**Review comments rebuttal**

We would like to thank all the reviewers for their time and appreciate their help in improving the manuscript. We have supplied additional representative results (figures and movies) showing applications of the technique for imaging live cells.

**Editorial comments:**  
 *1) All of your previous revisions have been incorporated into the most recent version of the manuscript. In addition, Editor may have made formatting changes and minor copy edits to your manuscript. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document.* ***Please use this updated version for any future revisions and track all changes using the track changes function in Microsoft Word****.  
  
2) Prior to peer review, the highlighted portion of your protocol exceeds our 2.75 page highlighting limit. After making all the requested changes to the protocol, please adjust the highlighting to identify a total of 2.75 pages or less of protocol text (which includes sub-headings and spaces) that should be visualized to tell the most cohesive story of your protocol steps. The highlighting should include complete statements and not portions of sentences. See JoVE's instructions for authors for more clarification.  
  
3) Formatting:  
a) A space is required between steps 4.4.2 and 4.4.3.*

A space has been added.

*b) References – Please abbreviate all journal titles.*

Reference style updated.

*4) Grammar: Please use the American English spelling for all words in the manuscript.*

Spelling has been changed to American English. *5) Visualization:  
a) Section 1 – Please provide labeled photographs of the setup as a supplemental file. Please upload these files to the "Supplemental File (as requested by JoVE)" section of the JoVE submission site. Please add the suffix "\_SW" for any files uploaded for this purpose.*

Two labelled photographs of the setup have been included as supplementary files.

*b) Please clarify “Separate the objective, lenses L3, L4, L5, and the SLM each by the sum of their respective focal lengths such that the SLM surface will be relayed onto the focal plane of the objective.” It is unclear what action will be filmed here.*This sentence does not require filming and we have removed the highlighting of corresponding text.

*6) Additional detail is required:*

*a) 1.9 – How is the position adjusted?*

Additional detail added.

*b) 3.6 – How is the spatial mask inserted?*

Additional detail added.

*7) Please remove branded term from the 4.6 note: LabVIEW*

Removed. *8) In Discussion, please include a discussion on the future applications of the technique.*

A discussion of the future applications of the technique has been included at the end of the manuscript.

*9) Please modify your references section to comply with JoVE’s instructions for authors, specifically when there are more than 6 authors, list only the first author then "et al." Currently, two authors are listed, then et al.*

Reference style has been updated.

*10) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.  
  
11) Please disregard the comment below if all of your figures are original.  
If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."***Reviewers' comments:**  
  
**Reviewer #1:**

We would like to thank the reviewer for their helpful comments and address the points raised individually below.

*Manuscript Summary:   
The manuscript detailed the parts and steps required for successfully building a TIRF-SIM setup.   
  
Major Concerns:  
From the diagram alone (Fig 1), it's hard to understand the SIM pattern-generation mechanism. What are the polarization states of the light incident on the SLM for the three orientations?*

We have added arrows showing the linear polarization state of the illumination light incident on the SLM. The light should be azimuthally polarized to produce the maximum modulation contrast in the interference pattern and this is explained in the Introduction under “Polarization control”.

*How is light modulated by the SLM such that it acts as a grating? These are crucial information because the mechanism here seems different from two major previous works (citations 13 and 16).*

We have added an additional paragraph in the introduction to clarify the pattern generation mechanism. The binary phase SLM operates as a phase diffraction grating as each pixel imparts either 0 or pi phase shift to the incident wave. Our implementation uses off axis illumination configuration (as previously used in citation 16, 18 and 28) compared to on axis as presented in citation 13. As citation 13 states, “Half of the power is lost in the final polarizing step” when using on axis illumination as the reflected beam from the SLM must traverse a polarizing beam splitter, but this is not the case for the off axis configuration as the polarization control is performed before the light is incident on the SLM. In citation 16, a QWP is used to produce circular polarization which is then passed via a fixed segmented polarizer to produce linear polarization for each of the 3 pattern angles. This method is passive and therefore removes the need for electro-optic control of polarization, but again half the power is lost due to the polarizer.  *Minor Concerns:  
-First paragraph: seems incomplete because it goes on about SMLM's pros and cons at length but then doesn't mention those for STED at all.*

We have added an additional paragraph mentioning the advantages of STED over SIM and SMLM. *-Line 79--80: statement not true any more because since OMX Blade version, no rotation or translation of the grating is done.*

This is of course correct and we have modified this statement. *-Line 200--203: "The arrangement shown in Figure 1" shows DM3 and DM4 both reflect around an axis that is normal to the paper. If that's the case, no such polarization compensation would occur. Use words to explain what 2D drawings cannot express.*

This was unclear and we have added further explanation in the note to step 1.3.We have added also axis labels to the figure which will also help to clarify the arrangement.

*-Step 1.4.2: Needs to emphasize that the pinholes have to be big enough to not clip the laser beam.*

The original wording was unclear. We referred to alignment disks (Thorlabs) used to steer the beam incorrectly as pinholes; beam clipping is not an issue here. We have corrected this point to avoid confusion.  *-Step 2.2: It's unclear what's being sought after here. Please provide more details including angular positions of the crossed polarizers, the retardance the LCVR is set to, etc. Also, explain the role of the polarizer "P" in-between "M" and "HWP" in Fig 1. Isn't the laser beam already linearly polarized?*

The LCVR can be physically mounted with its axis at 45°to the incident vertically polarized light, but this is only a rough alignment. The manufacturing tolerances of the device mean that the fast axis is actually slightly off when the device is mounted in the apparent “45°” position. The HWP is just used to slightly rotate the incident linear polarization and align it to exactly 45°*.*

The reviewer is correct to say that the laser diodes produce beams that are vertically linearly polarized, but we found that there is a very small amount of ellipticity introduced after reflection from the dichroic mirrors so we add an additional polarizer here to ensure perfectly linear polarization.

*-Step 4.4.4: What's "the calibration process"? When is it "complete"? A lot more details are needed.*

We have added an additional note in step 4.4.3 to clarify the purpose of this calibration procedure. The voltage applied to the LCVR is swept from minimum to maximum which has the effect of rotating the linear polarization incident on the sample. The modulation contrast is measured by calculating the ratio of the total signal in the first to the total signal in the zeroth-order Fourier components. We have included an additional MATLAB file (calculate\_contrast.m) to demonstrate this.  *Additional Comments to Authors:  
N/A****Reviewer #2:*** *Manuscript Summary:   
This manuscript describes implementation of a TIRF SIM microscope system using commercially available components, including construction and alignment of the optical system along with some simple testing procedures. For the most part the manuscript is appropriately detailed clearly written, however requires some additional detail on image reconstruction. In the opinion of this reviewer it is insufficient to simply reference existing literature covering SIM image reconstruction and, at the very least, the authors should include a high level overview of how their recommended reconstruction method. Once this point has been addressed, along with the minor concerns listed below the manuscript is suitable for publication in JoVE.  
  
Major Concerns:  
\*The authors should provide a description of the image reconstruction methodology, including accurate determination of the illumination pattern parameters and recombination of SIM information passbands  
  
[****Editorial recommendation:*** *The above comment may be addressed in the Introduction or Discussion.]*

We thank reviewer #2 for this comment and also the editors for allowing us to address this point and have added a few additional paragraphs in the introduction.

*Minor Concerns:  
\*Lines 59 - 60: replace microscope with microscopy in '…stimulated emission depletion microscopy…' and '...structured illumination microscopy.'*

Fixed. *\*Lines 81 - 83: I don't think it is correct that only custom-built SIM systems offer high temporal resolution - I think the later generations of the OMX SIM system offer single slice frame rates of several Hz*

As stated in the response to reviewer #1 to their similar comment, we have removed this statement. *\*Line 97: why is SIM TIRF only possible with 2 of the 3 lasers?*

TIRF-SIM is only possible with 488 and 640 due to the constraints on the SLM pattern: the grating period must be chosen to fit the foci in the TIR ring while also being divisible by 3 to allow 3 equally spaced phase shifts. For 561, the period 9 or 12 patterns used don’t fit within the TIR ring for this wavelength so TIRF-SIM isn’t possible. Note that this is not a fundamental limitation of the technique. *\*Line 174 - Choice of lenses: the stated critical angle presumably applies to a glass-water interface - this should be stated in the text. For samples of higher refractive index the critical angle will increase.*

Yes this is correct, we have clarified that this critical angle is for glass-water interfaces. For samples of higher refractive index, the critical angle increases and can result in scattering of the evanescent field, for example, at focal adhesions. This frustrated TIR seems to vary depending on the cell type, and we haven’t seen it in the COS7 or HEK cell samples presented here.

*\*Lines 313 onward: many of the instructions obviously relate to specific devices and software packages, for example line 314 refers to specific tabs in the SLM control software. I'm not sure that this level of detail is required and arguably makes the instructions more difficult to follow.  
  
[****Editorial recommendation:*** *Please keep JoV’sE protocol requirements in mind as you address the above comment - the protocol must contain sufficient details in order to enable users to accurately replicate your technique. In addition these details are required for our scriptwriters to most accurately plan and write for your video. We recommend NOT removing any details from the protocol text.]  
  
\*Line 358: it would be helpful to include images showing how the image of the fluorescent solution changes between TIRF and non TIRF*

Yes this is important and useful to highlight so we have expanded Figure 2 to include images of TIRF and non-TIRF illumination that clearly show the misalignment effects.

*\*Spherical aberration (for example caused by variations in coverglass thickness) can be a significant problem in SIM microscopy. It would be useful if the authors discuss this - presumably the objective lens has a correction collar?*

We agree that spherical aberration is a huge problem for SIM and have added a sentence in the introduction suggesting use of a correction collar. For 3D-SIM it is extremely important to reduce spherical aberrations to maintain high pattern contrast at depth but for TIRF-SIM it seems to be less of an issue.

*\*The caption to Fig. 2 is slightly misleading. Reduced overlap between the beams decreases the field of view over which the sinusoidal excitation pattern is formed.*

Yes you are correct and we have modified the caption. *\*Fig.5 - the image of carbocyanine labelled β-amyloid fibrils appears to suffer from artefacts (weak copies of filaments at either side of the main filament). These are not present in the image of the Rhodamine labelled fibrils. Could the authors comment on this.*

We have combined Figure 4 and 5 on the suggestion of Reviewer #3. The artefacts seen in the carbocyanine fibrils were ringing artefacts from the reconstruction due to the high dynamic range of the sample. The sidelobes result from the use of the Wiener filter reconstruction method and could have been reduced by empirically altering the Wiener parameter, or by using another reconstruction method which is less prone to this type of artefact.

*Additional Comments to Authors:  
N/A****Reviewer #3:*** *Manuscript Summary:   
The protocol by Young et al. describes the built up of a custom design TIRF-SIM setup enabling super-resolution imaging with down to 90-120 nm lateral resolution of specimen/features that are accessible in the range of <200 nm from the coverslip surface. The system outperforms commercial TIRF-SIM systems (Nikon N-SIM) in terms of acquisition speed through the replacement of a mechanical phase grating with a spatial light modulator. At the same time it should come with a considerably reduced price-tag (although the authors do not specifically mention this).  
The manuscript provides a potentially useful instruction for laboratories that have in-house expertise to build custom optical setups and want to study a suitable biological question that benefits from the 2-fold lateral resolution enhancement over conventional TIRF setups. That said, the manuscript still needs some improvement to firmly justify publication in the JoVE.*

These are valid comments and we have expanded the discussion both in the *future applications* and *potential modifications* sections of the manuscript. We have now included new data of dynamic biological processes (movies 1 and 2) that clearly demonstrate the capability of the instrument and the advantages that come with TIRF SIM, offering both increased resolution and contrast.

*Major Concerns:   
\*TIRF-SIM is geared toward particular samples and biological questions, typically addressing 2D cell surface biology that is within the reach of TIRF microscopy. The authors should mention this specialisation (or limitation) in applicability more thoroughly.   
On the other hand, if the system is not strictly restricted to operate in TIRF mode, the authors may consider describing or discussing options to use the instrument in other modes (e.g. far-field imaging, z-stack acquisition, or axial resolution increase by optional 3-beam interference).*

We have made this limitation more clear in the discussion, and have included further details on how the system presented can be extended to other imaging modalities such as fast 2D-SIM, MSIM and PA NL-SIM.

*\*One major advantage of SIM versus SMLM is the ability to study live samples with higher frame rates and lower photodamage. Although the authors claim frame rates of up to 20 Hz for their system, they do not show any live cell data to support that this is not only a theoretical possibility.*

We have included several examples of high speed live cell TIRF-SIM in a new figure and have provided movies that clearly resolve high speed microtubule dynamics and plasma membrane flow and demonstrate the fast acquisition rate.

*\*The description or discussion of camera, microscope stage, sample holder, temperature control, software for acquisition, reconstruction, data quality control, DSP is rather rudimentary. By following the protocol in its current form it may be hard to replicate an operational setup. If not all aspects can be discussed in detail for space restrictions, it should be at least very explicitly stated which additional instructions or readings have to be followed.*

This protocol is *not* intended for a lay audience, it requires prior optics expertise. It is suited to a laboratory that is already engaged in optical microscopy development: many biophysics laboratories for example have home built TIRF microscopy set-ups. It should be a relatively straightforward matter for an experienced microscopist with optics expertise to follow the instructions. We have now added a paragraph to the protocols to say that Labview code we have written for our system is available on request.

*Minor Concerns:  
\*(Line) 81 "Imaging with high  temporal resolution is therefore only achievable with custom-built systems"    
This is not true for all commercial systems. GE's OMX Blaze 3D-SIM system uses arrays of galvo scanner to change angle and phase positions of the two first order beams independently for each channel, which is in fact faster than any current SLM-based systems. GE's most recent OMX SR model also allows operation in 2D-SIM and 2D-SIM-TIRF mode.*

We have removed this statement and have instead emphasized the reduced cost and increased flexibility of this system compared to commercial instruments. *\*535ff "Custom-built TIRF-SIM systems are capable of multicolour super-resolution imaging at high  speed compared to commercially available microscopes. (see above) The inherent advantage of SIM as a  super-resolution technique is that the temporal resolution is not limited by the photophysics of the fluorophore… "   
One could argue that this also applies to SIM, as photophysical properties such as bleaching rate or quantum efficiency affect the minimum exposure time and the number of time points that can be recorded with sufficient signal-to-noise.*

The reviewer is correct and we have discussed this under “limitations of the technique”.

*\*541ff "Non TIRF-SIM systems can usually achieve resolution improvements of 1.7 times or less in practice as opposed to the  factor of 2 improvement reported here, and commercial systems are also slower and less  flexible than the system presented in this protocol."    
That is surely an overstatement. While this may be true for many instruments in the field (and maybe based on the personal experience of the authors), a well-calibrated and well-operated 3D-SIM system used on sufficiently contrasted samples can routinely achieve 2-fold resolution improvement, as has been demonstrated in many publications (see e.g.* *Demmerle et al. 2015, Methods 88).*

We have modified the sentence to reflect the reviewer’s concerns.

All other minor comments/edits have been addressed in the manuscript with appriopriate changes to text and figure captions.