse imaging experiments at a reduced cost and space relative to building separate microscopes for individual purposes.

INTRODUCTION

Fluorescence microscopes are important tools for the modern biological science research and fluorescent imaging is routinely performed in many biology laboratories. By tagging biomolecules of interest with fluorophores, we can directly visualize them under the microscope and record the time-dependent changes in localization, conformation, interaction, and assembly state *in vivo* or *in vitro*. Conventional fluorescence microscopes have a diffraction-limited spatial resolution, which is ~200 - 300 nm in the lateral direction and ~500 - 700 nm in the axial direction[1,2], and are, therefore, limited to imaging at the 100s of nanometers-to-micron scale. In order to reveal finer details in the molecular assembly or organization, various SR microscopies that can break the diffraction limit have been developed. Strategies used to achieve SR include non-linear optical effects, such as stimulated emission depletion (STED) microscopy[3,4] and structured illumination microscopy (SIM)[5-7], stochastic detection of single molecules, such as stochastic optical reconstruction microscopy (STORM)[8] and photoactivated localization microscopy (PALM)[9], and a combination of both, such as MINFLUX[10]. Among these SR microscopies, single-molecule detection-based SR microscopes can be relatively easily modified from a single-molecule microscope set-up. With repetitive activation and imaging of photoactivatable fluorescent proteins (FPs) or photo-switchable dyes tagged on biomolecules of interest, spatial resolution can reach 10 - 20 nm[11]. To gain information on molecular interactions and conformational dynamics in real-time, angstrom-to-nanometer resolution is required. smFRET[12,13] is one approach to achieve this resolution. Generally, depending on the biological questions of interest, imaging methods with different spatial resolutions are needed.

Typically, for each type of imaging, specific excitation and/or emission optical configuration is needed. For instance, one of the most commonly used illumination methods for single-molecule detection is through total internal reflection (TIR), in which a specific excitation angle needs to be achieved either through a prism or through the objective lens. For smFRET detection, emissions from both donor and acceptor dyes need to be spatially separated and directed to different parts of the electron-multiplying, charge-coupled device (EMCCD), which can be achieved with a set of mirrors and dichroic beam splitters placed in the emission path. For three-dimensional (3-D) SR imaging, an optical component, such as a cylindrical lens[14], is needed to cause an astigmatism effect in the emission path. Therefore, homebuilt or commercially available integrated microscopes are, usually, functionally specialized for each type of imaging method and are not flexible to switch between different imaging methods on the same set-up. Here we present a cost-effective, hybrid system that provides adjustable and reproducible switches between three different imaging methods: conventional epi-fluorescent imaging with diffraction-limited resolution, single-molecule detection-based SR imaging, and multi-color single-molecule detection, including smFRET imaging (**Figure 1A**). Specifically, the set-up presented here contains fiber-coupled input lasers for multi-color excitation and a commercial illumination arm in the excitation path, which allows programmed control of the excitation angle, to switch between epi-mode and TIR mode. In the emission path, a removable homebuilt cylindrical lens cassette is placed within the microscope body for 3-D SR imaging, and a commercial beam splitter is placed

before an EMCCD camera that can be selectively enabled to detect multiple emission channels simultaneously.

PROTOCOL:

1. Microscope Design and Assembly

1.1. Excitation path

NOTE: The excitation path includes lasers, differential interference contrast (DIC) components, the microscope body, and its illumination arm.

1.1.1. Prepare a vibration-isolated optical table. For example, a structural damping table of 48 x 96 x 12'' gives enough space for all the components.

NOTE: Build the set-up in a room with temperature control (*e.g.*, 21.4 ± 0.55 °C). Temperature stability is critical to maintaining the optical alignment.

1.1.2. Install a microscope body that is equipped with an illumination arm for optical fiber connection, a 100X oil-immersion TIRF objective lens, and DIC components.

1.1.3. Place four laser heads (647 nm, 561 nm, 488 nm, and 405 nm, encircled in **Figure 1B**) and their heat sinks on the optical table, and make sure the emitted laser beams have the same height and are as short as possible to ensure good stability (*e.g.*, 3'').

NOTE: If a laser head sits at a shorter height than other lasers, put an aluminum plate with adequate thickness underneath it. Always ensure maximum contact between the heat sinks and the optical table for the best heat dissipation (**Figure 1B**). The lasers need to be powerful enough for SR imaging. See **Table of Materials**. It is recommended to have laser clean-up filters in front of diode lasers.

1.1.4. Install a data acquisition card through a peripheral component interconnect (PCI) interface in a workstation and connect lasers with this card. Control the lasers' ON/OFF behaviors by transistor-transistor logic (TTL) output, and their power adjustment by the analog output of this card (**Figure 1C**). Install a proper microscopy imaging software (either commercial or homebuilt) to control the data acquisition card, as well as the microscope body.

1.1.5. Mount the mirrors and dichroic beam splitters (590, 525, and 470 long pass filters) to their respective mounts. Use very stable mirror mounts for the mirrors. Use circular splitters with retaining rings to avoid any bending of the dichroic beam splitters (**Figure 1D**).

1.1.6. Place the mirrors and dichroic beam splitters on the optical table to combine the laser beams (**Figure 1B**). To achieve the most stable alignment, make the whole arrangement as compact as possible, and use 1''-thickness optical posts. Arrange the lasers so that the shorter

wavelength lasers are closer to the optical fiber coupling (**Figure 1B**) since short-wavelength lights dissipate more in the air.

1.1.7. Combine laser beams into a single-mode optical fiber. To do so, build a fiber coupler in a cage system through the following steps:

1.1.7.1. Mount a fiber adapter plate in a z-axis translation mount (**Figure 1E**, leftmost panel).

1.1.7.2. Mount an achromatic doublet lens (focal length = 7.5 mm) in a cage plate (**Figure 1E**, second panel from the left).

1.1.7.3. Connect the two parts above by extension rods to form a cage. Mount the cage on the optical table with mounting brackets on the 1''-thick optical posts (**Figure 1E**, middle panel).

1.1.7.4. Align the 647-nm laser first with a single-mode optical fiber (FC/APC end to the coupler).

Note: A rough alignment using a multi-mode optical fiber before using the single-mode optical fiber may help the alignment process. Adjust the angles of the mirrors and dichroic beam splitters (**Figure 1D**, by adjustment knobs), as well as the distance between the achromatic doublet lens and the fiber adapter plate (**Figure 1E**, by adjusting z-axis translation mount) to gain maximum laser power output through the fiber.

1.1.7.5. Once the alignment of the first laser is done, temporarily install a pair of irises and align the rest of the lasers one by one (**Figure 1F**). Check the alignment efficiency with a power meter.

1.1.7.6. Leave one iris in the front of the adapter plate to reduce the reflections of the lasers.

NOTE: Strong back reflections can reduce the lifetime of laser sources. Optionally, an optical isolator can be installed in front of each laser head to remove the reflections completely.

1.1.8. Connect the other end of the optical fiber to the illumination arm of the microscope (**Figure 1H**).

1.1.9. Design and install the "magnification lens (mag lens)" through the following steps:

NOTE: The lasers can be used for epi-fluorescence imaging of the sample, but the narrow beam size of each laser limits the illuminated area of the sample to an area several times smaller than the actual size of the corresponding camera sensor, especially for the newer cameras (with 18.8 mm in diagonal length compared to the conventional 11.6-mm length). Thus, it is desirable to expand the beam to achieve a larger and flatter illumination of the sample.

1.1.9.1. Design the mag lens, which can fit into the illumination arm (**Figure 1H**).

NOTE: The design of the mag lens depends on where it will be installed. **Figure 1H** shows an example of the installation in the illumination arm, but it can be installed in any spot after the laser beams are collimated (see **Discussion**). Design it with a computer-aided design and drafting software.

1.1.9.2. Place two achromatic lenses, one concave (focal length = f_1), and one convex (focal length = f_2) in the home-made mag lens holder (**Figures 2A** and **2C**), with the distance equal to the sum of their focal lengths, $f_1 + f_2 = (-25) + 50 = 25$ mm (**Figure 2B**).

NOTE: With this choice of focal lengths, the mag lens expands the beam by $f_2/|f_1| = 2$ folds. The mag lens provides versatility. It can be removed to resume regular illumination without expanding the beams (**Figure 2D**) or inserted in the reverse direction to focus the laser beam to achieve a stronger excitation intensity.

2.2. Emission path

NOTE: The emission path is composed of a removable cylindrical lens, a barrier filter wheel, an emission splitter, and an EMCCD camera (**Figure 1G**). To attain the best point spread function (PSF) of single molecules, the DIC prism is put away from the objective lens.

2.2.1. Custom-design the cylindrical lens (3-D lens) cassette, which can fit into the manual DIC analyzer insert slot in the microscope body (**Figure 3C**).

NOTE: This design does not compromise the DIC analyzer since an analyzer block can be inserted in the filter turret.

2.2.2. Place the 3-D lens of 10 m of focal length in the cassette and insert it into the emission beam path to create the astigmatism effect necessary for extracting the z coordinate of every single molecule[14].

NOTE: Optionally, the 3-D lens cassette can be placed in or out of the emission path (**Figure 3C**).

2.2.3. Install a multi-band dichroic beam splitter in the filter turret inside of the microscope body.

2.2.4. Install emission filters.

NOTE: The emission filters are chosen depending on the preferred fluorophores. Depending on the imaging module, emission filters placed in different locations are used as described below:

2.2.4.1. For sequential multi-color epi-fluorescence imaging or SR imaging, use emission filters placed in the barrier filter wheel connected next to the microscope body to minimize the vibration in the microscope body during channel switch (**Figure 1G**).

2.2.4.2. For simultaneous multi-color detection (*e.g.*, smFRET experiments), place another filter set in an emission splitter (check step 4 for details).

NOTE: Usually, a commercial emission splitter has two switchable modes (*i.e.*, "engaged" or "bypass" modes). To separate emission lights chromatically for simultaneous multi-color imaging ("engaged" mode), a filter cube holding two dichroic beam splitters and three emission filters is used ("triple cube," **Figures 4C** and **4D**). An empty slot in the barrier filter wheel is used in combination with the triple cube. On the other hand, for sequential multi-color imaging, the triple cube is replaced by a cube that just has a mirror inside ("bypass cube," **Figures 4A** and **4D**).

2.2.5. Install the EMCCD camera as the last part of the emission path. Utilize the USB-PCI connection to achieve a fast frame rate.

NOTE: An EMCCD camera is recommended for the most sensitive single-molecule detection, but an advanced sCMOS camera can be an alternative.

3. Diffraction-limited Imaging with Epi-excitation

3.1. Adjust the excitation lasers' incidental angle to epi-mode in the illumination arm.

3.2. Disengage the 3-D lens if engaged (**Figure 3C**, right panel).

3.3. Insert the bypass cube in the emission splitter (**Figures 4A** and **4E**, bottom panel).

3.4. (Optional) Insert the mag lens for a broadened illumination area (**Figure 2D**, left panel).

NOTE: With the use of a mag lens and a 100X oil-immersion objective lens, about 91 x 91 μm^2 can be illuminated evenly, eliminating the need to use a white light source and multiple filter cubes.

3.5. Use a microscopy imaging software to take multi-channel, and/or Z-stack, and/or time-lapse images.

NOTE: There are multiple programs available for microscopy imaging, not only from microscope manufacturers but also from third-party companies or open source developers.

4. Multi-channel Single-molecule Imaging Including smFRET

NOTE: Move to an "empty" position in the barrier filter wheel, so that all the emission with any wavelength can reach to the second set of filters/dichroic beam splitters in the emission splitter.

4.1. To set up multi-color single-molecule detection of surface-immobilized molecules[15] using TIRF excitation, including smFRET measurement, adjust the excitation lasers' incidental angle to the TIRF angle. Disengage the mag lens and the 3-D lens.

4.2. Engage the three-channel mode in the emission splitter (**Figure 1G**) through the following steps:

4.2.1. Replace the bypass cube with a "calibration cube" that allows light to go through all channels (**Figures 4B** and **4E**).

4.2.2. Turn on the camera under DIC (*i.e.*, no emission filter in the barrier filter wheel) and adjust the aperture of the emission splitter until three fully separated channels appear on the screen.

NOTE: Conduct this step with the room light lit, to visualize all the channels.

4.2.3. Turn the vertical/horizontal adjustment control knobs on the emission splitter and roughly align the three channels (**Figures 4E** and **4F**).

4.2.4. Turn off the camera and replace the calibration cube with a triple cube (**Figures 4C** and **4E**).

4.2.5. Place a sample with 100-nm multichannel beads on top of the 100X objective lens and focus on the sample.

4.2.6. Turn on the camera and the 488-nm laser, zoom in on one of the bright beads, and finely align the three channels by turning the adjustment control knobs again (**Figures 4E** and **4G**).

NOTE: 100-nm multichannel beads emit different wavelengths of light upon 488-nm excitation, enabling the three-channel alignment.

4.3. Turn on the camera and lasers, focus, and find a good position with a reasonable spot density. Adjust the laser power and exposure time to achieve acceptable signal-to-noise and photobleaching levels. Use the microscopy imaging software to take time-lapse images.

5. SR Imaging

NOTE: This is single-molecule detection-based SR microscopy.

5.1. To set up SR imaging, insert the 3-D lens and remove the mag lens. Set the exposure time of the camera in the appropriate laser channels (*e.g.*, 5 - 60 ms). Determine and manually set the optimal excitation lasers' incidental angle to be the TIRF angle.

5.2. Place the sample in SR imaging buffer[16]. Allow the buffer to equilibrate for at least 10 min before imaging.

Note: SR imaging buffer expires after roughly 1 h, so make new SR imaging buffer accordingly.

5.3. Take a DIC image before SR imaging. In order to find the proper objective height for SR, which optimizes the astigmatism effect, use DIC imaging to find the middle plane of the cells. Identify the plane by the height at which the cells transition from "light" to "dark" images and appear to become transparent (**Figures 5A**, **5B**, and **5C**). Once the desired focal plane is determined, engage the z-drift correction system (**Figure 1A**).

5.4. Conduct SR imaging. Change the 405-nm laser power to maintain a reasonable density of 'blinking-on' spots.

NOTE: While it is possible to change the 405-nm laser manually, it is more convenient to run a programmed data acquisition code to maintain the density of "blinking-on" spots. Here is an example of how it is conducted automatically. The source code is available upon request (**Figure 6**).

5.4.1. Start the imaging acquisition with 0 W/cm^2 violet laser power.

5.4.2. Count the number of blinking-on spots in a certain period.

5.4.3. Modulate the violet laser power so that the number of blinking-on spots is kept above a user-defined "counting threshold" in the field of view. Increase the violet laser power when the number of blinking-on spots drops below the counting threshold.

5.4.4. Terminate the acquisition when the number of blinking-on spots drops below the counting threshold using the maximum violet laser power.

NOTE: The maximum can be set differently depending on the sample brightness, but no higher than 130 W/cm^2. Depending on the actual goal of the SR imaging, this automatic acquisition code can be manually terminated at any desired point.

5.5. Check the blinking behavior and PSFs of the spots soon after beginning the acquisition.

NOTE: If the blinking behavior is not ideal, change the excitation lasers' incidental angle or replace the imaging buffer. Expect a sampling of "vertical", "horizontal", and "diamond" shapes of PSF, representing fluorophores from below, above, and within the focal plane, respectively (**Figure 5D**). If most spots show either vertical or horizontal PSFs, then the focal plane is off from the center of the cells, so terminate the experiment and adjust the focal plane again. A presence of air bubble in the immersion oil or other local factors can affect the PSFs' quality, so it may be necessary to replace the oil or change to a different imaging area of the sample.

5.6. For the data analysis, use either open source (in NIH ImageJ plugins) or commercially available codes to detect centroids of each spot in each imaging frame and extract z-values of each spot from x- and y-widths[14].

NOTE: In this report, a source code originally developed in one of the earliest single-molecule detection-based SR[8] was modified for 3-D detection[16] and was used.

5.7. In the case of two-color imaging, image the fluorophore with the longer excitation wavelength, followed by the one with the shorter excitation wavelength. Run the automated acquisition code similarly to the one described in step 5.4, but with a different imaging laser.

NOTE: Chromatic aberration should be corrected between images with different fluorophores (*e.g.*, red dye and yellow-green dye). Here are the steps.

5.7.1. Immobilize multiple 100-nm multichannel beads on the glass coverslip, avoiding forming clusters.

5.7.2. Take images of them in different excitation channels.

5.7.3. Extract their (X, Y, Z) coordinates by software (step 5.6).

5.7.4. Plot $\Delta X_i = X_{1i} - X_{2i}$ and $\Delta Y_i = Y_{1i} - Y_{2i}$ (i is for different beads, and 1 and 2 are different color channels) separately and fit them with proper functions. Save the functions.

NOTE: Linear functions are sufficient in most cases. Once these functions are determined, this measurement does not have to be repeated each time of imaging.

5.7.5. In the actual two-color SR imaging of a sample of interest, apply the functions to correct (X, Y) chromatic aberration. For z-directional chromatic aberration, conduct it by obtaining $\Delta Z = Z_1 - Z_2$ for multichannel beads or known reference multichannel samples seeded together with the sample of interest.

NOTE: Unlike (X, Y) chromatic aberration, z-directional chromatic aberration is not well-reproducible in each experiment, mainly due to incomplete z-directional focus maintenance upon channel switching. Thus, it is recommended to conduct the correction each time. $\Delta Z = Z_1 - Z_2$ is mostly independent of (X, Y), so just a few beads or reference samples would be sufficient per each sample area of interest. Plot the constructed final two-color SR images in a 3-D visualization software and check ΔZ manually.

REPRESENTATIVE RESULTS:

This microscope allows flexible and reproducible switching between different imaging methods. Here we show sample images collected with each imaging module.

Figure 5D demonstrates the PSF of the blinking-on molecule during the SR acquisition. Thousands of such images are reconstructed to generate the final SR image (**Figure 5E**). **Figure 5E** shows the same bacterial regulatory RNAs as shown in the epi-fluorescence image in **Figure 7A**. **Figure 7** shows the representative epi-fluorescence images of a small regulatory RNA in *Escherichia coli* cells and an mRNA in U2OS mammalian cells. Both RNAs are labeled with fluorophore-tagged

DNA oligo through *in situ* hybridization[17]. **Figure 8** shows the representative smFRET measurement of folded RNA molecules labeled with donor dye (green dye) and acceptor dye (red dye). The complexes are immobilized on the microscope slide, and excited by TIRF illumination. Fluorescence intensity trajectories can be extracted from individual single molecules, generating FRET efficiency as a function of time.

FIGURE LEGENDS:

Figure 1: The design of the microscope set-up. (**A**) This panel shows a diagram of the set-up. M = mirror, DBS = dichroic beam splitter, LCF = laser clean-up filter, I = iris, L = lens, AP = adapter plate, ZTM = z-axis translational mount, OF = optical fiber, OFC = optical fiber coupling, CL = cylindrical lens, TL = tube lens, EF = emission filter(s), Obj = objective lens, and SM = step motor. The z-drift correction system moves the step motor on the nosepiece of the objective lens to the opposite direction of the z-drift, which is calculated by the infrared (IR) signal generated by its own LED and detected by its own detector. (**B**) This panel shows the laser excitation assembly. Four lasers are combined through mirrors and dichroic beam splitters and are then directed to an optical fiber through a focusing lens and an adapter plate sitting on a translational mount (bottom of the picture). (**C**) This panel shows the laser modulation. Two Bayonet Neill-Concelman (BNC) cables are connected to each of the laser (either the head or the controller, depending on the manufacturer) for TTL and analog modulation (leftmost panel). BNC cables are combined into a single cable (middle panel) which is connected to a data acquisition card in a workstation (rightmost panel) for computer control. (**D**) This panel shows the mirror and dichroic beam splitter mounts. (**E**) Building a fiber coupler in a cage system. A fiber adapter plate (AP) is mounted in a z-axis translation mount (ZTM) so that its distance to the achromatic double lens (L1, side view shown) can be modulated. AP and ZTM have matching threads, same as L1 and the cage plate. (**F**) A pair of irises (encircles) are installed during the alignment procedure for multiple lasers. They are used to ensure that the 647-nm laser (left panel) and the 561-nm laser (right panel) go through the same path. (**G**) A partial portion of the emission path is shown. Outside of the microscope body, the emission filter wheel, the emission splitter, and the EMCCD camera are installed in order. (**H**) This panel shows the illumination arm assembly. The mag lens is inserted into the illumination arm. The excitation laser beams are sent to the illumination arm through the optical fiber and the optical fiber coupling (at the right-hand side of the picture).

Figure 2: The mag lens. (**A**) This panel shows orthographic projections of the holder housing lenses for magnifying laser beams (unit: mm). This design is intended to be fit into the illumination arm. (**B**) This panel shows an internal drawing of the hole in the holder where two lenses get in. L1 is a concave lens and L2 is a convex lens, and the distance between them is the sum of their focal lengths. (**C**) This is a photo of the mag lens. (**D**) The mag lens can be inserted in the illumination arm to expand or focus the laser beams (left) or can be removed to keep the laser beams unchanged (right).

Figure 3: The 3-D lens. (**A**) This panel shows orthographic projections of the holder having a blank circular hole for the bypass mode and a rectangular hole for holding the cylindrical lens (unit: mm). This design is intended to be fit to the DIC analyzer slider in a microscope body. (**B**)

This is a photo of the 3-D lens. (**C**) The cylindrical lens can be engaged in the emission beam path to cause PSFs with astigmatism (left) or can be disengaged for the bypass mode, keeping the PSFs intact (right).

Figure 4: Multi-channel alignment of the emission splitter. Schemes of optics and light path are shown in the emission splitter with (**A**) a bypass cube, (**B**) a calibration cube, and (**C**) a triple cube. M = mirror. The bypass cube has a mirror inside (M5) and is supposed to be used with an emission filter (EF) in the barrier filter wheel. The calibration cube has beam splitters (BS) that allow lights to go through all channels. The triple cube has two dichroic beam splitters (DBS), as well as three emission filters. (**D**) These are photos of the three cubes. (**E**) The internal of the emission splitter is shown without a cube (upper panel) and with a cube (lower panel) sliding in. M3 is behind M4 and not captured in the photo. Control knobs for the mirrors are on top of the emission splitter (upper panel). (**F**) This panel shows channel alignment using the calibration cube under DIC. (**G**) This panel shows a fine alignment of the green (top panel) and red (middle panel) channels using 100-nm multichannel beads and a triple cube. A merged image of the two channels is also shown (bottom panel).

Figure 5: Representative SR image acquisition. These panels show a representative DIC image (**A**) below, (**B**) at, or (**C**) above the central plane of the cells. (**D**) This panel shows an example of the PSF of the blinking-on fluorophores. The orange circle surrounds a "vertical" PSF. The green circle surrounds a "horizontal" PSF. The blue circle surrounds a diamond-shaped PSF, representing fluorophores at a focused plane. (**E**) This panel shows a representative reconstructed SR image. A small regulatory RNA is labeled with fluorescence *in situ* hybridization with red dye. The white borders mark the edges of the cells.

Figure 6: A programmed data acquisition code for SR imaging. In this programmable acquisition module, various execution commands and microscope control functions are listed in the left panel and can be selected to create programmed acquisition code. The commands are executed sequentially from top to bottom. This screenshot shows an example where a predefined certain number of iterated imaging acquisitions are conducted until the acquisition is posed and the number of spots in three sequential image frames is calculated. If the average number of these spots are above the threshold (defined as 50% of the number of cells in bacterial imaging), the image acquisition continues in the same loop. If below the threshold, then the image acquisition continues in the next loop where stronger violet laser power is used (cut at the bottom of the screenshot).

Figure 7: Representative epi-fluorescence images. (**A**) This panel shows a small regulatory RNA in *E. coli* cells. (**B**) This panel shows an mRNA in U2OS mammalian cells. RNAs are labeled with red dye and blue dye through fluorescence *in situ* hybridization.

Figure 8: Example of smFRET imaging. (**A**) Samples were excited with the 561-nm laser. (**B**) Emissions from the green and red dyes are collected simultaneously and shown as green (right) and red (left) spots, respectively. (**C**) This panel shows the representative fluorescence intensity *vs.* time trajectories of green dye (green) and red dye (red) from one molecule. (**D**) This panel

shows the FRET efficiency *vs.* the time trajectory (solid blue line) calculated as $I_{Acceptor}/(I_{Donor} + I_{Acceptor})$ from one molecule of the sample. The FRET trace is fitted with the Hidden Markov Model (dashed red line).

DISCUSSION

This hybrid microscope system eliminates the need to purchase multiple microscopes. The total cost for all parts, including the optical table, table installation labor, software, and workstation, is about $230,000. Custom-machined parts, including the mag lens and 3-D lens, cost around $700 (the cost depends on the actual charges at different institutes). Typical commercially available integrated systems for single-molecule detection-based SR microscopy cost more than $300,000 ~ 400,000 and are not readily available for smFRET measurement, or epi-imaging for a reasonable field of view, without white light source. Thus, the approach presented here can achieve imaging capabilities equivalent to multiple microscopes at a significantly reduced cost.

This modulated microscope system can be based on microscope bodies from different manufacturers. The 3-D lens can potentially be installed in any position in the emission path, including inside the emission splitter, within the microscope body, or in available space between the filter wheel and the objective nosepiece. However, the physical location of the 3-D lens will determine its focal length for achieving the best astigmatism effect for 3-D SR imaging. We recommend starting with a 10-m focal length. The mag lens is essentially a beam expander installed in the excitation path. If the excitation lasers are air-coupled (*i.e*., without optical fiber), it is trivial to install such an expander in any spot after the laser beams are collimated. If the excitation lasers are fiber-coupled, then look for extra slots to install two lenses (one concave and the other one convex). For example, many commercial illumination arms have slots for neutral-density filters. Find two lenses with the sum of their focal lengths equal to the distance between the two slots. Then, they can be inserted to function as a mag lens. Alternatively, a mount for the field diaphragm slider can be another place, if available. Building the mag lens in a removable cassette has the additional advantage of enabling one to focus the excitation lasers to achieve higher power, which may be necessary for SR imaging, simply by flipping the orientation of the mag lens.

In this report, we demonstrated flexible switching between three different imaging techniques using a modulated microscope. This set-up can be used for broader and more advanced applications. For example, the SR configuration can be used for single-particle tracking in live cells, on photo-switchable or photoactivatable fluorescent protein-tagged biomolecules of interest[18,19]. The smFRET configuration can be used to study molecular interactions in *in vivo* systems[20]. The emission splitter-based multi-channel detection can be combined with the SR configuration to perform two-color SR imaging or single-particle tracking simultaneously[21].

This set-up can be further expanded to enable more modulated components. For example, another layer of filter turret and illumination compartment can be added, so that additional excitation/emission paths can be created for additional channels, such as one with an infrared (IR) laser for imaging samples labeled with IR dyes. In addition to allowing the added imaging channel, IR lasers can be used to correct the stage drift in SR imaging either during imaging

acquisition or during the post analysis. For drift correction during imaging acquisition, if the z-drift correction system is not available from the microscope manufacturer, an alternative system with IR laser can be either homebuilt[14] or acquired from third-party companies. For post-acquisition drift correction, an IR laser can be used for tracking fiducial markers.[22]

Single-molecule detection-based SR microscopy requires two sets of software, one for data acquisition and one for data analysis. For data acquisition, even a simple homebuilt software that takes images through the camera while controlling the lasers' ON/OFF behaviors can work to generate SR images, while it is possible to manually modulate the 405-nm laser power, *e.g.,* by rotating a gradient neutral-density filter installed in front of the laser. However, this way of conducting the experiment is dependent on the experimenter's subjective judgment of the density of blinking-on spots. Thus, this way is not objective and may not be suitable to be used for the quantification of SR imaging data. The data acquisition code used here includes spot detection/a counting algorithm while the data acquisition is ongoing, enabling automatic modulation of the 405-nm laser power based on the density of blinking-on spots (**Figure 6**). Nowadays, some microscope manufacturers provide programmable imaging modules, so one can utilize these, or look for plugins from open sources like Micro-Manager. For the data analysis, it is possible to use commercially available analysis modules from microscope manufacturers or look for plugins from open sources like ImageJ.

In summary, the set-up presented here provides high versatility and easy switching between different imaging configurations at a reduced cost and space. This set-up is relatively easy to adopt by any research laboratory in biological sciences with a need for routine and diverse imaging experiments.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure1

Click here to download Figure Fig1_setup_v7.tif

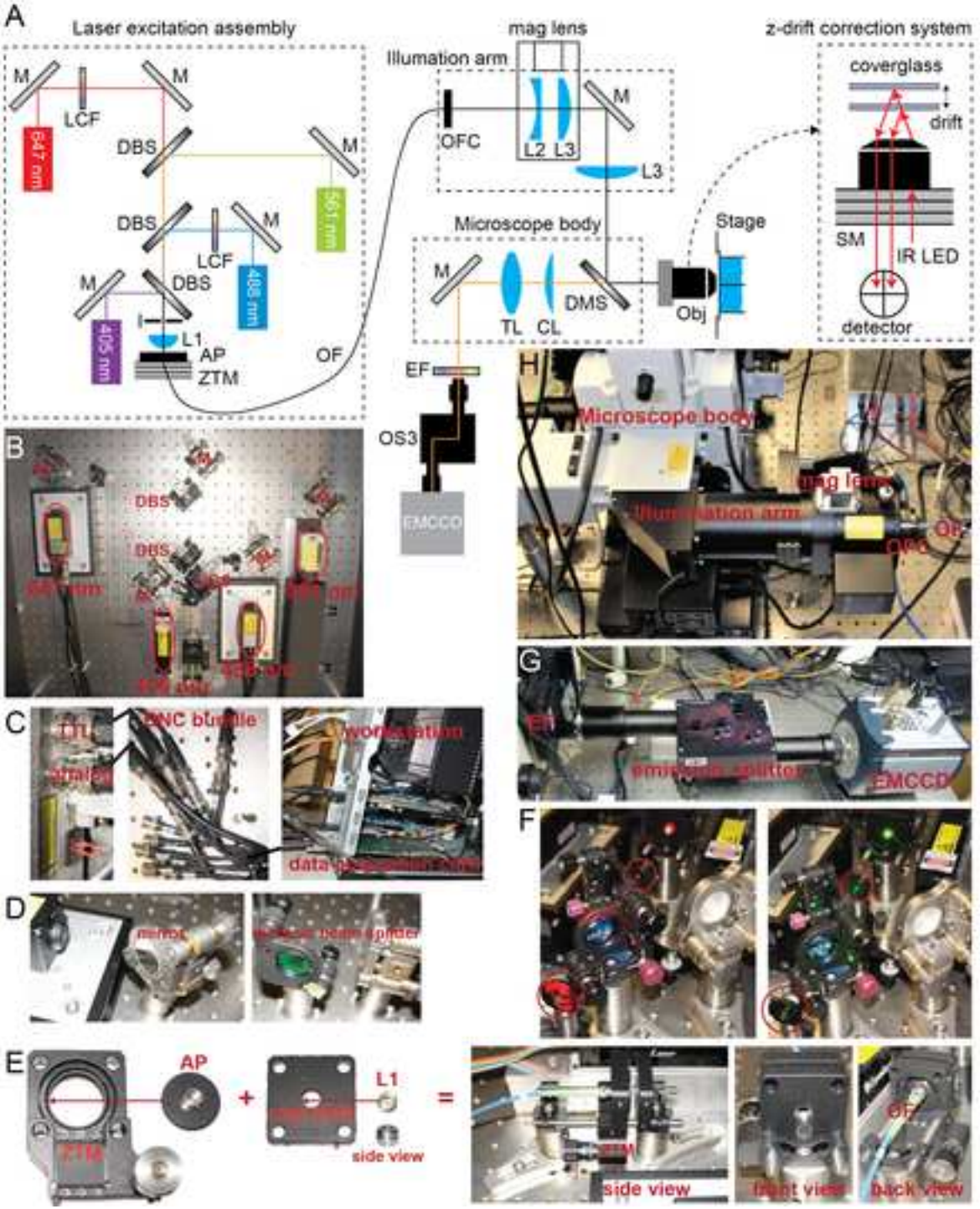

Figure 2

Click here to download Figure Fig2_Mag_lens_holder.tif

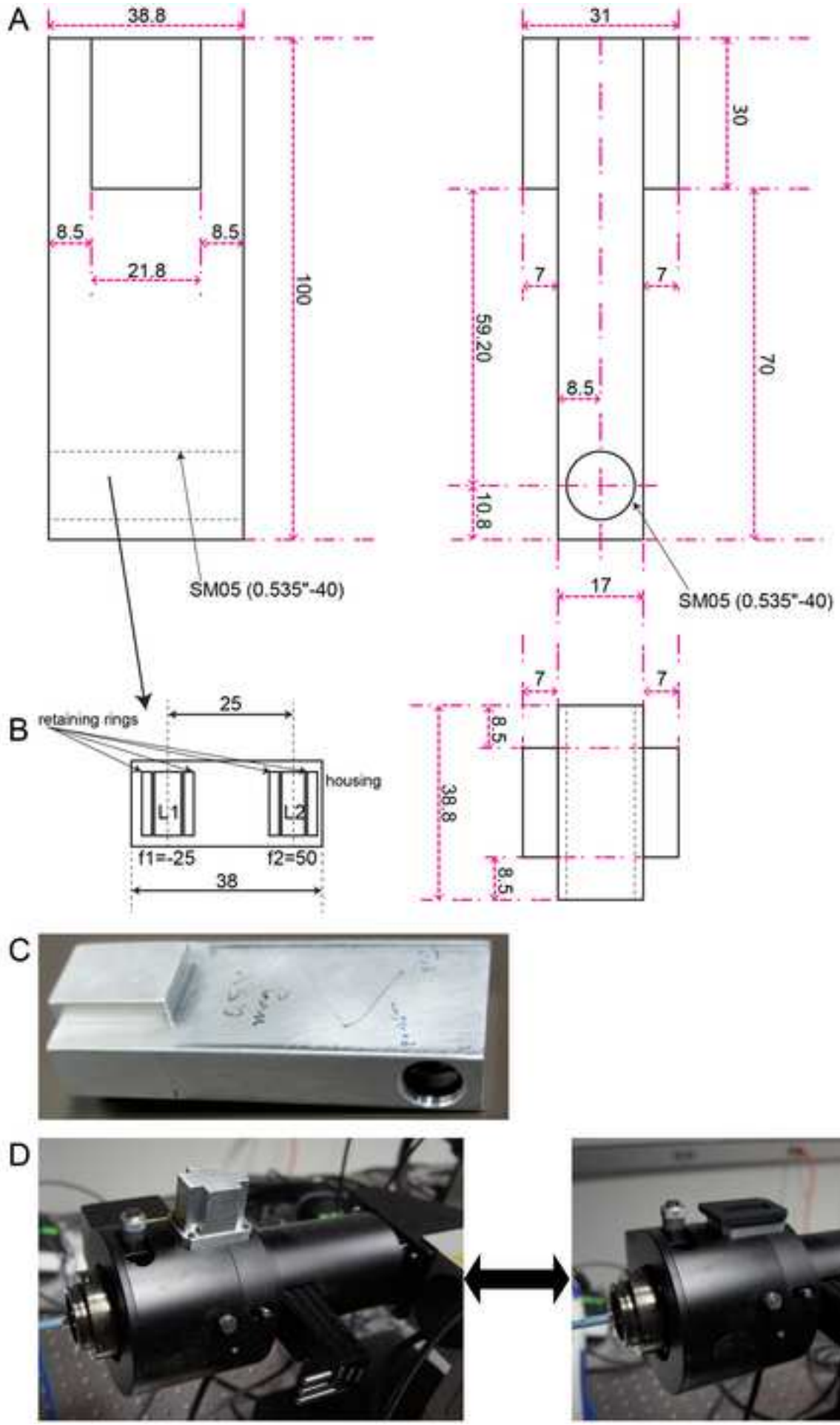

Figure 3

Click here to download Figure Fig3_3D_lens_update_v2.tif

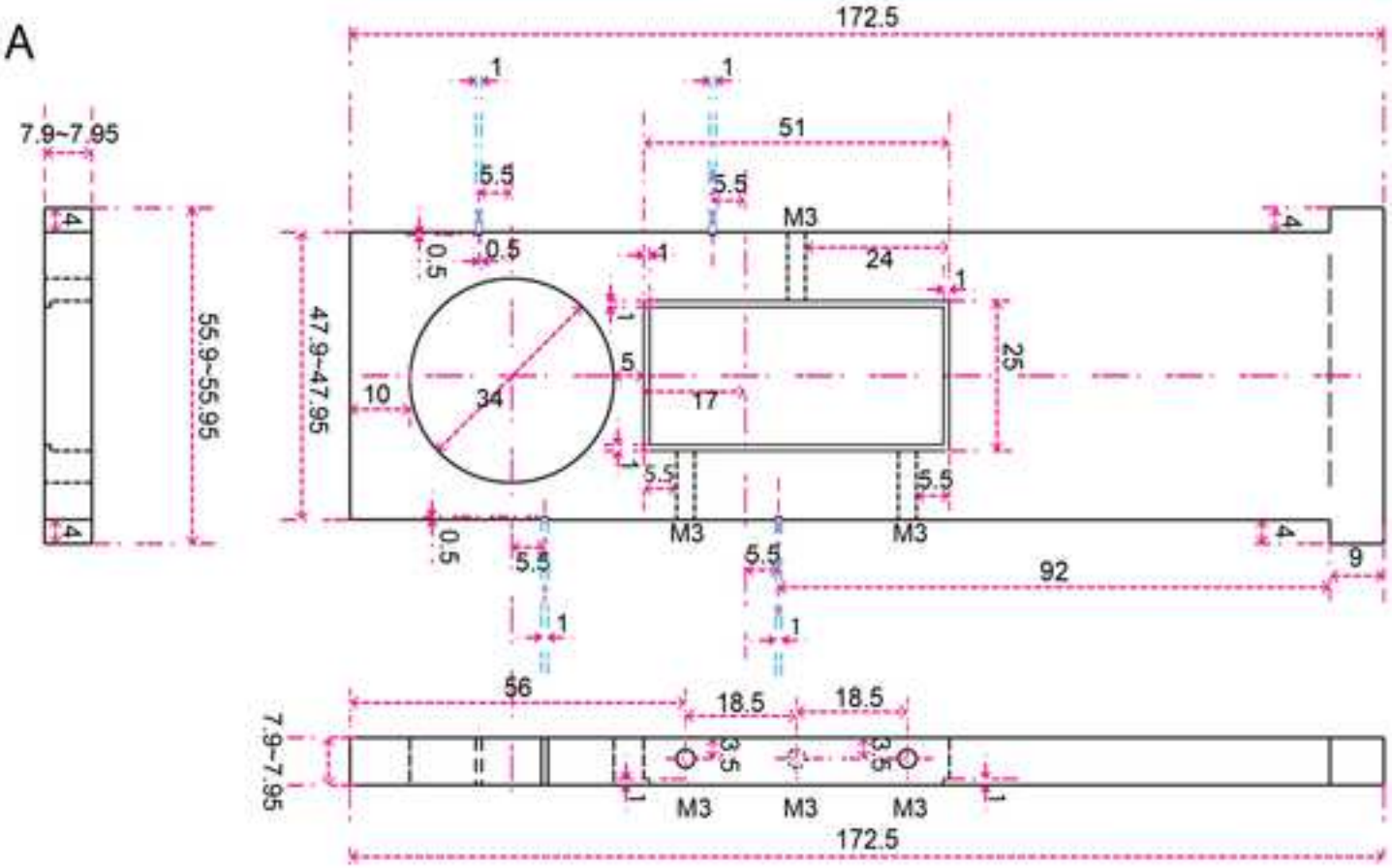

Figure 4

Click here to download Figure Fig4_Optosplit3_JF_v4.tif

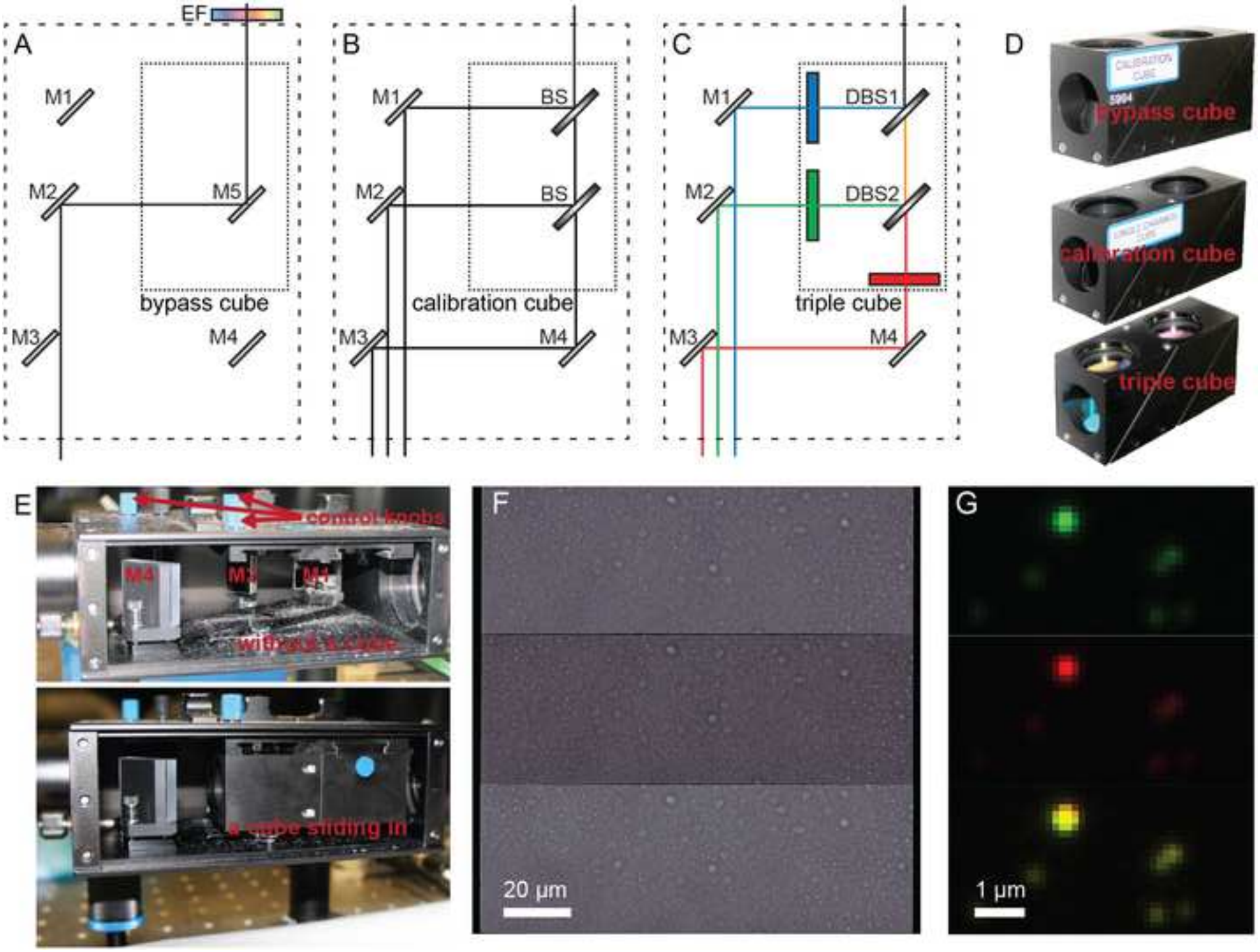

Figure5

Click here to download Figure Fig5_example of STORM_JFv2.tif

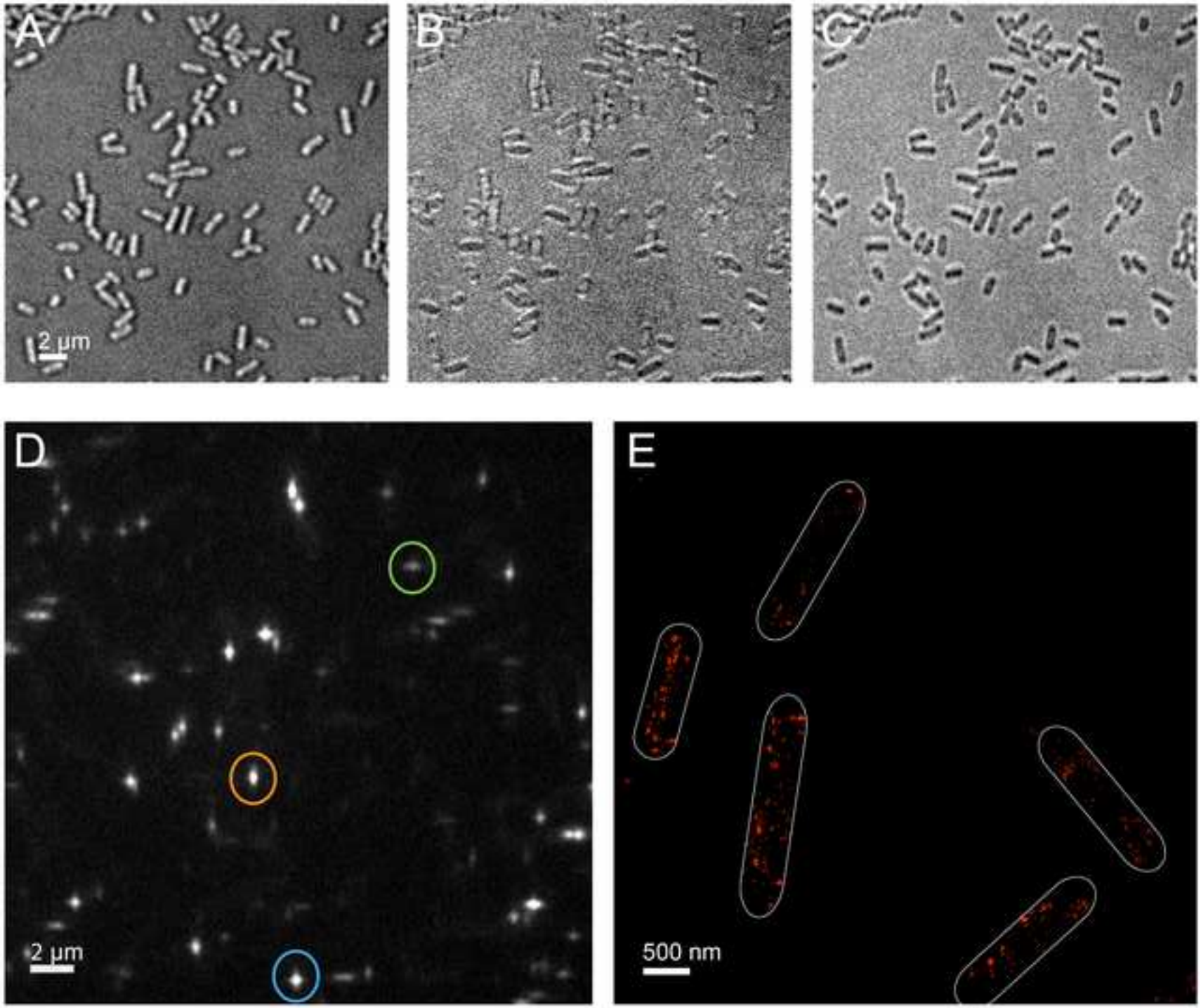

Figure 6

Click here to download Figure Fig6_JOBS1.tif

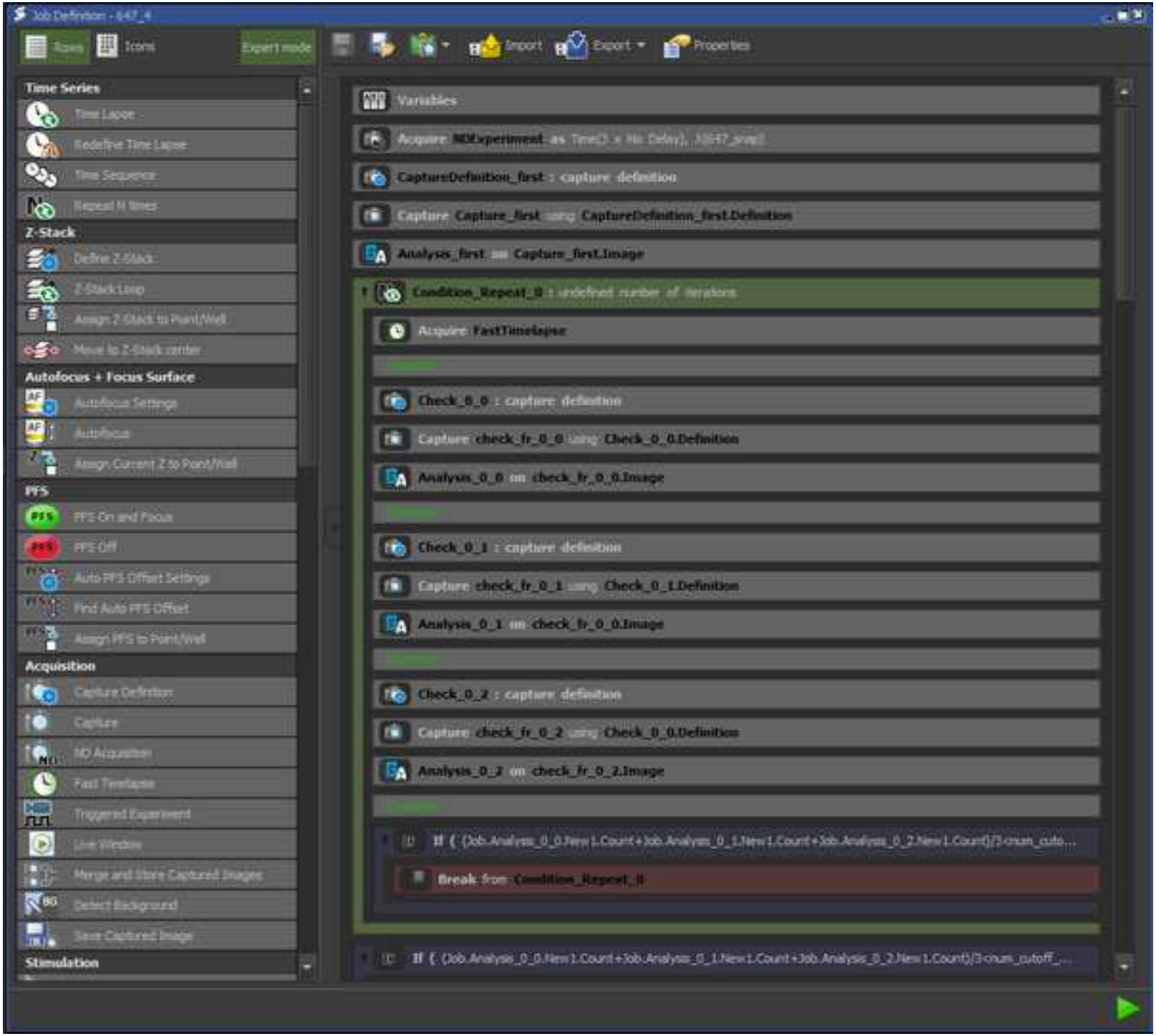

Figure 7

Click here to download Figure Fig7_jove_epi_data_JF.tif

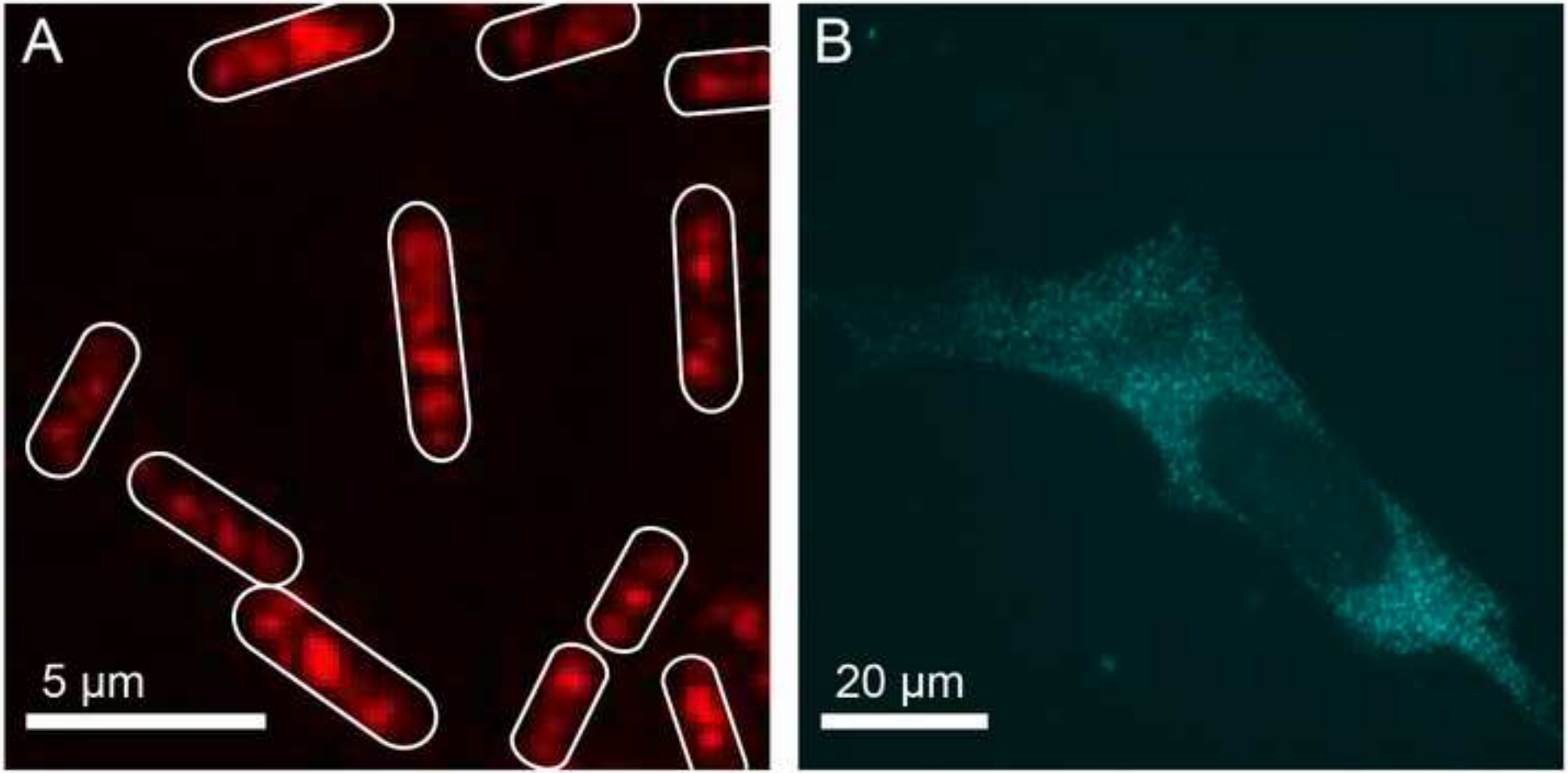

Figure8

Click here to download Figure Fig8_example of FRET _JF_v2.tif

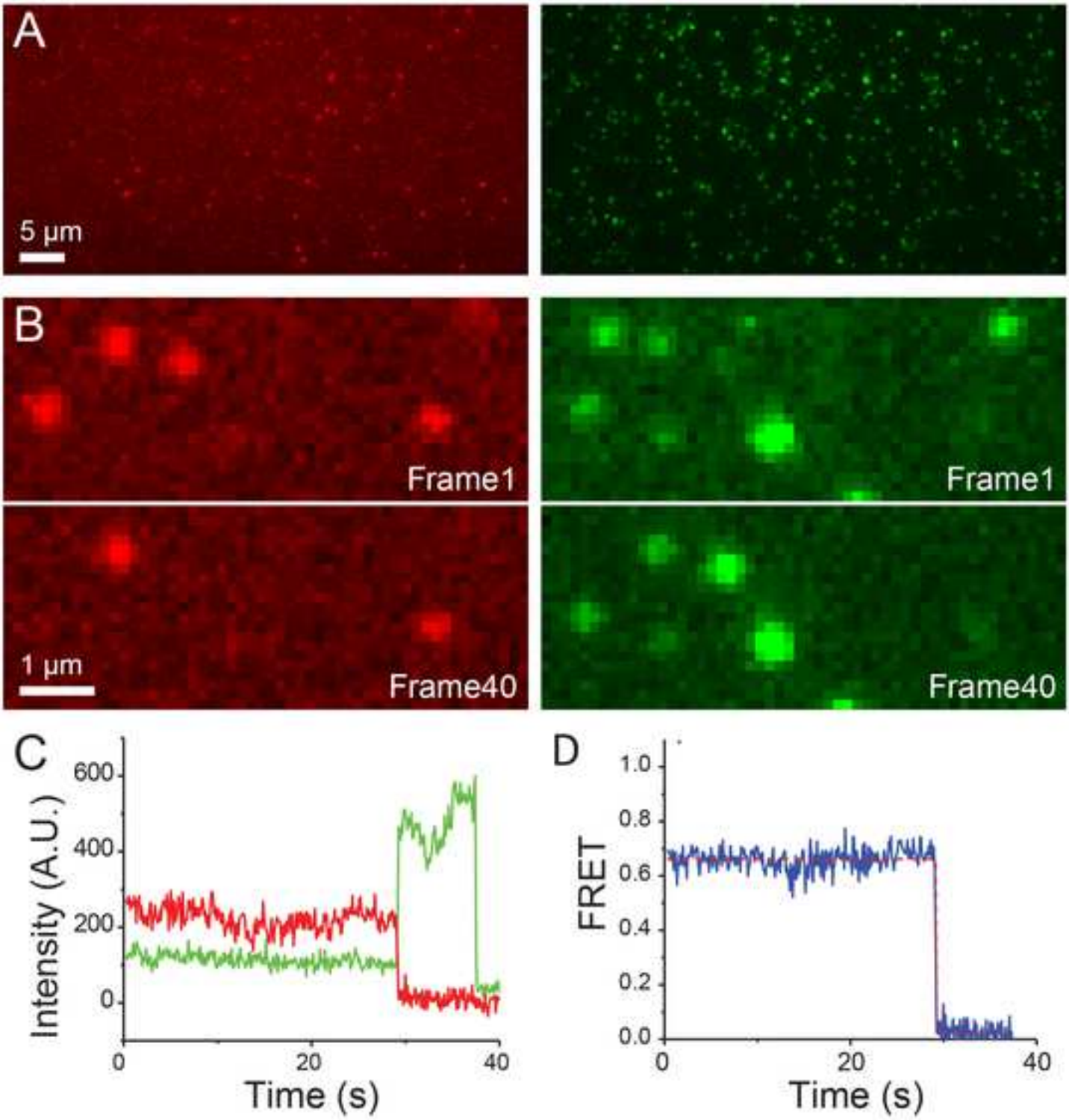

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
Nikon Ti-E microscope stand	Nikon	Ti-E
Objective lens	Nikon	100X NA 1.49 CFI HP TIRF
Microscopy imaging software	Nikon	NIS-Elements Advanced Research/HC
The illumination arm	Nikon	Ti-TIRF-EM Motorized Illuminator Unit M
Analyze block	Nikon	Ti-A
Z-drift correction system	Nikon	PFS
Optical table top	TMC	783-655-02R
Optical table bases	TMC	14-426-35
647 nm laser	Cobolt	90346 (0647-06-01-0120-100)
561 nm laser	Coherent	1280721
488 nm laser	Cobolt	90308 (0488-06-01-0060-100)
405 nm laser	Crystalaser	DL405-025-O
Heat sink	Cobolt	11658 (HS-03)
Heat sink	Coherent	1193289
CAB-USB-miniUSB	Cobolt	10908
aluminum for height adjustment	McMaster-Carr	9146T35
aluminum for height adjustment	McMaster-Carr	8975K248
BNC cable	L-com	CC58C-6
BNC adapter	L-com	BA1087
SMA to BNC Adapter	HOD	SMA-870
SMB to BNC Adapter	Fairview Microwave	FMC1638316-12
Data Acquisition Card	National Instruments	PCI-6723
Barrier Filter Wheel controller	Sutter Instrument	Lambda 10-B
Emission Splitter	Cairn	OptoSplit III
Dichroic beamsplitter	Chroma	T640LPXR-UF2
Dichroic beamsplitter	Chroma	T565LPXR-UF2
Emission filter	Chroma	ET700/75M
Emission filter	Chroma	ET595/50M
Emission filter	Chroma	ET525/50M
Emission filter	Semrock	FF02-447/60-25
Dichroic beamsplitter	Chroma	zt405/488/561/647/752rpc-UF3

DAPI Filter set	Chroma	49000
Nikon laser/TIRF filtercube	Chroma	91032
590 long pass filter	Chroma	T590LPXR-UF1
525 long pass filter	Chroma	T525LPXR-UF1
470 long pass filter	Chroma	T470LPXR-UF1
Laser clean-up filter (647)	Chroma	zet640/20x
Laser clean up filter (488)	Semrock	LL01-488-25
LED light source	Excelitas	X-Cite120LED
Mirror mount	Newport	SU100-F3K
Optical posts	Newport	PS-2
Clamping fork	Newport	PS-F
Power Meter	Newport	PMKIT
Dichroic beamcombiner mount	Edmund Optics	58-872
Retaining ring	Thorlabs	CMRR
Fiber Adapter Plate	Thorlabs	SM1FC
Z-axis translational mount	Thorlabs	SM1Z
Achromatic Doublet lens	Thorlabs	AC050-008-A-ML
Cage Plate	Thorlabs	CP1TM09
Cage Assembly Rod	Thorlabs	ER4
Cage Mounting Bracket	Thorlabs	CP02B
Single mode optical fiber	Thorlabs	P5-405BPM-FC-2
Multi mode optical fiber	Thorlabs	M42L01
Achromatic Doublet lens (mag lens)	Thorlabs	ACN127-025-A
Achromatic Doublet lens (mag lens)	Thorlabs	AC127-050-A
Retaining ring	Thorlabs	SM05PRR
Nylon-tipped screw	Thorlabs	SS3MN6
3D lens	CVI Laser Optics	RCX-25.4-50.8-5000.0-C-415-700
EMCCD camera	Andor	iXon Ultra 888
100 nm multichannel beads	Thermo	T7279, TetraSpeck microspheres
red dye	Thermo	Alexa Fluor 647
yellow-green dye	GE Healthcare	Cy3
green dye	GE Healthcare	Cy3B
blue dye	Thermo	Alexa Fluor 488

Comments/Description

HC includes "JOBS" module, the programmed acquisition module being used for SR imaging.
This arm has a slot for a magnification lens
This is installed in the filter turret.
This system is composed by the stepmotor on the objective nosepiece, IR LED, and a detector.

Modulated Laser Diode 647nm 120mW incl. laser head, CDRH control box, USB cable and PSU (Power Supply Unit)
OBIS 561nm LS 150mW Laser System
Modulated Laser Diode 488nm 60mW incl. laser head, CDRH control box, USB cable and PSU (Power Supply Unit)
405 (+/-5)nm, 25mW, Circular , M2 <1.3, Low Noise, CW, TTL up to 20MHz. 2 BNC connectors for TTL & Analog adjust
Two units, Heat sink without fan HS-03, Heat sink for 647 nm and 488 nm lasers
Obis heat sink with fan, 165 x 50 x 50 mm for the 561 nm laser
Two units, communication cable for 647 nm and 488 nm lasers
Multipurpose 6061 Aluminum, Rectangular Bar, 4MM X 40MM, 1' Long for raising 561 nm laser
Multipurpose 6061 Aluminum, 1-1/4" Thick X 3" Width X 1' Length for raising 405 nm laser
RG58C Coaxial Cable, BNC Male / Male, 6.0 ft
Coaxial Adapter, BNC Bulkhead, Grounded
Cobolt MLD lasers have SMA interface, so this adapter is used for BNC connection.
SMB Plug to BNC Female Bulkhead Cable RG316 Coax in 12 Inch for Coherent Obis lasers
13‑Bit, 32 Channels, 800 kS/s Analog Output Device for controlling lasers, DIC LED, and etc
Optical Filter Changer

Dichroic beamsplitter separating red emission from green emission in OptoSplit III
Dichroic beamsplitter separating green & red emission from blue emission in OptoSplit III
Two units, Emission filter for red emission (like Alexa Fluor 647) in OptoSplit III as well as in the Barrier filter wheel
Two units, Emission filter for yellow/green emission (like Cy3B) in OptoSplit III as well as in the Barrier filter wheel
Two units, Emission filter for blue emission(like Alexa Fluor 488/GFP) in OptoSplit III as well as in the Barrier filter wheel
Emission filter for violet emission (like DAPI/Alexa Fluor 405), installed in the Barrier filter wheel
Multiband dichroic beam splitter for 647, 561, 488, and 405 nm laser excitations inside of the microscope body

installed in the microscope body

for combining 647 nm laser and 561 nm laser
for combining already combined 647 nm and 561nm lasers with 488 nm laser
for combining already combined 647 nm, 561 nm and 488 nm lasers with 405 nm laser
for cleaning up other wavelengths from the 647 nm laser
for cleaning up other wavelengths from the 488 nm laser
used only for DAPI imaging

For measuring laser power
C-Mount Kinematic Mount, for holding dichroic beamcombiners in the laser excitation assembly
used for dichroic beamcombiner mounts
FC/PC Fiber Adapter Plate with External SM1 (1.035"-40) Thread
Z-Axis Translation Mount, 30 mm Cage Compatible
Ø5 mm, Mounted Achromatic Doublets, AR Coated: 400 - 700 nm
30 mm Cage Plate with M9 x 0.5 Internal Threads, 8-32 Tap
Cage Assembly Rod, 4" Long, Ø6 mm
30 mm Cage Mounting Bracket
Patch Cable, PM, FC/PC to FC/APC, 405 nm, Panda, 2 m
Ø50 µm, 0.22 NA, FC/PC-FC/PC Fiber Patch Cable, 1 m
ACN127-025-A - f=-25.0 mm, Ø1/2" Achromatic Doublet, ARC: 400-700 nm , a concave lens in the "mag lens"
f=50.0 mm, Ø1/2" Achromatic Doublet, ARC: 400-700 nm, a convex lens in the "mag lens"
SM05 Plastic Retaining Ring for Ø1/2" Lens Tubes and Mounts, for "mag lens"
M3 x 0.5 Nylon-Tipped Setscrew, 6 mm Long, for holding "3D lens"
f=10 m, rectangular cylindrical lens

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A practical guide to Merging merge multiple imaging modules into one fluorescence microscope Seongjin Park[1], Jiacheng Zhang[2], Matthew A. Reyer[2], Joanna Zareba[1,3], Andrew A. Troy[4], Jingyi Fei[1,2]

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Response to reviewers

Note to the editor: We rewrote most of the sentences in the protocol section, and in the process of editing back and forth between authors, some changes lost track. We added more sentences based on reviews.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. → completed.

2. Figure 8: Please provide a scale bar to provide context for the images. → done.

3. Please provide an email address for each author.

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4. Keywords: Please provide at least 6 keywords or phrases. → done.

5. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …" → done.

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7. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: X-Cite, Excelitas, Coherent Obis, Cobolt MLD, National Instruments, etc. → done.

8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). → done.

9. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” → done.

10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. → done.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript concisely illustrates how to customize a commercial microscope for multiple, real-world applications in biophysics. It is clear and very well-written. Moreover, it is a much needed addition to the field and will allow non-optics experts to enter the single molecule fluorescence field with confidence.

Major Concerns:
none

Minor Concerns:
Line 51, is it really true that information on molecular interactions "require" angstrom to nanometer resolution? Can't pull-down assays or mass spec also be informative of molecular interactions? →Pull-down assays or mass spec can reveal whether a certain molecule (=A)

interacts with another molecule (=B) or not. However molecular interactions between A and B can happen in various conformational dynamics with varying distances between them, and pull-down assay or mass spec cannot provide information of such dynamics. SmFRET can provide such dynamics in real time. But we agree with the reviewer that the original sentence can be misleading, we therefore revised it slightly as follows:

<To gain information on molecular interactions and conformational dynamics in real time, angstrom to nanometer resolution is required. >

line 98, the authors may want to explain why the laser beams should be set at a height of 3"→ It indeed doesn't have to be exactly at 3'', but any height not too tall above the optical table. Shorter heights are better for good stability. The text was modified as

<make sure emitted laser beams have the same height, as short as possible to ensure good stability, like 3''.>

line 109, the authors should explain why the shorter wavelength lasers are closer to the optical fiber coupling. Presumably this is just due to the order in which the laser pass through the dichroics. → Because of the design of dichroics, the order can be either ascending (shorter wavelength closer to the fiber coupling) or descending (longer wavelength closer to the fiber coupling). However, we recommended ascending order, since shorter wavelength lights dissipate more in the air. This effect may be very little due to short distances of optical elements, but for the sensitive nature of single mode optical fiber coupling, we propose that this order would be better for coupling. The text was modified as

< Arrange lasers such that shorter wavelength lasers are closer to the optical fiber coupling (Fig. 1B), since short wavelength lights dissipate more in the air. >

line 211, the authors use some custom JOBS code. is this available somewhere? The authors website or GitHub? → Yes, it is available upon request. We can provide the code as well as details guides of how to run it.

line 253, the authors may want to explain the difference between "fiber coupled" and "air coupled" → the text was modified as

< If the excitation lasers are air coupled (*i.e.*, without optical fiber), >

line 252 and in the parts list, does the 3D lens really have a focal length of 10 meters? Or is this

the f/#? → Yes, the 3D lens has 10 m focal length. The optical focal length for astigmatism depends largely on where the lens is placed. From the location where we put, f=10 m lens works very well.

Figure 1, what is the optic in between the two mirrors of the 647nm laser? Is this a laser line filter? Why don't the other lasers require one? → That is a laser clean-up filter. Indeed, we recently added one more laser clean-up filter for the 488 nm laser, so the picture and the diagram are updated. To our experience, some diode lasers tend to be “contaminated” with wavelengths other than the desired wavelength. DPSS lasers tend to be a lot cleaner. However, deciding whether a laser clean-up filter is necessary for a laser is largely dependent on the actual imaging tests. We found noticeable background reduction with the clean-up filter for 647 nm laser. For 488 nm laser, at TIRF mode the laser caused significant interference pattern, mainly caused by internal reflections in Nikon PFS filter cube. However, with the laser clean-up filter, the pattern was removed. On the other hand, we didn’t find a need for clean-up filter for 405 nm diode laser since it didn’t cause any pattern or high background. 561 nm DPSS laser used to have a leaking line to the camera, but it was eliminated by installation of OptoSplit III, so we don’t find a need for a clean-up filter for it either.

Figure 4, what are the dimensions of images in C-F? It may also be useful to report resolution in terms of nanometers per pixel. → scale bars are added.

The authors should check that each of the parts listed in their parts table are still available or provide alternates if no longer in stock. I was unable to find the 3D lens from CVI laser optics by doing a quick internet search. (or if this is a custom part it should be noted). → The cylindrical lens from CVI may not be listed in their website due to low demand. However, CVI may have one, or can manufacture one upon request. In our case, they didn’t have the size we originally wanted (1’’X1’’), so they cut a larger one (2’’X2’’) into half and sold it to us. Similarly, Chroma doesn’t have all their products in their webpage, so one needs to communicate their tech support to find the products. With the parts numbers in the table, that shouldn’t be a problem. A more impending issue is that Nikon is discontinuing their Ti-E body and the exact illumination arm we currently use. However we communicated with Nikon and confirmed that similar or identical modifications can be made in their new products. In the discussion session, we wrote the general guide for different microscope bodies. Especially for the mag lens, we added this line,

< Alternatively, a mount for the field diaphragm slider can be another place, if available. >

Reviewer #2:
The manuscript submitted by Park et al presents the implementation of microscope consisting in an home-made laser module connected to a commercial Nikon TIRF module and microscope and home-made spherical lens before a commercial Andor Optosplit multi-view system connected to an Andor emCCD camera. The whole system is controlled by NIS-Element software provided by Nikon for widefield, STORM/PALM or smFRET applications. If the work is well presented and may constitute an interesting piece of work for JOVE, I found several major weaknesses in this manuscript:
- The title is not adapted. I don't found this prototype as "multiple imaging modules". Every TIRF microscopes with an optosplit and a spherical lens can be considered to perform SMLM and smFRET and eventually wide-field… the work presented here is the implementation of a TIRF microscope for SMLM and smFRET application. →Theoretically that is right, but building the actual instrument that can switch different imaging modes smoothly and reproducibly isn't trivial. For example, deciding where to put the cylindrical lens of what focal length needs a lot of trials and errors. Also a regular TIRF microscope with lasers only cannot conduct epi-imaging with sufficiently large illumination area, unless with white light source (LED, mercury or other bulbs) and multiple filter cubes. Adding the LED light source as well as multiple filter cubes would cost up to ~$10,000 or more, but building the mag lens costs <$500 and provide very reasonable illumination area. Our approach has economic advantages.

On the other hand, we imagined purchasing a commercial SMLM setup (like Nikon's N-STORM) and converting it to have epi-fluorescence module as well as smFRET module. It is doable to do so, but due to the long N-STORM module in the emission path,

installing OptoSplit III will result in very long emission path. This will require more space for the microscope assembly. After all, this will be a much more expensive solution to what we have already achieved. Overall, our manuscript about providing a practical guide of building such a setup in a reduced cost, so we changed the title to,

< A practical guide to Merging merge multiple imaging modules into one fluorescence microscope>.

- The home-made laser module is a conventional solid laser module with conventional wavelengths. There is no particular tricks for this part. Alternative with commercially available modules such as Omicron, Oxyus and probably others has to be noticed since I'm not sure that the time of implementation, the ease of use, the service and eventually the cost goes for a home-made solution. → The initial cost will be very different. We didn't check many different products, but

such a system (Agilent MCL400B, for N-STORM) costs a lot more ($102,000, if purchased from Nikon.) than all of our lasers and fiber optics components. Our homebuilt laser module is very stable, and needs negligible maintenance, if any. We assume the most challenging part would be the initial fiber coupling alignment. If one was never experienced in fiber coupling, that will require some time to get trained. However, there are plenty of online learning resources, like Youtube. Once trained, then the whole laser module will take less than a weak to build. Then controlling the lasers through TTL and analog outputs are mostly trivial, through National Instruments' Data Acquisition Card and a program that controls the card (in our case, NIS-Element).

Another issue of a multiline laser box is that their power range may not be optimal for SMLM. For SMLM, the 405 nm laser has to be weak, and its power should be modulated from 0~5mW very precisely. On the other hand, 561 nm or 647 nm lasers need to be much stronger, at least 50 mW or higher (powers denoted after fiber coupling). We found some multiline laser boxes don't satisfy this power range requirement.

Examples:

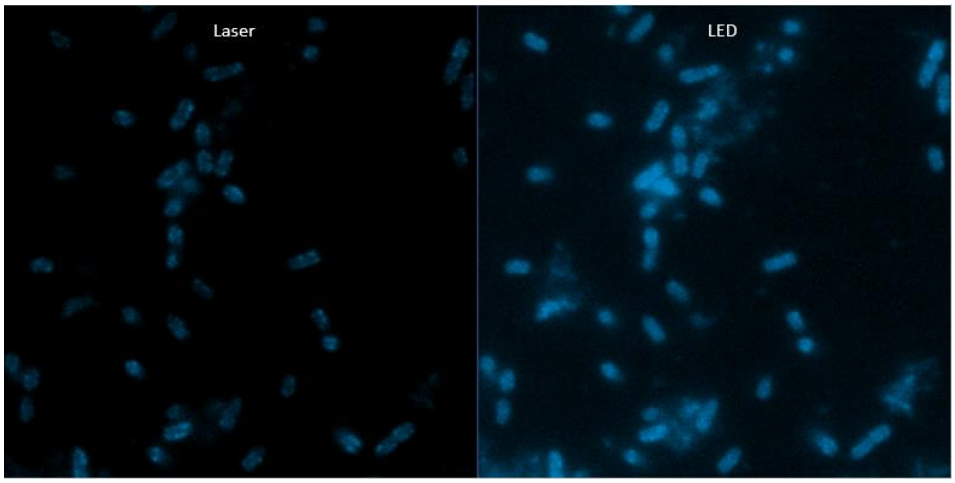
https://www.coboltlasers.com/lasers/multi-line-laser/ (mostly 50 mW for all wavelengths)

https://edge.coherent.com/assets/pdf/COHR_OBIS_CellX_DS_0117_3.pdf (50mW is the minimal power for 405 nm laser, and all the other lasers have the same power.)

Direct power modulation of a solid state laser is not very reliable under 10% of its maximum power, so unless a 405 nm laser is weak from the very beginning, modulating it in fine scales requires additional instruments like AOTF, resulting in increased cost as Agilent MCL400B which includes AOTF internally.

- In the excitation part, the authors present the possibility to change from TIRF to wide field using lasers. To have a larger field of view, it necessitates an additional expander. But in fact this part is not so trivial, since there is usually interference fringes on the field and Koller adjustments are not straight forward. What about fringes in your prototype? This has to be discussed. What advantages between the laser and conventional lamp? From my experience, a liquid fiber is sufficient to remove the coherence of the laser and to have really nice wide field illumination (using the conventional Koller port of the microscope. → As far as the design is correct for the mag lens, there isn't any interference patterns or fringes in our epi-imaging. We asked Nikon to provide necessary dimensions for the illumination arm slot. We built our custom mag lens based on Nikon's own mag lens. Theirs however can only shrink the laser beam, since it could not be placed in the opposite direction like ours. Our design can either expand or shrink the beam.

We didn't point out the advantage of laser over conventional lamp in the report. However, in our tests, lasers provide 'cleaner' images in general, with noticeably better signal compared to the background. Here is an example.



In these two images, the same sample (an inner membrane protein in E.coli cells) area was imaged with exactly same camera condition (100 ms exposure time and maximum gain). The contrast of these images are same so they can be compared side by side. Laser illumination was in Epi mode, with the use of mag lens, to be comparable to LED light.

The laser illumination clearly reveals the membrane localization of this protein. However with LED, the details are more smeared. We attribute this different to the monochromatic nature of laser, so that the emission is in general cleaner, not contaminated by the excitation light of many wavelengths.

Also lasers can provide stronger excitation. In the left image we only used 14% of the laser power, while for LED we used 100%. If a sample isn't bright enough to be well imaged by LEDs, a laser excitation (with the presence of oxygen scavenger, and just one frame image) can reveal more details.

- For the emission part, only the slide for spherical lens is an addition to other conventional TIRF microscopes. It is not clear for me what makes more… Moreover, the implementation of the multicolor is probably the interesting part since it is not so trivial. What is the difficulties to find the same corresponding pixel? What is the impact for single molecule detection? Is there the same resolution at different wavelength? What about the z resolution at different wavelength? Is the z-focus the same? All these aspects are not controlled and discussed in the result section. → We added the details of multicolor SR imaging in the protocol part (in section 5.7).

< In the case of two-color imaging, always conduct imaging fluorophores with longer excitation wavelengths to shorter wavelengths, since excitation spectrums have longer tails toward shorter wavelengths.

NOTE: Chromatic aberration should be corrected between images with different fluorophores, *e.g.*, Alexa Fluor 647 and Cy3B. Here are the steps.

3.1.1 Immobilize multiple 100 nm multichannel beads on the glass coverslip, avoiding forming clusters.

3.1.2 Take images of them in different excitation channels.

3.1.3 Extract their (X,Y,Z) coordinates by a software (section 5.6).

3.1.4 Plot $\Delta X_i=X_{1i}-X_{2i}$ and $\Delta Y_i=Y_{1i}-Y_{2i}$ (i is for different beads, and 1 and 2 are different color channels) respectively, and fit with proper functions. Save the functions.

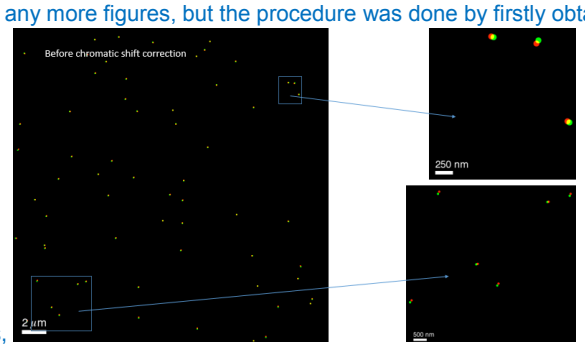
NOTE: Linear functions are sufficient for most cases. Once these functions are determined, this kind of measurement doesn't have to be repeated each time of imaging.

3.1.5 In the actual two-color SR imaging of a sample of interest, apply the functions to correct (X,Y) chromatic aberration. For z-directional chromatic aberration, conduct it by obtaining $\Delta Z=Z_1-Z_2$ for multichannel beads or known reference multichannel samples seeded together with the sample of interest.

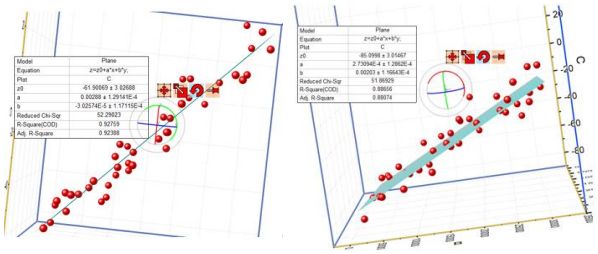
NOTE: Unlike (X,Y) chromatic aberration, z-directional chromatic aberration is not well reproducible in each experiment, mainly due to incomplete z-directional focus maintenance upon channel shifting. Thus it is recommended to conduct the correction each time. $\Delta Z=Z_1-Z_2$ is mostly independent of (X,Y), so just a few beads or reference samples would be sufficient per each sample area of interest. Plot the finally constructed two-color SR images in Matlab or VMD (Visual Molecular Dynamics) and check ΔZ manually.

>

We didn't add any more figures, but the procedure was done by firstly obtaining TetraSpeak beads images,

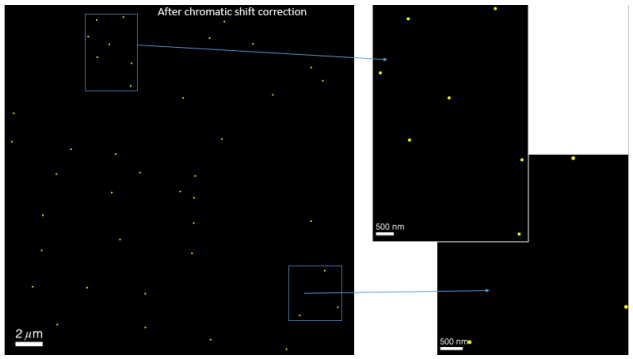


Then plotting ΔX and ΔY in Origin Pro.



(left one is ΔX, right one is ΔY) The bottom plane of these plots is X-Y plane.

Then applying these functions yield corrected images between two channels.



As far as there are these reference samples in the test sample (so immobilize beads to the test sample before imaging), then it is not difficult to align the two channels. Single molecule detection is mostly unaffected, since the beads are not 'too' bright to saturate the pixels, even with high laser powers. Also over time they photobleach too (getting dimmer), making them easier to be picked by the analysis software. We assume they have similar XY resolution since

the brightness and blinking behavior of Alexa Fluor 647 and Cy3B are comparable. We assume they have very similar Z resolution, since they showed very similar calibration curves (Single molecule PSF $width_x$ and $width_y$ as the function of z). To prove the points, we labeled the same mRNAs in E.coli cells with different sets of FISH probes (one set with Alexa Fluor 647 and the other with Cy3B or Alexa Fluor 568), and confirmed that the RNA localization distributions are similarly sized between two channels. Z-focuses are not the same. We added this text,

< Unlike (X,Y) chromatic aberration, z-directional chromatic aberration is not well reproducible in each experiment, mainly due to incomplete z-directional focus maintenance upon channel shifting… (skip) Plot the finally constructed two-color SR images in Matlab or VMD (Visual Molecular Dynamics) and check ΔZ manually. >

The correction has to be done for each set of two-color images. We use VMD to plot the final localizations and correct ΔZ.

- The part of software control is not presented in details. Authors choose NIS-element. Is it the only one? There is no details about the laser control? Is it through a DAC card? Which one? Is it possible to drive it with other software? Moreover, there is no details concerning the data analysis which is crucial for SR imaging.What about the module for data analysis (JOBS)? Is it online analysis? What is the algorithm for SML? How to implement it for multicolor? →Details about laser controls through DAC cards are put in the protocol section 1.1.4. PCI-6723 (National Instrument) was used, and is listed in the Materials Table. Of course it is possible to drive all the hardware parts of the microscope by alternative software, like Micro-Manager or Metamorph. However, the feature we wanted the most, the automated modulation of violet laser (explained in 5.4, and the second last paragraph in Discussion, as well as Figure 6 caption) was not readily achievable by Micro-Manager or Metamorph at that time when we built the setup. That's why we ended up using NIS-Element's JOBS feature. It is not online analysis. The algorithm of SMLM is based on the original STORM paper and 3D STORM paper, and these are referred multiple times in the text.

Rust, M. J., Bates, M. & Zhuang, X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). Nature Methods 3 (10), 793–795, doi:10.1038/nmeth929 (2006).

Huang, B., Wang, W., Bates, M. & Zhuang, X. Three-dimensional super-resolution imaging by stochastic optical reconstruction microscopy. Science 319 (5864), 810–813, doi:10.1126/science.1153529 (2008).

Fei, J., Singh, D., et al. RNA biochemistry. Determination of in vivo target search kinetics of regulatory noncoding RNA. Science 347 (6228), 1371–1374, doi:10.1126/science.1258849 (2015).

Prof. Taekjip Ha (JHU) has the copyright of the modified version of the code, so one can ask him for the code.

For multicolor, almost the same automated acquisition code can run, except that the imaging laser is different. (Section 5.7)

- Finally, the authors present "representative results" for widefield, SR image and smFRET. But there is no any details of the different protocols used for it. What about excitation/emission wavelengths? What abourt the preparation of the specimens? If the manuscript is a protocol, then you need to give the protocol for the obtained images… there is no details about SR imaging. Is it storm? Which fluorophore? Which sample preparation? What about the data analysis? And it is the same problem for smFRET. This part should be more detailed. → Our intention of writing this report was providing protocols of building the microscope modules and operation procedures. For the actual sample preparations, we used FISH for labeling, and all the general methods are referred to previous publications. The excitation wavelengths and fluorphores are also specified (In "Representative Results" as well as Figure 5, 7, and 8 captions). For SR imaging, added the text,

< NOTE: This is single-molecule detection based SR microscopy. >
Indicating that this is STORM/PALM type imaging. Fluorophores for STORM imaging was Alexa Fluor 647 for the example, and added in Figure 5 caption. Data analysis is referred to the original STORM paper as well as 3D STORM paper (Rust *et al,* and Huang *et al.*) For smFRET, we specified that the measurement was for folded RNA molecules. For all the exemplary samples, we didn't give further details because they are yet unpublished results.

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