Fluorescent speckle microscopy of microtubules: how low can you go?

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ABSTRACT Fluorescent speckle microscopy (FSM) is a new technique for visualizing the movement, assembly, and turnover of macromolecular assemblies like the cytoskeleton in living cells. In this method, contrast is created by coassembly of a small fraction of fluorescent subunits in a pool of unlabeled subunits. Random variation in association creates a nonuniform “fluorescent speckle” pattern. Fluorescent speckle movements in time-lapse recordings stand out to the eye and can be measured. Because fluorescent speckles represent fiduciary marks on the polymer lattice, FSM provides the opportunity for the first time to see the 2- and 3-dimensional trajectories of lattice movements within large arrays of polymers as well as identifying sites of assembly and disassembly of individual polymers. The technique works with either microinjection of fluorescently labeled subunits or expression of subunits ligated to green fluorescent protein (GFP). We have found for microtubules assembled in vitro that speckles containing one fluorophore can be detected and recorded using a conventional wide-field epi-fluorescence light microscope and digital imaging with a low noise cooled CCD camera. In living cells, optimal speckle contrast occurs at fractions of labeled tubulin of ~0.1–0.5% where the fluorescence of each speckle corresponds to one to seven fluorophores per resolvable unit (~0.27 μm) in the microscope. This small fraction of labeled subunits significantly reduces out-of-focus fluorescence and greatly improves visibility of fluorescently labeled structures and their dynamics in thick regions of living cells. Waterman-Storer, C. M., Salmon, E. D. Fluorescent speckle microscopy of microtubules: how low can you go? FASEB J. 13, S225–S230 (1999)

Waterman-Storer and Salmon (1) discovered that microtubules in migrating epithelial cells do not become uniformly fluorescent along their lengths but appear “speckled” after the cells are microinjected with tubulin bound with X-rhodamine. In past studies, fluorescent tubulin was microinjected into cells to make the fraction of labeled tubulin in the cellular pool 5–10%. At these concentrations, fluorescent speckle contrast is low (Fig. 1A) and not easily detected. In our studies, we acquired epifluorescent images with a microscope designed to give efficient light transmission to a camera with a cooled charge couple device (CCD), which has much lower noise than the intensified video cameras used in most previous studies (2). We found (3, 4) that reducing the concentration of labeled tubulin reduced the average fluorescence intensity of the microtubules and background in cells while significantly enhancing speckle contrast (Fig. 1B). With a wide-field epi-fluorescence microscope and sensitive cooled CCD camera, we were able to see microtubule growth and shortening dynamics within much thicker regions of the cell than at the higher fractions of labeled tubulin typically used previously in microtubule studies (compare Fig. 1A, B).

There is substantial evidence (3) that microtubules get fluorescent speckles from the stochastic association with growing ends of labeled and unlabeled subunits at small fractions of labeled subunits (Fig. 1C). In living cells, microtubules exhibit a random pattern of speckles for different microtubules and different regions of an individual microtubule. The speckle pattern changes only after microtubule shortening and regrowth. In vitro, microtubules assembled from mixtures of labeled and unlabeled pure tubulin exhibit fluorescent speckles in the absence of other cellular factors or organelles. In these experiments, the X-rhodamine-labeled tubulin is a dimer and not an oligomer induced by fluorescent labeling. Speckle contrast (measured as the standard deviation of fluorescence intensity along the microtubule divided by the mean fluorescence intensity) increases as the fraction of labeled tubulin decreases, and it is not altered by the binding of purified brain microtubule associated proteins (MAPs). Computer simulations of microtubule assembly based on random tubulin dimer association with a growing end closely predict the intensity variations measured for line-scans along

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microtubules assembled in vitro for fractions of labeled tubulin from 1.25 to 50%.

A significant aspect of FSM is that fluorescent speckles provide fiduciary marks on the lattice of polymers that can be used to measure the motility and discriminate which ends of the polymer contribute to growth and shortening. We initially used FSM to show within the cytoplasm of migrating epithelial cells that microtubule treadmilling occurs in a plus-end direction when the minus end is not centrosome bound. Treadmilling is produced by the differences in the dynamic instability between plus and minus ends.
ends: plus ends oscillate between persistent growth and brief shortening phases while free minus ends either pause or persist in shortening toward the plus end (1). By using very low fractions of fluorescently labeled tubulin or labeled actin subunits, remarkable wide-field views can be achieved of the movements and assembly dynamics of microtubules and actin filaments arrays at the leading edge and within the lamella of migrating tissue cells and of microtubules within the asters and central microtubule arrays of the mitotic spindle (4). The low background fluorescence and high speckle contrast at small fractions of labeled subunits allows detection of individual astral microtubule dynamics and the movements of microtubules in opposite directions within the dense arrays of the mitotic spindle (4). For example, time-lapse movies (http://www.unc.edu/depts/salmlab/) and kymograph trajectories show vividly speckle movements in opposite directions in the middle of the spindle produced by the movement of overlapping microtubules toward depolymerization sites at their poles. Fluorescent speckle images of microtubules in the budding yeast *Saccharomyces cerevisiae* can be seen by expressing tubulin fused with the GFP.

FSM can also be used to study protein binding dynamics to a site like the lattice of a microtubule. For example, when binding domain of the mammalian microtubule associated protein MAP ensconsin (7) is fused to multiple GFPs and weakly expressed in tissue cells, speckles can be seen to appear and disappear (“sparkling speckles”) as the fluorescent MAPs bind and release from the microtubule lattice (8).

In this study, we address the issue for microtubules of how few fluorophores can be detected with a cooled CCD camera within the resolution limit of the wide-field epi-fluorescent microscope and briefly discuss factors affecting the optimum fraction of labeled tubulin for the best speckle contrast in FSM. Previously, Kinosita et al. (9, 10) showed that an epi-fluorescence microscope using diffuse laser light to produce wide-field illumination and an intensified video camera can detect single fluorophores on actin filaments assembled *in vitro* and attached to the surface of a coverslip. This was accomplished by polymerizing filaments from an actin subunit pool containing a very small fraction of subunits attached to a single molecule of tetramethylrhodamine producing an average fluorophore density of 0.15 fluorophore per micrometer of actin filament (9). They used video sequences of the movements of single fluorophores to measure the translocation and rotation of individual actin filaments driven by myosin motor proteins in *in vitro* motility assays (9, 10). Single fluorophores attached to cytoskeletal associated proteins have also been imaged by video microscopy using the advantages of excitation by total internal reflection of laser illumination (11, 12). With this method, in contrast to conventional epi-fluorescence illumination, only fluorophores within ~100 nm of the coverslip surface are excited by the evanescent wave produced by the total internally reflected light beneath the coverslip.

Here we show by computer simulation and measurements of microtubules assembled *in vitro* that single fluorophores can be detected on tubulin subunits within microtubules using a conventional widefield epi-fluorescence microscope, illumination from a Hg arc lamp, and a sensitive cooled CCD camera. These findings provide some guidelines for achieving optimal microtubule speckle contrast in FSM when background fluorescence, photobleaching, and resolution inhibit single fluorophore detection.

**MATERIALS AND METHODS**

Computer simulations

Mathematica software (Wolfram Research, Champaign, Ill.) was used to calculate, as a function of the fraction $f$ of labeled tubulin, the probability density function (PDF) expected for the number of fluorophores in a resolvable unit (440 dimers) along a microtubule and the predicted fluorescent speckle pattern (See Results for other details). This is annotated Mathematica code:

<<Statistics’DiscreteDistributions’

Figure 1. A, B) Comparison of diffraction-limited conventional (A, ~10% labeled tubulin) and speckle (B, ~0.25% labeled tubulin) fluorescent images of microtubules in the lamella of a migrating newt lung epithelial cell injected with X-rhodamine-labeled tubulin. Bar = 10 μm. Reproduced with permission (2). C) A stochastic growth model for the origin of the fluorescent speckle distribution in light micrographs of microtubules grown from a tubulin pool containing a small fraction of labeled dimers. The model assumes that microtubule growth occurs by stochastic tubulin dimer association at the ends of the protofilaments in the microtubule cylindrical wall (only 7 of the 13 protofilaments are shown). The resolvable region at 620 nm wavelength is 270 nm long and contains 440 dimers. Reproduced with permission (3). D, E) Simulations using the Binomial Distribution of the probability density (D) and the distribution along a microtubule (E) of the number of fluorophores in a 0.27 μm resolvable region containing 440 tubulin dimers. For each simulation pair, the fraction of tubulin is given in parenthesis below the average (Avg.) and standard deviation (SD). See text for details. F, G) A time-lapse series of images of a single microtubule assembled *in vitro* from 0.1% fraction of tubulin labeled with X-rhodamine (F) and the corresponding standardized fluorescence intensity from line scans along the microtubule axis (G). The horizontal arrow in the first frame of panel F shows the position of the speckle at the origin of the line scan in panel G. The horizontal arrows in the second line scan show the approximate intensities estimated for one, two, and three fluorophores. See text for details.
RESULTS

Simulations

Our simulations of the fluorescent speckle distributions along microtubules are based on the binomial distribution. The binomial distribution also applies to the binding of MAPs to tubulin sites within a resolvable region along a microtubule, but the simulations here consider only fluorescent tubulin distributions.

In our simulations, N is the number of tubulins within a resolvable unit that can potentially contain fluorescent label. For X-rhodamine fluorescence at 620 nm, the diffraction-limited resolvable distance is \( \sim 0.27 \) \( \mu \)M (3). The number of microtubule dimers in this resolvable distance is \( n = 440 \) dimers, approximately one quarter of the 1625 dimers in 1 \( \mu \)M of microtubule length (3). The probability that a site in the microtubule lattice has a fluorophore depends on the fraction of labeled subunits, \( f \). Assuming one fluorophore per subunit, the expected mean \( (m) \) number of fluorophores per resolvable unit averaged from many microns of microtubule length is:

\[
M = fN
\]

Eq. 1

For a random stochastic process, the standard deviation \( (sd) \) from the mean is given by:

\[
sd = (fN(1 - f))^{0.5}
\]

Eq. 2

We have defined (3) speckle contrast \( (C) \) in the absence of background fluorescence to be:

\[
C = \frac{sd}{M}
\]

Eq. 3

The Binomial Distribution function in Mathematica software (see Materials and Methods) was used to calculate the probability of a given number of fluorophores per resolvable unit (Fig. 1D) and simulate expected distributions in the number of fluorophores per 270 nm along the length of microtubules as a function of the fraction of labeled subunits (Fig. 1E). Notice that for \( f = 50, 5, 0.5, \) and 0.05\%, the means and standard deviations of the number of fluorophores in the simulated data are \( 220 \pm 10.5, 22 \pm 4.6, 2.2 \pm 1.5, \) and 0.22 \pm 0.47, respectively. These simulation values are exactly the numbers calculated from the statistical Eq. 1 and 2 verifying the accuracy of the simulations. Notice also in the plots in Fig. 1E that speckle contrast increases substantially at the lower fractions of labeled subunits as expected from Eq. 3. Although contrast as defined by Eq. 3 gives a first approximation to speckle contrast, it is not very useful when the mean fluorescence approaches and falls below one fluorophore. For tubulins labeled with one fluorophore, the 0.5\% fraction of labeled tubulin appears to give the optimal contrast because the brightest speckles are predicted to have six or seven fluorophores while the weakest have none. Below that fraction of labeled tubulin, the maximum number of fluorophores per speckle decreases while the minimal number remains zero.

Images of individual microtubules

To test the sensitivity of our microscope and digital imaging system, we assembled microtubules from
pure tubulin in vitro using 0.1% tubulin labeled with X-rhodamine. Simulations predict that at this fraction, 64% of the resolvable regions along a microtubule will have no fluorophores while 28% will have 1, 6% will have 2, ~1% will have 3, and <1% will have 4. To see and measure this speckle distribution, assembled microtubules were stabilized by taxol and immobilized on the inner surface of a slide-coverslip perfusion chamber. Soluble tubulin was washed away with assembly buffer without tubulin then sealed. The field diaphragm was closed down to a ~25 μM field of view to reduce background fluorescence from the specimen and from the optical surfaces in the microscope. As seen in Fig. 1, this fraction of labeled tubulin produced, as predicted by the simulations, very punctate speckles along the microtubule axis are shown in Fig. 1, and the corresponding intensity line-scans along the microtubule. As seen in Fig. 1, F, G, this fraction of labeled tubulin produced, as predicted by the simulations, very punctate speckles along the microtubules with many resolvable regions having no fluorescence.

To test for the number of fluorophores in the speckles, we examined the pattern of speckle photobleaching. Although the oxygen scavenger Oxypore (1, 3) was present for reduction of photobleaching, we found that 2–3 s exposures with unattenuated illumination resulted in changes in the speckle pattern. If there were only one to four fluorophores per speckle as predicted by the simulations, then bleaching should occur asynchronously between different speckles and in discrete steps within an individual speckle as individual fluorophores photobleach at random times. This was the pattern seen in time-lapse recordings of microtubules during continuous illumination. An example of a sequence of images and the corresponding intensity line-scans along the microtubule axis are shown in Fig. 1, F, G. Individual speckles decreased in intensity at random times in a stepwise manner (arrows in Fig. 1G). The smallest intensity step height corresponded to the fluorescence contribution of a single fluorophore since this height was about the same as the intensity of the dimmest speckles prior to photobleaching. When photobleaching occurred for the dimmest speckles, they disappeared (e.g., Fig. 1, F, G, 32–42 s).

DISCUSSION

There are several conclusions from these results. First is that a conventional epi-fluorescence microscope in combination with a low-noise cooled CCD camera can detect the fluorescence of single X-rhodamine fluorophores within the microtubule lattice. The background noise from either the specimen, optics, or camera were surprisingly low compared to the fluorescence intensity of single fluorophores in the in vitro reassembled microtubule experiments. The second is that photobleaching is a potential problem for FSM because it changes the fluorescent speckle pattern. Photobleaching appears to be a more serious problem for microtubules assembled in vitro than for microtubules in living cells; the reasons for this are unclear (unpublished observations). Further, the effects of photobleaching are more severe for speckles with fewer fluorophores.

These results argue that the optimal fraction of labeled tubulin for FSM is probably ~0.5%, where the brightest speckles are predicted to have six or seven fluorophores while the dimmest have zero when each labeled tubulin subunit has only one fluorophore. At this fraction, minor photobleaching will not produce a substantial change in the pattern of fluorescent speckles along the lattice. In addition, it maybe much easier to detect the brightness of a speckle with seven fluorophores above the background fluorescence in cells in comparison with one or three fluorophores for speckles at 0.1% fraction of labeled tubulin.

Finally, another way to raise speckle contrast and reduce the negative effects of photobleaching is to attach multiple fluorophores per subunit. If each tubulin, for example, had four fluorophores, then the 0.05% fraction of labeled tubulin (see Fig. 1, E, 0.05%) would result in a maximal speckle brightness equivalent to a 10-fold higher fraction of subunits labeled with only one fluorophore (see Fig. 1, E, 0.5%). Thus, multiple fluorophores per subunit has major advantages for FSM for detectability and resistance to photobleaching.

This paper is dedicated to the memory of Dr. Keith Porter whose excitement for new discoveries about microtubules, unraveling the molecular and structural machinery of the cell, and clever thinking were a major stimulation in our scientific lives. We also thank Arshad Desai, Tim Mitchison, Kerry Bloom, Paul Maddox, and Chloé Bulinski for their contributions to FSM and Mike Caplow for teasing us about potential artifacts. Supported by NIH GM24364.

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