Journal of Visualized Experiments

Label-free imaging of single proteins secreted from living cells via iSCAT microscopy

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58486R1
Full Title:	Label-free imaging of single proteins secreted from living cells via iSCAT microscopy
Keywords:	iSCAT; label-free; single-protein; cellular secretion; imaging; scattering; dynamics; real-time
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Question	**Response**
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Dr. Jialan Zhang
Journal of Visualized Experiments

May 17, 2018

Dear Dr. Zhang,

Thank you for your invitation to submit our work to the Journal of Visualized Experiments (JoVE). Please find the manuscript enclosed, titled "Label-free imaging of single proteins secreted from living cells via iSCAT microscopy". In it, we outline a protocol for interferometric detection of scattered light (iSCAT) microscopy and show how this method can be used for real-time detection and imaging of single unlabeled proteins as they are secreted from individual living cells. We detail the process of building a functioning iSCAT microscope and demonstrate it with Laz388 cells—an Epstein-Barr virus (EBV) transformed B cell line. Compared to the established methods such as mass spectrometry or immunoassays, iSCAT detection provides orders of magnitude higher temporal resolution and is suitable for real-time investigation of living, active cells with single-protein sensitivity.

I believe that our work and this manuscript deserve the attention of the diverse audience of JoVE. I hope that you share our enthusiasm and request expert review for this manuscript. For your convenience, we have provided a list below of the names and contact information for three appropriate expert reviewers.

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

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TITLE:

Label-Free Imaging of Single Proteins Secreted from Living Cells via iSCAT Microscopy

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KEYWORDS:
iSCAT, label-free, single-protein, cellular secretion, imaging, scattering, dynamics, real-time

SUMMARY:
We present a protocol for the real-time optical detection of single unlabeled proteins as they are secreted from living cells. This is based on interferometric scattering (iSCAT) microscopy, which can be applied to a variety of different biological systems and configurations.

ABSTRACT:
We demonstrate interferometric scattering (iSCAT) microscopy, a method capable of detecting single unlabeled proteins secreted from individual living cells in real time. In this protocol, we cover the fundamental steps to realize an iSCAT microscope and complement it with additional imaging channels to monitor the viability of a cell under study. Following this, we use the method for real-time detection of single proteins as they are secreted from a living cell which we demonstrate with an immortalized B-cell line (Laz388). Necessary steps concerning the preparation of microscope and sample as well as the analysis of the recorded data are discussed. The video protocol demonstrates that iSCAT microscopy offers a straightforward method to study secretion at the single-molecule level.

INTRODUCTION:

Secreted proteins play a significant role in various physiological processes[1]. Because of this, they are routinely studied as a collective ensemble (proteomics) or as individual entities[2,3]. Proteomics traditionally investigates the entire set of proteins present in a particular biological system by way of *e.g.,* enzyme-linked immunosorbent assays (ELISA), flow cytometry, or mass spectrometry[4-6]. Single proteins, on the other hand, are generally detected using a variety of techniques that are based on fluorescence[7,8], plasmonics[9,10], or cryogenic electron[11] microscopies. All of these techniques use complex instruments, labeling, or both and often lack dynamics information as they only deliver long-term information about the system under study.

Here we use iSCAT[12,13] microscopy to sense individual secretory proteins with sub-second temporal resolution[14]. Importantly, the technique detects the weak scattered signal intrinsic to every protein[12,14]. The amount of light that a small bioparticle scatters scales with its polarizability. Assuming that the shape of a protein can be approximated by an effective scattering sphere[14-16], and that different proteins have very similar refractive indices, the measured signal can be directly connected to the molecular weight (MW) of the protein. The empirical calibration of iSCAT contrast *versus* molecular weight by reference measurements allows one to distinguish proteins of different sizes. iSCAT experiments can readily be complemented by fluorescence microscopy[17,18], immunosorbent reagents, as well as fluorescent or scattering labels to allow for a specific detection of any protein of interest[14,17,19].

In principle, iSCAT functions by amplifying a protein's weak scattered light via interferometric mixing with a secondary reference wave. The detected intensity (I_{det}) in an iSCAT microscope is described by

$$I_{det} = I_i(r^2 + s^2 + 2rs\ \cos\varphi)$$

where I_i is the incident intensity, r is a coefficient for the contribution of the reference wave, s signifies the scattering strength of the nano-object under study, and φ is the phase shift between the scattered and reference waves[14]. Either the transmitted or back-reflected incident light is typically used as a reference wave, where in each case r^2 accounts for the transmissivity or reflectivity of the sample chamber, respectively. The term s^2 is proportional to the protein's scattering cross section and can be neglected compared to the cross term. Thus, setting $\varphi = \pi$ for complete destructive interference, the detected light is given by $I_{det} \cong I_i(r^2 - 2rs)$ where $r^2 I_i = I_{ref}$ is the reference intensity and $2rsI_i = I_{int}$ is the interference intensity.

iSCAT microscopy offers an excellent method to study biological processes at the single-molecule level. As an example, we investigate Laz388 cells — an Epstein-Barr virus (EBV) transformed B lymphocyte cell line[20,21] — as they secrete proteins such as IgG antibodies[16]. However, the method is general and can be applied to a variety of other biological systems. iSCAT is inherently unspecific and can detect any protein or nanoparticle or it can be extended with common surface functionalization methods for specific or multiplexed detection. Its simplicity and ability to be combined with other optical techniques, such as fluorescence microscopy, make iSCAT a valuable complementary tool in cell biology.

PROTOCOL:

CAUTION: Please read all relevant material safety data sheets (MSDS) before using any chemicals, observe all appropriate safety practices, and wear personal protective equipment (laser safety goggles, eye protection, gloves, laboratory coats) as needed.

1. Building the iSCAT Microscope[16,18]

Note: The iSCAT microscope typically consists of a modified inverted microscope setup. In brief, a laser is focused onto the back focal plane of a high numerical aperture (NA) objective and an imaging lens is used to focus the particle's back-scattered light onto a camera chip. In general, this wide-field microscope can be built from scratch or based on an existing inverted microscope. This protocol covers the essential steps to realize the setup, while changes in the used hardware are possible. A more detailed description of the assembly of an iSCAT microscope can be found in the work of Arroyo *et al.*[18].

CAUTION: An iSCAT microscope involves a Class IIIB to Class IV laser light source. Appropriate eye protection is necessary when assembling and aligning the microscope optics.

1.1. Set up the illumination path of the microscope.

1.1.1. Using a damped optical table and a rigid metal block, build a microscope sample stage[18] that incorporates a high numerical aperture (NA) objective (100X/1.46 NA) and a translation unit that allows for lateral sample translation as well as change of focus position for the objective.

Note: Piezoelectric nanopositioners that allow for precise 3D movement are recommended. 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. **Figure 1** shows a suitable sample stage, including the piezo translation unit and the objective. 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.

[Place **Figure 1** here]

1.1.2. Use a 50 cm focal length singlet lens (wide-field lens) and a 45° (vertical) coupling mirror to focus the light of a diode laser at wavelength 445 nm onto the back focal plane of the objective. This creates a collimated beam at the objective's forward focus and will become the iSCAT illumination source. If necessary, filter the laser spatially prior to the 50 cm lens via a 30 µm pinhole or single mode fiber.

1.1.3. Apply a droplet of immersion oil to the objective and place a glass coverslip in the sample plane of the microscope stage. This will result in a beam that reflects back down through the imaging objective.

Note: The reflection arises from the air-glass interface at the upper surface of the sample coverslip and will serve as the basis for the iSCAT reference beam. Residual dirt or dust on the surface of the coverslip will give rise to scattering point sources, which aid in the correct focusing of the imaging objective in the next steps.

CAUTION: The majority of the laser light transmits through the coverslip and travels straight up from the objective. Place an opaque specular diffuser (*e.g.*, a paper card) above the coverslip to minimize the risk of injury from the laser light.

1.2. Set up the imaging path of the microscope.

1.2.1. Introduce an antireflection (AR)-coated beam splitter (70% reflection, 30% transmission) at a 45° angle relative to the incident beam, and approximately 10 cm after the wide-field lens. Point the AR coating towards the laser source. This transmits the incident beam, and reflects the reference and scattered beams at a 90° angle to the incident beam.

Note: AR-coatings or wedged beam splitters are recommended as significant ghosting and fringe effects can occur when using beam splitting cubes, or uncoated planar beam splitters. See the **Discussion** for more details.

1.2.2. At this point, focus the imaging arm of the interferometer and ensure that the sample plane and camera are parfocal. Place a concave f = -45 cm lens at a position 5 cm after the wide-field lens in the incident beam path. This will result in a collimated beam entering the back aperture of the objective.

1.2.3. With a screen placed in the reflected arm of the interferometer, move the objective in the vertical direction to find the coarse focal position. The objective is in focus when the beam hitting the screen is collimated.

Note: **Figure 2** shows a schematic of this process.

[Place **Figure 2** here]

1.2.4. Remove both the f = -45 cm lens and the screen when coarse focusing is complete.

Note: Instead of using a negative focal length lens, the wide-field lens itself may be placed on a movable mount and shifted out of the beam path for this step. However, to achieve the most stable microscope configuration, it is recommended to hold the wide-field lens in a fixed position.

1.2.5. Add a second f = 50 cm singlet lens to focus the scattered light and to collimate the reflected light onto the sensor of a CMOS camera. Ensure that the lens is placed 50 cm from the back focal plane of the objective so as to re-collimate the reference beam and focus the scattered light.

1.2.6. Place the CMOS chip 50 cm away from the f = 50 cm lens and position the beam directly onto the middle of the chip.

Note: The following parameters are typically used for imaging. The output power of the laser (wavelength 445 nm) is set to 100 mW. Pinhole and beam splitter attenuate the transmitted light so that the effective power entering the objective is about 9 mW. The beam diameter at the sample position amounts to 6 µm. With the used imaging lens, the effective magnification of the system is about 300X. The size of the image on the CMOS chip is set to 128 × 128 pixels within the illuminated area, resulting in a field of view of approximately 5 × 5 μm^2. **Figure 3** shows a schematic of the fully assembled iSCAT microscope.

[Place **Figure 3** here]

1.3. Set up additional imaging channels.

Note: This section adds another imaging path to the microscope that allows for the observation of a large area surrounding the iSCAT laser via bright-field microscopy, and to monitor cell viability via fluorescence microscopy.

1.3.1. Couple the output of an LED light source (approximately 500 nm < λ < 580 nm) into a long working distance 20X/0.4 NA objective, and install mechanical components above the sample chamber that allow for focusing and lateral positioning of the LED output onto the sample.

1.3.1.1. Ensure that the LED's output spectrum covers the excitation range of the cell death marker (propidium iodide (PI)) and does not interfere with its fluorescence (λ > 600 nm). Use optical filters if necessary.

1.3.2. Move the upper objective laterally so that the upper (wide-field) and lower (iSCAT) objectives are collinear. This is determined by placing a screen under the lower objective and maximizing the intensity of transmitted LED light on the screen. Place a λ = 550 nm short-pass dichroic mirror (SPDM) to split the transmitted LED light from the iSCAT laser path.

1.3.3. Split this beam into two channels with an 8% reflective/92% transmissive beam splitter (BS). The 92% path is the fluorescence channel and the 8% path is used for bright-field imaging.

1.3.4. Image the bright-field channel onto a CMOS camera using an f = 5 cm achromatic doublet lens.

1.3.5. Image the fluorescence channel onto a separate CMOS camera using an f = 5 cm achromatic doublet lens and a λ = 600 nm long-pass filter to block the excitation light. **Figure 4a** shows a schematic of the fully assembled microscope including all imaging channels.

[Place **Figure 4** here]

1.4. Set up the computer and software.

1.4.1. Connect all cameras to a computer. Install respective driver packages and obtain/write software for their control.

Note: Suitable hardware is necessary for high-speed acquisitions. At a minimum, a multi-core processor, 16 GB of RAM, a frame grabber card, and a solid-state disc for data storage are recommended.

1.4.2. Observe the iSCAT image on the CMOS camera and ensure that it is in focus by finding a residual dust or dirt particle on the glass coverslip. Verify that the particle's image is a circularly symmetric point spread function (PSF).

Note: The main reason for a non-circularly symmetric PSF is that the laser beam does not enter the objective straight but at a small angle with respect to the optical axis. This is corrected by adjusting the angle and position of the incident beam with the 45° coupling mirror.

1.4.3. Compare the camera images of the bright-field and the fluorescence channels. Ensure that both are in focus and display the same area. Verify that the position of the iSCAT laser is approximately in the center of the image and take note of its position for later reference. See **Figure 4b – 4f** for typical camera images.

Note: Use a cell sample or fluorescent beads to find the focus of the two channels. Temporarily remove the fluorescence long-pass filter to adjust the system. The focus for conventional imaging of the cells needs to be slightly higher than the iSCAT focal plane. To compensate for this without moving the objective, displace the cameras from their positions in the focus of the two respective f = 5 cm lenses.

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

Note: The following parameters are used: The iSCAT camera is set to 5000 frames per second (fps) with an exposure time of 80 µs. As mentioned above, the image size is 128 × 128 pixels. Both bright-field and fluorescence cameras operate at full frame size (1280 × 1024 pixels). 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 acquired at fixed 20 s time intervals.

2. Preparation of the Experiment

2.1. Prepare the stock microscopy medium.

2.1.1. Add 25 mL of HEPES Buffer Solution (1 mol/L) to 975 mL of RPMI 1640 medium to get a final 1 L of 25 mmol/L HEPES solution. Alternatively, use a buffer medium with HEPES already included.

Note: HEPES is used to maintain the pH value of the medium during a measurement in ambient conditions (*e.g.,* outside an incubator and without constant CO_2 supply).

2.1.2. Take an aliquot of the solution needed for an experiment and let it warm up to room temperature. 2 mL of medium is sufficient. Keep the remaining stock solution at 4 °C.

2.2. Prepare the microscope cuvette.

Note: The following steps describe the procedure for a custom-built sample holder consisting of an aluminum base plate and an acrylic cuvette dish that both fixes the coverslip and couples it to the piezoelectric 3D positioner. Commercially available sterile culture dishes with a glass bottom may also be used.

Note: **Figure 5** shows photographs of the custom-built sample holder.

[Place **Figure 5** here]

2.2.1. Take a new microscopy coverslip and rinse it with deionized water (DI-water) and ethanol. Air dry the slide with nitrogen or pressurized air.

2.2.2. Clean the coverslip in an oxygen plasma atmosphere (0.3 mbar gas pressure) for 10 min at 500 W RF power. This removes all organic impurities from the surface.

2.2.3. Clean the acrylic cuvette dish by immersing it in 0.2 mol/L NaOH solution for approximately 10 min. Rinse with DI water.

2.2.4. Assemble the sample holder and cover it with a plastic Petri dish until needed in the experiment.

2.3. Prepare the microscope.

2.3.1. Turn on the iSCAT illumination laser, the bright-field/fluorescence illumination LED, cameras, and the acquisition computer/software. Block the laser beam at a position before the objective.

2.3.2. Ensure that the 100X/1.46 NA objective is clean. If not, use lens cleaning wipes and ethanol to clean the objective in accordance with manufacturer guidelines.

2.3.3. Apply a drop of immersion oil on the microscope objective.

2.3.4. Take the sample stage (assembled in section 2.2) and carefully mount it on the piezo stage of the iSCAT microscope so that the sample coverslip is centered on the microscope objective. Be attentive and careful so as not to damage the objective lens. Fasten the unit to the piezoelectric positioner with thumb screws while ensuring that the manufacturer specified maximum torque is not exceeded.

2.3.5. Add 1 mL of stock microscopy medium (prepared in section 2.1.) into the cuvette.

2.3.6. Add 2 drops of propidium iodide stain to the medium as a cell death marker[16,22].

2.3.7. Unblock the laser and bring the system into focus. First, verify that the objective is positioned at the correct distance from the coverslip by repeating steps 1.2.2. – 1.2.4. (**Figure 2**). Then, fine tune the focus with the z-axis of the piezo stage.

2.3.8. Confirm that all settings for the light sources, cameras, and software are set correctly. This includes parameters such as laser power, LED intensity, camera frame rates, camera exposure times, or software saving paths.

Note: Saving videos at high frame rates can produce large file sizes. Ensure enough free disc space on the computer.

2.3.9. Block the laser beam again. The microscope is now ready for an experiment.

2.4. Prepare the cells.

Note: Laz388 cells[20] are cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), amino acids, pyruvate, and antibiotics. The cells are incubated at 37 °C and 5% CO_2 and are split and provided with fresh medium every 2-3 days[23].

2.4.1. Take the cell culture flask from the incubator and aspirate medium containing approximately 1 x 10^6 cells. To determine the correct volume, quantify the concentration of the cell culture by use of a hemocytometer.

2.4.2. Mix the cell solution with 10 mL of RPMI 1640 medium at room temperature and centrifuge the sample at 300 x g for 7 min.

2.4.3. Carefully extract and discard the supernatant while ensuring that the pellet of concentrated cells remains undisturbed.

2.4.4. Repeat steps 2.4.2. – 2.4.3. with the concentrated pellet of cells.

2.4.5. Re-suspend the cells in 0.5 mL stock microscopy medium (prepared in section 2.1.) and immediately use them in an experiment.

3. iSCAT Microscopy of Secreting Cells

3.1. Ensure that the laser beam is blocked to prevent the cells from being directly exposed to the iSCAT laser light.

3.2. Inject the cells into the sample cuvette.

3.2.1. Inject approximately 3 µL of the cell sample (prepared in section 2.4.) slightly off-center into the sample cuvette. Gently touch the pipette tip to the coverslip and slowly inject the cell solution. Allow the cells to settle on the coverslip.

Note: Use small volume pipette tips (10 µL) or long, flexible gel-loading tips.

3.2.2. Ensure that the density of cells is below approximately 1 cell per 500 µm² so that single-cell measurements are not influenced by multiple cells in the area surrounding the iSCAT laser.

3.2.3. If the number of cells is too low, repeat step 3.2.1. until a sufficient number is available.

3.2.4. If the coverage of cells is too dense, use an injection of approximately 20 µL of additional microscopy medium to disperse the cells across the coverslip.

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 exposure to the 445 nm laser light might be harmful for the cell.

3.4. Use bright-field and fluorescence images to locate and verify the cell's viability[25].

Note: 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[22].

3.5. Unblock the iSCAT laser beam and ensure that the coverslip surface is still in focus. Enclose the isolation table to minimize drift and acoustical coupling from the ambient surroundings.

Note: The latter is accomplished with heavy optical curtains or acrylic panels surrounding the optical table.

3.6. Start the measurement by acquiring images from the iSCAT, bright-field, and fluorescence cameras. Automate and control the process through software to maximize experimental efficiency. Periodically check the viability of the cell and the focus of the system.

Note: Depending on the laser intensity, optical components, and exposure time settings of the camera, the iSCAT laser might interfere with the fluorescence camera. If this behavior is observed, consider shuttering the iSCAT laser temporarily during fluorescence image acquisitions.

4. Data Analysis

Note: Experimental data is inherently noisy, and iSCAT images are no different. There are several sources of noise in a typical iSCAT measurement, including wavefront distortions in the incident light source, surface roughness of the coverslip, and camera noise. The section below presents some ways in which these noise sources are remedied via post processing. Additionally, lateral mechanical instabilities of the setup lead to noisy data and must be addressed accordingly, as described in the discussion section below. The described analyses are performed with custom MATLAB scripts.

4.1. Minimize camera noise by filtering the raw data with a two-dimensional Fourier filter that excludes high spatial frequencies. The size of the filter needs to be adjusted to fit the specific experimental configuration (mostly determined by the system's numerical aperture).

Note: Features in the image with higher spatial frequencies than the optical system originate from extraneous sources (such as camera read-out noise) and can be neglected.

4.2. Convert the images from raw camera counts to iSCAT contrast.

Note: The signal detected by the camera is $I_{det} = I_{ref} + I_{int}$. iSCAT contrast is defined as I_{int}/I_{ref} where I_{ref} is the intensity of the reference light, in this case the part reflected by the coverslip, and I_{int} is the interference between I_{ref} and the scattered intensity (I_{sca}).

4.2.1. Separate the signal into I_{int} and I_{ref} by computing the temporal mean of particular frames in which the particles of interest are not present. The resulting image provides the reference signal I_{ref}.

Note: Alternatively, an active background subtraction step may be performed as described in the discussion below.

4.2.2. Calculate contrast according to $(I_{det} - I_{ref})/I_{ref}$[12,14,16].

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.

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

4.5. Use the information collected in step 4.4 to create histograms of protein binding events and relate their extracted contrasts to protein mass through a calibration curve compiled from known protein samples[14,24].

REPRESENTATIVE RESULTS:

A schematic of an iSCAT microscope is shown in **Figure 4a**. Representative bright-field, fluorescence, and raw iSCAT images are shown in **Figures 4b**, **4c**, and **4d**, respectively[16]. **Figures 4e** and **4f** show the results of background removal and differential post processing; two adsorbed proteins are visible as diffraction-limited spots in **Figure 4f**. **Figure 6** shows a histogram of the detected proteins over the course of 125 s. These data were obtained by applying a peak-seeking algorithm to the captured images to count the binding events and catalog their contrast[16]. A total number of 503 proteins were detected.

Next, secreted species are identified by comparison with reference measurements carried out on purified protein solutions, or through additional measurements with functionalized glass surfaces[14,16]. The iSCAT data, thus, directly visualize cellular secretion dynamics on a subsecond scale[16]. As an example, we have previously found that IgG antibodies are a major fraction of the Laz388 secretome and are released from the cell at a rate of ca. 100 molecules per second[16]. Additionally, other particles spanning a range of 100 kDa – 1000 kDa are secreted by the cells[16]. The described method can be further employed *e.g.,* to investigate the spatial concentration gradient of secretions surrounding a cell[16], or to determine the temporal dynamics of cellular lysis[16].

FIGURE AND TABLE LEGENDS:

Figure 1: iSCAT sample stage. The photograph shows the massive aluminum block on which the piezo translation unit (black) is mounted as well as the centered 100X objective. The 3-axis piezo stage allows for precise positioning of the sample in the focal plane of the objective. Coarse focusing is carried out by rotating a threaded tube on which the objective is mounted (not depicted). The block is positioned on the optical table with four steel pedestals above the 45° coupling mirror.

Figure 2: Coarse focusing of the iSCAT microscope. The schematic shows the arrangement of the optics to help bring the system into focus. The AR-coated back side of the beam splitter (70/30 BS) is marked in red. Important distances are provided in green. The focal lengths (f) of the lenses

used are denoted. Components in the blue dashed box are added in steps 1.2.5 – 1.2.6. The concave lens (used to re-collimate the converging iSCAT beam) and the screen are removed later.

Figure 3: iSCAT microscope. The schematic shows the completely assembled iSCAT microscope. The AR-coated back side of the beam splitter (70/30 BS) is marked in red. Important distances are provided in green. The focal lengths (f) of the lenses used are denoted.

Figure 4: iSCAT microscopy of proteins secreted by single cells. (a) Schematic of the microscope described in the protocol. See section 1 for more information. Abbreviations: LED, light-emitting diode; SPF, short-pass filter; obj, objective; SPDM, short-pass dichroic mirror; BS, beam splitter; LPF, long-pass filter; BF, bright field; fluor, fluorescence; C1-C3, camera 1-3. **(b)** Bright field image of a single Laz388 cell about 4 µm away from the iSCAT field of view (depicted by a white square). Image taken by camera C3, scale bar: 10 µm. **(c)** Fluorescence image of the same region shown in (b) with the position of the cell marked by a white circle. The absence of fluorescence indicates that the cell is viable. Image taken by camera C2, scale bar: 10 µm. **(d)** Raw iSCAT camera image snapshot with 80 µs exposure time. Image taken by camera C1. **(e)** iSCAT image of the same region after spatiotemporal background subtraction as described in the discussion section. The image was integrated over 1000 sequential raw frames (d) with a final frame time of 400 ms and reveals the surface roughness of the glass coverslip. **(f)** Corresponding differential iSCAT image that shows the binding event of 2 proteins onto the coverslip. The image was constructed by subtracting two consecutive filtered images (e). Scale bars in (d), (e), and (f): 1 µm. This figure has been adapted from McDonald, M.P. *et al.*[16]. Copyright 2018 American Chemical Society.

Figure 5: Custom-built sample holder. (a) Sample holder components: (1) acrylic cuvette dish; (2) aluminum base plate; (3) holding screws; (4) silicone O-ring; (5) coverslip. **(b)** Fully assembled sample holder.

Figure 6: Quantification of secreted proteins by a single Laz388 cell. The histogram shows detected proteins during a time period of 125 s. Contrast values are accumulated in 1 x 10^{-4} contrast bins (blue bars). A total of 503 individual proteins were counted during this measurement. The experiment was repeated 10 times with similar results. This figure has been adapted from McDonald, M.P. *et al.*[16]. Copyright 2018 American Chemical Society.

Figure 7: Comparison of iSCAT images produced with high- and low-quality beam splitters. (a) Resulting raw iSCAT image by use of a 5 mm thick, AR-coated, and wedged beam splitter. **(b)** Resulting raw iSCAT image of the same area by use of a 1 mm thick planar beam splitter. Both beam splitters have the same splitting ratio (50% reflection, 50% transmission). Interference artefacts arising from Fresnel reflections are clearly observed in the image produced with the 1 mm thick planar beam splitter. Scale bars: 2 µm.

DISCUSSION:

One of the most crucial aspects to obtaining useful iSCAT data is the ability to find the correct focal position at the coverslip surface, and, furthermore, to hold this position for long periods of time. Failing to do so will result in broadened PSFs, weak iSCAT signals, and drift-associated

artifacts in dynamics analyses. It turns out that finding the focal plane on a clean, bare coverslip surface is not an easy task as surface features are not visible against the large reference beam background (see **Figure 4d**).

Raw iSCAT images are often obscured by background signals that arise from wavefront impurities in the excitation source, and can hinder one's ability to find the correct imaging plane. Active wavefront subtraction is a useful way to circumvent this issue and subsequently monitor the iSCAT focus during a measurement[16]. One way to accomplish this is through spatial sample modulation. In brief, a function generator applies a 50 Hz square wave to the external control port of the piezo stage, resulting in a spatial sample modulation at the applied frequency (290 nm amplitude). Synchronous camera acquisitions are triggered from the same source, and, when combined through lock-in principles, result in a wavefront-compensated image[14,16]. The resulting image typically shows the surface roughness of the coverslip (**Figure 4e**). Small features remaining on the glass after cleaning can be used to bring the microscope into focus. Parameters used for this active background subtraction step may be changed according to the frame rate, exposure time, or hardware.

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.

[Place **Figure 7** here]

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 area[14]. Another common approach is to use acousto-optic deflectors (AODs) and scan a confocal beam across the sample[12,17]. 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.

As discussed throughout the protocol, it is imperative to minimize lateral mechanical fluctuations in the sample stage of the microscope. Even nanometer deviations in the position of the sample can lead to variations in consecutive camera frames and induce significant extraneous noise in the differential image. It is therefore recommended to use a mechanically stable microscope

stage and a damped optical table (step 1.1.1.) and to cover the setup with optical curtains or panels during an experiment (step 3.5.).

An active focus stabilization scheme could also be considered for long-term measurements. In this approach, a second laser is incorporated into the microscope in a total internal reflection (TIR) arrangement, and subsequently imaged onto a quadrant photodiode. Changes in the system's focus translate into lateral displacements of the TIR laser spot on the quadrant diode, which can then be used in an active feedback loop to control the z-axis of the piezo stage[26]. Long-term vertical drift effects are thus eliminated.

Several modifications and extensions can be applied to the presented technique to address specific experimental needs. For example, commercial microscope stage incubators are available that could readily be incorporated into the iSCAT microscope for long-term imaging of cells. Other techniques can also be implemented to complement iSCAT imaging, such as confocal or TIR fluorescence microscopies[17]. To adapt on the system under study, iSCAT secretion measurements can be carried out in other cell media such as DMEM or DPBS, however, the pH indicator phenol red should be avoided as it can disturb the experiment due to absorption of the laser light. Additionally, supplements like fetal calf serum (FCS) or human platelet lysate (hPL) contain proteins that may interfere with iSCAT detection. Depending on the desired sensitivity of the experiment, these supplements should be excluded from the microscopy medium.

iSCAT relies on an analyte's ability to scatter light—a property that is intrinsic to all proteins—and is thus inherently nonspecific. Nevertheless, some degree of specificity is possible as iSCAT signals scale linearly with protein mass[14,27,28]. This allows for the calibration of an iSCAT system using standard protein samples, such as bovine serum albumin (BSA) and Fibrinogen[14,27,28]. In fact, very recently, Young *et al.*[28] have extended on the work of Piliarik & Sandoghdar[14] 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. Several conventional approaches can further complement iSCAT by providing an extra level of specificity. As an example, enzyme-linked immunosorbent assays (ELISA), and/or other surface modifications, restrict protein binding events so that only the target protein is detected[16].

In this protocol, we described how iSCAT microscopy can be used to investigate cellular secretions at the single protein level with subsecond temporal resolution[16]. The technique is general and can be implemented on any commercial or home-built microscope. In contrast to single-molecule fluorescence approaches, the method does not suffer from photobleaching or blinking effects but it still achieves single protein sensitivity. These features make iSCAT a powerful tool in the field of biosensing and microscopy. Future applications will focus on elucidating complex cellular interactions such as immunological response to a stimulus or cellular communication.

ACKNOWLEDGMENTS:
This work was supported by the Max Planck Society, an Alexander-von-Humboldt Professorship, and the Deutsche Forschungsgemeinschaft (CRC 1181). We thank Stefanie Schaffer at

Universitätsklinikum Erlangen for providing Laz388 cells and for useful discussions. We thank Simone Ihloff and Maksim Schwab at MPL for technical support.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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Figure 2

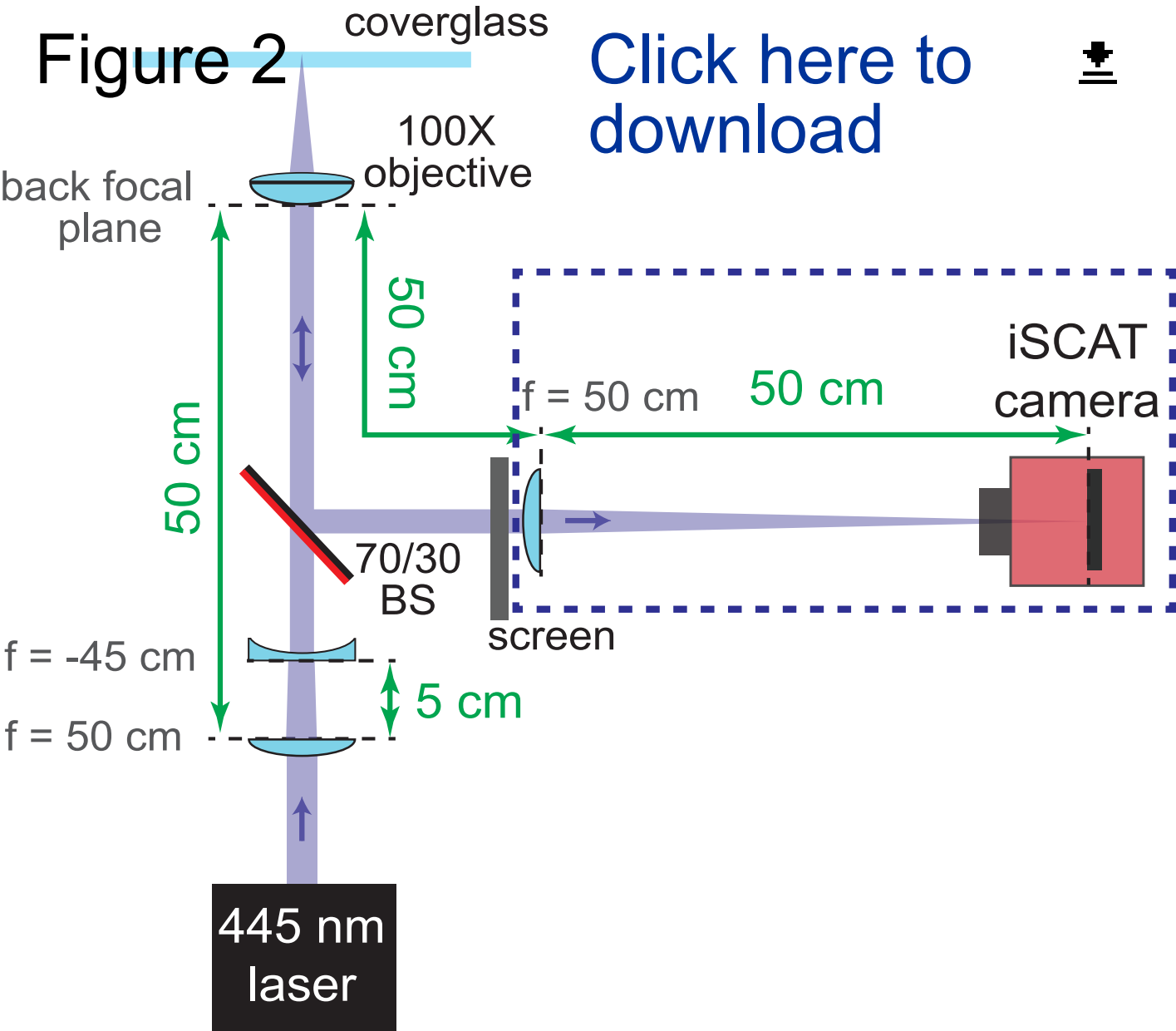

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Figure 3

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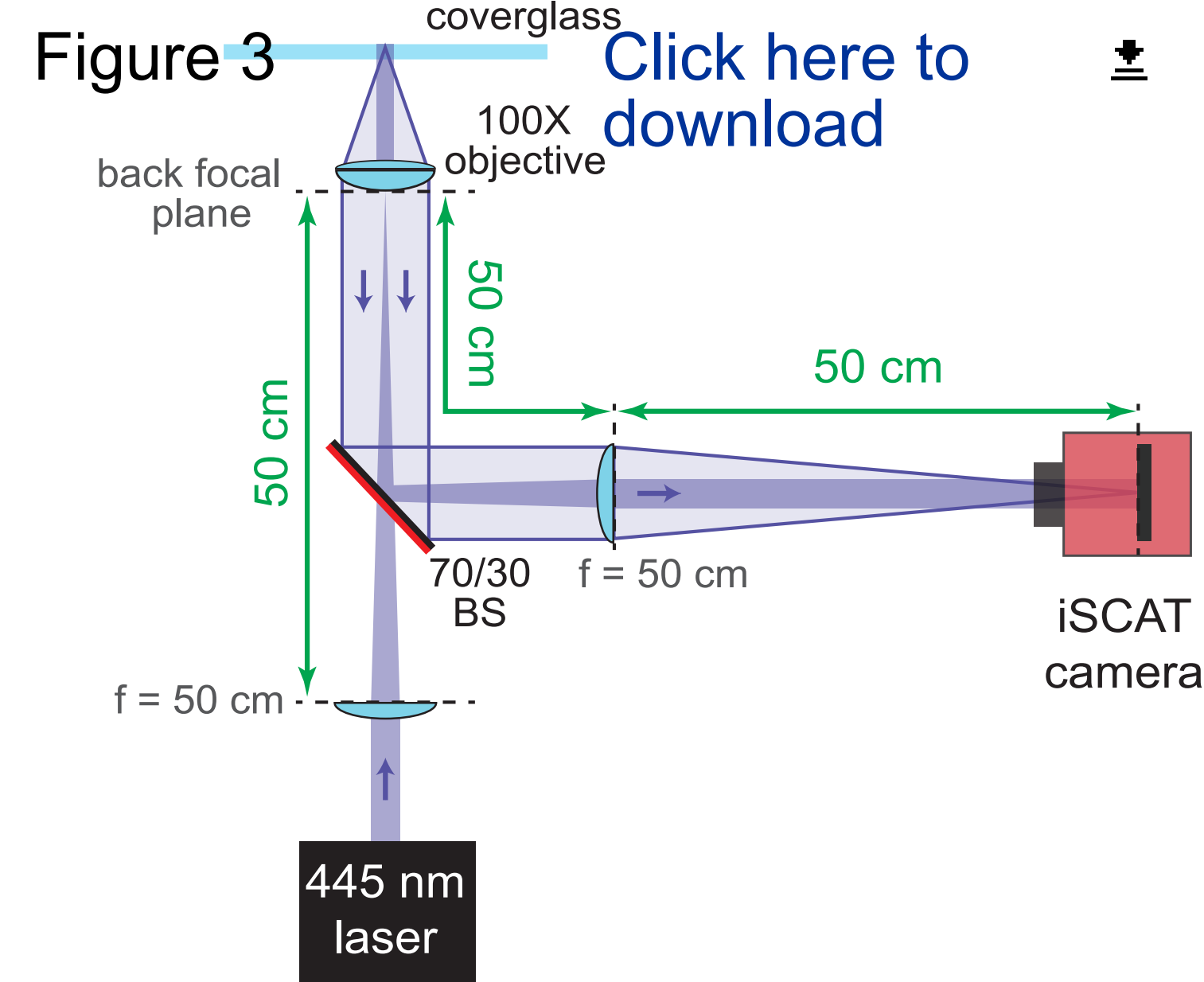


Figure 4

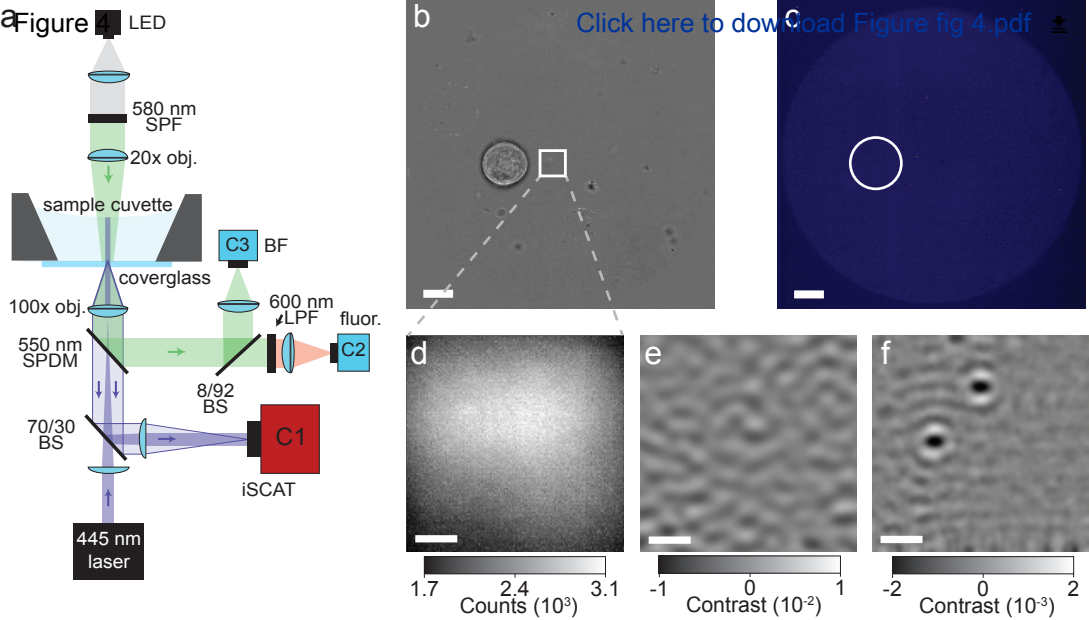
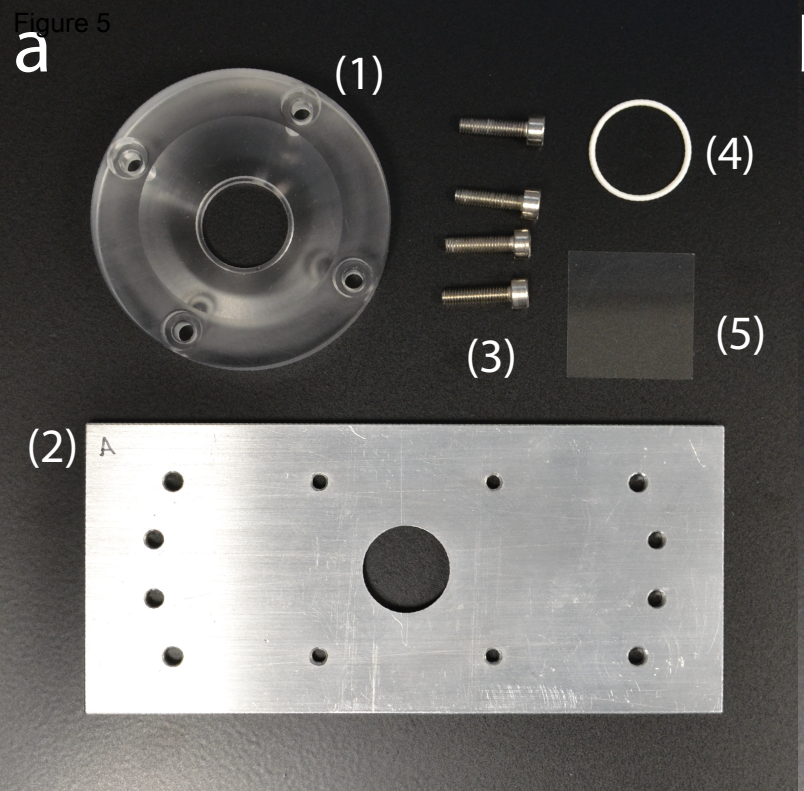


Figure 5



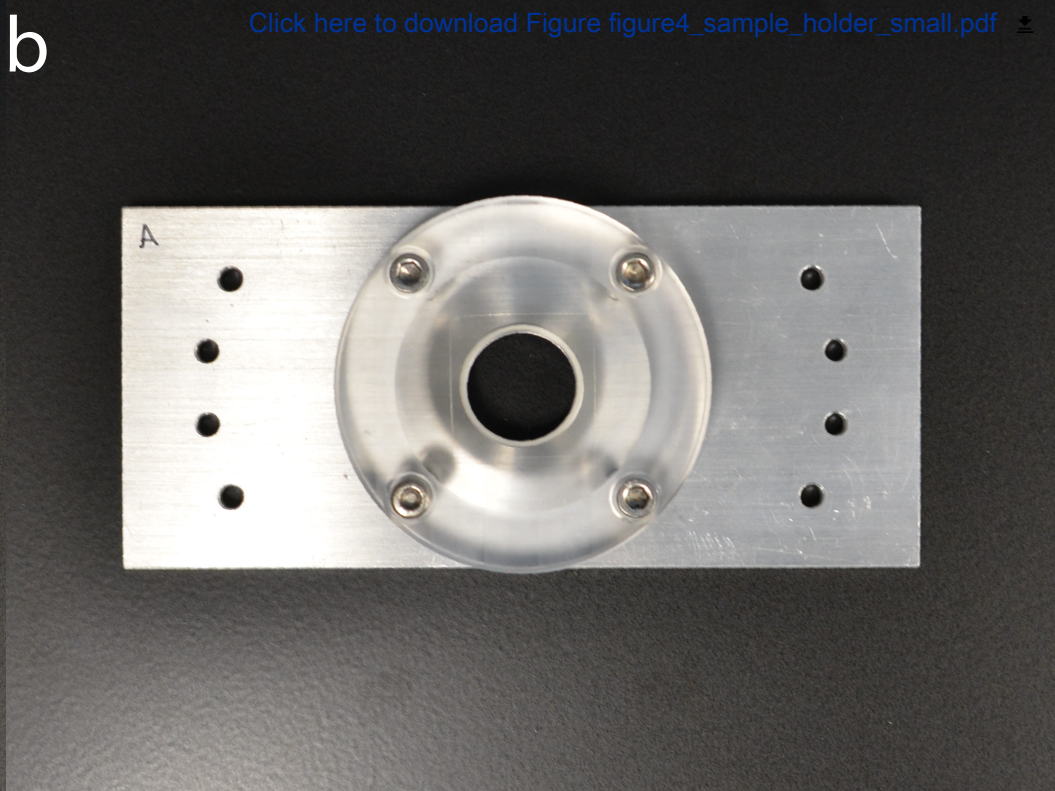


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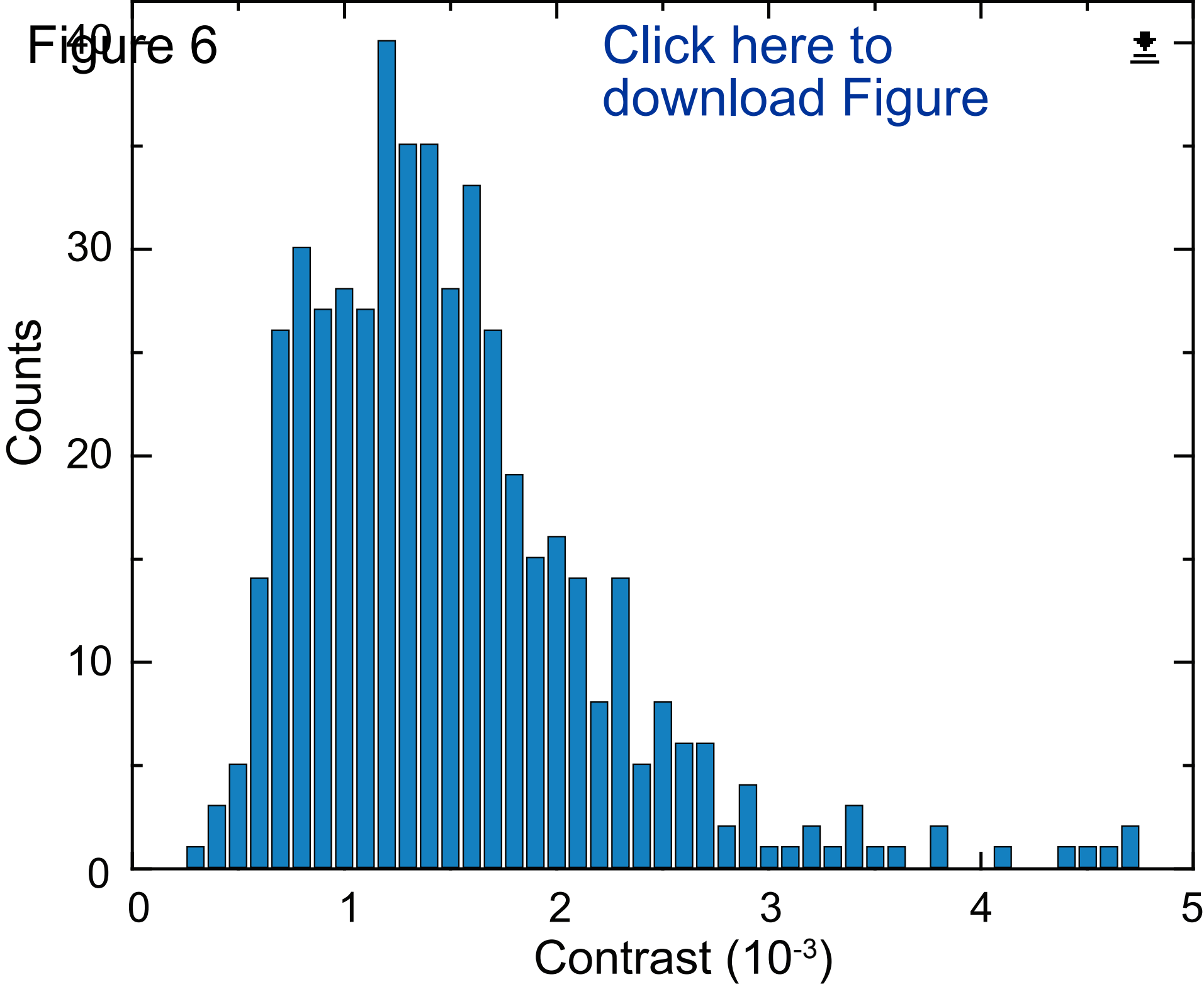
Figure 6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Contrast (10-3)

Figure 7

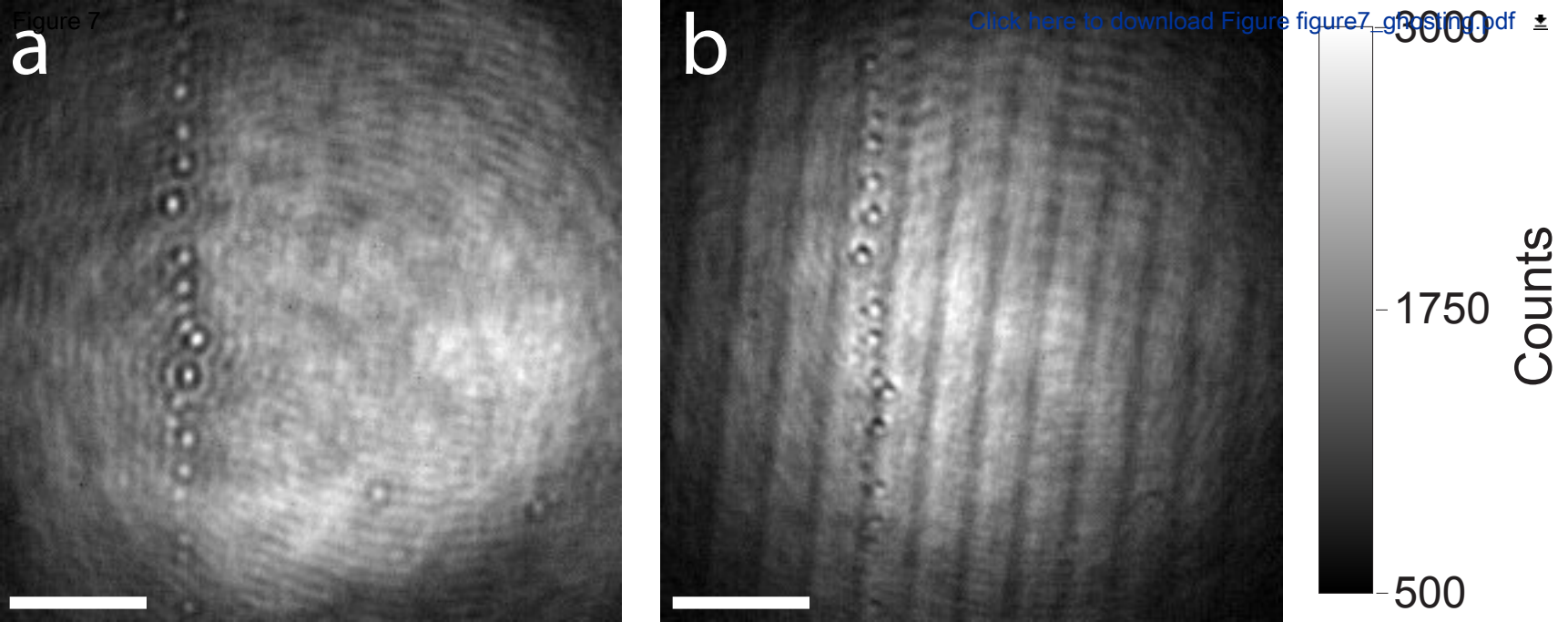

Table of Materials

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Name of Item/Device	Company	Catalog Number
100x / 1.46 NA objective	Zeiss	420792-9800-000
20x / 0.4 NA objective	Leica	566049
Piezo Stage	PI	P-517k020
Diode laser (445 nm)	Lasertack	PD-01236
Optics/Optomechanics	Thorlabs/Newport	-
Pinhole	Thorlabs	P30H
LED light source	Thorlabs	MCWHL5
Shortpass filter (580 nm)	Omega Optical	580SP
70R/30T beam splitter	Newport	20Q20BS.1
Economy beam splitter	Thorlabs	EBS1
Wedged plate beam splitter	Thorlabs	BSW26
Shortpass dichroic mirror (550 nm)	Edmund Optics	66249
8R/92T beam splitter	Thorlabs	BP108
CMOS camera	Photonfocus	MV1-D1024E-160-CL
CMOS cameras	Mightex	SCE-B013-U
Longpass filter (600 nm)	Thorlabs	FELH0600
Computer	Fujitsu Siemens	-
Acquisition Software	LabVIEW	-
Analysis Software	Matlab	-
Plasma Cleaner	Diener	Diener pico
Incubator	Binder	Model CB
Centrifuge	Eppendorf	5810R

Name of Reagent/Material	Company	Catalog Number
RPMI 1640 medium	Gibco	11835063
HEPES Buffer Solution (1M)	Sigma Aldrich	59205C
Cover slides	Marienfeld	107052
Glass bottom culture dishes	ibidi	81158
Fluorescent Microspheres	Invitrogen	F8834
Immersol immersion oil	Zeiss	444960
Propidium iodide stain	Invitrogen	R37108
Small pipette tips	Eppendorf	30075005
Flexible pipette tips	Eppendorf	5242956003
Ethanol, 99.8%	Fisher Scientific	E/0650DF/15
Sodium hydroxide, pellets	Sigma Aldrich	221465

Comments
alpha Plan Apochromat oil immersion
N Plan
3-axis stage with 100x100x10µm range

lenses, mirrors, posts, mounts

to modify the spectrum of the LED for fluorescence excitation

used for the comparison of fringe effects
used for the comparison of fringe effects

for iSCAT aquisition
for bright field / fluorescence aquisition

Core i7 Processor, 16 GB RAM, SSD
LabVIEW 2016 Suite
Matlab 2014 Suite

Comments
without phenol red

used for calibration

for preparing 0.2M NaOH solution

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Author(s): André Gemeinhardt, Matthew P McDonald, Katharina König, Michael Aigner, Andreas Mackensen, and Vahid Sandoghdar

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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 mass[14,27]. In fact, very recently, Young et al.[28] have extended on the work of Piliarik & Sandoghdar[14] 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 mass[14,27,28]. This allows for the calibration of an iSCAT system using standard protein samples, such as bovine serum albumin (BSA) and Fibrinogen[14,27,28]. In fact, very recently, Young et al.[28] have extended on the work of Piliarik & Sandoghdar[14] 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 area[14]. Another common approach is to use acousto-optic deflectors (AODs) and scan a confocal beam across the sample[12,17]. 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.".

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Title: Visualizing Single-Cell Secretion Dynamics with Single-Protein Sensitivity

Author: Matthew P. McDonald, André Gemeinhardt, Katharina König, et al

Publication: Nano Letters

Publisher: American Chemical Society

Date: Jan 1, 2018

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