Highly sensitive kinesin-microtubule motility assays using SLIM

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Abstract

We provide an experimental demonstration of Spatial Light Interference Microscopy (SLIM) as a tool for measuring the motion of 25 nm tubulin structures without the use of florescence labels. Compared to intensity imaging methods such as phase contrast or DIC, our imaging technique relies on the ratios of images associated with optically introduced phase shifts, thus implicitly removing background illumination. To demonstrate our new found capabilities, we characterize kinesin-based motility continuously over periods of time where fluorescence would typically photobleach. We exploit this new method to compare the motility of microtubules at low ATP concentrations, with and without the tagging proteins formerly required to perform these studies. Our preliminary results show that the tags have a non-negligible effect on the microtubule motility, slowing the process down by more than 10%.

Introduction

Microtubule mediated processes are ubiquitous to eukaryotic cells and related defects constitute the phenomenology of a large class of clinical disorders [1]. Although motile microtubules were first observed using conventional contrast enhancement techniques [2-4], cutting edge assays of microtubule behavior often rely on fluorescence imaging [5-8]. While fluorescence benefits from specificity, it introduces experimental difficulties, e.g., photobleaching, which motivate increasingly elaborate light dosage schemes (for example, [9]). Interestingly, the question as to whether the tagging proteins themselves affect the function of the cellular cytoskeleton has not been studied systematically, to our knowledge.

Here we attempt to address the issues typical of transmitted light modalities by solving some of the associated stability and contrast issues typical of darkfield ([3]) and video-enhanced differential interference contrast (VE-DIC [2]). We employ quantitative phase imaging (QPI, [10]) as an ultrasensitive, label-free imaging method to study structure and dynamics at the nanoscale. Specifically, we use Spatial Light Interference Microscopy (SLIM [11]) – a white light QPI system that attaches to an existing phase contrast microscope. Unlike darkfield, our system benefits from enhanced phase sensitivity enabling us to acquire high-contrast images of unlabeled microtubules. Unlike DIC, our system avoids the directional shading that makes parallel microtubules difficult to resolve.

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After application of a rolling window, we are able to track objects characterized by angstrom optical path length shifts, an order of magnitude smaller than reported in [12]. In contrast to the setup in [13] our system is completely integrated with a convention microscope, capable of imaging structures with no special preparation or processing at a comparably larger field of view.

Optical System

In SLIM (Fig. 1), the phase between scattered and transmitted light is modulated by way of a spatial light modulator (SLM) conjugate to the pupil plane. Under broadband illumination conditions discussed in detail in Ref. [14], the resulting system consists of four equations with four unknowns, which can be solved to obtain the per-pixel phase shift between the transmitted and scatted light (ϕ_{ac}). As shown in [15, 16], following further processing, it is possible to recover the phase associated with the object(ϕ). As noted in [17], the scheme is sufficient for framerates approaching real-time.

Measurements for this experiment were performed on a Zeiss Observer Z1 at 63x (NA=1.4, PN 420781-9910 PC3). The external SLIM module (Cell Vista SLIM Pro, Phi Optics., Inc.) includes a SLM (Meadowlark Optics) and a sCMOS camera (Zyla 5.5). The central wavelength was observed to be at 590nm (HLX 64625, NAED 54248 halogen lamp).

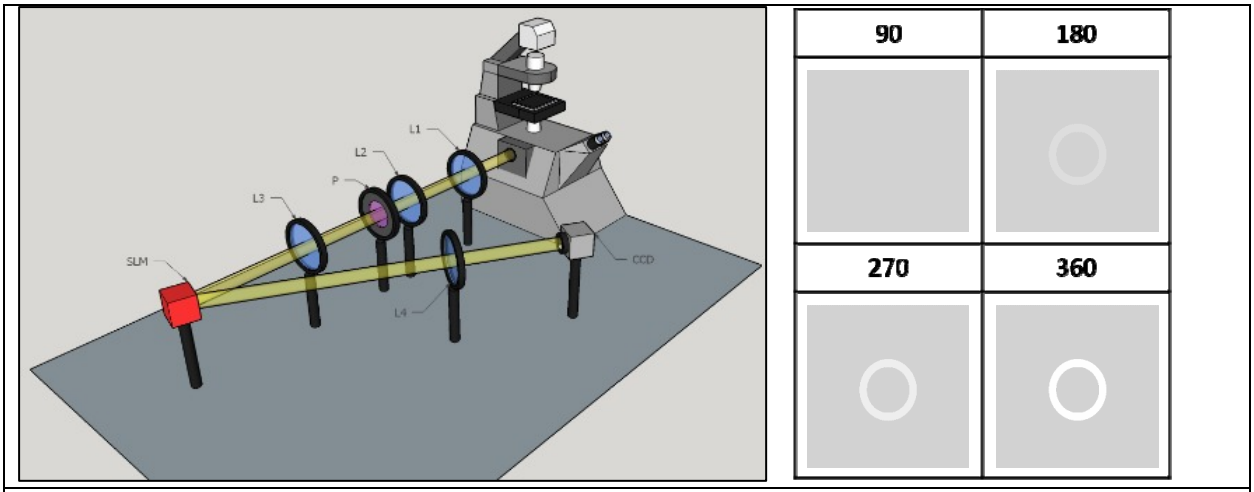

Figure 1: SLIM system. In SLIM (A) the phase shift between transmitted and scattered light is modulated with a phase mask externally matched to the conjugate plain of the objective. Right panel, shows the surface of the modulator.

Preliminary Results

In this work we image two samples, both of which are difficult to resolve without fluorescence tagging: microtubules tagged with biotin and microtubules without any tags. The two samples were imaged in succession with the biotin tagged microtubules imaged first.

In order to demonstrate the utility of our system, we assayed the speed of microtubule by manually tracking the tips using the MTrackJ plugin for Fiji([18, 19]). In our measurements, all microtubules in the field of view were moving, and thus we tracked all sizes. Fig. 2 summarizes the primary outcome of this work.

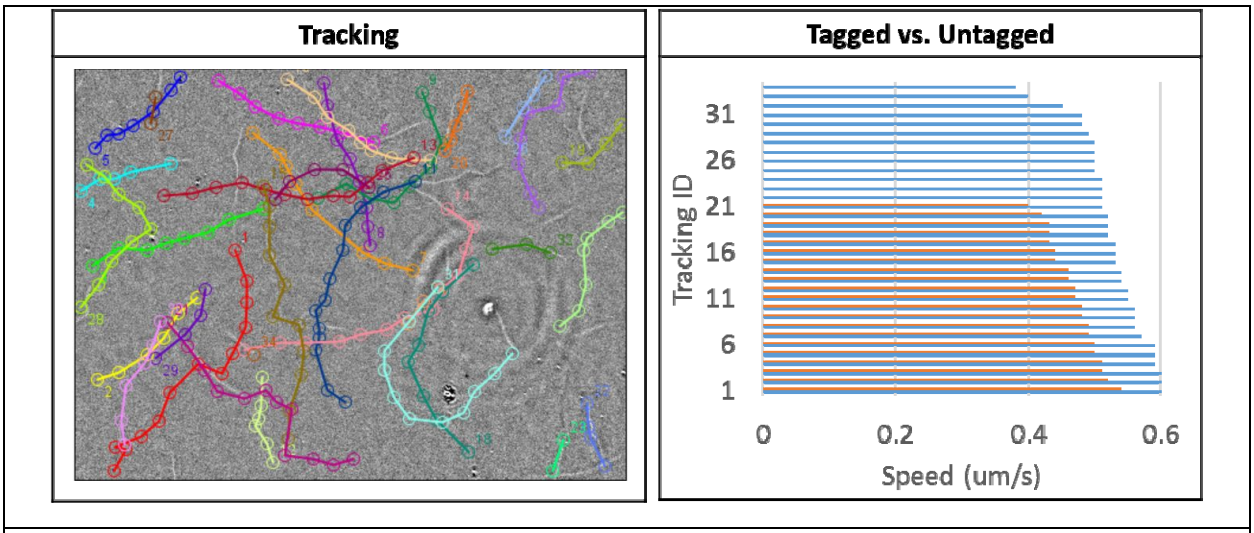

Figure 2: Biotin slows down microtubules. After tracking the speed of biotin tagged tubes was on average 0.47 um/s compared to 0.53 um/s for completely unlabeled tubes.

Discussion and Future Work

Foremost, the difference between tagged and untagged microtubules is on the same order as noted in [20] (Fig. 5 in that paper), although they report a 5% speed increase with a 6x HIS tag, we observe a 13% decrease with our biotin tag. Further, as tagged (slower) microtubules were imaged first, we would expect an even greater decrease due to tagging if both categories (tagged vs. untagged) were imaged simultaneously. Thus, we believe the results merit further investigation.

A further observation concerns the measured phase values of microtubules. Although the milliradians scale recorded here, to our knowledge, represent among the most sensitive measures of phase shifts in full-field imaging systems, the phase values deviate from those reported in [12]. We believe this difference can be explained, in part, by the differences between the comparably lower refractive index of their specimen compared to our microtubule forming solution. In summary we show that our real-time imaging platform is able to see biologically relevant structures on the order of miliradians under particular challenging imaging conditions.

Sample Preparation and Experimental Protocol

SLIM images used in the following experiment, were acquired at 4 FPS with 30ms for SLM stabilization and 10ms exposure. Although, in principle, microtubule are visible at higher frames rate such as 16 FPS, as limited by the refresh rate of the SLM.

Microtubule preparation protocol was similar to the one in [21]. In brief, microtubules were polymerized by adding 2 mM of GTP to 0.6 mg of tubulin or 1:8 ratio of biotin-tubulin: tubulin mixture in BRB80 buffer (80 mM PIPES, 1 mM MgCl2, 1 mM EGTA, pH 6.8). The sample was then incubated at 37° C for 30 minutes. 0.05 mM paclitaxel was then added to the sample with BRB80 buffer, and the sample was centrifuged at 15,000 g for 30 minutes to remove unpolymerized tubulins. The microtubule is stored in 0.05 mM paclitaxel solution with 1 mM GTP before the experiment.

For the microtubule gliding assay, the coverslip was coated with 5% biotin-PEG and PEG. Streptavidin in the concentration of 10 mg/ml was diluted in BRB80-BSA (80 mM PIPES, 1 mM MgCl2, 1 mM EGTA, 8mg/mL BSA pH 6.8) 10x and added to the PEG-Biotin channel. Approximately, 0.1 µM C-terminus biotinylated-K432 kinesin-1 was used to bind to the streptavidin diluted in BRB80-BSA. Thereafter, the chamber was washed with BRB80-BSA plus 0.2 mM of free biotin to saturate the unbound streptavidin on the surface. Imaging buffer was prepared by 90 µL BRB80-BSA, 1 µL Paclitaxol (microtubule stabilizing agent), 1 µL PCD (oxygen scavenging enzyme), 2 µL PCA (substrate for PCD), 0.2 µL THP (reducing agent). Next, previously prepared microtubules were diluted at 50x and flowed into the sample chamber with 2 mM of (final) ATP concentration.

Processing

Input data was de-noised from a mean subtracted cumulative moving average [22]. Specifically, the i^{th} frame in the time lapse, $f(x,y)^i$, is updated according to the recurrence formula:

$$f(x,y)_i = 0.1 f(x,y)_i + 0.9 f(x,y)_{i-1}$$

As the image was considered resolved, on the first try, we did not pursue further optimization of the average (Fig. 3).

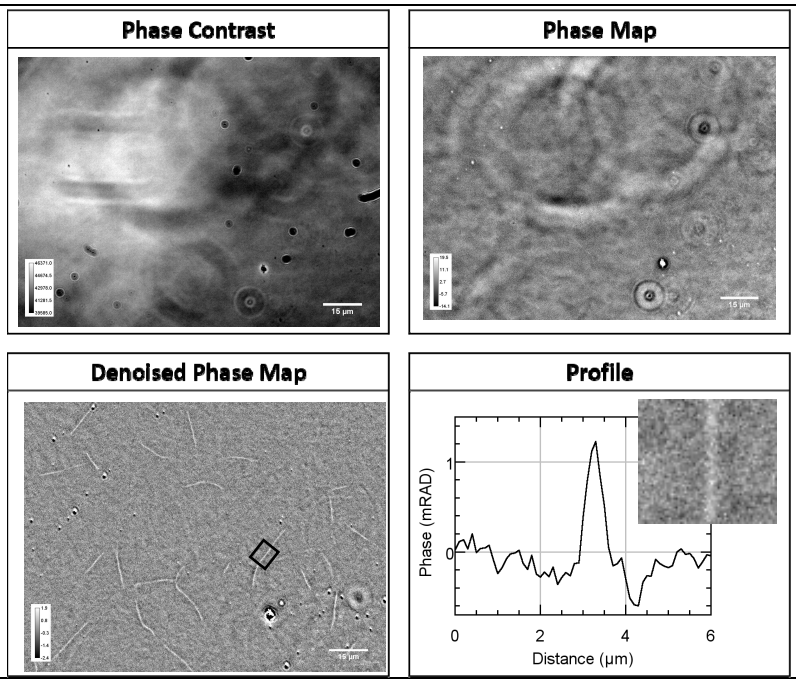

Figure 3: A typical frame used to assay microtuble motility. Image was de-noised as show in the previous section. From the cross section it is visible that a microtubule is approximately 1 miliradian in phase shift. In this case, the negative phase values are due to the impulse response of the system, a characteristic shared by all imaging instruments.

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