

Label-free imaging of microtubules with subnanometer precision using interferometric scattering microscopy

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ABSTRACT

Current in vitro optical studies of microtubule dynamics tend to rely on fluorescent labeling of tubulin, which limits imaging and tracking precision through finite achievable photon flux, photobleaching and blinking. Here, we demonstrate tracking of label-free microtubules with nm precision at kHz frame rates using interferometric scattering microscopy (iSCAT). With microtubules tethered to a glass substrate by low-density surface-bound kinesin, we readily detect sequential 8 nm steps characteristic of a single kinesin molecule moving a microtubule and demonstrate measurement of dynamic changes in filament length with <5 nm precision. Using the arbitrarily-long observation time enabled by label-free iSCAT imaging, we show continuous monitoring of microtubule disassembly over a thirty-minute period. The capability of iSCAT to image microtubules with up to single protein sensitivity and the possibility of simultaneous single molecule fluorescence imaging represent a platform for novel approaches to study microtubule dynamics.

INTRODUCTION

Microtubules (MTs) are tubular filaments with an outer diameter of 24 nm built via head-to-tail polymerization of tubulin heterodimers. Polymerization is coupled to GTP-hydrolysis, such that newly-polymerized GTP-tubulin forms a cap at the ends of the microtubule, whilst the older core of the filament is composed of less stable GDP-tubulin. As a result, microtubules built from pure tubulin grow steadily provided the stabilizing GTP-tubulin caps remain intact, whilst breaches in the cap expose the GDP-core of the microtubule and trigger rapid shrinkage. The cycle of growth, catastrophe, shrinkage and re-growth (rescue) is termed dynamic instability and is critical to the cellular function of microtubules.[1] In addition, MTs play numerous important roles in cellular function by acting as a scaffold for intracellular structure and serving as molecular tracks for a variety of motor proteins.[2] Biogenesis and subsequent motility of cilia and flagella requires MT assembly. The tight control of MT dynamics is essential for MT function in cell proliferation and differentiation.[3, 4] Direct imaging of MT assembly and kinesin-driven microtubule gliding motility has been demonstrated using dark field and a number of interference-based microscopy techniques.[5-8] More recently, it has been shown that MTs can also be visualized using computer-enhanced bright field microscopy.[9] The intrinsic background and noise characteristics of these contrast modalities, however, limit the achievable localization precision and therefore the ability to visualize nm scale dynamics.

Fluorescent labelling of tubulin enables super-resolution imaging of microtubules, but suffers from several shortcomings: fluorescent dyes photobleach, which limits the number of photons that can be collected from a single fluorophore and thereby the spatiotemporal resolution.[10, 11] The localization precision is further affected by residual background originating from free tubulin. For nm-precise imaging, more

sophisticated labelling schemes have been applied, such as the use of quantum dots.[12] Even for quantum dots, however, the time-resolution in tracking MTs is limited to several tens of milliseconds and continuous observation is hampered by photoblinking. Here, we show that interferometric scattering (iSCAT) microscopy [13, 14] can be used to track the position and length of individual, unlabelled MTs with simultaneous ms temporal and sub-nanometer lateral precision for arbitrarily long observation times.

MATERIALS AND METHODS

iSCAT microscope

Our experimental setup is similar to that reported recently.[15] Briefly, we image a 6 x 6 μm region at 1000 frames/s using a 445 nm diode laser scanned across the sample by two acousto-optic deflectors. The beam deflections generated by the AODs are imaged into the back focal plane of an oil immersion objective (Olympus PLAPON 1.42 NA, 60x) after passing through a polarizing beam splitter (PBS). A quarter wave plate before the objective causes any reflected and scattered light by the sample to be reflected by the PBS before being imaged onto a CMOS camera (Photonfocus MV-D1024-160-CL-8) at 333x magnification. The incident power was 17 kW/cm^2 at the sample adjusted to achieve near-saturation of the camera at an exposure time of 0.56 ms.

Sample preparation

Kinesin: We used a truncated dimer of kinesin-1, consisting of the first 430 residues of the rat kinesin heavy chain.

Microtubule preparation: Microtubules were polymerized from 20 µg porcine tubulin (Cytoskeleton) suspended in 20 µl GBRB80 (80 mM PIPES pH 6.9, 1 mM EGTA, 5% glycerol) with 1 mM NaGTP and 2.5 mM MgCl₂ and incubated for 30 minutes at 37 °C before adding taxol to 20 µM. Fresh aliquots were used every day.

Chamber preparation: A 50 mm x 25 mm cover glass was cleaned by washing with ultrapure water (MilliQ), ethanol and again with MilliQ. After drying, it was passed through a blue flame to remove dust and residual moisture. A channel was created between the cleaned cover slip and another 25 mm x 25 mm cover slip with double sided tape. The resulting volume of the chamber was between 15 – 20 µl.

Kinesin gliding assay: The experiments were performed in motility buffer (MB: 20 mM PIPES dissolved in KOH pH 7.4, 10 mM K-acetate, 4 mM MgCl₂ and 5 mM DTT). Two volumes of kinesin solution were pipetted into the flow chamber and incubated for 2 minutes each time. Next, two volumes of 10 mg/ml BSA (BSA diluted in MB buffer) were pipetted into the chamber, once again with an incubation time of 2 minutes each to block any remaining sites where kinesin motors did not bind. One volume of MT solution (diluted 50 times in MB containing taxol) was pipetted into the chamber; unbound MTs were washed away. At this stage, the iSCAT imaging precision could be tested by moving the piezostage in distinct steps. Finally, one volume of motility buffer supplemented with, 10 µM ATP and 20 µM taxol was added and MT gliding was observed.

Microtubule shrinking: Freshly prepared MTs were attached to the glass surface as described above and washed 3 times with motility buffer that did not contain taxol.

Microtubule tracking and step detection

Individual microtubules (MT) were tracked by fitting their PSF to a functional form resulting from the convolution of a 2D Gaussian with a unit box given by the following equation:

$$MT(A, x_0, y_0, s_x, s_y, L, t, \theta) = A \left[\left(\operatorname{Erf} \left(\frac{t}{2s_x} + \frac{x_m}{s_x} \right) + \operatorname{Erf} \left(\frac{t}{2s_x} - \frac{x_m}{s_x} \right) \right) \left(\operatorname{Erf} \left(\frac{L}{2s_y} + \frac{y_m}{s_y} \right) + \operatorname{Erf} \left(\frac{L}{2s_y} - \frac{y_m}{s_y} \right) \right) \right];$$

where $x_m = (x - x_0) \cos \theta - (y - y_0) \sin \theta$; and $y_m = (x - x_0) \sin \theta + (y - y_0) \cos \theta$.

A is an amplitude; x and y denote MT position, $s_{x/y}$ the width of the 2D Gaussian in x and y direction; L is the long axis of the MT and t its short axis (which was fixed to 24 nm corresponding to the diameter of the MT), θ refers to a rotation angle and Erf is the error function. Step detection was performed by a model- and parameter-free change point detection algorithm based on the minimization of the Schwartz information criteria.[16]

RESULTS AND DISCUSSION

A typical iSCAT image of an unlabelled MT bound to a microscope a cover slide is shown in Figure 1a. The contrast results from interference between light scattered by the MT and light reflected at the glass-water interface.[14] The indicated cross sections along and across the long axis of the MT illustrate the magnitude of the filament signal compared to the background caused by the roughness of the cover glass surface (8% vs. 0.3%). To determine the length and centre of mass position of such MTs, we fit the image to an elongated 2D Gaussian (see Materials and Methods). The standard deviation of the MT centre of mass position was on the

order of 1.4 nm at 1 kHz frame rate and dropped to 0.4 nm after 10-fold temporal binning (Fig. 1b). A single kinesin molecule is expected to move a microtubule in 8 nm steps in a hand-over-hand fashion.[17, 18] In order to test our ability to accurately and precisely measure such steps, we translated a surface-bound MT using a nanometric stage. We were able to resolve individual steps with 0.4 nm precision and determine the length of MTs with 1 nm precision at 100 Hz imaging speed (Fig. 1c).

Next, we tracked MTs bound to kinesin motors attached to the glass surface in the presence of 10 μ M ATP. We reduced the kinesin concentration until MT swivelling occurred, indicating that single kinesin motors are interacting with the MT.[19, 20] At this point we could clearly resolve sequential 8 nm steps (Fig. 2a, Supplementary movie 1) in agreement with previous reports. The data was recorded at 1000 frames/s, two orders of magnitude faster than the current state of the art.[12] A histogram of steps sizes agrees with the previously reported behaviour of single kinesin-1 molecules moving along a microtubule.[17, 18]

At higher motor densities, we frequently observed fractional steps on the order of 4 and even 2 nm, most likely resulting from several motors acting on a single MT (Fig. 2b). In order to resolve these steps, we averaged the original 1000 frames/s movie to 100 frames/s, which improved the localization precision from 1.4 to 0.4 nm (Fig. 1b). Although such small steps are difficult to discern in the time-distance traces alone, they are clearly visible in the corresponding XY-trajectories. We emphasize that we could not precisely control the number of motors interacting with a single MT in these experiments, a shortcoming that could be addressed in the future using fluorescently labelled kinesin together with correlative iSCAT and imaging as demonstrated by

tracking individual, dual-labelled myosin 5a molecules and quantum dot labelled virions.[21, 22]

Finally, we present label-free imaging of a single unlabelled MT shrinking after removal of free tubulin and taxol from solution (Fig. 3, Supplementary movie 2). This measurement was taken over 40 minutes; due to data storage constraints, we recorded 1 s movies every minute, even though the sample was constantly illuminated. Since iSCAT detection relies on light scattering instead of fluorescence emission, the illumination time can be arbitrary long without interference from photobleaching or blinking. In addition, non-resonant iSCAT illumination is less likely to cause photodamage to MTs due to sample heating and phototoxicity as often encountered in the presence of fluorescent dyes.

CONCLUSIONS

Our results demonstrate that light scattering from MTs when detected coherently in an interferometric geometry generates images of sufficient quality to enable high-speed centre of mass tracking and length measurements with subnanometer precision. These capabilities should enable detailed studies of how multiple motors interact with single MTs, how they coordinate and how their function scales with ATP concentration. Observation times and speeds are only limited by data storage and camera speed, respectively, enabling access to previously unattainable short (<ms) and long (hours) timescales while maintaining nm precision. In principle, nm-precise MT tracking could have been achieved at up to 1 MHz with an appropriate camera, given that the iSCAT contrast of individual MTs is comparable to 20 nm gold particles.[23] Combination with single molecule fluorescence and super-resolution

imaging could enable simultaneous localization of MTs and attached motors. Similarly, the recently demonstrated single protein sensitivity of iSCAT,[15, 24] could be used for real-time monitoring of MT assembly and disassembly down to the single protein level.

AUTHOR CONTRIBUTIONS

J.A. and K.L. performed research; J.O.A contributed analytic tools; J.A. analyzed the data, R.C. provided essential reagents; J.A. and P.K. wrote the paper.

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REFERENCES

1. Mitchison, T., and M. Kirschner. 1984. Dynamic Instability of Microtubule Growth. *Nature*. 312:237-242.
2. Vale, R.D. 2003. The molecular motor toolbox for intracellular transport. *Cell*. 112:467-480.
3. Kirschner, M., and T. Mitchison. 1986. Beyond Self-Assembly - from Microtubules to Morphogenesis. *Cell*. 45:329-342.
4. Howard, J., and A.A. Hyman. 2007. Microtubule polymerases and depolymerases. *Curr. Opin. Cell Biol.* 19:31-35.
5. Horio, T., and H. Hotani. 1986. Visualization of the Dynamic Instability of Individual Microtubules by Dark-Field Microscopy. *Nature*. 321:605-607.
6. Walker, R.A., E.T. O'Brien, ..., E.D. Salmon. 1988. Dynamic Instability of Individual Microtubules Analyzed by Video Light-Microscopy - Rate Constants and Transition Frequencies. *J. Cell Biol.* 107:1437-1448.
7. Howard, J., A.J. Hudspeth, and R.D. Vale. 1989. Movement of Microtubules by Single Kinesin Molecules. *Nature*. 342:154-158.
8. Amos, L.A., and W.B. Amos. 1991. The bending of sliding microtubules imaged by confocal light microscopy and negative stain electron microscopy. *J. Cell Sci. Suppl.* 14:95-101.
9. Gutierrez-Medina, B., and S.M. Block. 2010. Visualizing individual microtubules by bright field microscopy. *American Journal of Physics*. 78:1152-1159.
10. Gell, C., V. Bormuth, ..., J. Howard. 2010. Microtubule Dynamics Reconstituted In Vitro and Imaged by Single-Molecule Fluorescence Microscopy. *Microtubules, in Vitro*. 95:221-245.
11. Nitzsche, B., V. Bormuth, ..., S. Diez. 2010. Studying Kinesin Motors by Optical 3D-Nanometry in Gliding Motility Assays. *Microtubules, in Vitro*. 95:247-271.
12. Leduc, C., F. Ruhnaw, ..., S. Diez. 2007. Detection of fractional steps in cargo movement by the collective operation of kinesin-1 motors. *Proc. Natl. Acad. Sci. U.S.A.* 104:10847-10852.
13. Jacobsen, V., P. Stoller, ..., V. Sandoghdar. 2006. Interferometric Optical Detection and Tracking of Very Small Gold Nanoparticles at a Water-Glass Interface. *Opt. Express*. 14: 405-414.
14. Ortega Arroyo, J., and P. Kukura. 2012. Interferometric Scattering Microscopy (iSCAT): New Frontiers in Ultrafast and Ultrasensitive Optical Microscopy. *Phys. Chem. Chem. Phys.* 14:15625-15636.

15. Ortega Arroyo, J., J. Andrecka, ..., P. Kukura. 2014. Label-free, all-optical detection, imaging, and tracking of a single protein. *Nano Lett.* 14:2065-70.
16. Kalafut, B., and K. Visscher. 2008. An objective, model-independent method for detection of non-uniform steps in noisy signals. *Comp. Phys. Comm.* 179:716-723.
17. Svoboda, K., C. F. Schmidt, ..., S.M. Block. 1993. Direct Observation of Kinesin Stepping by Optical Trapping Interferometry. *Nature* 365:721-727.
18. Yildiz, A., M. Tomishige, ..., P.R. Selvin. 2004. Kinesin walks hand-over-hand. *Science.* 303:676-678.
19. Hunt, A.J., and J. Howard. 1993. Kinesin Swivels to Permit Microtubule Movement in Any Direction. *Proc. Natl. Acad. Sci. U.S.A.* 90:11653-11657.
20. Hua, W., J. Chung, and J. Gelles. 2002. Distinguishing inchworm and hand-over-hand processive kinesin movement by neck rotation measurements. *Science.* 295:844-848.
21. Kukura, P., H. Ewers, ..., V. Sandoghdar. 2009. High-Speed Nanoscopic Tracking of the Position and Orientation of a Single Virus. *Nat. Methods.* 6:923-927.
22. Andrecka, J., J. Ortega Arroyo, ..., P.Kukura 2015. Structural dynamics of myosin 5 during processive motion revealed by interferometric scattering microscopy. *Elife.* 10.7554/eLife.05413.
23. Lin, Y.H., W.L. Chang, and C.L. Hsieh. 2014. Shot-noise limited localization of single 20 nm gold particles with nanometer spatial precision within microseconds. *Opt. Express.* 22:9159-70.
24. Piliarik, M., and V. Sandoghdar. 2014. Direct optical sensing of single unlabelled proteins and super-resolution imaging of their binding sites. *Nat. Communications.* 5: 4495.

FIGURE LEGENDS

Figure 1. Label free imaging and tracking of a single microtubule with interferometric scattering microscopy. (a) iSCAT image of a single MT, including cross sections along and across (blue and orange arrow) its long axis. (b) Artificial steps performed by moving an MT bound to cover glass on a nanometric stage in 8 nm steps. The tracking precision was determined by calculating the positional fluctuation, σ , which varies with imaging speed, but is independent of MT orientation relative to the motion of the nanometric stage. (c) MT length, provided by the fit determined with 4 nm precision. Imaging speed: Raw data acquired at 1000 frames/s (grey dots) and averaged to 100 frames/s (red line).

Figure 2. Analysis of MT motion driven by surface bound kinesin. (a) Representative time traces showing 8 nm steps for MTs bound to single kinesins including a representative XY-trajectory acquired at 1000 frames/s. (b) Tracking results for higher motor densities exhibiting smaller but distinct, fractional steps including representative XY-trajectories acquired at 100 frames/s.

Figure 3. Microtubule shrinking upon the washout of taxol. (a) Time lapse images acquired over 40 minutes. Scale bar: 500 nm. (b) MT length as a function of time.

SUPPLEMENTARY MOVIE LEGENDS

SM_movie1. Microtubule gliding at low kinesin concentration when MT swivelling occurred. Image size 1.9 x 1.9 μm ; 60 x 60 pixels (31.8 nm/pixel). The movie was acquired at 1000 frames/s frame rate with every frame shown.

SM_movie2. Visualization of microtubule shrinking upon the washout of taxol. Image size 2.2 x 2.2 μm ; 70 x 70 pixels (31.8 nm/pixel). The movie was acquired for 1 second every minute over 40 minutes with the sample continuously illuminated. The original frame rate was 1000 frames/s but was averaged to 1 frame/s.





