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A guide to build a highly inclined swept tile microscope for extended field-of-view single-molecule imaging

Jialei Tang, Chun-Hung Weng, Jeffrey B. Oleske & Kyu Young Han

Dear Editor,

Enclosed is an article regarding detailed instruction on how to build a highly inclined swept tile (HIST) microscopy and its usage for single-molecule imaging. This has been invited by Dr. Jialan Zhang.

Single-molecule imaging has greatly advanced our understanding of molecular mechanisms in biological studies. However, it has been challenging to obtain large field-of-view, high-contrast images in thick cells and tissues. Here we introduce highly inclined swept tile (HIST) microscopy that overcomes this problem. A pair of cylindrical lenses was implemented to generate an elongated excitation beam that was scanned over a large imaging area via a fast galvo mirror. A 4f configuration was used to position optical components. A scientific complementary metal-oxide semiconductor camera detected the fluorescence signal and blocked the out-of-focus background with a dynamic confocal slit synchronized with the beam sweeping. We present a step-by-step instruction on building the HIST microscope with all basic components.

Our method should be of interest to many researchers, and I believe it will be a valuable tool for super-resolution fluorescence imaging for whole cells and tissues, single-molecule tracking and single-molecule RNA FISH for detecting rare transcriptional mutants. Note that our work was published on [Optica 5, 1063-1069 \(2018\)](#).

Thank you very much for kindly considering our work, and we look forward to hearing from you.

Sincerely,

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TITLE:

A Guide to Build A Highly Inclined Swept Tile Microscope for Extended Field-of-view Single-molecule Imaging

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KEYWORDS:

optical microscopy, fluorescence, single-molecule imaging, three-dimensional microscopy, illumination, medical and biological imaging

SUMMARY:

A detailed instruction is described on how to build a highly inclined swept tile (HIST) microscope and its usage for single-molecule imaging.

ABSTRACT:

Single-molecule imaging has greatly advanced our understanding of molecular mechanisms in biological studies. However, it has been challenging to obtain large field-of-view, high-contrast images in thick cells and tissues. Here, we introduce highly inclined swept tile (HIST) microscopy that overcomes this problem. A pair of cylindrical lenses was implemented to generate an elongated excitation beam that was scanned over a large imaging area via a fast galvo mirror. A 4f configuration was used to position optical components. A scientific complementary metal-oxide semiconductor camera detected the fluorescence signal and blocked the out-of-focus background with a dynamic confocal slit synchronized with the beam sweeping. We present a step-by-step instruction on building the HIST microscope with all basic components.

INTRODUCTION:

Single-molecule fluorescence imaging plays an important role in many biological studies that reveal ultrastructures, dynamics and the quantity of biomolecules¹⁻³. However, it has been challenging to study single-molecules inside cells or tissues. While confocal microscopy provides high sectioning capability⁴, it is not suitable for single-molecule imaging due to severe photobleaching by the high excitation intensity or slow imaging speed. Widefield microscopy uses weaker illumination but suffers from a poor signal to background ratio (SBR)⁵. Light-sheet microscopy, on the other hand, could show good sectioning and low photobleaching⁶; however, the available numerical aperture (NA) is greatly limited by the requirement of orthogonally placed objectives⁷. Alternatively, it requires special illuminators and sample chambers^{8,9}.

For these reasons, highly inclined and laminated optical sheet (HILO) microscopy has been widely used for 3D single-molecule imaging¹⁰. When an inclined beam encounters an interface of two media (glass and water, for example), the beam is refracted according to Snell's law. Importantly, the refracted beam gets thinner, and its thickness is described as $dz = R/\tan(\theta)$ where R is the diameter of the inclined beam and θ is the refraction angle of the transmitted beam. This simple implementation results in a good sectioning capability. Nevertheless, this relation indicates that a thin illumination (i.e., high sectioning capability) requires a small R and/or a large θ . For example, when $R = 20\ \mu\text{m}$ and $\theta = 72$ degrees, one can obtain $dz = 6.5\ \mu\text{m}$. Since there is a practical limit to increasing the refraction angle in order to image deep inside cells and avoid total internal reflection, there is a strong coupling of the illumination diameter and the beam thickness. For this reason, HILO imaging shows a relatively small field-of-view (FOV) that greatly restricts its applications in multicellular imaging.

Recently, we have overcome this problem by highly inclined swept tile (HIST) microscopy where the FOV is decoupled from the beam thickness in a very simple way¹¹. First, a beam elongated in one direction is generated via a pair of cylindrical lenses. This beam, termed as a tile, produces a thin illumination with $dz \sim 4\ \mu\text{m}$ while its FOV is $130 \times 12\ \mu\text{m}^2$. Then, the tile is swept across the sample using a rotating galvo mirror. Meanwhile, the fluorescence image is recorded on a scientific complementary metal-oxide semiconductor (sCMOS) camera that filters efficiently out-of-focus background by operating in a rolling shutter mode which serves as tunable confocal slit detection. In this way, HIST microscopy enables single-molecule imaging with a larger field of view ($\sim 130 \times 130\ \mu\text{m}^2$) and a thinner illumination than HILO imaging. We applied this new imaging technique to detect RNA transcripts with a single probe in cells or with a few probes in mouse brain tissues, which has significant potential for studying gene expression and diseases. Unlike other approaches, HIST employs only a single high numerical aperture objective without an additional illuminator or remote detection objectives and is fully compatible with inverted microscopes. These advantages along with a large FOV and high contrast will make HIST microscopy a prominent tool in biology and medicine. We present detailed instructions regarding instrumentation of the HIST microscope, and how to test and calibrate its performance as below.

PROTOCOL:

1. Setting up the microscope, lasers and alignment tools

1.1. Before building the microscope, prepare all the necessary components including optics, optomechanics and electronics as listed in **Table of Materials**.

1.2. Prepare a microscope body mainly composed of two parts: an objective holder with an RMS-threaded port and a piezo-stage mounted on an aluminum block (**Figure 1A**).

NOTE: The custom-made microscope body is used for the convenience and flexibility of instrumentation¹². Any commercially available microscope body can be used for HIST microscopy.

1.3. Combining multiple laser lines and coupling them to a single mode fiber

1.3.1. Install 405, 561, 638 nm lasers onto the optical table and combine the beams through a polarizing beam splitter and a long-pass dichroic mirror as shown in **Figure 1B**. Make sure that all laser beams pass through pinholes on the alignment tool. Insert half wave plates for power adjustment.

NOTE: Wear safety goggles for eye protection and use beam-blocks to absorb unwanted laser beams.

1.3.2. Install a fiber coupling lens ($f = 4.5$ mm) and a fiber adapter held in z-axis translator with a cage system.

1.3.3. Connect a multimode fiber (MMF, $\varnothing 62.5$ μm) to the fiber adapter. Adjust each pair of the steering mirror and the z-translator until the coupling efficiency of each laser is higher than 95%. The output beam has a near Gaussian-shaped profile with speckle patterns.

1.3.4. Take away the multimode fiber and connect a single mode fiber (SMF). Similar to MMF, fine-tune and maximize the coupling efficiency of three lasers.

1.4. Assemble a collimated light source that will be used for beam alignment in the excitation and detection paths. This device is composed of a temporally coherent light source (561 nm) connected to SMF, fiber adapter, achromatic lens ($f = 60$ mm), iris and $\varnothing 1''$ tube spacer in a cage system (**Figure 1C**). Adjust the distance between the fiber adapter and the lens using a shearing interferometer to ensure the collimation.

1.5. Prepare a beam alignment tool (**Figure 1D**). This is a pair of aluminum posts with pinholes at 2'' height from the surface of optical table, which allows for quick and precise beam alignment.

1.6. Assemble a double pinhole system which consists of two $\varnothing 1''$ ground glass alignment disks at each end and two $\varnothing 1''$ lens tubes (the bottom one is slotted) as shown in **Figure 1E**.

2. Setting up the detection path

2.1. Take out the objective and install the collimated light source. Adjust the knobs of the mirror (M1) beneath the objective holder so that the output beam from the microscope is roughly parallel to the optical table in height and aligned with threaded holes on the table. Refer to **Figure 2** for detailed positions of each optical component.

2.2. Insert a multiband dichroic mirror (DM) and reflect the beam by 90 degrees. Use the maximum size of the iris and make sure that the beam passes through the center of the dichroic mirror without clipping.

2.3. Use the leaky beam passing through the dichroic mirror to guide the alignment of detection path. Place an sCMOS camera into the beam and make sure that the beam hits the center of the camera chip by using two mirrors (M2 and M3).

2.4. Insert a tube lens (TL; $f = 300$ mm) approximately 300 mm away from the camera.

2.5. Remove the collimated light source and adjust the relative distance between the tube lens and the camera until a pattern on the ceiling is clearly resolved by the camera.

2.6. Insert a multi-band pass filter (BF) before the tube lens for multi-color fluorescence imaging.

3. Setting up the excitation path

3.1. Reinstall the collimated light source on the objective holder. Place a fold-mirror (M4) to redirect the beam output from the microscope by 90 degrees. Adjust the knobs of the dichroic mirror and the fold-mirror iteratively until the beam passes through pinholes in the beam alignment tool.

3.2. Detach the collimated light source and install it on the table, in which the beam points towards the microscope body. Align the beam using a beam alignment tool and a double pinhole system.

3.3. Insert a lens L4 ($f = 400$ mm; $\varnothing = 2$ ") to the optical path approximately 400 mm away from the objective holder. Install the objective lens and adjust the position of L4 along the optical axis until a perfect Airy disk pattern is formed on the ceiling.

NOTE: When inserting the lens, the beam position passing through the lens should be kept unchanged. The lens L4 has a SM2 thread that allows it to be attached/detached from the 60 mm SM2-threaded cage plate easily.

3.4. Unscrew the objective and reinstall the collimated light source with an open iris. Trace down the beam output from the microscope with a business card. Mount a mirror M5 at the place where the size of beam is the smallest and approximately 400 mm away from L4, which is a conjugated image plane (CIP).

3.5. Install a mirror M6 and reflect the beam by 90 degrees. Adjust M5 and M6 iteratively with the beam alignment tool.

3.6. Insert a lens L3 ($f = 150$ mm) roughly 150 mm away from M5. Use a shearing interferometer to ensure the collimation of the output beam.

3.7. Temporarily take away L4 and trace down the beam to find the focal position of L3. Put a single axis galvo mirror at this point, which is a conjugated back focal plane (cBFP). Supply 0 volts to the galvo mirror and rotate the holder of the galvo mirror so that it reflects the beam by 90

degrees.

3.8. Place a fold-mirror M7. Properly place L2 ($f = 100$ mm) the same way as step 3.6.

3.9. Remove the collimated light source from the object holder. Install a collimation lens L1 ($f = 100$ mm), fiber adapter and iris. Connect a single mode fiber to the adapter and send the beam through the imaging system.

3.10. Reinsert L4 and fine tune the system until a perfect Airy disk pattern appears on the ceiling.

4. Setting up the cylindrical lenses

4.1. Insert a cylindrical lens (CL1, $f = 400$ mm) after L1 and make sure that the cylindrical lens focuses the beam along the x-axis.

4.2. Insert another cylindrical lens (CL2, $f = 50$ mm) to the beam path. Use a shearing interferometer to ensure the output beam is collimated.

NOTE: The distance between the two cylindrical lenses is 450 mm. The output beam has a compression ratio of 8 and an elongated oval shaped Airy disk pattern is formed on the ceiling.

5. Testing tile imaging

5.1. Prepare 3D hydrogel sample. Mix 20 nm Crimson beads with a hydrogel solution, which consists of 12% acrylamide:bisacrylamide (29:1), 0.2% (v/v) TEMED and 0.2% (w/v) ammonium persulfate in 0.75x TAE buffer. Inject 50 μ L of the mixed solution into a flow chamber as described elsewhere¹¹. After 10 min, the 3D hydrogel sample is ready to for imaging.

5.2. Put the sample onto the sample holder. Turn on 638 nm laser and adjust the power to <1 mW for sample excitation.

5.3. Run the camera control software. In the camera acquisition-setting panel, select **Internal** in trigger mode and then click **Take video** for free running mode.

5.4. Slightly adjust the position of the camera so that the image is located at the center of the camera when 0 volts is applied to the galvo mirror.

5.5. Rotate the horizontal knob of the mirror M5 to achieve a highly inclined illumination.

NOTE: With the increase of the illumination angle, the hydrogel image becomes clearer than that of the Epi image as the beam gets thinner. However, the image keeps almost the same position.

5.6. Record the tile image. Calculate the effective illumination width¹¹. For example, **Figure 3** shows the effective illumination width of 12 μ m.

6. HIST imaging

6.1. Prepare a data acquisition board connected with a terminal block. Connect USER 1 BNC connector with P0.0 through an electrical wire. Use USER 1 as a digital output for the external triggering of sCMOS camera. Connect an analog output A00 to a galvo mirror driver.

6.2. Generate TTL pulse trains from P0.0 using a custom-made program (**Figure 4A**) and set the period time = 400 ms and $t_{ON} = 2$ ms. Check the generated pulses from USER 1 BNC terminal by a digital oscilloscope and then connect the BNC cable to the camera external trigger port.

NOTE: The control software used in this paper is available upon request. When imaging at different camera frame rates, the period time should be adjusted accordingly.

6.3. Start sweeping a galvo mirror via the custom-made program. Adjust V_{min} to -800 mV and V_{max} to 800 mV for full FOV imaging. Note that under this operation, 3D hydrogel samples still show high background similar to Epi illumination.

6.4. Changing the camera acquisition setting.

6.4.1. Select **External** in trigger mode and **Down (Sequential)** in LightScan Plus drop-down menu as shown in **Figure 4B**.

NOTE: In this setting, the camera does not take images unless a trigger signal is turned on.

6.4.2. Click **Scan Speed Control** for window height and line exposure time control and set the values to be **185 rows** and **32 ms**, respectively.

NOTE: When a tile width (W_{eff}) is 185 rows (12 μ m) and an integration time per line (T_{int}) is 32 ms, a delay time between lines (T_D) is determined as $T_D = T_{int}/W_{eff} = 0.173$ ms. For imaging 2,048 x 2,048 pixels, the total acquisition time is $2,048 \times T_D + T_{int} = 386$ ms, corresponding to ~ 2.6 fps.

6.5. Slightly adjust V_{max} and V_{min} to obtain clearer images.

6.6. Obtain 3D stack images using the custom-made program by switching on **3D stack ON** and specifying the number of stacks and the step size.

REPRESENTATIVE RESULTS:

As an example, single-stranded DNA labeled with Atto647N was imaged with an excitation wavelength of 638 nm in a 3D hydrogel. DNA was anchored to the hydrogel network via an acrydite moiety during gel polymerization. The images were taken at 5 μ m above the surface as shown in **Figure 5a**. The HIST image showed much less background compared to the Epi image, from which the signal to background ratio was calculated to be 1.9 ± 0.7 for the HIST image while most of the single molecule spots could barely be detected by Epi.

Single-molecule RNA fluorescence in situ hybridization (smFISH) was performed with 4 FISH probes. **Figure 5b** displays smFISH images of *EEF2* (eukaryotic translation elongation factor 2) labeled with AlexaFluor 647 on A549 cells in an imaging buffer (refer to our previous work regarding the sample preparation¹¹). A maximum intensity projection was performed on 20 z-stacks corresponding to 5 μm thickness. The HIST image showed not only much improved SBR but also more uniform illumination compared to Epi image. For Epi imaging, the exposure time was 400 ms while for HIST imaging the integration time per line was 32 ms, both of which had the same illumination power of 7.5 mW measured before the objective. The imaging speeds of Epi and HIST were 2.5 fps.

FIGURE LEGENDS:

Figure 1. Microscope body, lasers and alignment tools. (A) Objective and sample holder. (B) Photo of laser systems. LP, long-pass dichroic mirror; $\lambda/2$, half-wave plates; PBS, polarizing beamsplitter. (C) Collimated light source. (D) Beam alignment tool with two insertable pinholes. (E) Double pinhole system.

Figure 2. Detailed setup for highly inclined swept tile (HIST) microscopy. Photo (A) and schematic (B) of HIST microscope system. BF, multi-band pass filter; CL1-2, cylindrical lenses; DM, dichroic mirror; GM, galvo mirror; BF, band-pass filter; M1-7, mirrors; L1-4, lenses; SMF, single mode fiber; TL, tube lens; cIP, conjugated image plane; cBFP, conjugated back focal plane.

Figure 3. Tile illumination with a compression ratio of 8. (A) Fluorescence image of 20 nm beads in a 3D hydrogel. Scale bar, 20 μm . (B) Standard deviation projection along the y direction of A, smoothed by 10 data points. The red arrow indicates an effective illumination width of 12 μm .

Figure 4. Control and imaging software front panels. (A) A custom-made LabView program synchronously controls the scanning of the galvo mirror, the starting acquisition of sCMOS camera and the movement of the piezo stage. (B) Camera acquisition setting control panel.

Figure 5. (A) Images of Atto647N-labeled DNA in a 3D hydrogel with Epi and HIST illumination. (B) smFISH images of *EEF2* using 4 FISH probes on A549 cells by Epi and HIST microscopy. DAPI stain is shown in blue. Scale bars, 20 μm .

DISCUSSION:

There are two critical steps in this protocol. The first one is the proper placement of L4 in step 3.3, ensuring that the incident beam passes through the center of the lens and a perfect Airy disk pattern is formed on the ceiling. The position of L4 determines the placement of all the other optical components, including M5, L3, GM and L2. The second critical step is the synchronization process. To reject the out of focus background, active pixels whose effective detection width is equal to the tile width should be synchronized with the beam sweeping. Therefore, it is necessary to measure the effective illumination width of a tile beam (step 5.6) and set camera parameters accordingly in step 6.4.

When imaging with very large FOV, the presented method shows an increased background at one side compared to the other side. This is attributed to slightly altered angles of illumination at different imaging positions. Implementing a second galvo mirror instead of M5 alleviates this problem as demonstrated before by synchronously adjusting the position and the scanning angle¹¹. Instead of off-the-shelf achromatic doublets, a telecentric scan lens will be also helpful. However, for imaging an area of $<80 \times 80 \mu\text{m}^2$, single galvo mirror sweeping was sufficient. HIST microscopy has a limit of the imaging depth, however, it is able to obtain a good SBR when imaging up to $\sim 15 \mu\text{m}$ with a $12 \mu\text{m}$ tile beam and a NA 1.45 oil immersion objective lens¹¹.

In this protocol, we used a beam compression ratio of 8 to make a tile beam. A thinner illumination can be used in HIST microscopy to achieve higher SBR, which might be powerful for single-molecule tissue imaging¹¹. However, in this case, photobleaching effect should be considered by an increased excitation intensity while the current beam compression ratio showed reduced photobleaching in 3D imaging compared to Epi¹¹. Compared to light-sheet microscopes with two orthogonally placed objectives, HIST microscopy is simple to implement and compatible with conventional sample preparations. The enhanced SBR and large FOV of HIST microscopy is suitable for studying the interactions and dynamics of single biomolecules in multiple cells and can be used further in super-resolution imaging and single-molecule tracking.

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DISCLOSURES:

University of Central Florida has filed a patent application covering the work described in this paper.

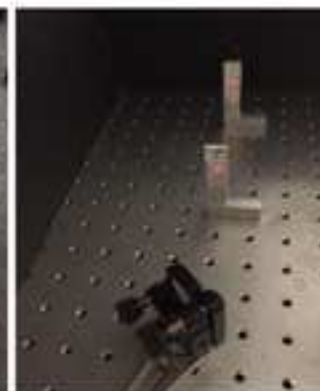
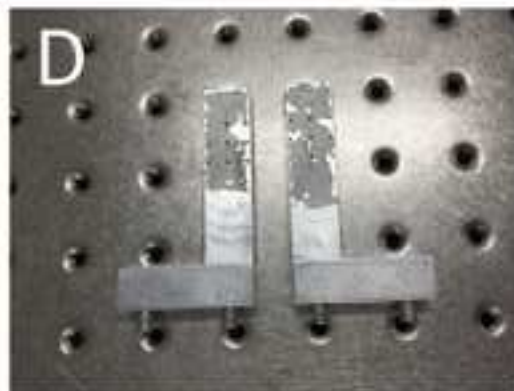
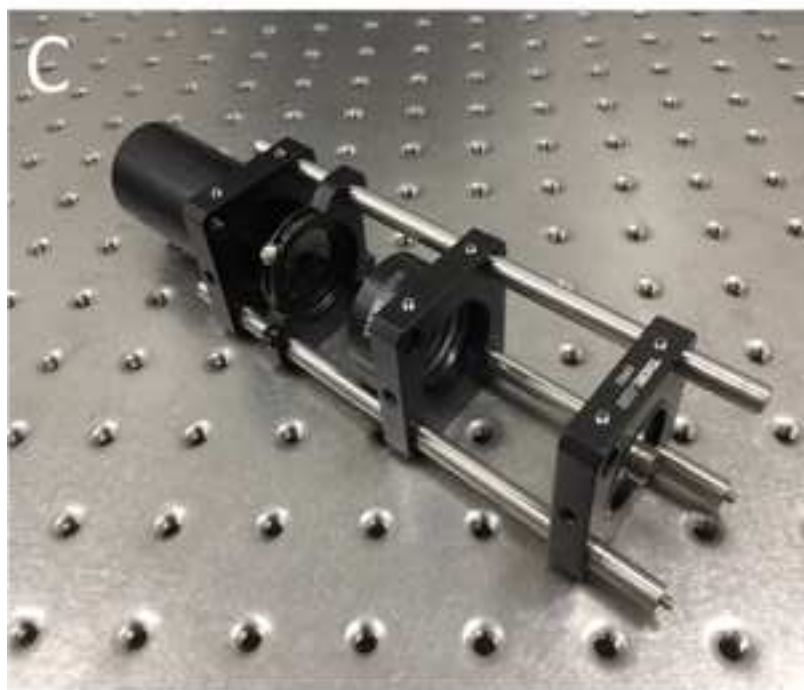
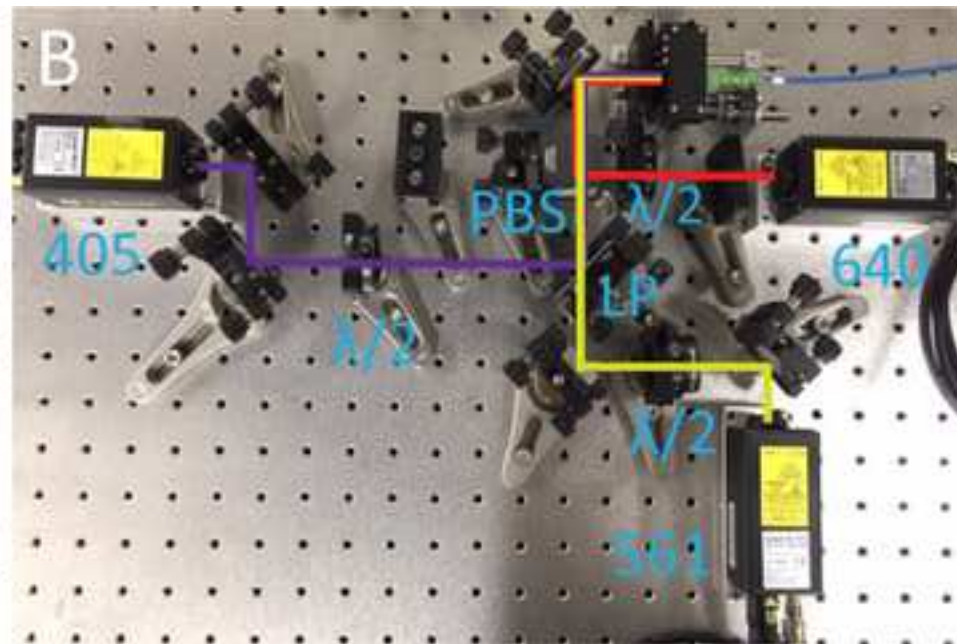
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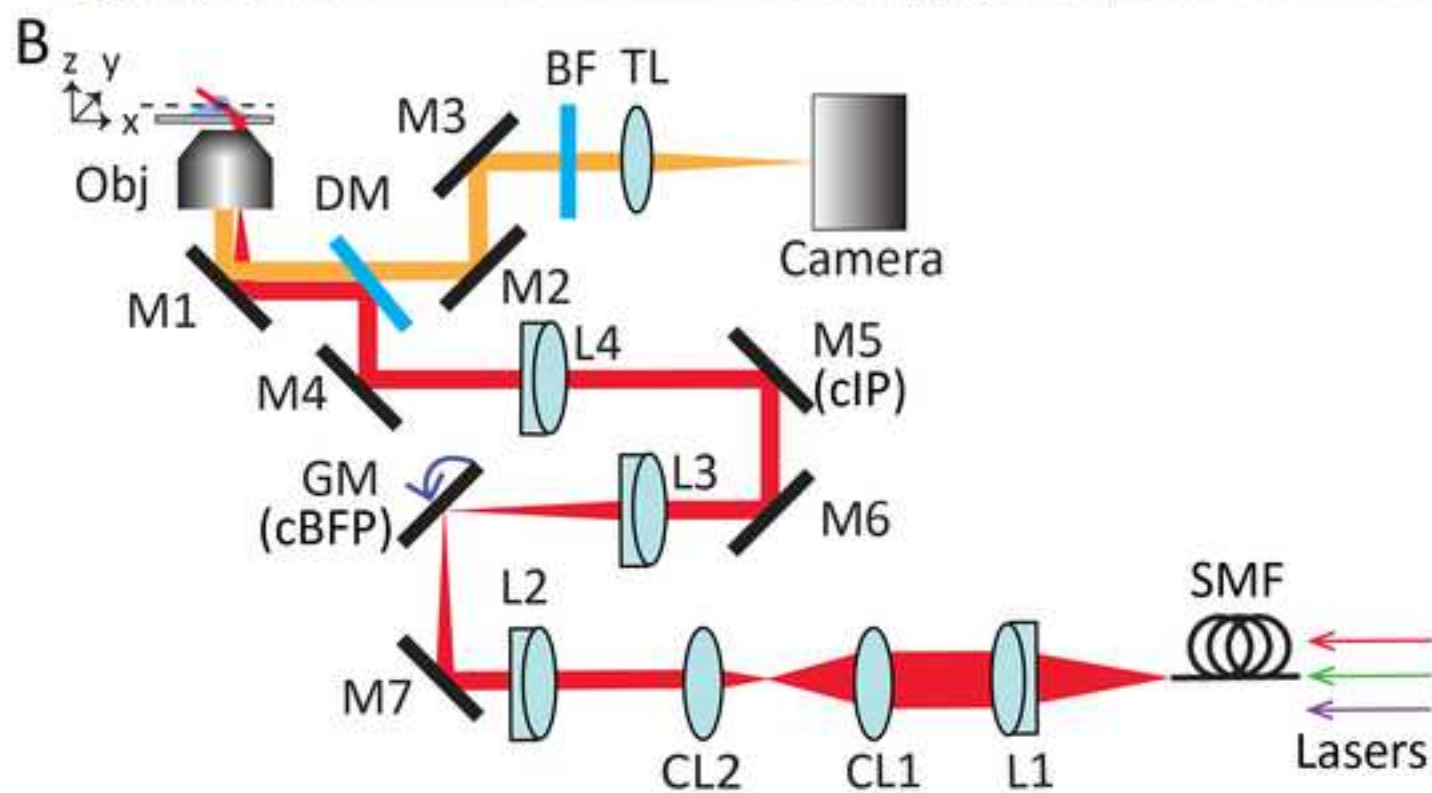
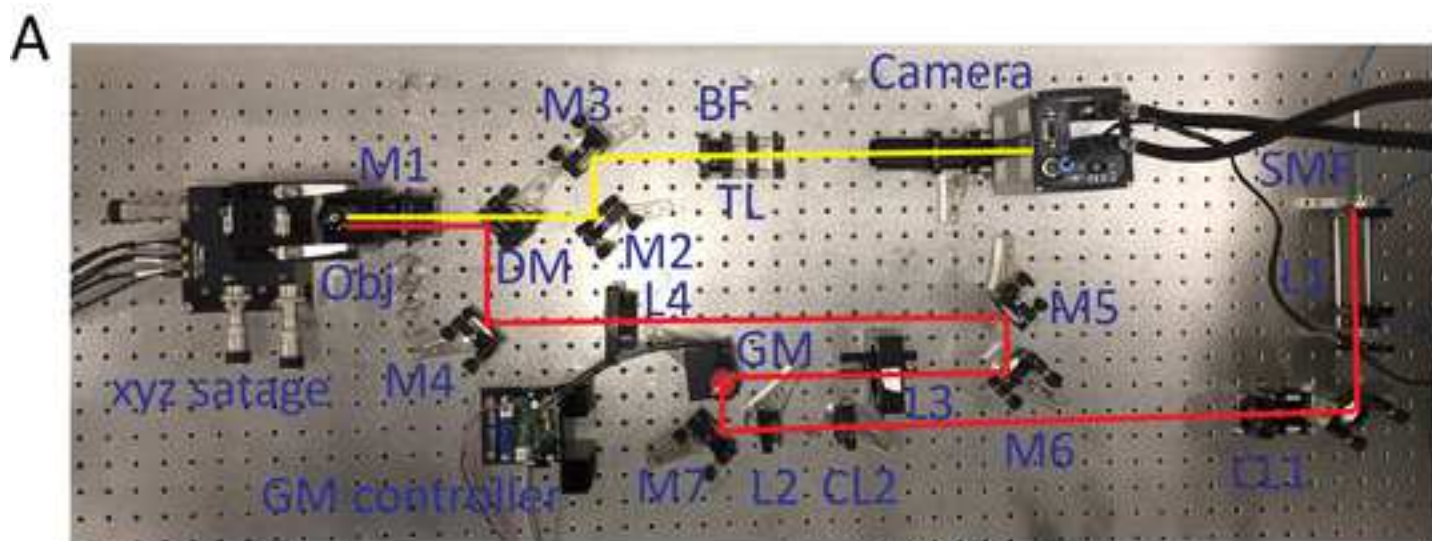
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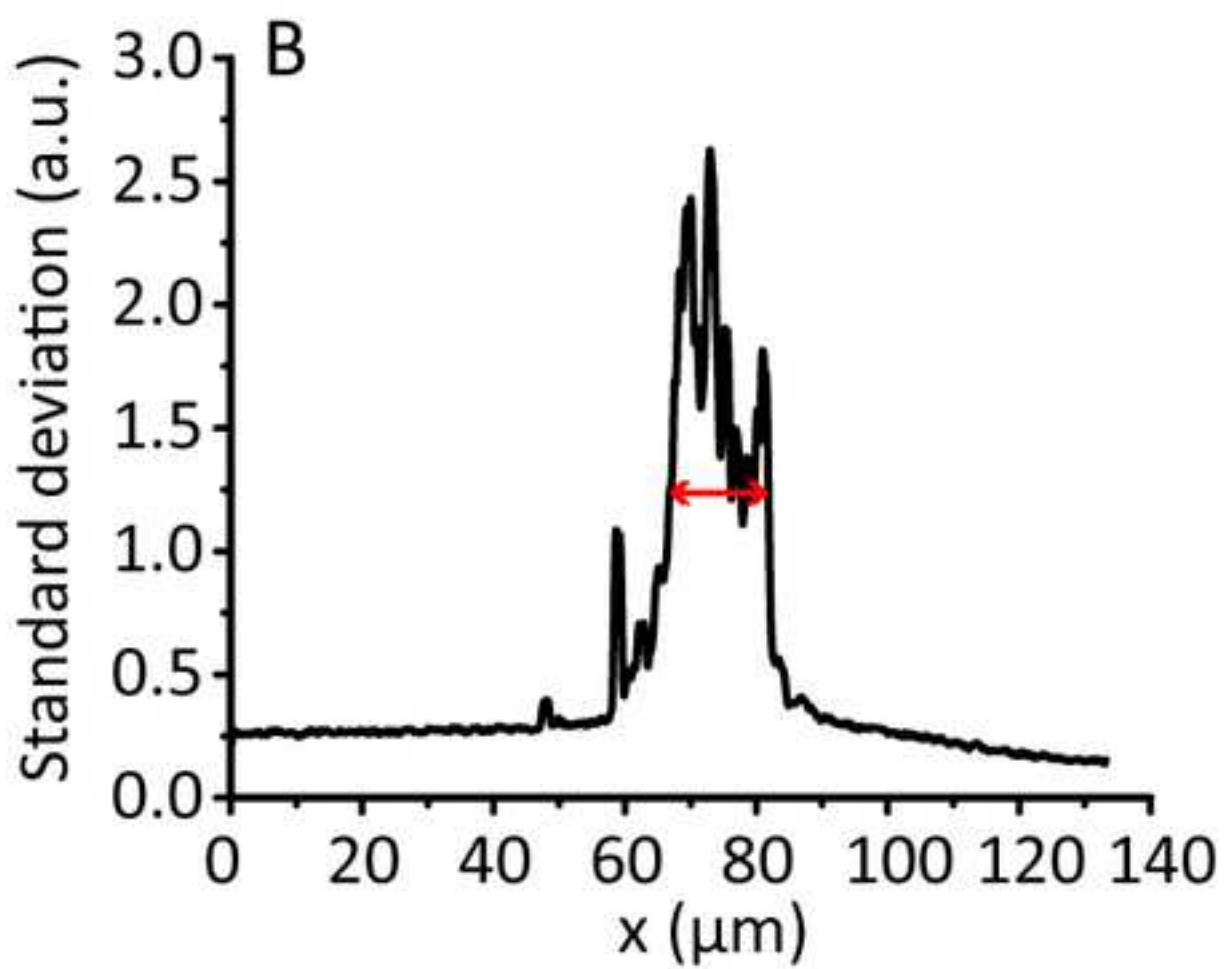
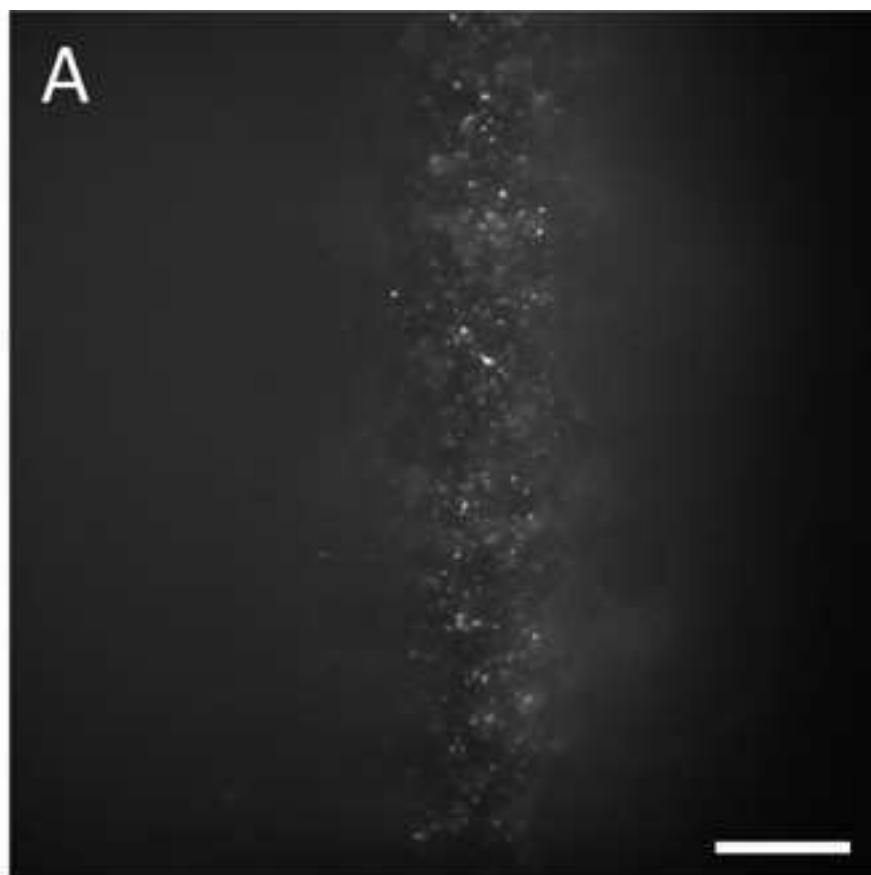
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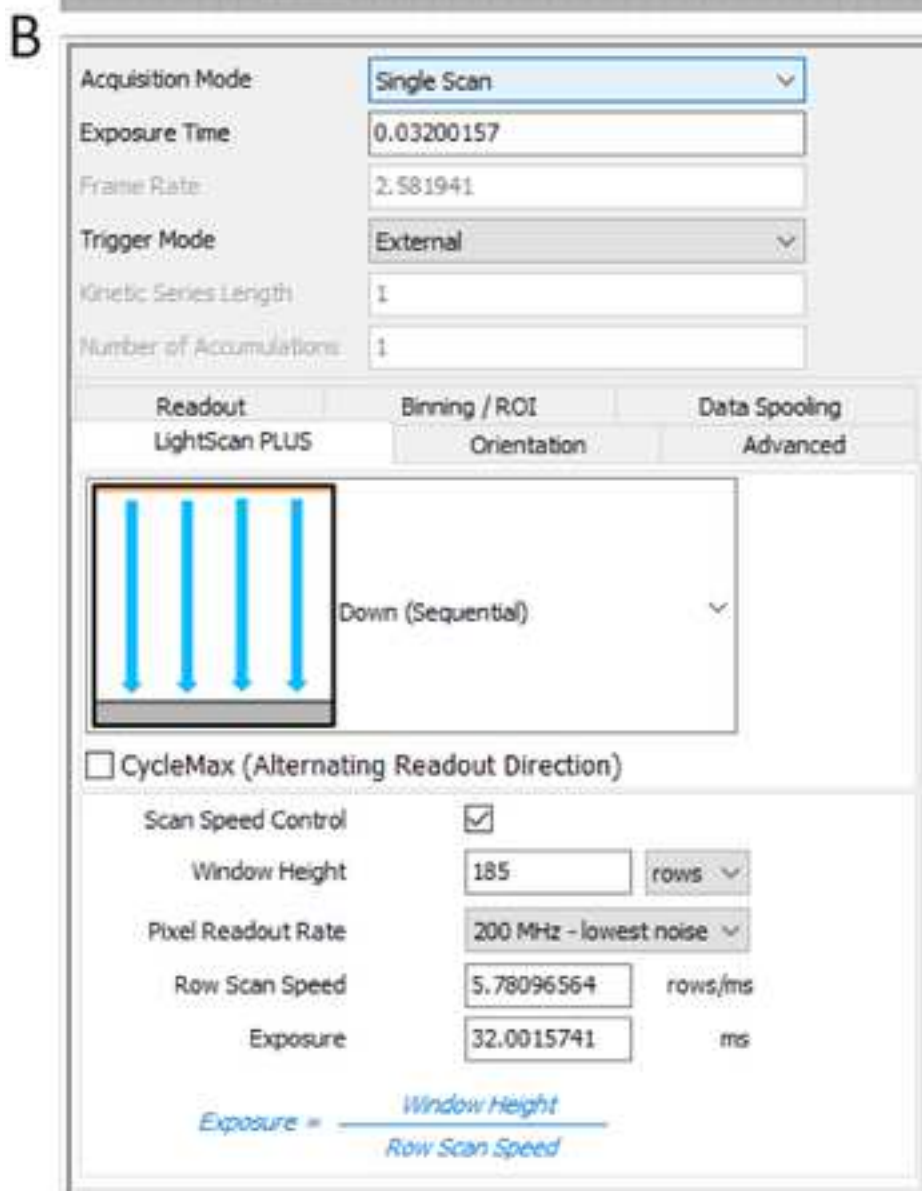
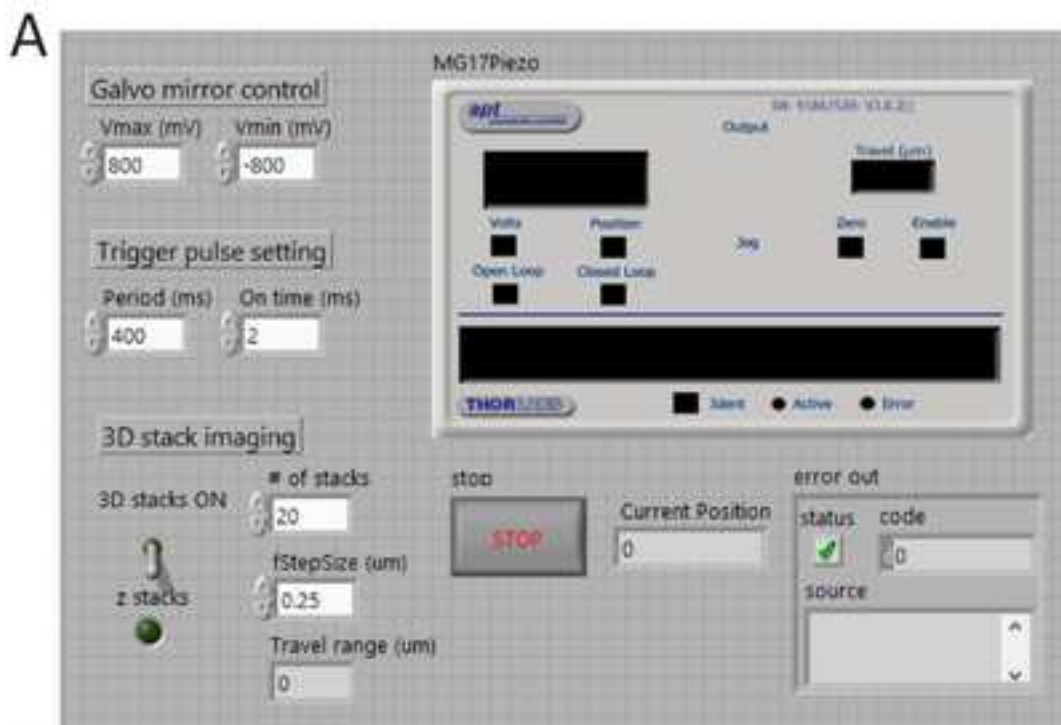
Figure1

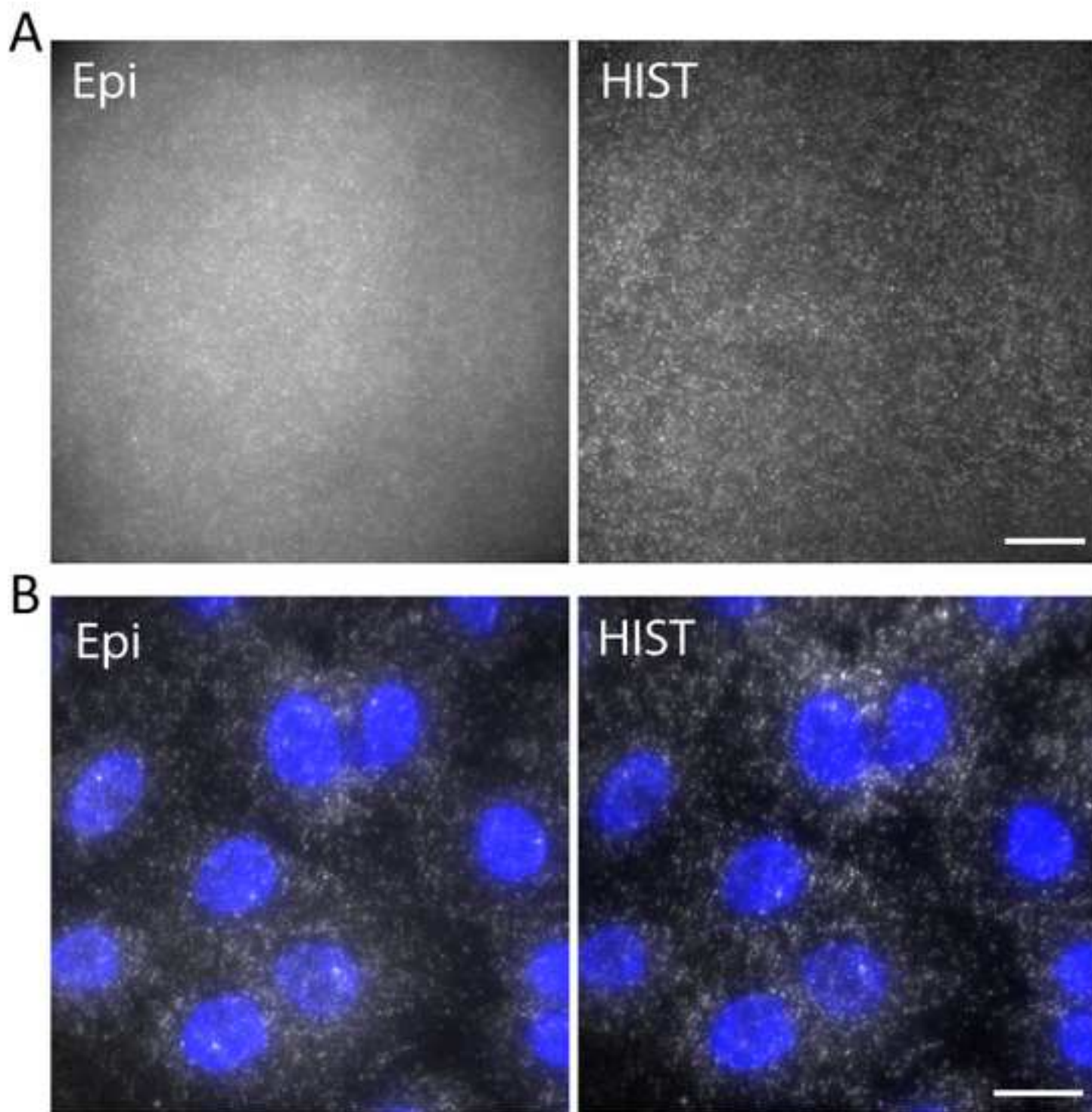
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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1" Achromatic doublet	Thorlabs	AC254-060-A-ML	Collimator
1" Achromatic doublet	Thorlabs	AC254-100-A-ML	L1,L2
1" Achromatic doublet	Thorlabs	AC254-300-A-ML	TL
1" Broadband Dielectric Mirrors	Thorlabs	BB1-E02-10	M1~M7
1" Cylindrical Lenses	Thorlabs	LJ1363RM-A	CL1
1" Cylindrical Lenses	Thorlabs	LJ1695RM-A	CL2
1" square kinematic mount	Edmund Optics	58-857	For dichroic mirror mounting
1" Threaded Cage Plate	Thorlabs	CP02	For holding other lenses
2" Achromatic doublet	Thorlabs	AC508-150-A-ML	L3
2" Achromatic doublet	Thorlabs	AC508-400-A-ML	L4
2" Threaded Cage Plate	Thorlabs	LCP01	For holding L4
2" Threaded Cage Plate	Thorlabs	LCP01T	For holding L3
2% Bis Solution	Bio Rad	64085292	hydrogel component
20 nm fluorescent beads	Thermo Fisher	F8782	For testing imaging
30 mm Cage Right-Angle Kinematic Mirror Mount	Thorlabs	KCB1	For objective & camera mounting
30mm Cage System Iris	Thorlabs	CP20S	
3-Axis NanoMax Stage	Thorlabs	MAX311D	
40% Acrylamide Solution	Bio Rad	64148001	hydrogel component
405 nm laser	Cobolt	Cobolt 06-MLD	
50x TAE buffer	Bio-Rad	161-0743	hydrogel component
561 nm laser	Cobolt	Cobolt 06-DPL	
638 nm laser	Cobolt	Cobolt 06-MLD	
Ammonium persulfate	Sigma	A3678-25G	hydrogel component
Beam alignment tool	custom made		
BNC terminal blocks	Natural Instruments	BNC-2110	

Cage plate with M9 x 0.5 internal threads	Thorlabs	CP1TM09	For holding aspheric lens
Cage System Rods	Thorlabs	SR series	
Cell culture & smFISH			See a reference [11]
Double side tape	Scotch	515182	Flow chamber
Epoxy	Devcon	14250	Flow chamber
Galvo mirror	Thorlabs	GVS211	GM
Galvo System Linear Power Supply	Thorlabs	GPS011	
Half wave plate	Thorlabs	WPH10M-405/561/633	Power adjustment
long-pass dichroic mirror	Chroma	T550lpxr	For combining lasers
Microscope slides	Fisherbrand	12549-3	Flow chamber
Mikroskopische Deckglaser	Hecht Assistent	990/5024	Flow chamber
Mounted Frosted Glass Alignment Disk	Thorlabs	DG10-1500-H1-MD	For double pinhole system
Mounted rochester aspheric lens	Thorlabs	A230TM-A	
Multi-band dichroic mirror	Semrock	Di03-R405/488/561/635-t3	DM; 3 mm thickness
Multi-band filter	Semrock	FF01-446/523/600/677-25	BF
Multimode fiber	Thorlabs	M31L02	MMF
N,N,N',N'-tetramethyl ethylenediamine	Sigma	T7024-25ML	hydrogel component
NI-DAQ board	Natural Instruments	PCI-6733	
Ø1" Kinematic Mirror Mount	Thorlabs	KM100	For holding mirrors
Objective lens	Olympus	PLANAPO N 60X	60X 1.45NA oil
Pedestal Base Clamping Forks	Newport	9916	
Pedestal Pillar Posts	Thorlabs	RS1P8E	
Piezo controller	Thorlabs	BPC303	
Polarized beam splitter	Thorlabs	PBS251	For combining lasers
RMS-SM1 adapter	Thorlabs	SM1A3TS	For objective lens
Rod holder	custom made		

Rotation cage mount	Thorlabs	RSP1/CRM1/CRM1P	For HWP & cylindrical lens mounting
sCMOS camera	Andor	Zyla-4.2P-CL10	
Shearing interferometer	Thorlabs	SI100	Beam collimation test
Single mode fiber	Thorlabs	P5-405BPM-FC-2	SMF
SM1 Lens Tubes	Thorlabs	SM1S25	For double pinhole system
SM1 Slotted Lens Tube	Thorlabs	SM1L30C	For double pinhole system
Stage mount	custom made		
threaded fiber adapter	Thorlabs	SM1FC	
Z-Axis Translation Mount	Thorlabs	SM1Z	Fiber coupling



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A guide to build a highly inclined swept tile microscope for extended field-of-view single-molecule imaging

Author(s):

Jialei Tang, Chun-Hung Weng, Jeffrey B. Oleske & Kyu Young Han

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Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

[We have proofread it.](#)

2. Summary: Please revise it to be a complete sentence.

[We have changed the summary as follows:](#)

[“A detailed instruction is described on how to build a highly inclined swept tile \(HIST\) microscope and its usage for single-molecule imaging.”](#)

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[We have removed all the commercial phrases used in the manuscript.](#)

4. Please revise the protocol text to avoid the use of any personal pronouns (e.g., “we”, “you”, “our” etc.).

[We have changed all the sentences used in the protocol with personal pronouns.](#)

5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). 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.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

[We have changed the sentences to imperative tense in the protocol.](#)

6. 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. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

[We have simplified the steps and if necessary, made sub-steps.](#)

7. Sections 2-4: Please reference Figure 2 to guide the readers about the positions of different optical parts.

[We have added a sentence in the beginning of the section 2.1 as follows.](#)

[“... Refer to Figure 2 for detailed positions of each optical component.”](#)

8. 6.2, 6.3, 6.6: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).

Following editor's advice, we have added a detailed software control panel in Fig. 4A and 4B and revised the software instruction in step 6.

9. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Done.

10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Done.

11. Please include all relevant details that are required to perform the step in the highlighting. 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 highlighted.

Done.

12. Please remove the embedded figure(s) from the manuscript.

Done.

13. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

- a) Critical steps within the protocol
- b) Any modifications and troubleshooting of the technique
- c) Any limitations of the technique
- d) The significance with respect to existing methods
- e) Any future applications of the technique

We have revised it accordingly.

14. References: Please do not abbreviate journal titles.

We have changed the format of the references.

15. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Done.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this article titled "A guide to build a highly inclined swept tile microscope for extended field-of-view single-molecule imaging", the authors present some really useful tricks to allow high contrast, extended field of view, deep tissue imaging using relatively simpler optical components. Their article is very useful for researchers performing single-molecule imaging, and microscope alignment, and additionally add HIST capabilities to allow cells and tissue

imaging. I highly recommend the publication of this paper.

Major Concerns:
None

Minor Concerns and suggestions:

1. I understand that it is out of scope for the authors to provide detailed instructions on 1.2 section of Protocol, where they talk about combining lasers, and fiber coupling. They could instead provide citations of published literature that provides such instructions.

Thank you for this valuable advice. We have added the details for combining lasers and fiber coupling in section 1.3.1 to 1.3.4 as follows:

“1.3. Combine multiple laser lines and couple them to a single mode fiber.

1.3.1. Install 405, 561, 638 nm lasers onto the optical table and combine the beams through a polarizing beam splitter and a long-pass dichroic mirror as shown in Figure 1B. Make sure that all laser beams pass through pinholes on the alignment tool. Insert half wave plates for power adjustment.

Note: Wear safety goggles for eye protection and use beam-blocks to absorb unwanted laser beams.

1.3.2. Install a fiber coupling lens ($f = 4.5$ mm) and a fiber adapter held in z-axis translator with a cage system.

1.3.3. Connect a multimode fiber (MMF, $\varnothing 62.5$ μm) to the fiber adapter. Adjust each pair of the steering mirror and the z-translator until the coupling efficiency of each laser is higher than 95%. The output beam has a near Gaussian-shaped profile with speckle patterns.

1.3.4. Take away the multimode fiber and connect a single mode fiber (SMF). Similar to MMF, fine-tune and maximize the coupling efficiency of three lasers.”

2. It was unclear in certain steps of the protocol, in which direction the alignment is happening. Is the laser alignment being performed by tracing the beam back from the objective holder or by forward tracing the beam from the fiber-coupled laser source. The authors can clarify this in the manuscript.

Thanks for the suggestion. We have clarified the direction of the laser beam for each step.

3. In 6.6, authors mention they collected 3D stack in figure 4. Which image are they referring to? Could the authors also mention the tissue/cell penetration depth they were able to achieve with the 12 μm tile?

This was a typo. The 3D stack images should indicate Figure 5B. We have discussed the penetration depth in the revised manuscript as follows.

“HIST microscopy has a limit of the imaging depth, however, it is able to obtain a good SBR when imaging up to ~ 15 μm with a 12 μm tile beam and a NA 1.45 oil immersion objective lens¹¹.”

Reviewer #2:

The paper presents a detailed instruction on how to build a highly inclined swept tile (HIST)

microscope. The manuscript is well written and I enjoyed reading it. The instruction is clear and complete for others to replicate the setup. Therefore I recommend publication.

There are some minor issues though:

1) Figure 3 and Figure 4 are in the wrong order;

We are sorry for the mistake. We have corrected this.

2) The camera setting could be presented clearer. After a little research, it seems to me that Zyla 4.2 PLUS has Sequential Readout Direction Options such as 'top down' and 'bottom up'. I assume this is what was used in this paper. Is this the case?

We have clarified the camera software controlling part in step 6.4:

“6.4. Change the camera acquisition setting.

6.4.1. Select “External” in trigger mode and “Down (Sequential)” in LightScan Plus drop down menu as shown in Figure 4B.

Note: In this setting, the camera does not take images unless a trigger signal is turned on.

6.4.2. Click “Scan Speed Control” for window height and line exposure time control, and set the values to be 185 rows and 32 ms, respectively.

Note: When a tile width (W_{eff}) is 185 rows (12 μm) and an integration time per line (T_{int}) is 32 ms, a delay time between lines (T_{D}) is determined as $T_{\text{D}} = T_{\text{int}}/W_{\text{eff}} = 0.173$ ms. For imaging 2,048×2,048 pixels, the total acquisition time is $2,048 \times T_{\text{D}} + T_{\text{int}} = 386$ ms, corresponding to ~2.6 fps.”