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TITLE:

Implementation of interference reflection microscopy for label-free, high-speed imaging of microtubules

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Interference reflection microscopy, label-free imaging of large biomolecules, in vitro surface assays, microtubule dynamics, high-speed imaging, image analysis

SUMMARY:

This protocol is a guide for implementing interference reflection microscopy on a standard fluorescence microscope for label-free, high-contrast, high-speed imaging of microtubules using in vitro surfaces assays.

ABSTRACT:

There are several methods for visualizing purified biomolecules near surfaces. Total-internal reflection fluorescence (TIRF) microscopy is a commonly used method, but has the drawback that it requires fluorescent labeling, which can interfere with the activity of the molecules. Also, photobleaching and photodamage are concerns. In the case of microtubules, we have found that images of similar quality to TIRF can be obtained using interference reflection microscopy (IRM). This suggests that IRM might be a general technique for visualizing the dynamics of large biomolecules and oligomers in vitro. In this paper, we show how a fluorescence microscope can be modified simply to obtain IRM images. IRM is easier and considerably cheaper to implement than other contrast techniques such as differential interference contrast microcopy or interferometric scattering microscopy. It is also less susceptible to surface defects and solution impurities than darkfield microscopy. Using IRM, together with the image analysis software described in this paper, the field of view and the frame rate is limited only by the camera; with a sCMOS camera and wide-field illumination microtubule length can be measured with precision up to 20 nm with a bandwidth of 10 Hz.

INTRODUCTION:

Label-free imaging of microtubules is of interest as it circumvents the need for fluorescent labeling of tubulin to generate contrast in the images. Fluorescent labeling has several

drawbacks: it not feasible if the protein concentration is low[1] and photobleaching and photodamage limit the observation time. Several techniques have been used to image label-free microtubules, including video-enhanced differential interference contrast microscopy (DIC) and darkfield microscopy[2–5]. Recently, interferometric scattering microscopy (iSCAT)[6], rotating-coherent-scattering microscopy (ROCS)[7] and spatial light interference microscopy (SLIM)[8], have also been used. All these techniques are capable of imaging microtubules and have proved to be valuable for studying microtubule dynamics. However, each have their own limitations. In DIC the contrast depends on the angle between the microtubule and the Nomarski prism axis. In darkfield, the microtubule signal is degraded by scattered light from impurities or surfaces defects. Though iSCAT exhibits extraordinary sensitivity (down to single proteins) and ROCS can image microtubules deeper into the sample, both methods are technically demanding, requiring laser scanners.

This protocol demonstrates how interference reflection microscopy (IRM)[9, 10] can be set up as an alternative technique for label-free imaging of microtubules. IRM is easy to implement as it requires only the addition of an inexpensive 50/50 mirror to a standard fluorescent microscope. When used in conjunction with the software described here, IRM produces high contrast microtubule images, can image large fields of view at high speed, requires a onetime alignment, and can easily be combined with other techniques such as fluorescence imaging.

PROTOCOL:

1. Microscope modification and objective lens

1.1. Insert a 50/50 mirror into the filter wheel of the fluorescent microscope using an appropriate filter cube (**Figure 1**). Handle the 50/50 mirror with care as often they have anti-reflection coating.

NOTE: We used a 50/50 mirror in an empty filter cube of the microscope. The 50/50 mirror is inserted where the dichroic mirror is located.

1.2. Use a high magnification/high NA oil objective.

NOTE: In this protocol, we used a 100x/1.3 NA objective.

2. Chamber preparation to adhere microtubules to the surface

2.1. Clean the microscope slide and 22 mm x 22 mm coverslips (for upright microscope) or 22 mm x 22 mm and 18 mm x 18 mm coverslips (for inverted microscopes). Modify the surface as needed. For example, clean the coverslips using an alkaline detergent (see **Table of Materials**) followed by 100% ethanol with distilled water washes in between and after[11].

2.2. Cut 3 mm wide strips of plastic paraffin film (see **Table of Materials**) using a razor blade and another microscope slide as an edge.

2.3. Place two plastic paraffin film strips 3 mm apart on a clean 22 x 22mm cover slip. Then place an 18 mm x 18 mm coverslip to form a channel. If using an upright microscope, place the strips on top of a clean microscope slide and then place a coverslip on top.

2.4. Place the coverslip (or slide) on a heat block at 100 °C for 10-30 s for the paraffin film to form a sealed channel.

2.5. Flow in 50 µg/mL of antibody (in Brinkley Buffer 1980 (BRB80)) by perfusion using a pipette. Incubate for 10 min.

NOTE: We use anti-rhodamine antibodies to bind rhodamine-labeled microtubule seeds to the surface. Alternatively, avidin can be used to bind biotin-labelled microtubule seeds or to biotinylated gold particles. To simply look at microtubules in the absence of tubulin in solution (i.e., a non-dynamic assay), an anti-tubulin antibody can be used.

2.6. Wash five times with BRB80 buffer. It is recommended to filter the solutions using a 0.22 µm filter.

2.7. Flow in 1% poloxamer 407 (a triblock copolymer) in BRB80 to block the surface against non-specific binding. Incubate for 10 min.

2.8. Wash five times with BRB80 buffer.

2.9. The sample is ready to be placed on the microscope. To prevent the sample from drying out, add two droplets of BRB80 buffer at the ends of the channel and replenish when needed.

3. Microscope alignment

3.1. Place the sample on the microscope stage. Turn on the epi-illumination light source.

3.2. Focus on the sample surface. Observe multiple surfaces as the objective is moved up and down due to back reflection of light from optics within the optical path. A good method to find the sample surface is to focus on the paraffin film edge. Once the surface is found, set the field of view to the center of the chamber.

3.3. Center the field diaphragm in the field of view by closing it half way and using the adjustment screws. Once aligned, reopen the diaphragm.

NOTE: Screw adjustment only needs to be done sporadically, perhaps every 3-6 months or when troubleshooting.

3.4. Slide in the Bertrand lens to view the exit pupil (back focal plane) of the objective.

3.5. Close the aperture diaphragm (AD) beyond the edges of the exit pupil of the objective.

3.6. Use the adjustment screws to center the AD with the exit pupil. Double check by opening the AD and matching its edges with those of the exit pupil.

NOTE: This adjustment only needs to be done sporadically.

3.7. Set the aperture diaphragm to about 2/3 of the NA of the objective. See section 7 for detailed procedures for optimizing the aperture diaphragm.

4. Imaging stabilized microtubules or 40 nm gold particle

NOTE: Stabilized microtubules and gold nanoparticles serve as good control samples. It is recommended to image surface attached microtubules or gold nanoparticles as a first step to assess IRM performance and help in setting the optimal aperture diaphragm opening (section 7).

4.1. Set the exposure time of the camera to 10 ms and adjust the illumination to nearly saturate the camera dynamic range.

4.2. Flow in 10 µL of guanylyl-(alpha, beta)-methylene-diphosphonate (GMPCCP)- or taxol-stabilized microtubules in BRB80[4,12,13] (or 40 nm gold particles) by perfusion using a pipette to a fresh sample and monitor binding by imaging the surface. Once 10-20 microtubules are bound within the field of view, wash the sample 2x with BRB80.

Note: With a well aligned microscope, the microtubules should be visible without background subtraction.

4.3. Acquire 10 images by setting a time lapse with 1 second delay period for 10 seconds (at 10 ms exposure).

4.4. Acquire a background image. To do so, move the stage using the stage controller or computer software along the channel long axis while acquiring 100 images with no delay (i.e. streaming close to 100 fps @ 10 ms exposure).

NOTE: The background is the median of the 100 images. By taking the median, the illumination profile and other stationary features like dirt on the optics are preserved while everything else is filtered out. There should be no tilt on the sample as this will lead to change in the axial position as the stage is moved and ultimately degrade the background image. If the tilt cannot be avoided, then an alternative method is to acquire averaged background images before flowing the seeds.

4.5. For processing and analyzing the images, go to step 6.

5. Imaging microtubule dynamics

5.1. For microtubule dynamics using brain tubulin, set the sample heater temperature to 37 °C.

5.2. Flow in 10 µL of GMPCCP-stabilized microtubule seeds in BRB80[11] by perfusion using a pipette to a fresh sample and monitor them binding to the surface by imaging the surface live (i.e., while streaming). Once 10-20 seeds are bound with field of view, wash the sample using 2x channel volume with BRB80 (BRB80 should be prewarmed to 37 °C or at least at room temperature).

Note: With a well aligned microscope, the seeds should be visible without background subtraction.

5.3. Flow in the polymerization mix (7.5 µM unlabeled tubulin + 1 mM guanosine triphosphate (GTP) + 1 mM Dithiothreitol (DTT) in BRB80 buffer). For measuring microtubule growth, set a time-lapse using the acquisition software to acquire an image every 5 seconds (0.2 frames per second (fps)) for 15 minutes.

5.4. To enhance the contrast, acquire 10 images (instead of one) at each time point and average them. For microtubule shrinkage, acquire images at 100 fps by setting the time delay to 0 and an exposure time of 10ms (higher fps is possible with smaller regions of interest depending on the camera used).

5.5. Acquire a background image as in step 4.4.

6. Image processing and analysis

NOTE: For analysis, this protocol uses Fiji[14] but the reader is free to use any software she/he find suitable.

6.1. Open saved background images.

6.2. Calculate the median image (i.e. background) using **Image > Stack > Z project > Median**.

6.3. Open microtubule dynamics movie as a stack (same for non-dynamic microtubules) using **File > Open**.

6.4. Subtract the background image from the stack using **Process > Image calculator**. Make sure to check the “32bit (float) result” option.

6.5. For dynamic microtubules, using the **line tool** draw a line along the microtubule of interest and add to the region of interest manager by pressing “t”. Repeat for all microtubules of interest.

6.6. For dynamic microtubules, run the Multi-Kymo macro (**Supplementary File 1**). The macro will generate a video and a kymograph for every microtubule in the ROI manager. Every microtubule will get a unique identifier.

6.7. For dynamic microtubules, run the Kymo-Analysis macro (**Supplementary File 2**) and follow its steps to measure the growth rates and shrinkage rates of the microtubules.

7. Aperture diaphragm size

NOTE: An important factor for acquiring high contrast images of microtubules using IRM is setting the illumination numerical aperture (*INA*) correctly[10,15]. The *INA* can be changed by changing the size of the incoming illumination beam at the objective's exit pupil which is controlled by the size of the AD (the AD is located at a conjugate plane with the exit pupil (back focal plane) of the objective, **Figure 1**):

$$INA = D_{\mathrm{AD}} / (2 * f_{\mathrm{objective}}) \quad \text{, for } D_{\mathrm{AD}} < D_{\mathrm{ep}}$$

where D_{AD} is the diameter of the aperture diaphragm, $f_{\mathrm{objective}}$ is the focal length of the objective and D_{ep} is the objective's exit pupil diameter. Typically, the AD is left fully open for fluorescence imaging, so the *INA* equals the objective's *NA*. In a fluorescence microscope, the AD scale does not indicate its diameter, thus the *INA* can't be calculated. It's possible to calibrate the AD size with the help of an objective. Yet, it's not necessary since the AD size would be fixed to the size that produces the highest contrast.

7.1. Prepare a sample of fluorescently labelled stabilized microtubules stuck to the surface (steps 4.1-4.2).

NOTE: We used tetramethylrhodamine labeled microtubules[16] (Ex: 550 nm, Em: 580 nm).

7.2. Bring microtubules into focus using the microscope focusing knob while fluorescently imaging them (if microtubules are not labelled, image them with IRM[15] or DIC[5]).

7.3. Set camera exposure to 10 ms using the camera software.

NOTE: This exposure is arbitrary and an exposure of 100 ms would also work.

7.4. Close down the AD to its smallest opening. Adjust the illumination to nearly saturate the camera's dynamic range or to the maximum possible.

NOTE: As a guide, use the look up table typically provided by the acquisition software.

7.5. Acquire 10 images (by streaming or taking an image every second) of a field of view containing 10+ microtubules.

7.6. Acquire a background image as in step 4.4.

7.7. Change the size of the AD and repeat steps 7.5-7.6 for the whole AD opening range (from closed to the exit pupil size, **Figure 2**). Every time the AD size is changed, adjust the illumination intensity to match that of step 7.4.

7.8. For every field of view acquired, subtract the corresponding background using **process > image calculator** and choosing "subtract" from the drop-down menu. Make sure the "32bit (float) result" option is checked. Then average the resulting images using **image > stack > Z project > average**.

7.9. Measure the signal-to-background noise ratio (SBR) of the microtubules defined as the average intensity of the microtubule signal (intensity of the microtubule minus the intensity of the background) divided by the standard deviation of the background (**Figure 3**).

7.10. Determine the optimal AD size (i.e. optimal *INA)* by calculating the average SBR noise ratio of the microtubules for every opening size and set the AD size to the one producing the highest SBR (**Figure 2**). It is possible that there is a range of AD sizes that produce comparable contrast[15].

REPRESENTATIVE RESULTS:

As mentioned above, with a well aligned microscope, microtubules should be visible without background subtraction (**Figure 4A**). Subtracting the background (**Figure 4B**) enhances the contrast of microtubule (**Figure 4C**). To further enhance the contrast, averaging or Fourier filtering or a combination of both can be used (**Figure 4D,F,E**). The line scans in **Figure 4G** shows the incremental improvement of image quality. Notice the reduction of background noise with each processing step.

Examples of kymographs of microtubule dynamics generated from time-lapse movies are shown in **Figure 5**. The videos were acquired at two frame rates: 0.2 fps (slow) and 100 fps (fast). The former is suitable for measuring growth rates while the latter is more suitable for measuring shrinkage rate that is an order of magnitude faster than the growth rate.

For the case where gold nanoparticles are used for setting up the microscope, an example image is shown in **Figure 6**. Gold nanoparticles were passively attached to the surface. While 40 nm particles are recommended, it is also possible to image 20 nm particles, yet at a lower contrast.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic representation of IRM. **(A)** Epi-illumination from the light source passes through the aperture diaphragm before reaching the 50/50 mirror. The aperture diaphragm sets the beam width thus the illumination NA. The 50/50 mirror partially reflects the light up to the objective to illuminate the sample. Light reflected from the sample is collected and then projected onto the camera chip (by the tube lens) where it interferes to generate the image. Image contrast is the result of the interference between the light reflected from the glass/water interface (I1) and the light reflected from the water/microtubule interface (I2). Depending on the microtubule/surface distance (h), the optical path difference between I1 and I2 will result in a

constructive (bright signal) or destructive (dark signal) or anything in between. For example, if light with a wavelength of 600 nm is used for imaging, the contrast will switch between dark and bright when the microtubule height changes by about 100 nm. The asterisk indicate conjugate planes (modified from[15]). **(B)** Example of the 50/50 mirror installation. A suitable filter cube was opened and the mirror was inserted where a dichroic mirror usually sits. The mirror was oriented as per manufacturer instructions. Then the cube was inserted in the filter wheel which was inserted back to the microscope (not shown). During the installation, gloves were used, and the mirror was only held by the edges.

Figure 2. Optimal Aperture diaphragm setting. (A) Same field of view was imaged at different aperture diaphragm openings without background subtraction. Visually, the contrast increased as the size of the aperture diaphragm increased till it reached a plateau and started to degrade afterwards. This was confirmed by **(B)** SBR measurements of background subtracted images error bars are standard deviation. Scale bars are 500 µm (AD) and 3 µm (microtubules).

Figure 3. Measuring signal-to-background noise ratio. Microtubules were isolated in regions of interest. Each region of interest was thresholded to separate the microtubule from the background. The average microtubule signal was obtained from a line scan across the microtubule. The scan line width was set to equal the microtubule length. This way, every point on the scan is an average of the signals of all pixels along the microtubule axis that are parallel to that point. The background noise is the standard deviation of all the pixels below threshold cut off.

Figure 4. Image processing. After acquiring raw images **(A)**, the background **(B)** was subtracted **(C)** to enhance the microtubule contrast. To further improve the contrast the images were either averaged **(D)** or Fourier filtered **(E)** or both **(F)**. The line scans **(G)**, whose location is indicated by the dashed red line in **(A)** are color matched to the various images in **(A)** to **(F)**. The numbers at the lower corner are average SBRs measured for the whole field of view. The scale bar is 5 µm (modified from[15]) .

Figure 5. Examples of kymographs. (**A**) Kymograph examples of microtubule dynamics generated from time-lapse movies acquired at 0.2 fps. (**B**) Kymograph depicting an example of a shrinkage event generated from a movie acquired at 100 fps. Dashed lines mark the seeds.

Figure 6. Example of gold nanoparticles imaged with IRM. Gold nanoparticles of sizes 20 and 40 nm were passively attached to the surface. 10 images were acquired. After background subtraction, the images were averaged to enhance contrast.

Figure 7. Microtubule length Tracking precision in IRM images. Stabilized microtubules (i.e., fixed lengths) were imaged 200x at 100 fps then averaged to 10 fps to enhance contrast. Next, the microtubules' lengths were measured using Fiesta[17] tracking software. For every microtubule the mean length and standard deviation were calculated as shown in the figure (dashed line represents the mean and solid red lines represents the standard deviation, length = 3971 ± 20

nm. The overall tracking precision was the average of the standard deviation of all tracked microtubules (*n* = 6 microtubules x 20 data points = 120 data points)

DISCUSSION:

This protocol demonstrated the successful use of IRM for imaging and measurement of the microtubule dynamics. Care should be given to correctly set the illumination numerical aperture as it has the strongest impact on image contrast. Also, using high numerical aperture (NA) objectives is important for getting high resolution/high contrast images, as higher NA objective have higher light collecting power compared to low NA objectives. The cleaner the surface and solutions used the lower the noise as dirt ends up attaching to the surface and adding (over the course of the experiment) speckle like noise to the images. Acquisition of a background image is important as well as it removes illumination inhomogeneities, static noise and surface irregularities.

A recommended modification is to introduce a long pass filter (>600 nm) in the illumination path. The spectrum of white light sources typically contains wavelengths in the UV which can damage microtubules. In addition, using long wave length for IRM comes in handy when combining IRM with fluorescence (e.g., when studying the effect of microtubule associated proteins (MAPs) on microtubule dynamics. Be aware that when imaging for expended period of times, sample drift (especially along the optical axis) decreases image contrast due to the deviation of the image plane from the background plane. Modern microscopes are often equipped with stabilization mechanisms (e.g., perfect focus (Nikon), Definite focus.2 (Zeiss), IX3-ZDC2 (Olympus)). An alternative solution is to thermally stabilize the setup either passively or actively[18] or by correcting for drift[19–21]. Finally, microtubule contrast can be increased by reducing the size of field diaphragm (a 70% opening is good choice as it is a balance between increasing contrast and field of view size)[15].

While IRM is suitable for imaging microtubules it is not sensitive enough to detect single proteins. For such application, iSCAT is a more suitable technique. Similarly, fluorescence and iSCAT are better suited if tracking precision of less than 10 nm is needed. For IRM, the measured length tracking precision is ~20 nm as shown in **Figure 7**.

Use of IRM in surface assays can go beyond microtubules; for example, molecular motors can be labelled with gold nanoparticles and tracked as they interact with microtubules. In addition a more advanced form of IRM known as reflective interference contrast microscopy (RICM)[22] can, in principle, be used to further enhance microtubules contrast and obtain higher tracking precision.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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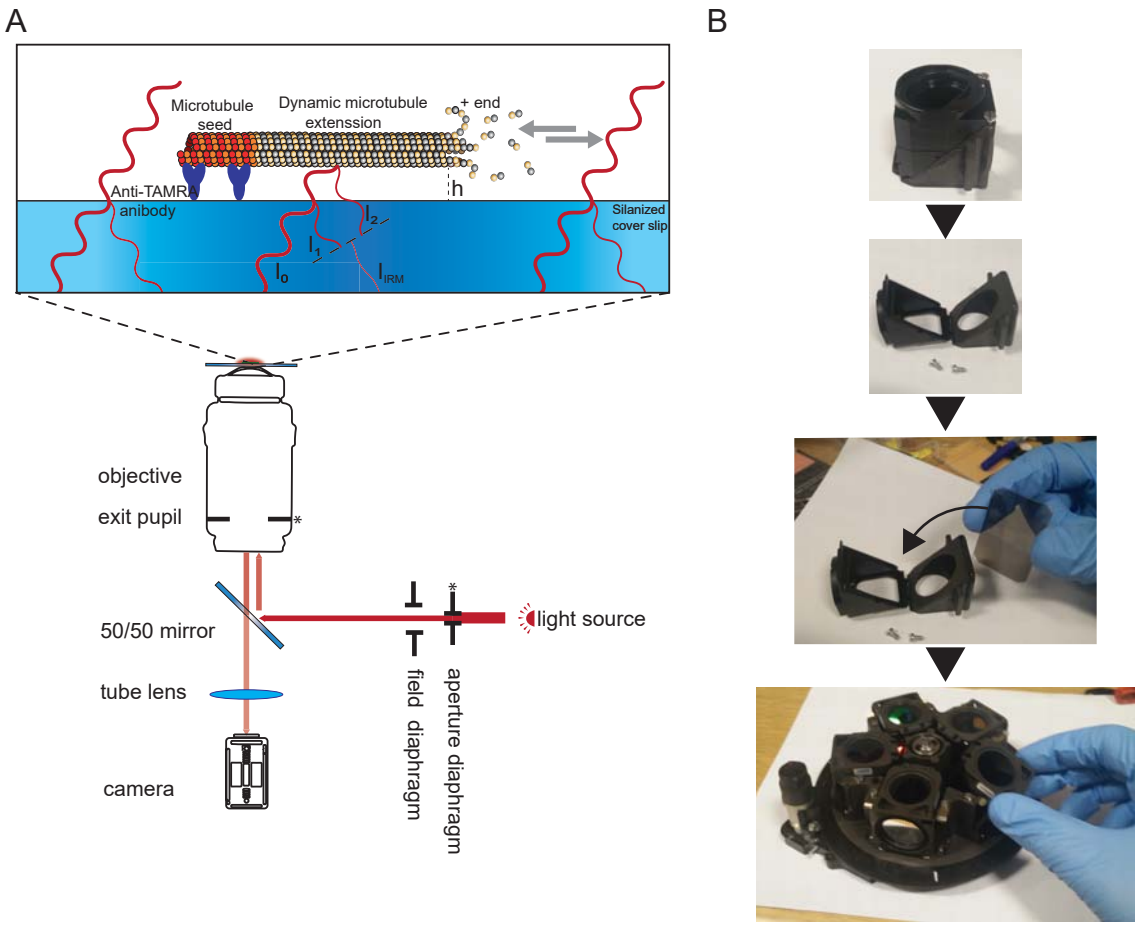

Figure

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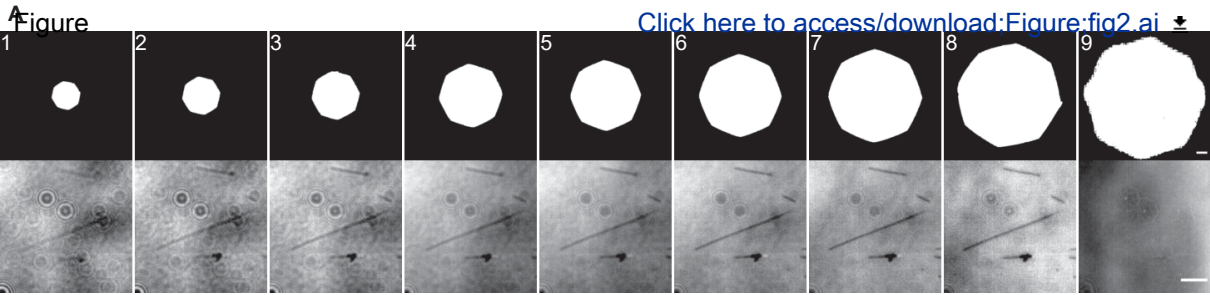

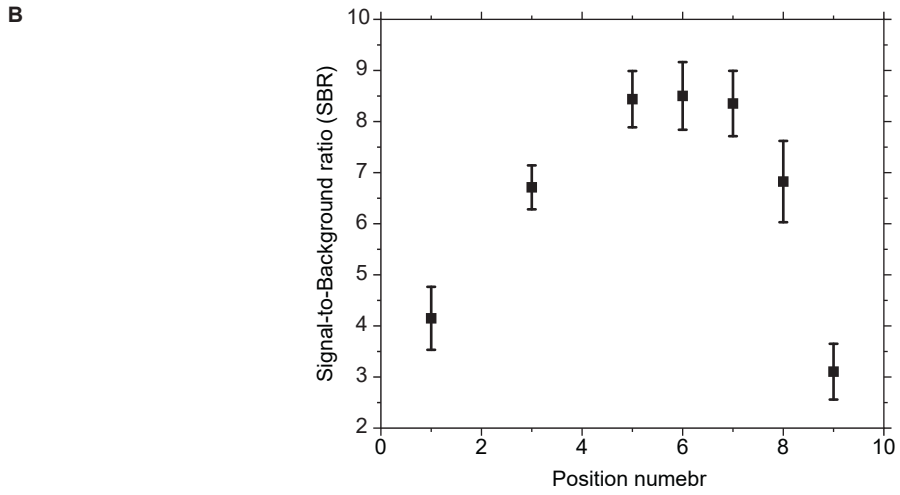

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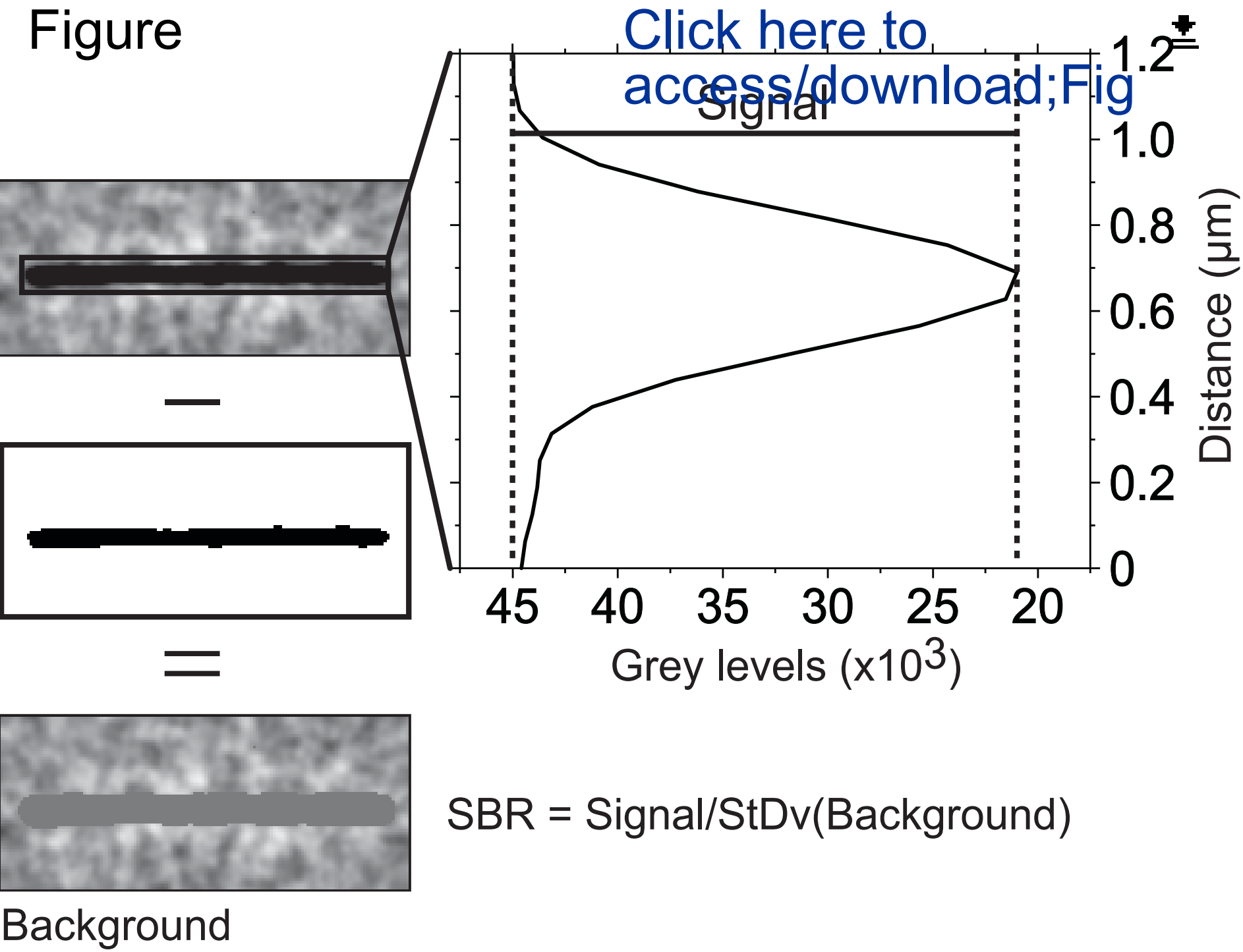

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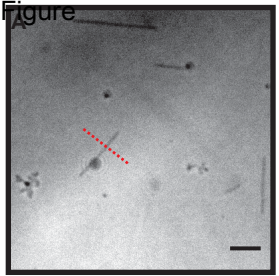

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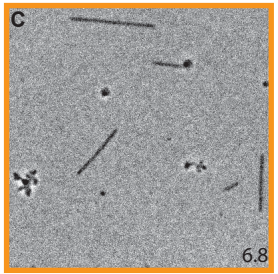

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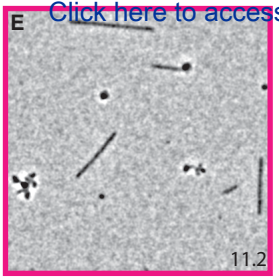
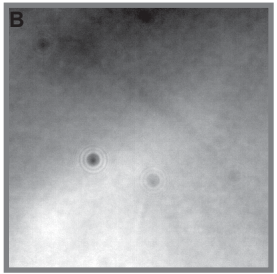


FFT low pass filter



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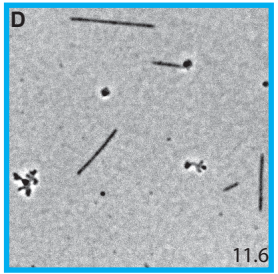
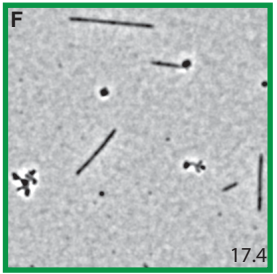
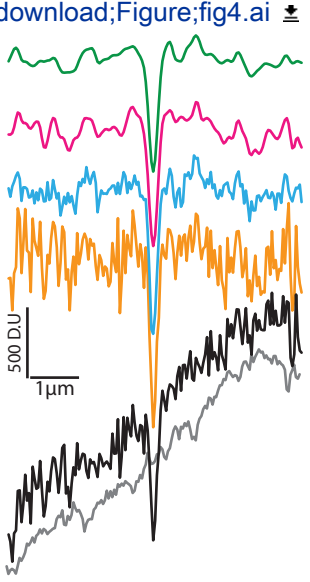


10 Averages

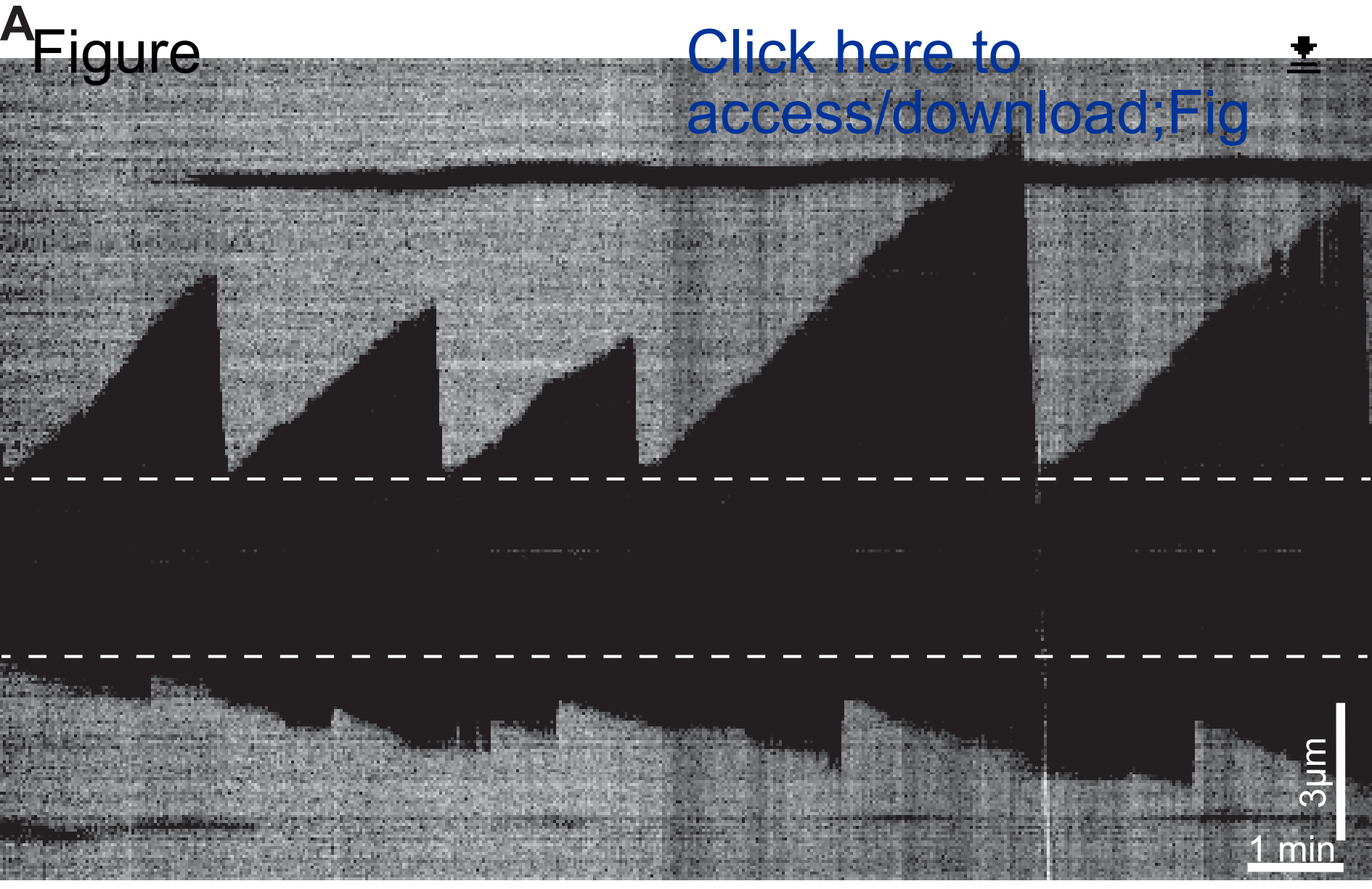


10 Averages + FFT low pass

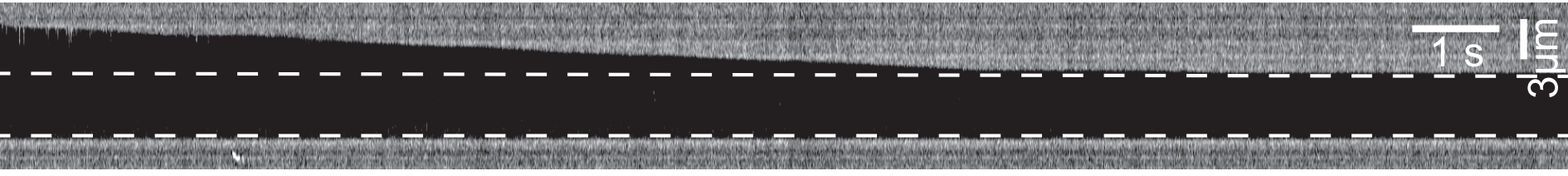


A Figure

Click here to access/download;Fig



B



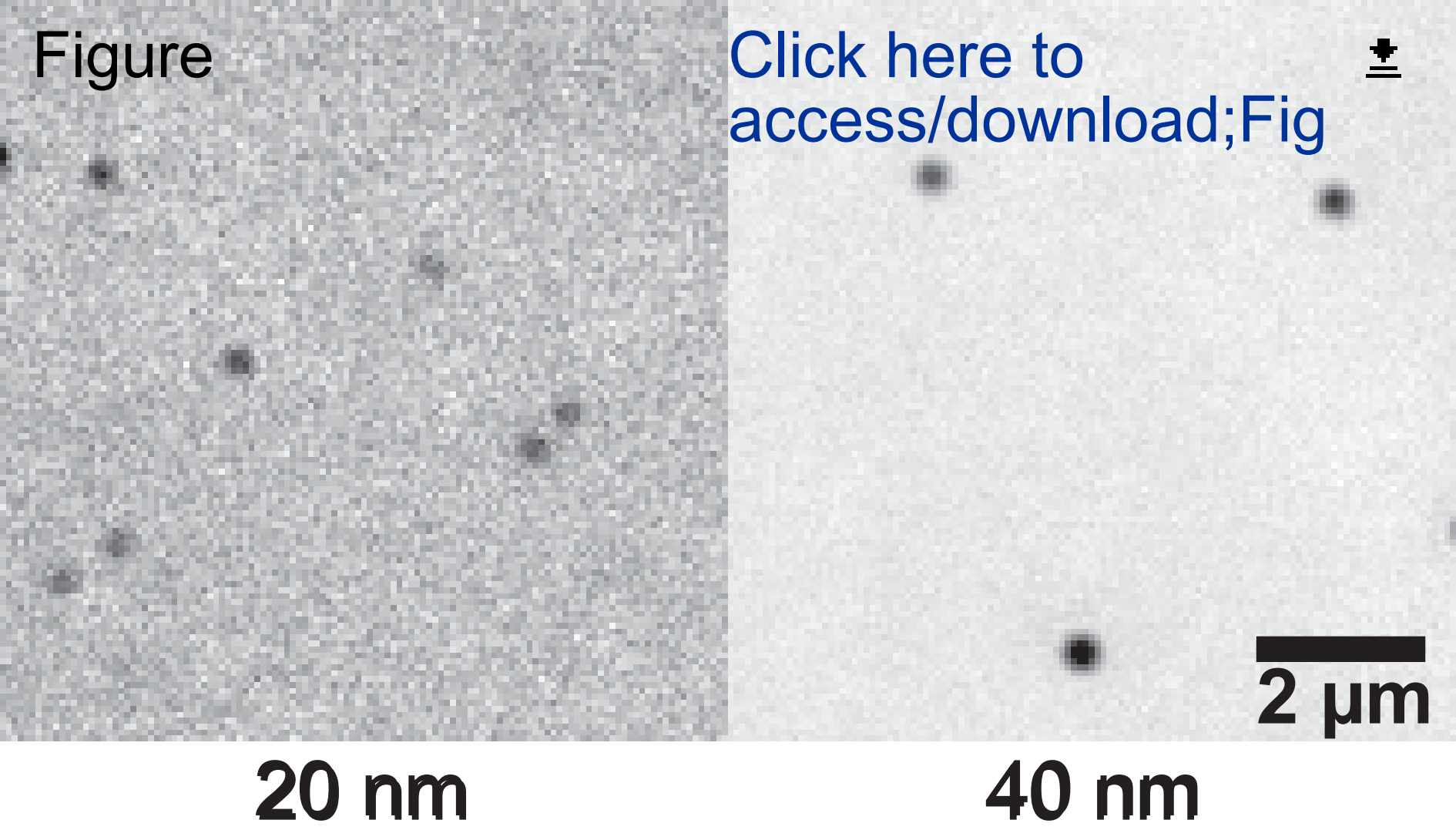
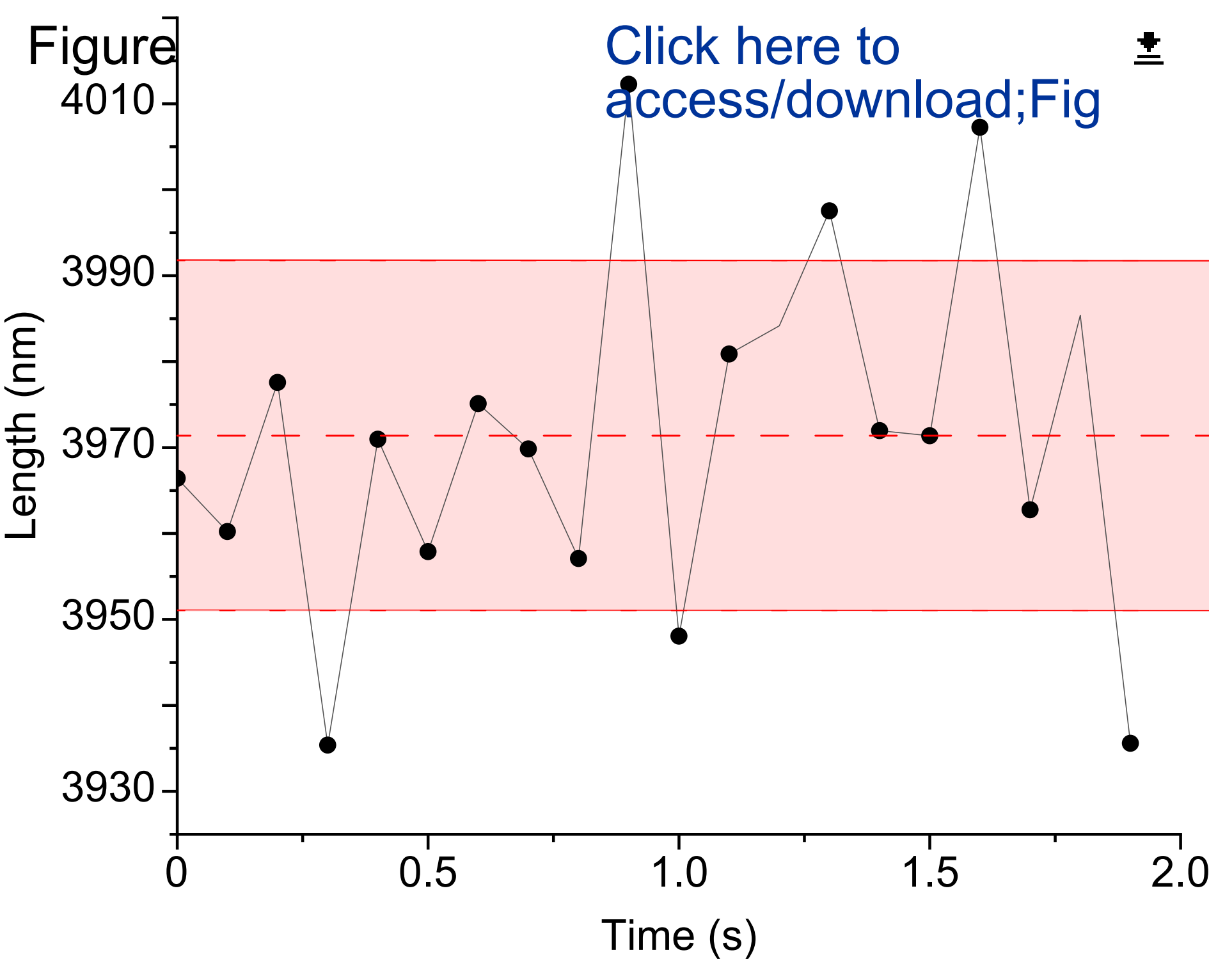


Figure
Click here to access/download;Fig
2 µm
20 nm
40 nm

Figure

Click here to access/download;Fig



Name of Reagent/ Equipment	Company	Catalog Number
Microscope	Nikon	Ti-Eclipse
50/50 beam splitter	Chroma	21000
NIKON PLAN FLUOR 100X/0.5-1.3 Iris objective	Nikon	MRH02902
Mucasol universal detergent	Sigma-aAldrich	Z637181-2L
plastic paraffin film (commerical name Parafilm M)	Sigma-aAldrich	P7793
Anti-TAMRA antibody	Invitrogen	A-6397
Poloxamer 407 (commercial name Pluronic F-127)	Sigma-aAldrich	
40 nm gold nanoparticles	Sigma-aAldrich	753637
20 nm gold nanoparticles	Sigma-aAldrich	753610
Zyla 4.2 Camera	Andor	Zyla 4.2
Feista tracking software		
Stabilized microtubles		

Comments/Description

An inverted microscope used for perfoming the expriments
When buying make sure to choose the splitter dimensions that fit the cube used in the microscope

Imaging objective. This objective has a NA adjusting iris that was opened to NA 1.3
Used for cleaning coverslips and slides

Used for constructing flow channels

Used to bind TAMRA labeled molecules (e.g. microtubules) to the sample surface. RRID (AB_2536196)

Used for blocking the channel surface to prevent nonspecific binding
Used as a control sample
Used as a control sample
2048x2048 pixles (6.5µm pixel size) with quantum efficiency of 72% and 16bit dynamic range
https://www.bcube-dresden.de/fiesta/wiki/FIESTA
prepared in house (see references in text)

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Author(s): Mohammad Mahamdeh and Jonathon Howard

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CORRESPONDING AUTHOR:

Name: Jonathon Howard

Department: Molecular Biophysics and Biochemistry

Institution: Yale University

Article Title: Implementation of interference reflection microscopy for label-free, high-speed imaging of microtubules

Signature: Jonathon Howard

Date: 12/7/2018

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We would like to thank the reviewers for their comments and feedback. We believe that we have addressed all their questions and concerns, and in doing so the manuscript has been improved considerably.

Reviewer comments:

Reviewer #1:
This work by Mahamdeh and Howard describes a method to implement interference reflection microscopy (IRM), specifically applied to imaging the dynamics of microtubules reconstituted in vitro. This method is particularly useful for two reasons:
1. The paper describes a way to implement IRM on any commercial fluorescent following a simple modification
2. The resulting imaging method is label-free, saving time and costs to produce fluorescent tubulin, and eliminating concerns about the photo-bleaching of the specimen.
The paper is to the point and succinct, however I feel that it is too succinct to present a useful guide to a wide range of readers who might want to adapt it to their specific experimental assays and equipment. Below I listed a few specific points that need to be addressed.

We thank the reviewer.

Line 36 - The field of view and acquisition speed are defined by the combination of the objective and the camera, not by the IRM method itself. Unless there is a specific reason to require big FOV and fast acquisition rate for the method to work, these constraints should be loosened.

We thank the reviewer for pointing this out. We modified the abstract. Now it reads “Using IRM, together with the image analysis software described in this paper, the field of view and the frame rate is limited only by the camera; with a sCMOS camera and wide-field illumination, microtubule length can be measured with a precision up to 20 nm with a bandwidth of 10 Hz.”

Line 37 - Axial resolution of 20 nm claimed here in the abstract is not addressed anywhere in the text. The authors should provide a method to achieve this resolution.

We modified the abstract and discussion to clarify it is length precision and we also included an example figure (Fig. 7).

Line 71 - Is there a difference in resolution/SNR resulting from using an objective with lower NA? Please comment and compare directly.

We imaged the same field of view of microtubules with the following objectives 100x/1.3, 100x/1.45 and 100/1.49 (without background subtraction). Visually, the microtubules’ contrast looked similar in all three images. Line scans supported that as well. Being said, we understand that high end TIRF objectives are not readily available in every lab so we will redo the presented measurements using a 100x/1.3 objective as this is the more common/more affordable objective.

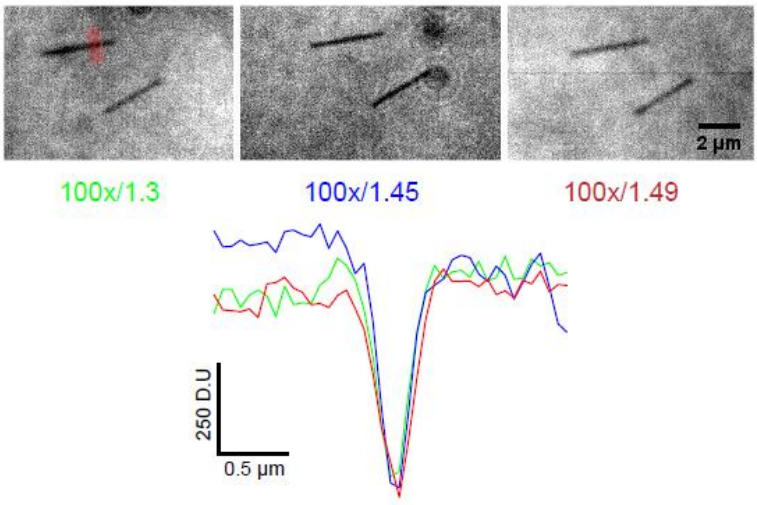

Figure 1, Same field of view was images using three 100x objectives at 3 different NAs: 1.3, 1.45 and 1.49. Each image is an average of 10 images without background subtraction. The line scans (matched by color to the corresponding NA) are made across the upper microtubule where the red transparent line is.

Line 93 - Spell out BRB80

We added “Brinkley Buffer 1980 (BRB80)”.

Line 101 - Is the sample supposed to be sealed to protect from water evaporation? Any recommendations on the sealant?

We understand the reviewer concerns. We keep the sample ends open especially when doing dynamic assays as we need to add protein to the sample. To prevent the sample of drying out we add droplets of buffer at the channel ends. We added this comment to the protocol.

Line 107 - This sounds too simple to be true. Will any epi-illumination light source work? Hg lamp, LED array, laser? Does it require a certain range of the spectrum or any white lamp should work?

A lamp or an LED light source is better than a laser because of the interference patterns (speckles) of the laser. Lasers can be used in point scanning mode, in which case the technique is referred to as confocal reflection microscopy[1] or interferometric scattering (iSCAT)[2]

Lines 109-112 - It would be helpful if the authors provided a few sample images illustrating the good way to find the right imaging surface.

Finding the surface using the parafilm border will be demonstrated in the video.

Line 132 - Gold nanoparticles are mentioned here and in other places in the text, but no data are provided. Please include typical sample images to illustrate the use of IRM as a good imaging method for gold particles, as well as some considerations of the useful range for particle size.

We have included images of 20 nm and 40 nm gold particle (Fig 6).

Lines 138-142 - Reading this description I assume that the authors' microscope is upright (otherwise it would be impossible to change solution in a simple way, while imaging). Since the majority of fluorescent microscopes are inverted, the authors should describe a procedure that is suitable for these.

We modified section 2 (sample preparation) to include two options: i) upright using a 22x22 mm^2 cover slip and slide and (ii) inverted using a 18x18 mm^2 and 22x22 mm^2 coverslips using custom-made cover-slip holders or holders are available commercially.

Line 146 (point 4.4) - Isn't it better to acquire the background images before introducing contrast objects like gold beads or microtubules?

The problem is that introducing solution with a pipette can change the axial position of the sample (compared to the background). Therefore, we recommend here to acquire background images by translating the sample right after finishing the experiment.

Line 167 - Since the contrast in IRM depends on the sample depth, and long microtubules can fluctuate a lot in Z direction, it seems to be useful to include a small percentage of a crowding agent (for example, 0.05-0.1% methylcellulose) to push the microtubules towards the glass surface.

Methylcellulose keeps microtubules at the surface, but there are potential problems with it affecting microtubule dynamics and nucleation.

Line 220 - Is it required to use exactly 10ms exposures and not any other exposure times?

We have changed the wording to:
“Set camera expose to 10 ms using the camera software. This exposure is arbitrary and an exposure of 100 ms would also work.”

Lines 230-243 - Since the sample contrast will depend on microscope alignment, I suggest to move this section to the beginning of the paper, before describing the data acquisition

We appreciate the reviewer suggestion, but we find it more suitable to keep the current sections order. While setting the optimal INA produces the highest image quality, closing the aperture down to 2/3 its full opening (step 3.8) produces image quality that is acceptable for non-demanding experiments. We consider section 7 to be an optimization that some researcher may not choose to do.

Line 324 - Provide a reference showing the use of IRM to image protein complexes

We have decided not to include other protein complexes in the paper at this stage.

Line 329 - Provide a reference or, even better, a sample dataset showing the use of gold nanoparticles to visualize the motility of microtubule-interacting proteins.

we plan on doing these experiments and, if so, will include them in the final version of the paper.

Reviewer #2:

Manuscript Summary:

This is a short, useful article describing implementation of interference reflection microscopy to visualize microtubules. In fact, I am quite impressed at the quality of images achievable. The manuscript is well organized, and provides clear, point-by-point instructions for modifying a standard fluorescent microscope for IRM, then applying it to the microtubule system. The article is impartial and well referenced, and clearly worthy of publication.

We thank the reviewer.

Major Concerns:

Some mention is made of tracking precision (20 nm, according to the abstract), but I am a bit unclear on what precisely is being tracked-- the microtubule center line? The microtubule end-to-end distance? I also would like to see some comment on how that tracking precision is determined-- is this a simple frame-to-frame imprecision that is directly measured from the standard deviation across multiple frames? Or an estimate based on, e.g., fitting a gaussian to the microtubule intensity profile?

We tracked the microtubule length using Fiesta tracking software[3] and determined the precision from the standard deviation across multiple frames. We modified the text and added Figure 6 detailing how we reached the value of 20 nm.

Minor Concerns:

Spelling should be checked; there is an issue with the word 'number' in the axis of a plot in Figure 2.

We fixed the typo.

References

1. Amos, L.A., Amos, W.B. The bending of sliding microtubules imaged by confocal light microscopy and negative stain electron microscopy. *J Cell Sci*. **1991** (Supplement 14), 95–101, doi: 10.1242/jcs.1991.Supplement_14.20 (1991).
2. Andrecka, J., Ortega Arroyo, J., Lewis, K., Cross, R.A., Kukura, P. Label-free Imaging of Microtubules with Sub-nm Precision Using Interferometric Scattering Microscopy. *Biophysical Journal*. **110** (1), 214–217, doi: 10.1016/j.bpj.2015.10.055 (2016).
3. Ruhnow, F., Zwicker, D., Diez, S. Tracking Single Particles and Elongated Filaments with Nanometer Precision. *Biophysical Journal*. **100** (11), 2820–2828, doi: 10.1016/j.bpj.2011.04.023 (2011).

We would like to thank the editor again for the comments on the manuscript. Below we address the new comments:

Line 65. I have made some edits for clarity and structure to meet jove's style requirements.

We thank the editor.

Line 90. Editor: This step is confusing. Please restructure for clarity and avoid the numbering within the step.

We rewrote the step to clear any confusion.

Line 100. Editor: I've adjusted non-imperative voice content into notes that are not filmable. If you prefer to film these details, please re-write in imperative voice.

This is a note, we will not film it.

Add to the table of materials with RRIDs

Antibody was added with RRID.

Line 152. Editor: define

We defined GMPCCP.

what is the diluent? What is the suspension concentration? How much of the sample is flowed in?

We added missing information.

add to the table of materials

We added the microtubules to table of materials.

perfusion?

We fixed the typo.

Line 159. Editor: Unclear. What is the frame rate? What is the shutter speed?

We added the exposure time (shutter time).

Line 162. Editor: At what frame rate?

We added the frame rate.

Line 170. Editor: This is only if tilt cannot be avoided correct?

We rewrote the sentence to clarify that the reader can jump to section 6 if she/he would like to process

and analyze the images.

Line 176. Editor: what is the diluent? What is the suspension concentration? How much of the sample is flowed in? mention size and other specifications of microtubules if relevant

We added missing information.

mention any imaging settings used.

We pointed out that observing the microtubule binding is done while streaming.

Line 184. Editor: What is the suspension concentration? What is the diluent?

We added missing information.

define

We defined GTP

define

We defined DTT

mention button clicks and software selections to set this up.

As software interface will vary from setup to setup we include the acquisition parameters that are necessary for setting the time-lapse regardless of what software used. In addition, this step is going to be filmed which will cover the clicks for the software we used.

Line 189. Editor: Fig 4 is called out before figure 2 and 3, please reorder.

We fixed ordering problem.

Line 237. Editor: Add to the table of materials if commercially acquired. If synthesized in house please cite a reference. Please mention the fluorescent entity and microtubule used (mention size and other specifications if relevant).

The choice of what labeled microtubules to use is up to the reader. We added a note and a refence for the labeling method that we used in this protocol.

Line 240. Editor: Mention fluorescence excitation and emission filter settings.

Please cite the steps where you have described this or cite references to previous publications.

The excitation and emission of the fluorophore were noted in the step before. We added references to IRM and DIC imaging of microtubules.

Line 247 Editor: Manually? If using software please provide button clicks and menu selections.

Yes, manually as will be shown in the video.

Laser intensity?

There are no lasers used. When lasers are used, they are combined with point scanning and the technique is called interferometric scattering (iSCAT) or reflective confocal microscopy. In addition, as mentioned in the same step, the illumination needs to be adjusted to nearly saturate the camera dynamic range. Thus there is no preset value for illumination intensity.

Add the camera you used to the table of materials.

We added the camera to the table of materials.

Line 305. Editor: Remove the product name from here and add to the table of materials.

We removed the product name.

Line 341. Editor: Add to the table of materials.

We added the tracking software to the table of material.

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Title of new article	Implementation of interference reflection microscopy for label-free, high-speed imaging of microtubules
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