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Dr. Alisha DSouza

Journal of Visualized Experiments

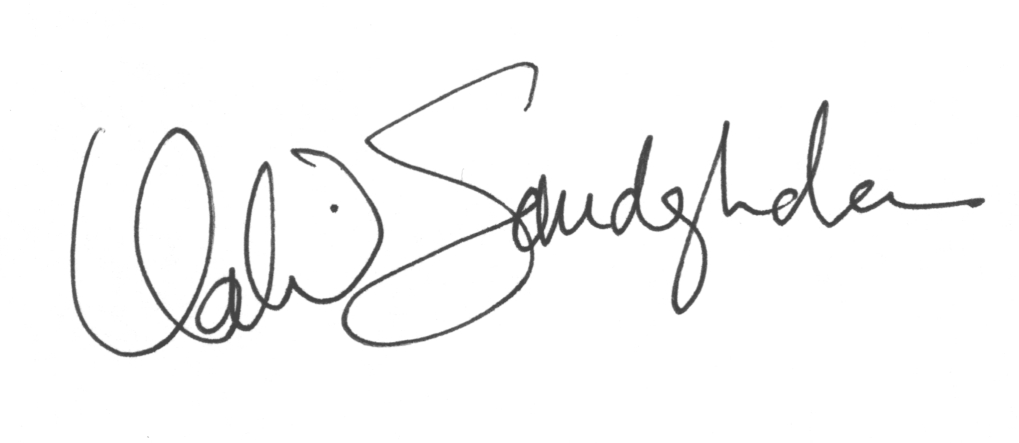
July 9, 2018

Dear Dr. DSouza,

Thank you for securing detailed and timely reviews. We have addressed the concerns raised by the reviewers and editorial staff and have revised the manuscript accordingly. Below, we provide a detailed explanation for how we have addressed each issue.

We hope that the manuscript is now acceptable for publication in the Journal of Visualized Experiments. Should you have any questions, or require my assistance in connection with this submission, please do not hesitate to contact me right away.

Sincerely,



Prof. Vahid Sandoghdar

**Details of Manuscript Revision**

**Editorial comments**:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

*We have proofread the manuscript.*

2. Figure 5: Please include a space between numbers and their units (580 nm, 550 nm, 600 nm).

*Please find an updated Figure 5 attached.*

3. Please rephrase the Abstract to more clearly state the goal of the protocol.

*We have now rephrased the abstract to make it clearer.*

4. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

*We have now rephrased the introduction to make the goal of the method clearer.*

5. Please remove the brackets enclosing the superscripted reference numbers.

*The brackets have been removed.*

6. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

*The units have been updated.*

7. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

*Spaces have been placed between all numbers and their corresponding units.*

8. Please use centrifugal force (x g) for centrifuge speeds.

*We have replaced “300 g” with “300 x g”.*

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

*We have removed all personal pronouns from the protocol.*

10. Please revise the protocol so that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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 move the discussion about the protocol to the Discussion.

*We revised the protocol to use the imperative tense.*

11. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

*We have re-ordered some of the notes to be sub-steps and have moved some of the notes to the discussion section.*

12. Line 106/529: Please make the reference number a superscript.

*The reference number is now a superscript.*

13. 1.1.1: Please add more details to this step. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action.

*We have included a reference (Ref. 18) to a detailed description on how to build a microscope sample stage.*

14. Please number the figures in the sequence in which you refer to them in the manuscript text.

*The figures are now numbered according to their position in the manuscript text.*

15. References: Please do not abbreviate journal titles.

*The journal titles are now complete.*

16. Please do not number the Table of Materials in the article.

*The Table of Materials is no longer numbered.*

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

In this paper, Gemeinhardt et al. describe a protocol for label-free imaging of proteins using iSCAT. Label-free imaging of proteins is highly relevant to biology and medicine. The authors are experts in the field and Sandoghdar group is known for their development of iSCAT technology. I recommend publication of the paper.

*Major Concerns:*

No major concern.

*Minor Concerns:*

It would be nice to include an analysis to show the use of this approach in resolving proteins based on their MWs. It is mentioned in the paper that this can be done but it would be nice to show some data using some model proteins with distinct MWs and to add a discussion on associated limitations.

*We and others have already published several papers on the subject of resolving proteins based on their MW (cf. Piliarik & Sandoghdar, Nat. Comm. 2014, 5, 4495, Liebel, et al. Nano Lett. 2017, 17, 1277–1281, and Young et al., Science 2018, 360, 423-427), including the associated limitations of mass resolution. In fact, Piliarik & Sandoghdar (Nat. Comm. 2014), and more recently Young, et al. (Science, 2018) have created calibration curves with which to empirically relate a protein’s mass with its iSCAT signature.*

*Thus, we think that adding more data to this protocol would be beyond the scope of the paper. We have, nevertheless, modified the discussion to include more reference to our and others previous works, and have included more discussion about how different proteins can be used to calibrate an iSCAT system. Specifically, on p. 11 we have changed the text from*

*“Nevertheless, some degree of specificity is possible as iSCAT signals scale linearly with protein mass14,27. In fact, very recently, Young et al.28 have extended on the work of Piliarik & Sandoghdar14 and have shown that the sensitivity of iSCAT in determining particle mass can reach the kDa regime. ”*

*to*

*“Nevertheless, some degree of specificity is possible as iSCAT signals scale linearly with protein mass14,27,28. This allows for the calibration of an iSCAT system using standard protein samples, such as bovine serum albumin (BSA) and Fibrinogen14,27,28. In fact, very recently, Young et al.28 have extended on the work of Piliarik & Sandoghdar14 and have shown that iSCAT can be used to determine the molecular weight of proteins as small as streptavidin (53 kDa) with a mass resolution of 19 kDa and an accuracy of about 5 kDa.”.*

**Reviewer #2:**

I have read carefully through this manuscript and have not really found anything urgent that comes to mind that should be changed. The protocol is detailed, clear and well written, in particular in its connection to the relevant manuscript published in Nano Letters and accurate in so far as I can tell. The only section I found somewhat lacking detail was the one discussing the 'rolling differential image'. I could not quite tell what the key parameters here were and how this image was supposed to be created from the raw data.

This manuscript will be highly beneficial to the field, enabling other researchers to take advantage of the capabilities of iSCAT.

*We thank the reviewer for the encouraging words. We have modified the manuscript to more clearly describe our imaging procedure. Specifically, in section 4 of the protocol, we have modified the text from*

*“4.3. Create a rolling differential image to retrieve the binding events of particles whose contrast is smaller than the contrast resulting from other sources (like the surface roughness of the coverslip). This step also removes any wavefront distortions present in the incident beam.”*

*to*

*“4.3. Create a rolling differential image by subtracting each consecutive frame from its successor.*

*Note: Residual signals from the surface roughness of the coverslip and wavefront distortions are effectively removed in this step as they are constant within consecutive frames. The rolling differential removes these residual signals, leaving only the protein bindings that occur from one frame to the next. This dynamic background subtraction is beneficial as it is not sensitive to long term sample drifts.”.*

**Reviewer #3:**

Manuscript Summary:

This paper provides the protocol for the authors' recent publication regarding label-free imaging of single proteins secreted from living cells via iSCAT microscopy. This paper, as a protocol paper, is well written by addressing essential points directly related to the aforementioned topic with no significant redundancy with published method papers for iSCAT. To further improve the utility of this paper, I would like to recommend the authors to revise the manuscript to reflect the following minor concerns:

*Minor Concerns:*

(1) In p 2, the authors recommended a centrosymmetric piezo stage to prevent acoustic excitations. For unfamiliar readers, explain what the stage is and why it is important.

*We have changed the text on p. 2 of the protocol to more clearly state the purpose of the centrosymmetric system. Specifically, we have changed the text from*

*“Operation of an iSCAT microscope at the limit of detecting single proteins is highly susceptible to external vibrations. A centrosymmetric piezo stage is recommended to limit acoustic excitations of the sample.”*

*to*

*“Operation of an iSCAT microscope at the limit of detecting single proteins is highly susceptible to external vibrations. A centrosymmetric piezo stage that supports the sample from all sides is recommended to limit acoustic excitations of the sample that would otherwise compromise focal and lateral stability.*

(2) In p 2, they used a 445 nm laser for iSCAT. For cell study, isn't it too short for cell viability? Make a comment on use of 445 nm regarding cell viability.

*Indeed, 445 nm laser light can be toxic for cells. However, we are not directly irradiating the cells, but rather a small section of the bare coverslip adjacent to the cells, as we mention in steps 3.1 and 3.3. The cell under study is kept at least 10 microns away from the iSCAT laser and is thus exposed to very little 445 nm light. To make this clearer, we have extended step 3.3 from*

*“3.3. Using the piezo positioner, move the sample laterally to position a cell close (approximately 10 µm) to the iSCAT field of view (FOV).”*

*to*

*“3.3. Using the piezo positioner, move the sample laterally to position a cell close (approximately 10 µm) to the iSCAT field of view. Ensure that the cell does not enter the iSCAT field of view as direct 445 nm laser light might be harmful for the cell.”*

(3) In p 3, the authors mentioned two recommended ways to split the incident beam to avoid ghosting and fringe effects. Compare these cases with less desirable situations by showing representative images side by side to clearly show how crucial this point is.

*We have now included an image with a side-by-side comparison of a poor beam splitter (showing fringe effects) and a high quality wedged beam splitter with an anti-reflective coating. We have added this as Figure 7 in the discussion section together with the following paragraph:*

*“As mentioned above, the use of a high-quality beam splitter in the iSCAT setup (step 1.2.1.) is recommended, as imaging artefacts like ghosting or interference arising from thin planar beam splitters will influence the image and disturb the measurement. Figure 7 shows a comparison between a high-quality and low-quality beam splitter. Both raw iSCAT images show the same area on the coverslip containing some residual particles. The same iSCAT setup was used to capture both images, only the beam splitter was exchanged. Figure 7a shows the image formed on the camera by use of a thicker (5 mm), AR-coated, and wedged beam splitter. Due to the wedged design, the reflected beam from the back surface of the beam splitter is anti-parallel to the reflection arising from the front surface and is not entering the objective. No interference artefacts occur. Figure 7b shows the same field of view on the sample but this time a thinner (1 mm) planar beam splitter was used. The two reflections from front and back surfaces of the beam splitter are parallel and propagate to the camera. Interference artefacts are clearly visible.”.*

(4) The iSCAT scheme used here is different from a better known AOD-based scheme. Comment on this and inform readers of technical differences and (dis)advantages of the current scheme.

*The principle of iSCAT microscopy is unchanged whether an AOD is used or not. All of the measurements discussed herein could be carried out in either confocal or wide-field schemes. We choose a wide-field configuration here as was done in the first report on the detection of unlabeled proteins (cf. Piliarik & Sandoghdar, Nat. Comm. 2014, 5, 4495) because it is significantly less complex than confocal designs and can be intrinsically faster.*

*We have added a section discussing differences between the two methods in the discussion section as follows:*

*“In this protocol we describe a wide-field illumination scheme for iSCAT as it is fast, easy to realize and allows for parallel sensing over a large area14. Another common approach is to use acousto-optic deflectors (AODs) and scan a confocal beam across the sample12,17. This approach avoids the need for high-quality wavefronts but is more experimentally complex than conventional wide-field imaging. Furthermore, the speed of confocal illumination is limited by that of AODs. Depending on the desired experimental parameters, either confocal or wide-field illumination schemes can, in principle, be utilized to detect single proteins secreted from living cells.”*

(5) In p 5, it is mentioned that an adjustable 45 coupling mirror is used to direct the beam to the objective. Comment on the issue of stability arising from use of the adjustable mirror.

*The reviewer is right that we need to include a statement about mount stability in the manuscript. Indeed, not only the 45˚ coupling mirror, but all mirrors and optical components should be installed on massive and stable adjustable mounts. We would like to point out that such high quality and adjustable components are readily available from commercial suppliers. We have modified the text to include the following on p. 2*

*“In addition, it is recommended that massive and stable mounts are used for all optical components discussed in the following steps. Such components are readily available from commercial optics suppliers.”*

(6) In p 5, for concreteness, provide the fps for bright-field and fluorescence measurements.

*We have clarified by changing the text on p. 5 from*

*“Bright-field imaging is carried out with a 20 ms exposure time. The fluorescence camera is set to 750 ms exposure time, and 5 consecutive frames are accumulated to form one final image.“*

*to*

*“Bright-field imaging is carried out with a 20 ms exposure time. The fluorescence camera is set to 750 ms exposure time, and 5 consecutive frames are accumulated to form one final image. Bright-field and fluorescence images are captured at fixed 20 s time intervals.”.*

(7) In p 5, provide a brief description for usage of phenol red. It seems out of context.

*We have modified the manuscript on p. 5 from*

*“However, phenol red should be avoided as it can disturb the experiment due to absorption of the laser light.”*

*to*

*“However, the pH indicator phenol red should be avoided as it can disturb the experiment due to absorption of the laser light.”*

(8) In p 6, it is clear that the authors used an open cell scheme. Why? It should be more subject to environmental noises. Can they use a close cell scheme?

*We have chosen to have an open air scheme for our measurement so that we have easy and simple access to the cells for injection, stimulation, and manipulation. To ensure cell viability over longer measurement periods, it is necessary to enclose the cell culture in a microscope incubator as mentioned in the discussion section (p. 11). This is certainly possible, but removes easy access to the cell sample under study. Regarding the second point, the entire optical table is enclosed (as mentioned in section 3) which minimizes any environmental noises.*

(9) In p 8, (in 3.3) the authors used bright-field and fluorescence images to verify the cell's viability. I suppose that it should be from the shape of cells and the fluorescence of propidium iodide. For unfamiliar readers, explain in more details (with images) the criteria for cell viability.

*We have modified the manuscript to make this clearer. Specifically, we have changed the text on p. 8 from:*

*“3.3. Using the piezo positioner, move the sample laterally to position a cell close (approximately 10 µm) to the iSCAT field of view (FOV). Use bright-field and fluorescence images to locate and verify the cell’s viability.”*

*to*

*“3.3. Using the piezo positioner, move the sample laterally to position a cell close (approximately 10 µm) to the iSCAT field of view.*

*3.4. Use bright-field and fluorescence images to locate and verify the cell’s viability. A viable cell has a round shape in the bright-field image and is not fluorescent, whereas cell death is indicated by strong fluorescence signals arising from the presence of propidium iodide inside the cell.”*

(10) In p 9, (in 4.3) the authors mentioned that they created a rolling differential image. Explain what it is.

*In response to Reviewer 2, we have modified this section to more clearly state the rolling differential imaging method. Specifically, we have changed*

*“4.3. Create a rolling differential image to retrieve the binding events of particles whose contrast is smaller than the contrast resulting from other sources (like the surface roughness of the coverslip). This step also removes any wavefront distortions present in the incident beam.”*

*to*

*“4.3. Create a rolling differential image by subtracting each subsequent frame with its predecessor.*

*Note: Residual signals from the surface roughness of the coverslip and wavefront distortions are effectively removed in this step as they are constant within consecutive frames. The rolling differential removes these residual signals, leaving only the protein bindings that occur from one frame to the next. This dynamic background subtraction is beneficial as it is not sensitive to long term sample drifts.”.*

(11) In p 9, (in 4.4) what is 'index'?

*The index we refer to is the frame index, i.e. which frame the particle is registered in. We have modified the text to clarify this point. Specifically, we have changed the text from*

*“4.4. Apply a peak-seeking algorithm to detect single particles for each frame and determine their specific contrast, index, and position (if desired).”*

*to*

*“4.4. Apply a peak-seeking algorithm to detect and index single particles for each frame and determine their specific contrast and position.”.*

Other minor points:

In p 5, (in 1.4.4) 'make sure to' sounds clumsy. Change to 'be sure to'.

*We have modified the text from*

*“1.4.4. Set the necessary camera parameters. Make sure to use a fixed frame rate and to disable software gain and correction tools.”*

*to*

*“1.4.4. Set the necessary camera parameters. Use a fixed frame rate and disable software gain and correction tools.”.*

In p 6, (in 2.2.4) 'it' is missing: ... and cover it with ...

*We have corrected the typo.*

In p 8, (line 382) 'self-written' sounds a little bit strange. Change to a better expression.

*We have changed the text from*

*“We perform the described analysis with self-written scripts in MATLAB.”*

*to*

*“The described analyses are performed with custom MATLAB scripts.”.*