Tubulin Dynamics in Microtubules of the Mitotic Spindle

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A critical factor in understanding the mechanisms that move chromosomes and regulate the assembly of mitotic spindles is identifying the pathways by which tubulin subunits exchange with microtubules in mitotic spindle fibers. The spindle is composed of a bipolar arrangement of microtubules, as diagrammed in Figure 1A for a metaphase spindle in a first-division sea urchin embryo. Mitotic microtubules that extend away from the region of the centrosome and spindle poles in vivo all have "plus" distal structural polarity (Fig. 1A), similar to the structural polarity of microtubules that have been nucleated from centrosomes in vitro (Telzer and Haimo 1981; McIntosh and Euteneuer 1984). Four possible pathways for the exchange of tubulin with spindle microtubules in vivo have been proposed, and these are summarized diagrammatically in Figure 1B. All but one of the proposed pathways (see no. 4 in Fig. 1B) (Inoué and Sato 1967; Bajer and Molé-Bajer 1975; Inoué and Ritter 1975) are based on end-dependent subunit exchange models, the mechanism by which microtubules apparently assemble in vitro (Hill and Kirschner 1982; Mitchison and Kirschner, this volume).

In this paper, I discuss recent findings from two experimental approaches that are directed toward elucidating the pathways of tubulin exchange with spindle and cytoplasmic microtubules in living cells. In the first approach, the rate of tubulin dissociation from nonkinetochore spindle microtubules was measured and compared to rates of tubulin association expected from the characteristic kinetic properties of end-dependent microtubule polymerization in vitro (Salmon et al. 1984a). The second approach involves measurements of microtubule dynamics in living cells using tubulin fluorescent analog cytochemistry and fluorescence redistribution after photobleaching (FRAP). This work has been done in collabo-
Investigating Tubulin Dissociation Rates In Vivo by Microinjecting Colchicine and Tubulin-Colchicine Complex into Living Cells

When microtubule polymerization is abruptly blocked at metaphase or early anaphase, the intrinsic rate of tubulin dissociation from microtubules is revealed. In experiments that are described in detail elsewhere (Salmon et al. 1984a), colchicine, Colcemid, or nocodazole at high intracellular concentrations was used to bind tubulin rapidly, thus blocking polymerization. Nonkinetochore microtubules depolymerized within a characteristic time of 20 seconds in sea urchin embryos (Fig. 2) and 10–15 seconds in PtK, tissue-culture cells; these values are based on measurements of changes in birefringence retardation of spindle fibers.

Colchicine-like drugs bind to the tubulin dimer at a site that is not exposed when the dimer is assembled into microtubules (Wilson and Meza 1973; Margolis and Wilson 1977; Deery and Weisenberg 1981; Schiff and Horwitz 1981). Tubulin dimer bound with colchicine (T-C) is the active intermediate for the rapid depolymerization of spindle microtubules following the injection of colchicine into sea urchin cells. P. Wadsworth has found in preliminary studies conducted in my laboratory that microinjecting mitotic cells with >2 μm intracellular concentration of T-C complex (made from porcine brain tubulin; Engelberghs and Lambeir 1980) produces loss of spindle birefringence retardation within 20 seconds, the same characteristic time as for spindle disassembly caused by injected colchicine at high concentrations. Colchicine binds to and dissociates from tubulin very slowly (Boris and Taylor 1967); 2 μm free intracellular colchicine has little effect on spindle assembly during mitosis.

In the sea urchin embryo, following microinjection of 0.1–5.0 mm intracellular colchicine, the birefringence retardation of nonkinetochore microtubules dimin-

![Figure 1](image)

Figure 1
(A) Sketch of the current concept of microtubule arrangements in a metaphase first-division spindle. Such a cartoon is based on detailed structural studies but must still be considered preliminary and merely indicative of the true state of affairs. The structure of the spindle poles, in particular, is poorly understood. Only one chromosome pair is drawn for clarity. Kinetochore microtubules are defined as microtubules attached to kinetochores. (B) Four possible pathways of tubulin exchange with spindle microtubules: (1) subunit exchange by reversible association-dissociation events at one (a) or both (b) ends of a microtubule; (2) treadmilling or head-to-tail polymerization as a consequence of GTP hydrolysis; (3) dynamic instability as a consequence of the loss of tubulin-GTP caps at the plus ends; (4) subunit exchange all along the apparent length of a microtubule either by insertion or deletion of tubulin within the microtubule wall (a) or by rapid breaking and reannealing (b). (Reprinted, with permission, from Salmon et al. 1984b.)

![Figure 2](image)

Figure 2
Polarization micrographs showing the rapid loss of spindle birefringence following the microinjection of 0.5 mm intracellular colchicine (for details, see Salmon et al. 1984a). Time is given in hr:min:sec on each frame. By 20 sec, most nonkinetochore fiber birefringence retardation has disappeared. Magnification, 202 x; scale, 20 μm.
ished uniformly throughout the spindle (Fig. 2). The birefringence-retardation decay followed exponential kinetics with a first-order rate constant of \( k = 0.11 \pm 0.023 \text{ sec}^{-1} \) and a corresponding half-time of \( t_{1/2} = 6.5 \pm 1.1 \text{ sec} \). The initial rate of tubulin dissociation from nonkinetochore spindle microtubules is in the range of \( k_{\text{dissoc}} = 180-992 \text{ dimers/sec/microtubule} \) (Salmon et al. 1984a). This range is based on \( k = 11\% \) of the initial polymer/sec and the average length of a half-spindle microtubule, which is estimated from electron micrographs to be between 1 \( \mu \text{m} \) and 5.5 \( \mu \text{m} \). If all half-spindle microtubules have one end in the centrosome or pole region, the so-called “minus” end, then the 5.5-\( \mu \text{m} \) estimate, corresponding to \( k_{\text{dissoc}} = 992 \text{ dimers/sec/microtubule} \), appears to be most likely for microtubules in the sea urchin half-spindle, which is typically 11-12 \( \mu \text{m} \) long.

This estimate of non-steady-state \( k_{\text{dissoc}} \) appears to approximate the steady-state rate of tubulin exchange with the nonkinetochore spindle microtubules. Several birefringence studies of spindle reassembly or augmentation (for review, see Salmon et al. 1984a) demonstrate that the rate of microtubule polymerization in marine embryos is at least 30\% as fast as the rate of disassembly observed after injection of high intracellular colchicine. The differences in the rates of disassembly and assembly, however, may be due in part to delays caused by initiation events during assembly.

The Rate of Tubulin Dissociation Is Much Faster Than Expected from In Vitro End-dependent Association Parameters

How does the value of \( k_{\text{dissoc}} \) compare with rates of tubulin association expected from kinetic parameters measured for end-dependent polymerization in vitro? Values for the bimolecular rate constant for tubulin association with microtubules in vitro range from \( 1 \times 10^{9} \) to \( 20 \times 10^{9} \text{ M}^{-1} \text{sec}^{-1} \) (summarized in Salmon et al. 1984a). The association rate constant in vivo will be smaller than the in vitro rate constant because the diffusion rate of tubulin in sea urchin cytoplasm is eight to ten times slower than in vitro (Salmon et al. 1984c). The expected association rate constant for tubulin in the sea urchin embryo is \( k_{\text{a,embryo}} = 0.1 \times 10^{9} \) to \( 2.5 \times 10^{9} \text{ M}^{-1} \text{sec}^{-1} \). This range of values predicts significantly slower steady-state microtubule polymerization-depolymerization than seen in vivo if the critical concentration for spindle tubulin assembly in vivo is about 2 \( \mu \text{M} \), the value measured for in vitro reassembly buffers (Keller and Rebhun 1982): \( 0.1-2.5 \times 10^{6} \text{ M}^{-1} \text{sec}^{-1} \times 2 \mu \text{M} = 0.2-5.0 \text{ dimers/sec} \). Even for the highest estimated concentration of tubulin in the egg (range 5-27 \( \mu \text{M} \); Pfeffer et al. 1976), the rate of tubulin association-dissociation does not balance if microtubules assemble only by addition of subunits at the ends.

Increasing the number of exchange sites along the apparent length of microtubules solves this problem (see no. 4 in Fig. 1B). If microtubules are constantly breaking and reannealing, then not all microtubule "minus" ends are located near the spindle poles or in the centrosome. On the other hand, the concentration and mobility of tubulin in the spindle region may be significantly different than in the surrounding cytoplasm.

Investigating Microtubule Dynamics with DTAF-Tubulin and FRAP Techniques

A powerful approach for analyzing microtubule dynamics in living cells is the combination of fluorescent analog cytochemistry (Kreis and Birchmeier 1982; Wang et al. 1983; Taylor et al. 1984) and measurements of FRAP (Jacobson et al. 1983). In our approach, tubulins labeled with dichlorotriazinyl amino fluorescein (DTAF-tubulin) were microinjected into cells where they mimicked the behavior of endogenous tubulin, thus serving as tracers of the cellular tubulin pool during the assembly and disassembly of microtubules. FRAP techniques are well established for measuring the mobility of fluorescent molecules in cells (Jacobson et al. 1983). With laser microbeaming, fluorescence can be bleached in a restricted, well-defined region of a cell or organelle. Because photobleaching is permanent, recovery of fluorescence in a bleached region requires redistribution of unbleached, labeled tubulin subunits into the irradiated area. The instrumentation procedures we have used for laser photobleaching and quantifying temporal changes in the spatial distribution of fluorescence using low-light-level video cameras and photographic procedures (video FRAP) are described in detail elsewhere, along with a description of measurements of the rate of tubulin diffusion in the cytoplasm of sea urchin embryos (Salmon et al. 1984c).

Procedures developed in McIntosh’s laboratory for purifying bovine or porcine neurotubulin labeled with DTAF (DTAF-to-protein ratio \( \sim 1.0 \)) and the in vitro assembly characteristics of DTAF-tubulin are described by Leslie et al. (1984). DTAF-tubulin has assembly-disassembly characteristics in reassembly buffers in vitro that are typical of unlabeled tubulin. Normal microtubules grow from DTAF-tubulin nucleated from the ends of axonemal fragments and purified centrosomes, and DTAF-tubulin does not appear to bind to the walls of microtubules in vitro. When DTAF-tubulin was injected at low concentrations into living sea urchin embryos (Fig. 3), or mammalian tissue-culture cells, fibrous patterns of fluorescence formed, similar to anti-tubulin immunofluorescence and polarization im-

![Figure 3](image-url)

**Figure 3**
Micrograph of the DTAF-tubulin distribution in metaphase mitotic cells of a second-division embryo of *Lytechinus variegatus*. The embryo was injected with DTAF-tubulin about 30 min before first mitosis. The pattern of DTAF-tubulin fluorescence in the spindle-aster complex is similar to birefringence images seen in polarization microscopy, as described by Salmon et al. (1984b). Magnification, 310 \( \times \); scale, 20 \( \mu \text{m} \).
ages of microtubule arrays at similar stages in the cell cycle (for photographs and details, see Salmon et al. 1984b and Saxton et al. 1984). Changes in fluorescence intensity of microtubule arrays in living cells injected with DTAF-tubulin followed the normal fluctuations in assembly of cytoplasmic and mitotic spindle microtubules throughout the cell cycle, and fluorescence disappeared upon application of nocodazole, as happens with normal microtubules in vivo.

Spindle FRAP

In the sea urchin spindle experiments, cells were injected with DTAF-tubulin before mitosis, so the DTAF-tubulin equilibrated with the endogenous tubulin pool and the labeled tubulin dimers were incorporated uniformly into the microtubules that formed the mitotic spindle. After about 50% of the initial fluorescence was bleached, Salmon et al. (1984b) found that the recovery of fluorescence in either large or small bleached regions of the sea urchin mitotic spindle had four striking characteristics: it was exponential, rapid, nearly complete, and uniform (Fig. 4).

As measured by the video FRAP methods, the recovery of fluorescence in the bleached region was an exponential function of time. The time for 50% recovery ($t_{1/2}$) was 19.4 ± 8.2 seconds. Maximum recovery, about 93% of initial fluorescence intensity, was reached within 60–90 seconds. Fluorescence recovered uniformly throughout the spindle, but no distinct geometrical pattern could be resolved.

More recently, Wadsworth and Salmon have analyzed the recovery of fluorescence using photon-counting, photomultiplier recording methods that provide better sensitivity and temporal resolution than the video recording methods used previously (Salmon et al. 1984b). The recovery of fluorescence in the bleached region of a half-spindle was seen to involve fast and slow exponential phases (Fig. 5a,b). The fast phase is due to diffusion of tubulin within the bleached region at rates similar to the diffusion of tubulin in the cytoplasm (cf. Fig. 5c). The second phase is due to the incorporation of unbleached DTAF-tubulin into microtubules of the spindle fibers. We have not analyzed these kinds of data in detail, but the half-times for the second phase of recovery of fluorescence in the

![Figure 4](image)

**Figure 4**
Fluorescence recovery after photobleaching the lower half-spindle of a tripolar metaphase spindle in a first-division embryo of *Lytechinus variegatus*. Diameter of the bleaching beam was about 4 μm. Video images were recorded as described by Salmon et al. (1984b,c). Magnification, 175 x; scale, 20 μm.

bleached region are in the range of 14–20 seconds, similar to the half-times measured by the video FRAP method. Again, recovery of fluorescence was nearly

![Figure 5](image)

**Figure 5**
Computer records of FRAP of the central half-spindle region of metaphase spindles (a,b) and a cytoplasmic region adjacent to the spindle in b for first-division embryos of the sea urchin *Lytechinus variegatus* (c). Fluorescence excitation and bleaching were produced by a laser microbeam ($\lambda = 488$ nm and 4.2 μm dia). During the measurement phase, the laser microbeam intensity was attenuated by a factor of about $5 \times 10^{-4}$ compared with the bleaching intensity. Bleaching period was about 300 msec. The number of photon counts was recorded every 0.5 sec using an EMI 9883A photomultiplier, C-10 photomultiplier (EMI) and an Apple computer to sample and store the data. Initial fluorescent intensities before bleaching were about 5000 pulses per second (pps) for the central half-spindle region (a,b) and 2000 pps for the adjacent cytoplasm (c). A total of 750 samples were taken in each experiment. The first 200 samples were plotted, then samples 450–475 (between the first and second vertical bars), and then samples 720–750 (second vertical bar to end). Note that in a, the spindle goes into anaphase about 2 min after bleaching. In b, the spindle goes into anaphase shortly after bleaching, and the normal decrease in microtubule assembly during anaphase is seen in later stages of recovery. In c, cell cleavage occurs during the late stages of recovery, and the optical measurement path through the cell is reduced.
complete (>90%) within 60 seconds. The exact extent of recovery is difficult to measure in sea urchin spindles because of the normal changes in assembly that occur as the spindle progresses through mitosis (see Fig. 5b).

Does the rapid rate of fluorescence recovery that we observed represent steady-state microtubule dynamics or reassembly of microtubules following depolymerization induced by the high-intensity photobleaching light? The 14–20-second half-time for recovery of fluorescence is similar to the reassembly rate following depolymerization of spindles by various agents (for review, see Salmon et al. 1984a). However, evidence thus far shows that microtubules in vivo (Salmon et al. 1984b; Saxton et al. 1984) and in vitro (Leslie et al. 1984) do not depolymerize significantly when fluorescence is photobleached. Photobleaching can cross-link microtubule proteins in vitro under some conditions, and the possibility that photobleaching does submicroscopic damage to the tubulin lattice has not yet been ruled out (Leslie et al. 1984). Nevertheless, the available evidence is positive that the FRAP results reflect steady-state microtubule dynamics.

This conclusion is also supported by the difference in FRAP between spindle and cytoplasmic microtubules. For PKI- and BSC-cultured cells, Saxton et al. (1984) have measured half-times of recovery of fluorescence in bleached regions of metaphase spindles of about 12 ± 7 seconds, similar to the forward incorporation rate of DTAF-tubulin into the spindle fibers when injected at metaphase (Saxton et al. 1984), to the rate of incorporation of tubulin labeled with DTAF into sea urchin astral fibers (Wadsworth and Slobooda 1983), and to the sea urchin spindles FRAP rates. Bleached regions of cytoplasmic microtubule arrays in interphase cells also recover fluorescence exponentially with time, but the half-time of the nondiffusional recovery is 235 ± 80 seconds, or about 19 times slower. The difference in FRAP rates between spindle and cytoplasmic microtubules resembles the different rates of depolymerization for nonkinetochore spindle microtubules and interphase cytoplasmic microtubule arrays when assembly is blocked by nocodazole (M. de Brabander, pers. comm.; E.D. Salmon et al., unpubl.).

Tubulin Exchange Is Not a Simple Equilibrium at Microtubule Ends

The observed 20-second half-time for turnover of tubulin within microtubules of sea urchin spindle fibers in our FRAP studies clearly cannot be accounted for by simple equilibrium exchange of tubulin at the ends of microtubules (see no. 1 in Fig. 1B). At steady state, the average number of tubulin dimers, \( m \), replaced inward from one end of a microtubule as a function of time, \( t \), and dissociation constant, \( k_d \), is given by (Zeeberg et al. 1980; Hill and Kirschner 1982): \( m = (k_d t)^{1/2} \). If \( k_d = 992 \text{ dimers/sec} \), corresponding to an estimated average length of 5.5 \( \mu \)m for a nonkinetochore half-spindle microtubule, then \( m = 159 \) dimers replaced within 20 seconds at each end, which corresponds to 2% of the number of tubulin dimers in a 5.5-\( \mu \)m microtubule (Salmon et al. 1984b). Thus, simple equilibrium exchange of subunits at the ends of spindle microtubules should lead to very slow FRAP rates, but this was not what we observed in living spindles. In contrast, the simple equilibrium model does predict the extremely slow recovery of fluorescence that we observed in vitro after photobleaching astral microtubules that were generated by nucleating DTAF-tubulin assembly from purified centrosomes (Leslie et al. 1984).

Analysis of Other Proposed Pathways

High-efficiency treadmilling (see no. 2 in Fig. 1B) could, in principle, exchange all the tubulin molecules in the spindle fiber microtubules with a half-time of 20 seconds (Salmon et al. 1984b). However, if treadmilling does occur and microtubules are organized by the centrosomes and the spindle poles, as diagramed in Fig. 1A, then we should see the bleached region transported poleward during recovery, as predicted by the model of Margolis and Wilson (1981). So far, the evidence for poleward transport during fluorescence recovery is negative.

The spindle FRAP results are consistent with either the exchange of tubulin subunits at sites all along the length of spindle fiber microtubules (see no. 4 in Fig. 1B) or rapid, continuous depolymerization and repolymerization of spindle fiber microtubules as proposed by Mitchison and Kirschner (this volume) (see no. 3 in Fig. 1B). Some form of microtubule “breathing” and tubulin intercalation or rapid breaking and reannealing of microtubules would produce the uniform, exponential recovery of fluorescence in spindle fibers observed after photobleaching. However, there is no direct evidence yet that tubulin subunits can exchange at sites along a microtubule other than at its ends. Perhaps unknown proteins seve microtubules or disrupt the tubulin lattice in a manner analogous to the action of some proteins on actin filaments (Bonder and Mooseker 1983). Mitchison and Kirschner (this volume) propose that whole microtubules can be turning over rapidly at steady-state assembly equilibrium, a so-called “dynamic instability” (see no. 3 in Fig. 1B). They postulate that the hydrolysis of GTP, bound to tubulin at the ends of microtubules, regulates rapid, catastrophic depolymerization of entire microtubules (Carlier et al. 1984). If microtubules are continuously depolymerizing, then regrowing from the centrosome, our FRAP results indicate that the whole process must occur rapidly, but nonsynchronously, among the population of nonkinetochore spindle microtubules with a half-time of about 20 seconds. The colchicine injection studies described above show that nonkinetochore spindle microtubules do depolymerize rapidly (as their scheme requires) when elongation ceases. The major reservation with this scheme is the same puzzle raised from the colchicine injection experiments. The rate of end-dependent microtubule elongation, based on the highest estimated tubulin concentration in the egg and the largest rate constant measured for end-dependent association of tubulin (corrected for the rate of tubulin diffusion in the cytoplasm), is too slow to polymerize a 5-\( \mu \)m microtubule within a half-time of 20 seconds (e.g., 27 \( \mu \)m \( \times 2 \times 10^8 \) M\(^{-1}\)sec\(^{-1}\) \( \times 20 \) sec/1635 dimers \( \mu \)m\(^{-1} = 0.66 \mu \)m).

SUMMARY

Rates of tubulin dissociation and flux with microtubules in nonkinetochore spindle fibers have been measured in living cells. The spindle FRAP rates are clearly inconsistent with simple equilibrium exchange of tubulin subunits at microtubule ends (see no. 1 in Fig. 1B). The uniform pattern of fluorescence recovery that we
observed is inconsistent with the treadmilling model proposed by Margolis and Wilson (1977) (see no. 2 in Fig. 1B). The FRAP data are, in principle, consistent with the dynamic instability model of Mitchison and Kirschner (see no. 3 in Fig. 1B), but, based on in vitro kinetic parameters and the rate of tubulin diffusion in the cytoplasm, end-dependent microtubule elongation does not appear to be fast enough. The dynamic equilibrium exchange of tubulin subunits at sites all along microtubules (see no. 4 in Fig. 1B; Inoué and Sato 1967; Inoué and Ritter 1975), however, is consistent with the current in vivo rate data, although the mechanism(s) underlying this process cannot yet be explained.

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REFERENCES


