Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - Author Produced Video
Manuscript Number:	JoVE60378R1
Full Title:	High-throughput total internal reflection fluorescence and direct stochastic optical reconstruction microscopy using a photonic chip
Section/Category:	JoVE Biology
Keywords:	Fluorescence; total internal reflection fluorescence; Super-resolution optical microscopy; nanoscopy; waveguides; Integrated optics
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Additional Information:	
Question	**Response**
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TITLE:

High-Throughput Total Internal Reflection Fluorescence and Direct Stochastic Optical Reconstruction Microscopy Using a Photonic Chip

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KEYWORDS:

Fluorescence, total internal reflection fluorescence, Super-resolution optical microscopy, nanoscopy, waveguides, integrated optics

SUMMARY:

Chip-based super-resolution optical microscopy is a novel approach to fluorescence microscopy and offers advantages in cost effectiveness and throughput. Here, the protocols for chip preparation and imaging are shown for TIRF microscopy and localization-based super-resolution microscopy.

ABSTRACT:

Total internal reflection fluorescence (TIRF) is commonly used in single molecule localization based super-resolution microscopy as it gives enhanced contrast due to optical sectioning. The conventional approach is to use high numerical aperture microscope TIRF objectives for both excitation and collection, severely limiting the field of view and throughput. We present a novel approach to generating TIRF excitation for imaging with optical waveguides, called chip-based nanoscopy. The aim of this protocol is to demonstrate how chip-based imaging is performed in an already built setup. The main advantage of chip-based nanoscopy is that the excitation and collection pathways are decoupled. Imaging can then be done with a low magnification lens, resulting in large field of view TIRF images, at the price of a small reduction in resolution. Liver sinusoidal endothelial cells (LSECs) were imaged using *direct* stochastic optical reconstruction microscopy (*d*STORM), showing a resolution comparable to traditional super-resolution microscopes. In addition, we demonstrate the high-throughput capabilities by imaging a 500 µm x 500 µm region with a low magnification lens, providing a resolution of 76 nm. Through its compact character, chip-based imaging can be retrofitted into most common microscopes and can be combined with other on-chip optical techniques, such as on-chip sensing, spectroscopy,

optical trapping, etc. The technique is thus ideally suited for high throughput 2D super-resolution imaging, but also offers great opportunities for multi-modal analysis.

INTRODUCTION:

Since the initial demonstration of single molecule localization microscopy, many variations have been developed to solve different challenges[1–3]. One challenge that has remained, however, is large field of view dSTORM imaging. Many *d*STORM setups use the same objective lens to both excite the sample and to image it. In order to increase the field of view, a low magnification lens is needed. Low magnification and low numerical aperture (NA) objective lenses typically have a large depth of field, resulting in an increased out-of-plane signal that will reduce the localization precision. TIRF objectives are commonly used to increase the image contrast by reducing out-of-plane fluorescence. Through TIRF, the excitation is limited to an optical thickness of approximately 150 nm from the surface by means of an evanescent field[4]. TIRF objective lenses require a large NA resulting in a small field-of-view (FOV) (e.g., 50 x 50 μm^2), which limits the throughput significantly. There are, however, alternative ways to generate an evanescent field.

An optical waveguide is a structure that will confine and guide light if it is coupled into the structure. Most commonly, waveguides are used in fiber-based telecommunication. Great effort has been made in order to develop 2D integrated waveguides as a main component of photonic integrated circuits. The technology has advanced to a point where fabricating low-loss nano-structured optical waveguides can be routinely done[5]. Today, several foundries around the world can be used to develop photonic integrated circuits. Waveguides guide light through total internal reflectance also exhibiting an evanescent field at the surface. By careful design of the waveguide structure, a high intensity can be achieved in the evanescent field. A sample placed directly on top of the waveguide surface can thus also be illuminated by the evanescent field for imaging applications. The evanescent field will be generated along the entire length and width of the waveguide, and thus it can be made arbitrarily large[6].

We present a novel approach to TIRF *d*STORM that offers an arbitrarily large field of view. Instead of using a TIRF lens for both excitation and collection, we excite using the evanescent field from optical waveguides. This decouples the excitation and collection light pathway, allowing for total freedom along the collection light path without compromising the optical sectioning for a given wavelength provided by the waveguide chip illumination. Low magnification lenses can thus be used to image very large regions in TIRF mode, although a smaller NA will reduce the lateral resolution. Furthermore, multicolor imaging is also greatly simplified using waveguides[7], as several wavelengths can be guided and detected without readjusting the system. This is advantageous for *d*STORM, as low wavelengths can be used to enhance fluorophore blinking and for multicolor imaging. It is worth noting that the penetration depth of the evanescent field will change as a function of wavelength, although it does not affect how the imaging procedure is performed. The chip is compatible with live cell imaging[8] and is ideal for applications such as the integration of microfluidics. Each chip can contain tens of waveguides, which can allow the user to image under different conditions or apply optical trapping[9] and Raman spectroscopy[10].

The chip-based system works equally well for both diffraction-limited and super-resolution

imaging. A similar approach was introduced in 2005 using a prism to generate evanescent field excitation[4]. The photonic chip also excites through the evanescent field, but with modern waveguide fabrication techniques, one can generate exotic light patterns with waveguides. The present chip-based nanoscopy implementation is limited to 2D imaging only, as the excitation field is locked inside the waveguide surface. Future development will aim for 3D applications. Additionally, other super-resolution techniques such as structured illumination microscopy are being developed using the same chip-based microscope[11].

PROTOCOL:

1. Preparation of the polydimethylsiloxane (PDMS) layer

1.1. Prepare a 10:1 mix of Sylgard 184 monomer and curing agent.

1.2. Place the mixture in a vacuum chamber until air bubbles are gone.

1.3. Pour 1.7 g of PDMS mixture in the center of a 3.5 inch (diameter) Petri dish.

1.4. Place the Petri dish on the vacuum chuck of a spin coater.

1.5. Spin coat the Petri dish for 20 s at 900 rpm, with an acceleration of 75 rpm/s.

1.6. Cure the dish on a hotplate at 50 °C for at least 2 h.

2. Sample preparation

2.1. Waveguide cleaning

2.1.1. Prepare 100 mL of a 1% dilution of cleaning detergent concentrate (**Table of Materials**) in deionized (DI) water.

2.1.2. Place the chip in a glass Petri dish using a wafer tweezer and cover completely with the detergent solution.

2.1.3. Place the Petri dish on a hot plate at 70 °C for 10 min.

2.1.4. While still on the hot plate, rub the surface with a cleanroom tissue swab.

2.1.5. Remove the chip from the Petri dish. Rinse with at least 100 mL of DI water.

2.1.6. Rinse with at least 100 mL of isopropanol, taking care that solvent does not dry on the surface to prevent evaporation stains.

2.1.7. Rinse with at least 100 mL of DI water. Blow the chip dry with a nitrogen gun.

2.2. Chamber preparation

2.2.1. Prepare a layer of 150 µm polydimethylsiloxane (PDMS) in a Petri dish (section 1).

2.2.2. Use a scalpel to cut a 1.5 cm x 1.5 cm frame from the PDMS layer.

2.2.3. Lift the frame from the Petri dish with a tweezer. Deposit it flat on a clean and polished chip. The sample is now ready for cell seeding.

2.3. Fluorescent labeling

2.3.1. Prepare the following chemicals: phosphate-buffered saline solution, dye solution(s), *d*STORM imaging buffer.

2.3.2. After cell seeding, remove the chip from the media.

2.3.3. Use a pipette to remove any excess fluid from outside the PDMS chamber.

2.3.4. Remove the current fluid from inside the PDMS chamber with a pipette while adding approximately 60 µL of clean PBS at the same time.

NOTE: The amount added to the chamber will have to be changed according to the chamber size. Be careful not to remove all media from the cell surface.

2.3.5. Replace the PBS with 60 µL of clean PBS and let it incubate for 1 min.

2.3.6. Repeat the previous step, letting it incubate for 5 min this time.

2.3.7. Remove the PBS and replace it with 60 µL of the dye solution. Leave the sample to incubate for around 15 minutes, shielding it from light.

NOTE: This step might have to change significantly, depending on the fluorescent dye used. We use CellMask Deep Red to label the cell membrane for this experiment

2.3.8. Wash the sample with PBS as in steps 2.3.3–2.3.5.

2.3.9. Remove the PBS and replace it with 40 µL of the imaging buffer at the same time.

NOTE: There are several different imaging buffers for different fluorescent dyes.

2.3.10. Place a coverslip on top, preventing air bubbles from forming underneath. Gently press the coverslip against the imaging chamber to remove any excess media.

2.3.11. Use a pipette to remove any excess media outside the coverslip. Clean the area outside the coverslip with a water moist swab to avoid crystals formed by dried immersion media residues.

3. Imaging procedure

3.1. Component setup

NOTE: This version of the setup consists of three main components: the microscope, coupling stage, and sample stage. See the **Table of Materials.**

3.1.1. Use a microscope with a filter holder, white light source, camera, and objective revolver.

3.1.2. Use a 3-axis piezo coupling stage with a fiber-coupled laser and a coupling lens.

3.1.3. Use a one-axis manual sample stage with tip and tilt and a vacuum holder.

3.1.4. Mount both the coupling and the sample stage on a 2-axis motorized stage for sample translation.

3.2. Waveguide coupling

3.2.1. Place the chip on the vacuum chuck with the coupling facet towards the coupling objective. Make sure the chip is approximately one focal length away from the coupling objective.

3.2.2. Turn on the vacuum pump.

3.2.3. Turn on the laser to 1 mW. Roughly adjust the chip height so that the beam hits the edge of it. Turn off the laser.

3.2.4. Turn on the white light source. Choose a low magnification objective lens (e.g. 10x). Focus the microscope on a waveguide.

3.2.5. Translate the microscope along the waveguide to see if it is well aligned with the optical path. Move the microscope to the coupling edge.

3.2.6. Turn on the laser at 1 mW or less. Translate the microscope along the coupling edge to find the laser light. Focus the beam on the chip edge.

3.2.7. Adjust the coupling stage along the optical path in the direction that reduces the laser beam spot size until it disappears. The beam is now either above or below the chip surface.

3.2.8. Adjust the coupling stage height until the beam spot reappears and is maximized.

3.2.9. Repeat the two previous steps until the laser forms a focused spot.

3.2.10. Move the focused spot to the waveguide of interest.

3.2.11. Translate the microscope a short distance away from the edge so that the focused beam spot is no longer visible. Turn off the white light.

3.2.12. Adjust the contrast. If the waveguide is guiding, the scattered light along the waveguide should be clearly visible.

3.2.13. Adjust the axes of the coupling stage to maximize the scattered light intensity. Turn off the laser.

3.2.14. Turn on the white light. Adjust the contrast if necessary.

3.2.15. Navigate to the imaging region.

3.3. Diffraction limited imaging

3.3.1. Focus with the desired imaging objective. Turn the white light off.

3.3.2. Insert the fluorescence filter and turn the laser power to 1 mW.

3.3.3. Set the camera exposure time to approximately 100 ms. Adjust the contrast as needed. Ensure that the coupling is still optimized.

3.3.4. Locate a region of interest for imaging. Turn on the piezo stage looping to average out modes.

NOTE: 20 µm scan range with a step size of 50 nm is suitable for most waveguide structures.

3.3.5. Capture at least 300 images.

3.3.6. Load the captured image stack to Fiji using a virtual stack. From the image menu in Fiji, choose **Stacks** and **z-project**.

3.3.7. Calculate the TIRF image by choosing projection type **average intensity**.

3.4. *d*STORM imaging

3.4.1. Turn on the laser to 1 mW and set the camera exposure time to 30 ms.

3.4.2. Adjust the contrast and focus.

3.4.3. Increase the laser power until blinking is observed.

NOTE: This might take a while, depending on evanescent field intensity.

3.4.4. Zoom in on a small region of the sample.

3.4.5. Adjust the contrast.

3.4.6. Capture a few images to see if the blinks are well separated.

3.4.7. Adjust the camera exposure time for optimal blinking.

NOTE: Optimizing blinking is a complex task, but a lot of suitable literature is available[12].

3.4.8. Turn on the piezo stage looping.

3.4.9. Record an image stack of at least 30,000 frames, depending on the blinking density.

3.5. *d*STORM image reconstruction

3.5.1. Open Fiji and load the *d*STORM stack as virtual images.

3.5.2. Adjust the contrast, if necessary.

3.5.3. Use the rectangle tool to select the area to reconstruct.

3.5.4. Open **Run analysis** in the Thunderstorm[13] plugin in Fiji.

3.5.5. Set the basic camera settings in Thunderstorm corresponding to the device. The remaining default parameters are usually satisfactory.

3.5.6. Start the reconstruction.

NOTE: For the full field of view, the data might need to be divided in substacks, due to the large file size.

3.5.7. Filter the localization list provided by the reconstruction software to remove unspecific localizations. Apple an additional drift-correction if necessary.

REPRESENTATIVE RESULTS:

TIRF microscopy is a popular technique as it removes out-of-plane fluorescence, increases contrast and thus improves image quality, and is less phototoxic compared to other fluorescence based microscopy techniques. Compared to the traditional objective-based approach, chip-based microscopy offers TIRF excitation without the limited throughput that is usually accompanied

with a TIRF lens. An overview of the presented setup can be found in **Figure 1A**. We present diffraction-limited as well as *d*STORM images of liver sinusoidal endothelial cells (LSEC) extracted from mice. A large field of view image of LSECs with labelled microtubulin is also presented, demonstrating the capabilities of high throughput imaging. A conventional *d*STORM setup using an oil immersion TIRF lens (either 60x or 100x magnification) typically images an area of 50 µm x 50 µm, which is 100 times smaller than the chip-based image in **Figure 2**, imaged with a 25x, 0.8 NA objective.

In this method, we use multi-moded Si_3N_4 waveguides for excitation. The utilized chips consist of a strip-etched guiding layer of 150 nm Si_3N_4 deposited over a 2 µm oxidized layer of a silicon chip. A schematic of the chip can be found in **Figure 1B**. Waveguide widths can vary between 200 and 1000 µm. Fabrication details can be found elsewhere[8]. Through interference between the propagating modes the excitation light will not have a homogeneous intensity distribution, but rather a spatially varying pattern. **Figure 2A** presents an image with clearly visible mode patterns. This interference pattern will change with the position of the laser beam at the edge of the waveguide. In order to achieve homogeneous excitation in the final images, we use a piezo stage to oscillate along the coupled facet. Over the course of the imaging procedure, enough variation of the interference patterns exists so that they can be averaged, removing intensity fluctuations in the image. The image stack will consist of several images such as in **Figure 2A**, although with different patterns, but when averaged, the stack will yield an image with homogeneous excitation such as **Figure 2B**. An alternative approach is to use adiabatic tapering to achieve wide, single moded waveguides[8,14], which removes the necessity of mode averaging. However, several millimeters of tapering length are necessary to maintain the single-mode condition to achieve a 100 µm waveguide width. Multi-moded waveguides circumvent this tapering necessity and leave no limitations on the structure width. Beyond the illumination pattern, the highly effective refractive index of the modes allow for unprecedented possibilities towards structured illumination microscopy[11] and fluctuation microscopy methods[7].

The first step in imaging is to collect a diffraction limited image. The experiment results in a stack of around 300 images and the final image is made by taking the average of the stack. In **Figure 2**, we present diffraction limited and *d*STORM imaging of LSECs labelled with CellMask Deep Red using a 60x, 1.2 NA water immersion objective. **Figure 2A** shows inhomogeneous illumination caused by insufficient mode averaging. Successful mode averaging is displayed in **Figure 2B**. **Figure 2C** is a *d*STORM image of the same region, with the marked region shown in **Figure 2D**. Liver sinusoidal endothelial cells have nano-sized pores in the plasma membrane[15], which can be seen here. A Fourier Ring Correlation analysis provided a resolution of 46 nm.

Figure 3 presents a *d*STORM image of a 500 µm x 500 µm region, demonstrating the high throughput capabilities of the technique. A zoomed image of **Figure 3A**, corresponding to a typical dSTORM field-of-view, is presented together with the diffraction limited image in **Figure 3B**. A Fourier ring correlation to estimate the resolution was performed, yielding a value of 76 nm.

FIGURE LEGENDS:

Figure 1: Imaging system and waveguide. (**A**) Photograph of the imaging system. The sample is placed on a vacuum chuck on the sample stage, with the coupling facet of the waveguide towards the coupling objective. A fiber coupled laser and a coupling objective is placed on top of a 3D piezo stage. A lens turret with imaging lenses captures the image from above and relays it to a camera. (**B**) Schematic of the waveguide with coupling and imaging lenses. The coupling lens couples light into the waveguide. The samples (orange beads) are kept inside a sealed PDMS chamber. The evanescent field along the waveguide will excite the sample and the imaging objective will capture the emitted fluorescence.

Figure 2: Diffraction-limited and dSTORM images. (**A**) Image of liver sinusoidal endothelial cells with insufficient mode averaging, resulting in a clearly visible excitation pattern. (**B**) The same region as in (**A**), but with sufficient mode averaging, resulting in homogeneous excitation. (**C**) Diffraction limited image of the inset in (**B**); (**D**) dSTORM image of the same region. (**E**) Inset of (**D**), clearly showing the fenestrations in the plasma membrane of the cell.

Figure 3: dSTORM image of rat LSECs. (**A**) Large field of view dSTORM image of Alexa 647 stained tubulin in rat LSECs. Scale bar = 50 µm. (**B**) Larger marked region from (**A**) comparing diffraction-limited (bottom left) and dSTORM image (top right). (**C**) Smaller marked region from (**A**). Scale bar = 1 µm. The image has a resolution of 76 nm. Adapted with permission from Helle et al. 2019[6].

DISCUSSION:

Chip-based imaging is similar to conventional *d*STORM imaging. Image quality can thus be gauged using the same approaches as for traditional *d*STORM imaging. The main difference for the user is that the transparent glass slide is exchanged with an opaque Si-wafer. Although they appear very different, the sample handling is practically analogous to a glass slide. The chips are quite sturdy and can easily be handled using wafer tweezers. The imaging procedure and image reconstruction is the same as in a regular *d*STORM experiment. Setting up a functional chip-based microscope requires no special components, except for the photonic chips. Further details of the set-up can be found in previous work[6,7]. The chips used in this work have been fabricated using standard photolithography[8].

Sample preparation encompasses the preparation of the sample chamber. When attaching the PDMS frame to the chip, it is crucial to avoid any small folds or rips where air might enter. If the PDMS folds when attaching it, simply remove it carefully with a tweezer and reattach it. When the sample is ready inside the PDMS chamber, the coverglass must be pressed against it, sealing the region. It is important to avoid any air bubbles that might form when attaching the coverglass. If an air bubble is formed, gently remove the coverglass and add PBS to the sample chamber to ensure that the sample is covered. The preparation and attachment of the cover slip can then simply be redone.

Coupling light into the waveguide is simplified using the protocol proposed in this paper. There are, however, a few common challenges that can limit coupling. Firstly, if the chip was not cleaned properly and any leftover PBS removed completely, there might be dirt or crystallized PBS on the waveguide. This can introduce major losses, resulting in very little power in the

imaging region. Using a moist swab to clean the region outside the cover glass can improve the power significantly. Secondly, if the coupling facet of the waveguide is damaged (e.g., by improper handling), the coupling loss can increase drastically. Optical inspection of the edge will usually reveal any damages easily. The entire coupling facet of the chip can be polished carefully, much like an optical fiber, and will give a smooth coupling facet, which then increases the coupled power.

After the light has been coupled, the imaging procedure is the same as in any conventional *d*STORM setup. If the image has inhomogeneous excitation, as demonstrated in **Figure 2A**, then most likely the mode averaging did not work well. The two most common reasons for this are: 1) too few images captured in order to create an average stack and 2) too short of an oscillation distance/too big of a step size. Collecting too few images can leave out some excitation patterns and the average will thus be inhomogeneous. This can easily be resolved by increasing the number of images in the average stack. Too short of an oscillation distance can also result in an inhomogeneous image, as not enough mode patterns are excited. This can also easily be resolved by increasing the oscillation distance and/or decreasing the step size. In this work we have used a piezo stage to scan the input laser beam over 20 µm and acquire at least 300 images. Another approach could be to use high-speed galvo-mirrors to scan the light across the input waveguide facet within a single acquisition time, such as 10-30 ms. This option is suitable for live cell TIRF imaging, where sub-cellular organelles are in constant motion.

Chip-based *d*STORM offers an unprecedented large area TIRF excitation, which makes it ideally suited for high throughput imaging. The compact character allows for retrofitting to commercial systems, where the chip can be placed upside down for inverted setups or transparent substrates can be developed. The chips are mass fabricated and can be modified to suit many needs. Currently, the main restriction is that it is limited to 2D. The evanescent field is only available approximately 200 nm away from the waveguide surface, so only fluorophores within this region will be excited. Altogether, the field of integrated optics offers many opportunities for chip-based microscopy in the near future, by tackling new imaging questions as well as providing new possibilities to existing ones.

ACKNOWLEDGMENTS:

The authors would like to acknowledge The European Research Council (grant no. 336716 to B.S.A.) The authors would also like to thank Irati Lagfragua for her invaluable assistance with recording and editing the video.

DISCLOSURES:

B.S. Ahluwalia has applied for patent GB1606268.9 for chip-based optical nanoscopy. The other authors declare no competing financial interests.

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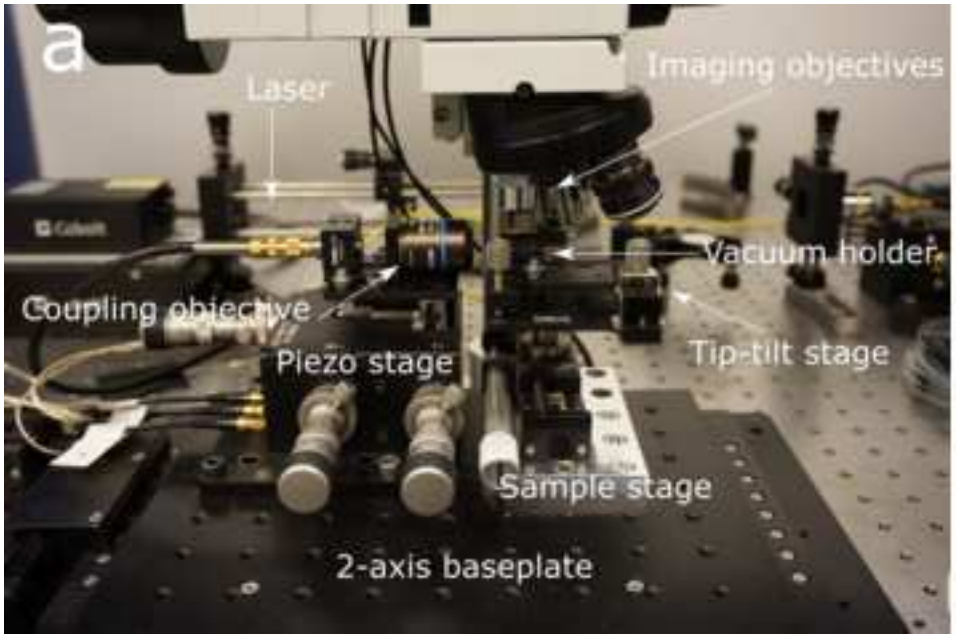
Figure 1



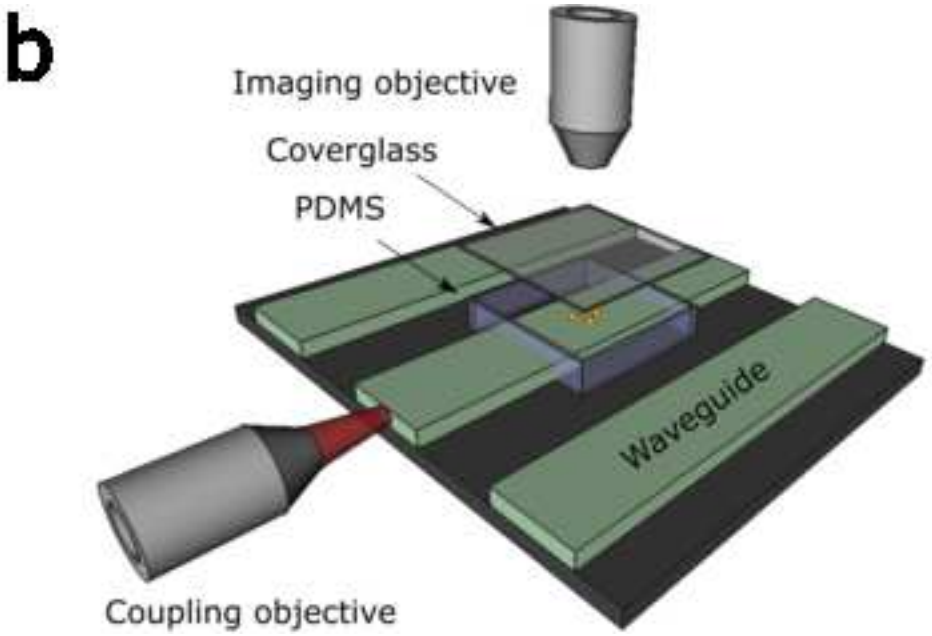


Figure 2

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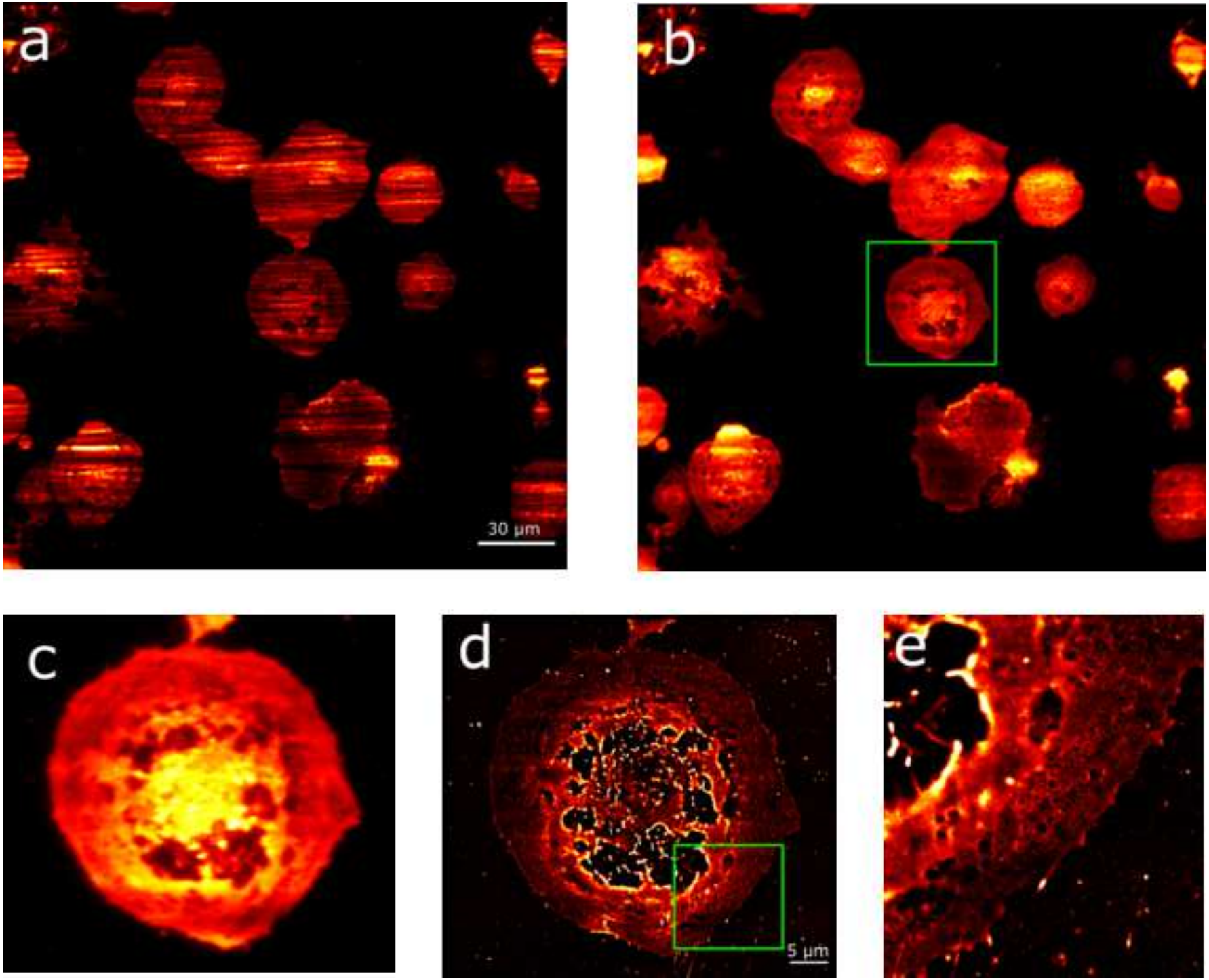

Figure 3

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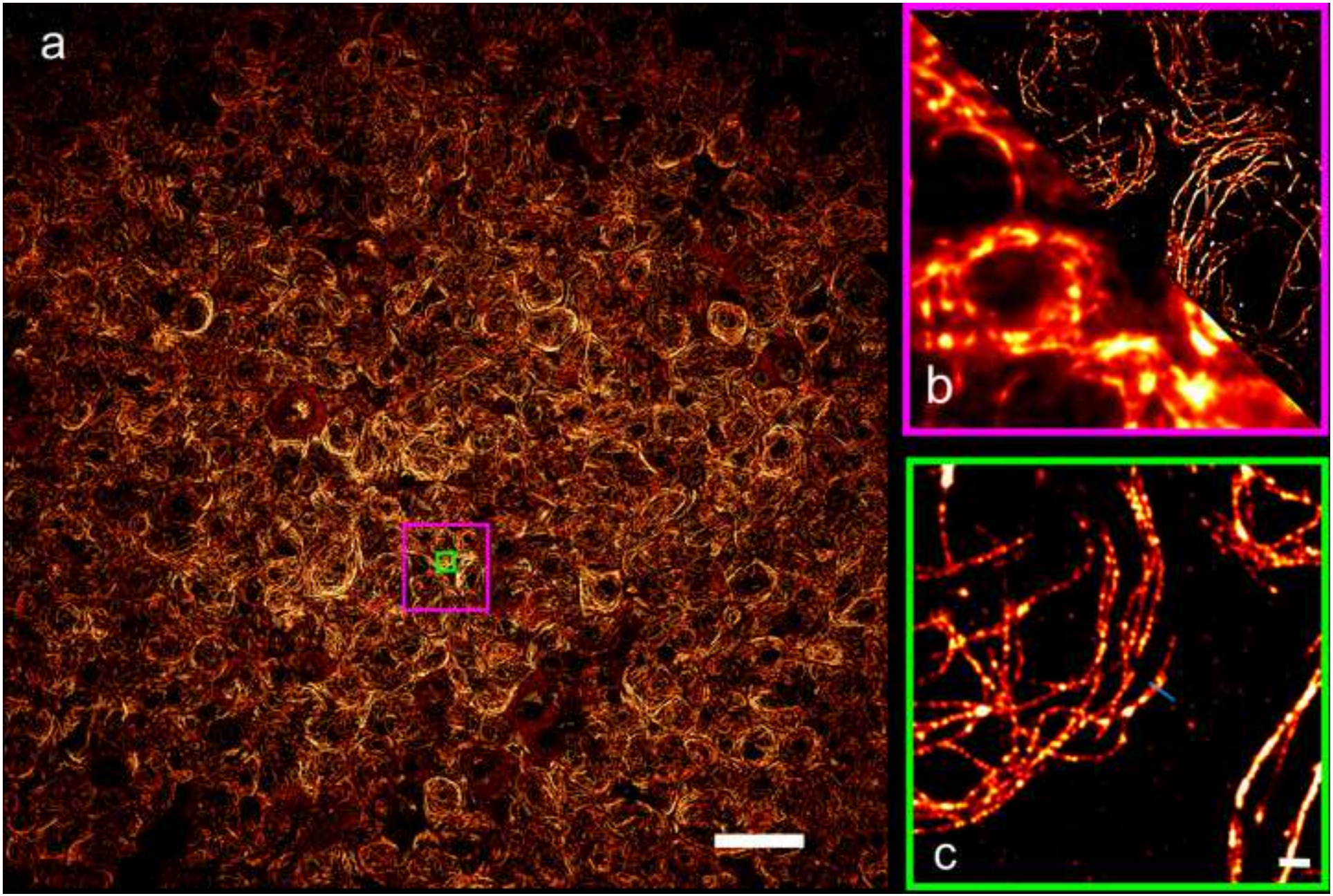

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1-axis sample stage	Standa	7T173-20	
2-axis sample translation stage	Mad City Labs		Custom order
3-axis NanoMax stage	Thorlabs	MAX311D	
BXFM microscope body	Olympus	OLY-LSM-037018	
CellMask Deep Red, Life technologies	ThermoFisher	C10046	
Cleanroom grade swabs	MRC Technology	MFS-758	
Fiber-coupled laser	Cobolt	Flamenco	
Filter Holder	Homemade		
Hellmanex III, Hellma Gmbh	Sigma-Aldrich	Z805939	Cleaning detergent concentrate
Isopropanol	Sigma-Aldrich	563935-1L	
KL 1600 LED	Olympus	OLY-LSM-E0433314	
Olympus Coupling lens	Olympus	LMPLFLN 50x/0.5	
Orca Flash 4.0 V2	Hamamatsu		
PBS tablets	Sigma-Aldrich	P4417-50TAB	Mix according to descriptions
SYLGARD 184 Silicone Elastomer 1.1 kg kit	Dow	1673921	
Tip-tilt stage	Thorlabs	APR001	
Vacuum holder	Thorlabs	HWV001	
Wafer Tweezers Type 2W	Agar scientific	AGT5051	

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Dated: 20th Sept-2019

Dr. Phillip Steindel,
The Review Editor
Journal of Visualized Experiments (JOVE)

Subject: Submission of our revised manuscript "*High-throughput total internal reflection fluorescence and direct stochastic optical reconstruction microscopy using a photonic chip*", by David André Coucheron, Øystein Ivar Helle, Cristina Ionica Øie, Jean-Claude Tinguely, and Balpreet Singh Ahluwalia

Article reference: JoVE60378

Dear Sir
We thank the reviewers for providing detailed and constructive feedback on our manuscript. Enclosed below is our point-by-point response to all the comments raised by the reviewers. This has helped us to further polish the paper.

We have made major revisions in several sections of the paper to clarify the doubts raised by the reviewers. In addition, the video has been changed significantly, in particular by replacing the mock experiment with an actual experiment. We hope the changes made are found satisfactory. If further inputs are sought please don't hesitate to contact us.

Best regards,

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Editorial and production comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Corrected

2. Please include email addresses for all authors within the manuscript itself.

Corrected

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For example: Hellmanex, Hellma Analytics

Corrected

Protocol:
1. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Corrected

Specific Protocol steps:
1. 1.2: Where does the chip come from? Please include a citation or include it in the Table of Materials, as applicable. Also, what is used to handle the chip?

Authors Response:

We have fabricated the chips together with collaborators and have added the following to the end of the first paragraph in the discussion to address this point:

The waveguides used here have been fabricated using standard procedures[13]

There are, however, many commercial foundries all over the world that offer fabrication and design assistance to users. We have added the following line in the second paragraph of the introduction:

Today, photonic integrated circuits are made around the world in state of the art foundries.

Your question regarding handling of the chips is very good one. Normal tweezers can be used, but it is much easier to use specialized wafer tweezers. With the correct tweezers, the chips are easy to work with and will not get damaged. We have added the following to the discussion to address this point:

The chips are quite sturdy and can easily be handled using wafer tweezers.

Additionally, we have added the tweezers to the list of materials. We have also changed protocol step 1.2. to reflect the use of wafer tweezers:

1.1. Place the chip in a glass petri dish using a wafer tweezer and cover completely with the detergent solution.

Specific Protocol steps:

1. Please remove the embedded figures from the manuscript.

Corrected

References:

1. Please do not abbreviate journal titles.

Corrected.

2. Does reference 8 refer to your arXiv manuscript? If so, that is acceptable, but please make that clear. If this is instead an article that is in preparation or under review (i.e., not accepted yet), please remove this reference.

Reference 8 is an arXiv manuscript from our group. We have changed the reference list to reflect that it is an arXiv paper.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Corrected.

Video:

1. Please ensure that the video and written protocol line up as much as possible; e.g.:

a. 1.3-1.4: The heating and cleaning happen on a hot plate, which is not apparent in the protocol.

Authors:

We have changed the steps to clearly show that the cleaning happens on a hot plate. The protocol steps now read:

1.2. Prepare 0.1 L of a 1% dilution of Hellmanex III (detergent from Hellma Analytics) in deionized water.
1.3. Place the chip in a glass petri dish using a wafer tweezer and cover completely with the detergent solution.
1.4. Place the petri dish on a hot plate at 70 ^{o}C for 10 minutes.
1.5. While still on the hot plate, rub the surface with a cleanroom tissue swab.

b. There is a description of the instrumentation between 3 and 4 (manuscript numbering) that isn't present in the manuscript.

Authors:

The description of the instrumentation has now been added to the manuscript in the protocol section and the Table of Materials.

c. 4.21: Adjusting contrast is not mentioned in the text.
Corrected.

2. The audio volume is significantly higher in the left audio channel than it is in the right audio channel. The audio should be equal volume in each channel and should be peaking between -6 and -12 dB.

Corrected.

3. There is no discrete Results section.

Authors:
Our revised video now presents real samples instead of mock experiments and a results section has now been added.

Reviewer 1 – Minor concern 1:

Note, the authors could perhaps add a line how users can get access to commercial wave-guides.

Authors:

Thanks to the reviewer for pointing this out. We fabricate our own waveguides in collaboration with the Institute of Microelectronics of Barcelona IMB-CNM. There are today many foundries that can assist with the entire process, from design to fabrication. To highlight this, we have added the following to the introduction:

Today, several foundries around the world can be used to develop photonic integrated circuits.

We have also added the following to the discussion to address how we fabricate our waveguides:

The chips used in this work have been fabricated using standard photolithography[8].

Reviewer 2:

Major concern 1:
For a better understanding of their protocol the authors should present a scheme of their chip/waveguide as positioned in the microscope.

Authors:
We have included a new figure with a photograph of the imaging system, as well as a schematic showing the coupling objective, photonic chip and collection objective. This figure will serve as the new Figure 1.

Minor concern 1:
It would be worth mentioning that low magnification objective lenses can also be used in prism type TIRFM (see e.g. Schneckenburger, Curr. Opin. Biotechnol. 16, 2005, 13-18);

Authors:
We agree that the reader would benefit from adding prism type TIRFM to the introduction. Using prisms offers large area excitation, similar to our chip based system, but the real advantage of the chip based system is the endless possibilities for integration. The waveguides can e.g. easily be tailored to generate exotic light patterns. The following has been added to the introduction to include prism type TIRFM:

A similar approach was introduced in 2005 using a prism to generate evanescent field excitation[4]. The photonic chip also excites through the evanescent field, but with modern waveguide fabrication techniques one can e.g. generate exotic light patterns with waveguides.

Minor concern 2:
Introduction, line 78: The authors describe multi-color imaging. However, they should mention that the depth of the evanescent field depends on the wavelength of light.

Authors:
This is a very good point by the reviewer. There certainly is a change in penetration depth with wavelength, although we have not experienced any challenges associated with that. We have added the following sentence to the end of the paragraph (line 83):

It is worth noting that the penetration depth of evanescent field will change as a function of wavelength, although it does not affect how the imaging procedure is performed.

Minor concern 3:
Protocol / Chamber preparation: Define "PDMS" and "Spinning in a Petri dish" (how? how fast?)

Authors:
We have added all parameters needed to fabricate the PDMS frames we use to define the sample area. The entire protocol is under C) Additional protocols and reads:

C) Additional protocol:

9. Preparing a 150 µm polydimethylsiloxane (PDMS) layer

9.1. Prepare a 10:1 mix of Sylgard 184 monomer and curing agent (Dow Corning).
9.2. Place the mixture in a vacuum chamber until air bubbles are gone.
9.3. Pour 1.7 g PDMS mixture in the center of a 3.5 inch (diameter) petri dish.
9.4. Place the petri dish on the vacuum chuck of a spin coater.
9.5. Spin coat the petri dish for 20 s at 900 rpm, with an acceleration of 75 rpm/s.
9.6. Cure the dish on a hotplate at 50 ^{o}C for at least 2 h.

Minor concern 4:
In their Discussion the authors mention the use of a piezo stage for increasing the number of images. Can this step also be documented in the protocol?

Authors:
The reviewer has a good point. We have added information about the piezo stage looping parameters in the protocol. Protocol step 5.8. now reads:

Turn on the piezo stage looping to average out modes.
NOTE: 20 µm scan range with a step size of 50 nm is suitable for most waveguide structures.

Further information to the oscillation process of the piezo stage is present in the results section.

Reviewer 3:

Minor concern 1:

Line 84 - The authors need to clarify that in the diffraction limited case, the lateral resolution is traded off for FOV. But the axial resolution is un-compromised.

Authors:

The reviewer makes a great point. The chip based approach allows us to image very large regions with diffraction limited imaging and dSTORM, although the price for the increase in FOV is a loss of resolution. As the reviewer points out, the optical sectioning is unaffected by the increased FOV. This is one of the main advantages with the technique, and to clarify this we have added the following from line 77:

This decouples the excitation and collection light pathway, allowing for total freedom along the collection light path without compromising the optical sectioning for a given wavelength provided by the waveguide chip illumination. Low magnification lenses can thus be used to image very large regions in TIRF mode, although a smaller NA will reduce the lateral resolution.

Minor concern 2:

Line 208 - Is "thunderstorm" a pre-installed add on to Fiji? if not mention that it's a prerequisite.

Authors:

The reviewer has an important remark: thunderstorm is not included in the base Fiji installation. We have changed protocol step 8.4 to add the following citation:

- Ovesný, M., Křížek, P., Borkovec, J., Švindrych, Z. & Hagen, G. M. ThunderSTORM: a comprehensive ImageJ plug-in for PALM and STORM data analysis and super-resolution imaging. Bioinformatics 30, 2389–2390 (2014).

The paper is where Thunderstorm was inroduced and it gives a great introduction to the plugin, as well as how to install it. Protocol step 8.4. now reads:

8.4 Open "Run analysis" in the thunderstorm plugin[13] in Fiji.

Minor concern 3:
Discussion - Practically, coupling light in to the waveguide can also be challenging. It's good to discuss this fact.

Authors:

We agree with the reviewer that although an experienced person can usually couple easily, it can be quite daunting to the readers that have never worked with waveguides before. Our future goal is, as we briefly state, to completely automate the coupling process, but this version requires manual coupling. To address this point, we have added a paragraph discussing common challenges and how to resolve them:

Coupling light into the waveguide is simplified using the protocol proposed in this paper. There are, however, a few common challenges that can limit coupling. Firstly, if the chip was not cleaned properly and any leftover PBS removed completely, there might be dirt or crystallized PBS on the waveguide. This can introduce major losses, resulting in very little power in the imaging region. Using a moist swab to clean the region outside the cover glass can improve the power significantly. Secondly, if the coupling facet of the waveguide is damaged (e.g. by improper handling), the coupling loss can increase drastically. Optical inspection of the edge will usually reveal any damages easily. The entire coupling facet of the chip can be

polished carefully, much like an optical fibre, and will give a smooth coupling facet, which then increases the coupled power.

Reviewer 4:

Minor concern 1:
Video - In the video, at 3:16 I'm just looking at a box top that says "Sample below" on it. I'm not sure what I'm supposed to get from this and it is quite distracting. Additionally, during the video there were no super resolution images shown and that was rather strange given that it is the point of the technique. Those should be added to give readers and idea of what they should get assuming they did everything right.

Authors:
We appreciate the reviewers comments about the video and have made a few changes that we believe will help the reader/watcher to better grasp the process. At 3:16 we have reedited the video to make it align better with the manuscript. The box is used for light protection when incubating and in the changed video, this should be much clearer. We have also added a separate results section in the video where we present the super-resolution images captured.

Minor concern 2:
Page 3: In general this was written quite well, however, I do have a few questions regarding the protocol.

1. In many places throughout the protocol the authors state things such as "Record an image stack of up to tens of thousands of frames, depending on the blinking density", "Rinse with plenty of DI water", "Rinse with DI water", "Rinse with isopropanol,...", "Turn on the laser at minimum power" and similar statements. These are extremely vague instructions, particularly when comparing "Rinse with plenty of DI water vs. Rinse with DI water". These are distinct instructions but I'm not sure how they should be functionally different or even if they should be functionally different. Please inform the reader of the exact values used. (i.e. rinse with 274 ml of DI water, etc.) If an exact value isn't used because it doesn't matter that much as long as it is washed visually well then at least give a minimal value (rinse with at least 100 ml of DI water, etc.) so that this can be replicated by other groups. How much laser power is "minimum power"? Wouldn't that be different for each laser/group who tries this? As for the image stack that depends on the blinking density, the authors need to go back through the manuscript and make sure that someone who is skilled in the art of TIRF and/or waveguide technology would know how many images to take based upon the blinking density. I have a lot of background knowledge in this subject area but I do not know off the top of my head how many frames would be needed but this is a clearly important aspect of the protocol that needs explanation.

Authors:

The reviewer makes a great point and we have done the following changes to try to make the protocol more specific and clear:

- Protocol step 1.1. has been changed to:
  Prepare 100 mL of a 1% dilution of Hellmanex III (detergent from Hellma Analytics) in deionized water
- Protocol step 1.6. has been changed to:
  Rinse with at least 100 mL of DI water.
- Protocol step 1.7. has been changed to:
  Rinse with at least 100 mL of isopropanol, taking care that solvent does not dry on the surface to avoid stains.
- Protocol step 1.8. has been changed to:
  Rinse with at least 100 mL of DI-water.
- Protocol step 3.6. has been changed to:
  Remove the current fluid from inside the PDMS chamber with a pipette while adding approximately 60 µL clean PBS at the same time.
  NOTE: The amount added to the chamber will have to be changed according to the chamber size.
- Protocol step 3.7. has been changed to:
  Gently aspirate the PBS while simultaneously adding 60 µL of the dye solution. Be careful not to remove all media from the cell surface.

- Protocol step 3.10. has been changed to:

  Now gently remove the PBS by replacing it with 40 µL the imaging buffer at the same time
- Protocol step 5.4. has been changed to:

  Turn on the laser to 1 mW.
- Protocol step 5.8. has been changed to:

  Choose a low magnification objective lens, for example a 10x.
- Protocol step 4.12. has been changed to:

  Turn on the laser at 1 mW or less.
- Protocol step 5.12. has been changed to:

  Insert the fluorescence filter and turn the laser power to 1 mW.
- Protocol step 6.3. has been changed to:
  Insert the fluorescence filter and turn the laser power to 1 mW.
- Protocol step 6.7. has been changed to:

  Capture at least 300 images.
- Protocol step 7.1. has been changed to:
  Turn on the laser to 1 mW and set the camera exposure time to 30 ms.
- Protocol step 7.9 has been changed to:
  Record an image stack of at least 30000 frames, depending on the blinking density.

The final part of the comment is very well pointed out – there are not enough details for the reader to perform dSTORM imaging at a high level. We have rewritten protocol step 7 entirely to clarify the process to the reader. We have also added a reference to the following JoVE video, as it is very useful for imaging:

9. *Metcalf, D. J., Edwards, R., Kumarswami, N. & Knight, A. E. Test Samples for Optimizing STORM Super-Resolution Microscopy. JoVE (Journal of Visualized Experiments) e50579 (2013). doi:10.3791/50579*

The entire protocol step now reads:

7.1. Turn on the laser to 1 mW and set the camera exposure time to 30 ms.
7.2. Adjust the contrast and focus.
7.3. Increase the laser power until blinking is observed.
NOTE: This might take a while, depending on evanescent field intensity.
7.4. Locate a region of interest.
7.5. Adjust the contrast.
7.6. Capture a few images to see if the blinks are well separated.
7.7. Adjust the camera exposure time for optimal blinking.
NOTE: Optimizing blinking is a complex task, but a lot of suitable literature is available[9].
7.8. Turn on the piezo stage looping.
7.9. Record an image stack of at least 30000 frames, depending on the blinking density.

Finally, we have also added the following to the first paragraph of the discussion (line 317):

Image quality can thus be gauged using the same approaches as for traditional dSTORM imaging.

We hope that these changes have resulted in a manuscript that will be easy for the readers to understand and use in their own research.

Minor concern 3:
Instruction 3.11 "Place a coverslip on top, adding air bubbles which might form underneath.". I don't think I understand. I thought air bubbles were bad for this experiment. Why would you want to add air bubbles here?

Authors:

The reviewer's confusion is well grounded – there is a very unfortunate typing error, as it should be *avoiding* instead of *adding*. Any potential air bubbles can be detrimental to the sample, so it is crucial to *avoid* them. We have changed the sentence in protocol step 3.11 to:

"Place a coverslip on top, preventing air bubbles from forming underneath"

Minor concern 4:
3. Piezo stage loop: What "averaging" procedure was used here? Was it a simple moving average? Weighted average? This makes a difference and it is crucial for averaging out the signal. How many times is it averaged?

Authors:
The reviewer raises an important point. Since all our current waveguides are multi-moded, any single image captured will have a visible inhomogeneous excitation pattern. The excitation field can be changed simply by adjusting the coupling of the waveguides. Using a piezo stage to loop the coupling objective along the coupling facet while imaging will result in an image stack where each image has a different excitation pattern. For dSTORM, the data can simply be reconstructed as it is, since areas with no/low excitation power will have no blinks. It is, however, important that all the different patterns result in a homogeneous excitation, to ensure that the entire sample is imaged. In TIRF, this is simply done by averaging the captured stack using "z-project" with "average intensity" in FIJI. This entire procedure would, however, be superfluous if we had single moded waveguides, as they would give a homogeneous excitation pattern. We have added the following to clarify this point:

The image stack will consist of several images such as the figure 2a, although with different patterns, but when averaged will yield an image with homogeneous excitation such as figure 2b.

Additionally, we have added several steps to protocol 5 to clarify the averaging process performed in FIJI. The protocol steps now read:

6.10 Load the captured image stack to Fiji (open source imaging software) using a virtual stack.
6.11 From the image menu in Fiji, choose "Stacks" and "z-project".
6.12 Calculate the TIRF image by choosing projection type "average intensity".

Minor concern 5:
In several areas the laser power is turned on and off again. In my experience and it is widely known that it takes a certain amount of time after a laser is turned on (regardless of the type) for it to thermally equalize with the surroundings. During this time the laser power and many other parameters can fluctuate until the laser obtains thermal equilibrium with the surroundings. Given how important keeping the laser power relatively constant is, why do the authors turn the laser on and off constantly during their protocol? Is there no way to simply block the laser instead of turning it on and off? Doesn't this alter things like coupling into the chip given that this can change the modes over time into the waveguide?

Authors:

The reviewer makes an excellent point regarding the laser stability. It is preferential to avoid powering the laser down. We have simply expressed us inaccurately, as most commonly, we use a beam stopper to block the beam when we write "turn off". For our fibre coupled laser the beam stopper is integrated and the laser stopped by the press of the button. We therefore chose "turn off" to avoid confusion for the reader whether to insert a physical stop or not. Regarding the coupling stability, we have found that the laser can be powered down and left for a long time and the coupling will still be good when turning it back on since the entire system is very mechanically stable.

Minor concern 6:
Cleaning steps: The authors use a lot of cleaning methods to remove contaminants from the surface of the waveguide including hellmanex, DI water, etc. This isn't really an issue with the paper but, have you considered using this material instead? https://www.photoniccleaning.com/ I only ask because I have seen it used as a standard for not only cleaning optics but storing them as well and many companies ship their optics to customers using it as well. It seems like this would help a lot with this project and simplify the cleaning process quite a bit.

Authors:

We would like to thank the reviewer for this interesting suggestion. We do not have any experience with the suggested product, but it does seem promising and we are eager to test it out. The waveguide structures can be quite sensitive and we have found that the procedure we currently use cleans the surface very well, without being too destructive. It is, however, always interesting to test out new procedures that might both improve the result and perhaps limit the chip degradation further. In the future, we aim to make the chips single use as they can be fabricated cheaply in large quantities.

Reviewer 5:

Major concerns

The protocol can be significantly improved if the authors include a few more schematics. To start with if the authors can reproduce something like Figure 1 from reference 2, it will be very helpful for the reader. I understand that there will eventually be a video, but a schematic helps the reader understand the system better. Likewise another schematic demonstrating the microscope setup will be helpful. Reading through imaging procedure (B), the reader is lost as to where the laser beam is being directed and how. Can the authors comment on the high throughput nature of the chip. How many wave-guides are possible on one chip and if different conditions can be imaged on each wave-guide, is it possible to make it a perfusion type chamber for live cell imaging? Also it would be important to mention where can users obtain such chips from? A materials section is missing in the protocol.

Authors:
We would like to thank the reviewer for many good points that certainly have helped us shape what we hope is a much clearer description of the technique. The changes we have made include:

- Added a new figure containing both an image of the system, as well as a schematic of coupling lens, chip and collection lens. We believe this will clarify the process for the readers.
- Made several changes to the entire protocol. In particular we have specified many vague expressions, as well as rewritten several steps for clarity.
- We have added information on how we fabricated our chips, as well as how the user can obtain chips, in lines 67 and 324.

We have also added an entire paragraph in the introduction to present how the chip based platform is ideal for high-throughput imaging. The paragraph reads as follows:

We present a novel approach to TIRF dSTORM that offers an arbitrarily large field of view. Instead of using a TIRF lens for both excitation and collection, we excite using the evanescent field from optical waveguides. This decouples the excitation and collection light pathway, allowing for total freedom along the collection light path without compromising the optical sectioning for a given wavelength provided by the waveguide chip illumination. Low magnification lenses can thus be used to image very large regions in TIRF mode, although a smaller NA will reduce the lateral resolution. Furthermore, multi-colour imaging is also greatly simplified using waveguides[7], as several wavelengths can be guided and detected without readjusting the system. This is advantageous for dSTORM, as low wavelengths can be used to enhance fluorophore blinking and for multi-colour imaging. It is, however, worth noting that the penetration depth of evanescent field will change as a function of wavelength. The chip is compatible with live cell imaging[8] and is ideal for applications such as the integration of microfluidics. Each chip can contain tens of waveguides, which can, e.g., allow the user to image different under conditions or apply optical trapping[9] and Raman spectroscopy[10].

Minor concern 1:
Is Hellmanex III some kind of detergent?

Authors:
Hellmanex III is an optical cleaning agent mostly used for things such as cuvettes, but it works very well with waveguides as well. We have changed protocol step 1.1. to the following:
“Prepare a 1% dilution of Hellmanex III (detergent from Hellma Analytics) in deionized water.”

Minor concern 2:
On line 3.11 it reads adding "air bubbles which might form underneath", are we trying to add or remove air bubbles?

Authors:

This is a very unfortunate typing error, as it should be *avoiding* instead of *adding*. Any potential air bubbles can be detrimental to the sample, so it is crucial to *avoid* them. We have corrected this is the text.

Minor concern 3:
Mention that Fiji is an imaging software

Authors:
We have added the specification that Fiji is an imaging software in protocol step 5.8, as not all readers might be familiar with it. We have changed the latter part of protocol 5 to give a better description of the TIRF image reconstruction. From step 6.10. it now reads:

6.10. Load the captured image stack to Fiji (open source imaging software) using a virtual stack.
6.11. From the image menu in Fiji, choose "Stacks" and "z-project".
6.12. Calculate the TIRF image by choosing projection type "average intensity".