**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.”

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Spectrolight, Zyla, etc.

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: Ware 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, Ø 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 12um 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 (Weff) is 185 rows (12 μm) and an integration time per line (Tint) is 32 ms, a delay time between lines (TD) is determined as TD = Tint/Weff = 0.173 ms. For imaging 2,048×2,048 pixels, the total acquisition time is 2,048×TD + Tint = 386 ms, corresponding to ~2.6 fps.”