17 Fluorescence Studies of Tubulin and Microtubule Dynamics in Living Cells

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Fluorescent analog cytochemistry (FAC) [Taylor et al., 1984] and fluorescence redistribution after photobleaching (FRAP) [Jacobson et al., 1983] are providing new insights about the polymerization and behavior of spindle and cytoplasmic microtubules within living cells [Keith et al., 1981; Leslie et al., 1984; McIntosh et al., 1985; Salmon, 1984; Salmon et al., 1984b,c; Saxton et al., 1984; Scherson et al., 1984; Wadsworth and Salmon, 1985a,b, 1986; Wadsworth and Sloboda, 1983, 1984]. FAC enables us to “tag” tubulin dimers, the subunits from which microtubules are polymerized, making them visible with fluorescence light microscopy under conditions that are not harmful to the cells, and FRAP techniques allow us to observe the mobility of tubulin in the cell and within cell structures that are composed of microtubules.

Microtubules are dynamic components of the cell cytoskeleton which participate in many processes including ciliary motility, vesicle transport, cell morphogenesis, and mitosis [Dustin, 1984]. As a cell progresses through the cell cycle, the spatial organization and the extent of assembly of microtubules change [Vandre et al., 1984] (Fig. 1). During interphase most microtubules originate at the centrosome region near the nucleus, and they extend toward the cell periphery in a stellate, astral array, the “cytoplasmic microtubule complex” (CMTC). The centrosome is a “microtubule organizing center” (MTOC) in the cell and an efficient nucleation site for microtubule polymerization in vitro. When the cell enters mitosis, the CMTC disassembles, and during mitosis all microtubule assembly is associated with the
central spindle and asters of the mitotic apparatus. The spindle is formed from a bipolar, overlapping array of microtubules (Fig. 2A). Microtubules attached to a kinetochore are termed “kinetochore microtubules,” as opposed to the other “nonkinetochore microtubules” of the half-spindle and asters. During anaphase, chromosomes move poleward as the kinetochore microtubules shorten. Concurrently, the distance between the poles increases, and microtubules grow outward from the centrosomes toward the cell surface to form a new CMTC. A parallel bundle of overlapping microtubules, “the midbody,” extends between the dividing daughter cells.

The basic subunit of microtubules is the tubulin dimer [Dustin, 1984]. Each 100-kD dimer is composed of one molecule of α tubulin and one of β tubulin, both polypeptides having nearly equal molecular weight. Tubulin dimers arrange themselves into 13 protofilaments that form the cylindrical wall of a microtubule. All microtubules have an intrinsic structural polarity which is specified by the tubulin lattice [McIntosh and Euteneuer, 1984; Telzer and Haimo, 1981]. This polarity is reflected in different polymeriza-

Fig. 1. Rearrangements of microtubule arrays during the cell cycle. The sketch shows major features of the typical distribution of microtubules inferred from immunofluorescent microtubule patterns and electron micrographs. It does not provide a comprehensive picture of the distribution of all microtubules in the cell.

Fig. 2. A. Sketch of the current concept of microtubule arrangements in a metaphase first-division sea urchin 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) simple equilibrium 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) dynamic equilibrium 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 annealing (b). Reprinted with permission, from Salmon et al. [1984c], Salmon [1984].
tion rates at the two ends of a microtubule: polymerization is favored at the (+) end, depolymerization at the (−) end [Purich and Kristofferson, 1984]. Microtubules in the mitotic apparatus and CMTC have the same polarity, (+) distal to the centrosomes.

In vitro purified tubulin self-assembles into microtubules in low-calcium buffers near neutral pH at physiological temperature [Purich and Kristofferson, 1984]. When purified centrosomes are used to nucleate assembly in vitro, the resulting microtubules have the same structure and polarity as microtubules in vivo [Evans et al., 1985]. The mechanism by which microtubules assemble in vitro has been identified as a nucleated, linear condensation polymerization reaction [Purich and Kristofferson, 1984]. The binding of guanosine triphosphate (GTP) to the tubulin dimer greatly promotes polymerization. Hydrolysis of the bound GTP follows incorporation of the dimer into the microtubule. The free energy of GTP hydrolysis frees microtubule polymerization from the thermodynamic constraints of simple helical polymerization and provides the opportunity for more dynamic pathways of assembly (Fig. 2B) [Margolis et al., 1978; Margolis and Wilson, 1981; Mitchison and Kirschner, 1984a-c].

Our principal interest has been the mechanism by which microtubules assemble and disassemble within the mitotic spindle. Observations of spindle birefringence with polarization microscopy have shown that the spindle microtubules can be rapidly disassembled (within 10–30 sec) by cold, increased hydrostatic pressure, and drugs, such as colchicine, which bind to tubulin and prevent assembly [Inoué and Sato, 1967; Inoué, 1981; Salmon, 1975; Salmon et al., 1984a]. The spindle reassembles rapidly after these conditions are returned to normal, which demonstrates that most of the spindle and astral microtubules are in a rapid, labile equilibrium with the cellular pool of tubulin subunits [Inoué, 1981]. The kinetochoore microtubules, usually a small fraction (<30%) of the microtubules in a half-spindle, are differentially stable to such treatments [Rieder, 1982], perhaps because both ends of these microtubules are engaged [Salmon et al., 1976; Mitchison and Kirschner, 1984c]. The lability of most microtubules in the spindle asters and the interphase CMTC is similar to that of the nonkinetochore spindle microtubules. The midbody microtubules, however, are even more stable than kinetochore microtubules [Salmon et al., 1976].

For analysis of microtubule dynamics in living cells, we have used tubulin labeled with a fluorescein derivative, 5-(4,6-dichlorotiazin-2-yl) amino fluorescein (DTAF)-tubulin, as a tracer of the endogenous tubulin pool (normal intracellular concentration about 20 μM). In living cells endogenous tubulin and injected DTAF-tubulin appear to intermingle randomly when microtubules form. A laser microbeam (488-nm wavelength) can then be used to photobleach the fluorophores in selected regions of microtubule arrays at steady-state assembly without producing structural damage to the cells. Polymerization characteristics of microtubules can be inferred from the pattern of fluorescence recovery and redistribution after photobleaching. This work was initiated in collaboration with J.R. McIntosh’s laboratory (University of Colorado, Boulder, CO) and our initial findings have been published [Leslie et al., 1984; Salmon et al., 1984b,c; Saxton et al., 1984].

As will be discussed more fully below, there are several proposed models for microtubule assembly (Fig. 2B) which make specific predictions about the rate and pattern of FRAP for the incorporation of tubulin in microtubule arrays at steady-state assembly. For example, very slow FRAP is expected for “simple equilibrium” tubulin subunit exchange solely at the free ends of microtubules [Hill and Kirschner, 1982; Zeerberg et al., 1980]. The “treadmilling” model proposes that GTP hydrolysis drives the preferential assembly and disassembly of tubulin subunits at opposite ends of microtubules, which results in uniform flow of tubulin along the spindle microtubules toward the poles [Margolis et al., 1978; Margolis and Wilson, 1981]. Consequently, FRAP should be detected first near the spindle equator, bleached spots should move poleward during FRAP, and the rate of FRAP will depend inversely on the diameter of the bleached spot [Wadsworth and Salmon, 1985a,b]. Both the “dynamic equilibrium” and “dynamic instability” models predict that FRAP will occur rapidly and uniformly [Salmon et al., 1984c].

DTAF-TUBULIN

Criteria for useful fluorescent analogs of any cellular molecule have been described in detail by Taylor et al., [1984]. The major criterion for a tubulin analog, however, is native assembly ability both in vivo and in vitro.

Only a few fluorophores and labeling procedures have been found that will label tubulin without denaturing the protein. Although there are various methods for preparing fluorescent analogs to tubulin [Wadsworth and Salmon, 1986b], we routinely use a procedure developed in J.R. McIntosh’s laboratory by Leslie et al. [1984] to label purified porcine brain tubulin with DTAF, a fluorescein derivative that reacts with ε amino groups and N-terminal amino acids near neutral pH.

DTAF-tubulin is the most extensively characterized fluorescent analog of tubulin [Leslie et al., 1984; McIntosh et al., 1986; Wadsworth and Salmon, 1985b]. After purification of labeled tubulin from denatured protein and microtubule-associated proteins, the stoichiometry of the DTAF/tubulin dimer has varied for different preparations from 0.5 to 1.8. Both α and β tubulin are labeled, with more DTAF on β tubulin. DTAF-tubulin has been shown to have nearly normal characteristics of microtubule assembly and
disassembly in vitro. DTAF-tubulin polymerizes onto microtubule ends and does not bind adventitiously to the walls of microtubules in standard in vitro microtubule reassembly buffers.

We have microinjected DTAF-tubulin into tissue culture cells and sea urchin embryos using glass microneedles [Graessmann et al., 1980; Salmon et al., 1984b,c; Saxton et al., 1984]. Typically, we injected an amount of 15–55 μM DTAF-tubulin (concentration in the needle), equal to 1–10% of the cell volume. DTAF-tubulin incorporates into microtubules at all phases of the cell cycle, and the cells develop normally when the intracellular concentration of DTAF-tubulin is less than 2–5 μM [Salmon et al., 1985c; Saxton et al., 1984] (Fig. 3).

**FLUORESCENCE MICROSCOPY, PHOTOBLEACHING, AND RECORDING**

The optical system we have recently used in our fluorescence studies is diagrammed in Figure 4. There are three illumination and three imaging optical pathways through a custom-made inverted optical bench microscope [Inoué, 1961; Wadsworth and Salmon, 1986a, 1986d]. The rationale for the

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Fig. 3. Time-lapse photographs from a video monitor showing the DTAF-tubulin distribution from prometaphase through telophase and cell cleavage in a first-division embryo of the sea urchin *Lytechinus variegatus*. Full metaphase occurs near 17:25:54, while anaphase begins near 17:27:30. Time in hours:minutes:seconds is given on each frame. Bar, 20 μm x 275. Reprinted with permission from Salmon et al. [1984c].

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Fig. 4. Optical pathways for fluorescence microscopy, laser photobleaching, and video and photomultiplier recording. Major components are described in the text, in Salmon et al. [1984b] and in Wadsworth and Salmon [1985a, 1986]. Symbols: L1, argon-ion laser; L2, 100-W Hg illuminator; L3, 100-W Hg or 60-W tungsten illuminator; C, condenser; O, objective; EF, fluorescence excitation filters; DM, dichroic mirror; BF, fluorescence blocking filters; AIP, aerial image plane; FL, lens that focuses laser beam at AIP; PMT, photomultiplier; S1, low-transmission shutter for PMT-FRAP; S1, S2, S3, shutters, LLL, low light level; CAM, camera; Apple, Apple II computer; VLA, video line analyzer. For polarization microscopy, a polarizer and a compensator are inserted above the condenser, an analyzer is inserted below the objective, and DM and BF are removed.
design is described in detail elsewhere [Salmon et al., 1984b]. Transillumination is used for polarization and phase contrast microscopy. A DC-regulated 100-W Hg burner provides uniform epi-illumination of large regions of the specimen. Local regions of the specimen are illuminated or photobleached by using a 488-nm laser microbeam (Spectra-physics model 164). Zeiss fluorescein excitation and barrier filters (#487709) are used for DTAF analogs. Fluorescent images are projected to the eye, to a low-light-level video camera (Venus DV2 or DAGE-MTI model 68 SIT), or through a pinhole in a conjugate image plane to a photomultiplier (EMI 9863A). Images from the low-light-level camera are photographed using 0.5-sec exposures [Salmon et al., 1984b]. Electronic shutters (Uniblitz, Vincent Associates) limit whole field epi-illumination during photographic exposures to 0.6 sec to prevent photobleaching and to block illumination of the video camera and photomultiplier tubes during laser photobleaching.

An Apple computer records video voltages along lines scanned through the image to provide line profiles of fluorescence and FRAP (Video-FRAP) [Wadsworth and Salmon, 1986a]. Line profiles have also been obtained from calibrated densitometry of photographic negatives recorded from the video monitor [Salmon et al., 1984b; Saxton et al., 1984]. The Apple computer is also used to sample, store, and plot data obtained from the photomultiplier through a photon counter (EMI model C-10).

We have performed fluorescence excitation and photobleaching using two patterns. In most of our recent experiments, we have analyzed FRAP for circular photobleached regions. For photomultiplier measurements (PMT-FRAP), a spherical focusing lens (17.5-mm focal length in most experiments) was used to focus the laser beam on the field diaphragm plane of the Zeiss 4 FL epi-illuminator. The characteristic 1/e beam radius [Axelrod et al., 1976; Schneider and Webb, 1981] at the point of focus at the specimen depends on the objective and is 5.8 μm for a Zeiss 10× Acromat, 2.25 μm for the Zeiss 25× Plan Neofluor, 1.4 μm for the Zeiss 40× Plan Neofluor, and 0.76 μm for the Zeiss 63× Plan Apochromat objective. For bleaching, the beam power into the objective was about 100 mW, attenuated by 1-1.8 OD depending on the objective. During the measurement phase of photometric recordings of FRAP, the beam was further attenuated by a factor of 20,000 compared to the bleaching beam intensity with an electronic shutter (S2 in Fig. 4) [Yguerabide et al., 1982]. Spindle fluorescence was usually bleached by 40-60% by a 0.1-sec exposure to the unattenuated laser beam. We have also used a bar pattern of photobleaching in our studies of spindle microtubule dynamics. An 18-mm focal length cylindrical focusing lens and a Zeiss 63×/1.4 NA objective produced the bar pattern [Wadsworth and Salmon, 1986a, 1986d].

**TUBULIN MOBILITY IN THE CYTOPLASM**

The tracer diffusion coefficient (D) for tubulin has been measured in two types of cells: sea urchin embryos and BSC-1 tissue culture cells. Initially, the diffusion coefficient was measured in sea urchin embryos by using video measurements of FRAP (Video-FRAP) [Salmon et al., 1984b]. Profiles of fluorescence through circular bleached regions were obtained by calibrated densitometry of video images before and after photobleaching. The diffusion constant was obtained by analysis of fluorescence recovery profiles by using Fick's second law of diffusion, assuming that only radial diffusion occurred within the cylindrical bleached region through the cell. More recently we have used conventional photomultiplier spot measurements (PMT-FRAP) [Axelrod et al., 1976; Jacobson et al., 1983] to obtain values for D in both sea urchin and BSC-1 mammalian tissue culture cells from half-times of fluorescence recovery (Fig. 5). Both techniques yielded similar values for D in the sea urchin cytoplasm, about 4-6 × 10⁻⁸ cm²/sec at 25°C. Tubulin diffusion in the cytoplasm of BSC-1 cells is 1-2 × 10⁻⁸ cm²/sec at 32°C, two to six times slower. As with other proteins, the diffusion coefficient of tubulin strongly depends on cell type [Salmon et al., 1984b]. Jacobson and Wojcieszyn [1984] provide an interesting analysis and discussion of the structural and chemical factors which may determine the rate of diffusion of proteins through the cytoplasm.

The major limitation on tubulin mobility in the cell is incorporation into microtubules. As seen in Figure 5, about 65% of the DTAF-tubulin in a region of an interphase BSC-1 cell near the centrosome (see Fig. 1) is relatively immobile compared to the diffusible fraction of DTAF-tubulin in the cell. Depolymerization of the great majority of the CMTG by 10 μM nocodazole [Debrabander et al., 1981] correspondingly reduces the relatively immobile fraction to less than 15%. The rate of diffusion of nonpolymerized tubulin does not appear to be greatly altered after microtubule depolymerization. Other components of the cytoplasm or cytomatrix affect the mobility of soluble tubulin more significantly [Jacobson and Wojcieszyn, 1984].

**SPINDLE FRAP**

When a region between the chromosomes and a pole of a metaphase spindle is photobleached, the majority of bleached fluorescence recovers rapidly, as illustrated by the video images in Figures 6 and 7. In Figure 6, a substantial amount of the initial fluorescence in a large region of the upper half-spindle and aster have been photobleached. Sixty seconds later spindle fluorescence and morphology have returned to near normal. Fluorescence recovery appears to occur uniformly throughout bleached regions of spindles.
Fig. 5. The lateral diffusion of DTAF-tubulin in the cytoplasm of interphase BSC-1 tissue culture cells measured by PMT-FRAP of (a) a normal cell and (b) a cell pretreated for 30 min with 10 μM nocodazole to induce the depolymerization of labile microtubules in the cytoplasmic microtubule complex (CMTC). A 50-cm spherical focusing lens (FL in Fig. 3) plus 40×0.75 NA Zeiss Neofluor phase objective produced a 6.6-μm-diameter Gaussian laser beam at the specimen. Measurements were made in the cytoplasm about 10 μm from the centrosome region and 20 μm from the cell periphery. Photon counts were sampled over 0.1-sec intervals. The first five samples were averaged by the computer and used to normalize the remaining samples which are plotted. Samples are plotted for the time periods 0–20 sec, 45–47.5 sec (between the vertical bars), and 72–75 sec. The percentage of bleached fluorescence recovered in 75 sec in a is 38% and in b is 85%.

Fig. 6. FRAP in a first-division metaphase spindle in an embryo of the sea urchin *Lytechinus variegatus*. Bleaching for 8 sec with a 12-μm diameter laser beam, focused on the specimen using a Zeiss 25×0.6 NA Neofluor phase objective, ended at 14:06:03. Fluorescence in the region of the upper half-spindle and aster, almost completely bleached by laser irradiation, recovered substantially within 60 sec. The fluorescence (a,c,e,f,h) and polarization (b,d,g) micrographs demonstrate that photobleaching does not significantly alter the normal amount and distribution of spindle and astral birefringence, morphological changes in assembly during mitosis, or the timing of mitotic events. Bar, 20 μm. ×275. Reprinted with permission from Salmon et al. [1984c].

Fig. 7. FRAP of a metaphase BSC-1 cell microinjected with DTAF-tubulin. The lower half-spindle was photobleached midway between the chromosomes and the pole for 0.1 sec with a bar bleach pattern (approx. 1.6 μm wide; see text for details). Cell before bleaching (a); immediately after bleaching (b); and after 73 sec (c), 200 sec (e), and 271 sec (e) of recovery. Arrows mark the position of the bleached region. Bar, 10 μm. ×700. Reprinted with permission from Wadsworth and Salmon [1986d].
and asters [Salmon, 1984; Salmon et al., 1984c, Saxton et al., 1984]. A similar result is seen when a 1.6-μm-wide bar pattern is bleached across the half-spindle of the BSC-1 spindle in Figure 7 [Wadsworth and Salmon, 1985a, 1986d].

We have quantitated the kinetics of FRAP using standard PMT-FRAP methods [Axelrod et al., 1976; Wadsworth and Salmon, 1986d], since translation of the bleached regions during fluorescence recovery was not apparent from video FRAP images. Figures 8 and 9 illustrate FRAP records for metaphase sea urchin and BSC-1 spindles. For each record, there are three major phases of fluorescence recovery. There is an initial, rapid phase which corresponds to the rate of diffusion of tubulin subunits in the cytosol within the spindle and in the cytoplasm above and below the spindle along the optical path of the laser beam. The sea urchin embryo is much thicker along the optical path of the laser beam than is the BSC-1 cell (about 6 times) and the magnitude of the rapid diffusion phase in the FRAP kinetics is greater. Both the sea urchin and BSC-1 central spindles are about the same size, about 24 μm long and 6 μm wide at the equator.

The second phase of fluorescence recovery we term the “fast incorporation phase” because it appears to correspond with the rate of tubulin incorporation into the majority of spindle fiber microtubules at steady state. Fluorescence recovery during the incorporation phase can be fit closely with an exponential function [Salmon et al., 1984c; Saxton et al., 1984; Wadsworth and Salmon, 1986a]:

\[
\bar{F}_m - \bar{F}(t) = \left[ \bar{F}_m - \bar{F}(0) \right] e^{-kt}
\]

(1)

where \(\bar{F}_m\) is the normalized fluorescence in the bleached region 365 sec after bleaching, \(\bar{F}(t)\) is the normalized fluorescence after bleaching where time \(t = 0\) immediately after bleaching, and \(k\) is a first-order rate constant (Fig. 8b). The half-time of fluorescence recovery is given by

\[
t_{1/2} = \ln 2 / k
\]

(2)

where \(k\) is determined from the shape of a best-fit straight line through plots of \(\ln [\bar{F}_m - \bar{F}(t)]\) vs. time [Wadsworth and Salmon, 1986a, 1986d]. Table 1 lists half-times measured for sea urchin and BSC-1 spindles. At physiological temperature the half-time of fluorescence recovery for the sea urchin (16 sec) is similar to the value for BCS-1 cells (estimated to be 24 sec at 37°C from an Arrhenius plot of the half-time data in Table 1 [Wadsworth and Salmon, 1986a]). Similar half-times have been determined by video FRAP for BSC-1 and PtK2 metaphase spindles.

The third phase of FRAP is a very slow incorporation phase. About 12% of the initial bleached fluorescence in the sea urchin spindle (about 25% for

Fig. 8. Computer-generated records of FRAP for a metaphase sea urchin spindle at 25°C obtained by photometric measurements. A Zeiss 25×/0.6 NA Neofluor phase objective was used to produce a 4.2-μm-diameter Gaussian laser beam through a central half-spindle region. The number of photon counts as recorded every 0.5 sec. Fluorescence measurements, \(F(t)\), were normalized by dividing by the average number of photon counts for five samples preceding the recording data. A total of 7680 samples were taken in the 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 the end). At the end of the record, the spindle has gone into metaphase and fluorescence increases because microtubule assembly increases. Fluorescence was about 5,000 pulses per second (pps) in the spindle, compared with 2,500 pps in the adjacent cytoplasm. \(F_\infty\) is the average prebleach fluorescence and \(F_m\) is the average recovered fluorescence after bleaching for 0.2 sec at 17 sec. B. The rapid incorporation phase of fluorescence recovery was analyzed according to Eq. 1 by plotting \(\ln([F_m - F(0)]\) vs. time and fitting a straight line to the data as described in the text.
TABLE 1. Comparison of FRAP\textsuperscript{1} for Metaphase Spindles in Sea Urchin Embryos and BSC-1 Culture Cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Temperature (°C)</th>
<th>Beam Diameter (μm)</th>
<th>t\textsubscript{50} (sec)\textsuperscript{2}</th>
<th>R%\textsuperscript{2}</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sea urchin\textsuperscript{3}</td>
<td>23–25</td>
<td>4.5</td>
<td>16 ± 4.5</td>
<td>88 ± 11</td>
<td>12</td>
</tr>
<tr>
<td>BSC-1\textsuperscript{4}</td>
<td>32</td>
<td>4.5</td>
<td>41 ± 10</td>
<td>69 ± 15</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>2.8</td>
<td>43 ± 13</td>
<td>74 ± 22</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>2.8</td>
<td>75 ± 33</td>
<td>53 ± 25</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>2.8</td>
<td>107 ± 9</td>
<td>39 ± 20</td>
<td>4</td>
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<tr>
<td></td>
<td>20</td>
<td>2.8</td>
<td>144 ± 18</td>
<td>47 ± 26</td>
<td>5</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Fluorescence redistribution after photobleaching.
\textsuperscript{2}Calculated as described in the text.
\textsuperscript{3}Wadsworth and Salmon [1986a].
\textsuperscript{4}Wadsworth and Salmon [1986d].

BSC-1 spindles at 32°C (physiological temperature) does not recover within 365 sec. Part of this unrecovered, bleached fluorescence, $R\% = (\bar{F}_w - \bar{F}_0)/(\bar{F}_w - \bar{F}_0)$, may be the result of bleaching a significant fraction of the total cell tubulin, particularly when the half-spindle fluorescence is bleached greater than 50% in the BSC-1 cells [Saxton et al., 1984]. A large fraction of the unrecovered fluorescence is probably also due to the slow exchange of tubulin within the differently stable kinetochore fiber microtubules. We have not yet tried to measure the rate of fluorescence recovery from the very slow phase.

Both $t_{50}$ and $R\%$ are sensitive functions of temperature [Wadsworth and Salmon, 1986a] (Table 1). This suggests that the microtubules which persist at lower temperatures are more stable than the majority of microtubules at physiological temperature. We are currently exploring further the energetics of spindle microtubule dynamics.

Our spindle FRAP results thus far have shown that at steady state, tubulin throughout a great majority of spindle fiber microtubules (probably the labile nonkinetochore microtubules) exchanges with the tubulin pool with a halftime of about 20 sec at physiological temperature, an extremely rapid rate.

**FRAP OF OTHER MICROTUBULE ARRAYS**

The rate and percentage of FRAP was found initially by Saxton et al. [1984] to depend on the type of microtubule in living cells, and they are different for microtubules in vivo and in vitro [Leslie et al., 1984]. Figure 9 compares the FRAP for a BSC-1 metaphase spindle and a midbody complex, and Figure 10 compares the FRAP for the interphase CMTc of BSC-1 cells and for a preparation of reassembled microtubules in vitro. The $t_{50}$ we have measured using PMT-FRAP for the CMTc is 200 ± 61 sec at 32°C, and $R\% = 77 ± 17 (N = 15)$. This value, similar to the half-time obtained by Saxton et al. [1984] using Video-FRAP, is about ten times greater than the half-time for spindle FRAP. The incomplete recovery of bleached fluorescence suggests that about 25% of the microtubules in the CMTc are much more stable than the majority of microtubules.

Steady-state incorporation of tubulin into either midbody microtubules or reassembled microtubules in vitro is extremely slow (Figs. 9, 10). Since microtubules of the midbody are highly resistant to microtubule depolymerization conditions [Salmon et al., 1976], little FRAP is expected. Although in vitro microtubules in reassembly buffer are rapidly depolymerized by cool-
ing, as are labile microtubules in the living cell, FRAP is extremely slow compared to the majority of microtubules in the spindle and CMTC.

Leslie et al. [1984] found a similar result for microtubules assembled in vitro by nucleated growth from purified centrosomes. The microtubules were grown from purified brain microtubule protein and immobilized at their (−) ends by the attachment of the centrosome to the coverslip [Evans et al., 1985]. The whole aster was photobleached, and less than 10% of the initial bleached fluorescence was recovered after 60 min.

For self-assembled microtubules in suspension, the rate of FRAP decreases with time, after the initiation of assembly. At early stages of assembly, FRAP that is not associated with subunit diffusion appears due to the diffusion of whole microtubules, which becomes slower as the average length of a microtubule in the preparation becomes longer (apparently because of end-to-end microtubule annealing) at steady state and approaches zero when gelation occurs [Pryer, Wadsworth, and Salmon, unpublished observations]. FRAP has been used successfully to analyze actin polymerization dynamics in vitro [Lanni et al., 1981], and we are currently exploring further this use of FRAP to characterize the dynamics of in vitro microtubule assembly. Qualitatively, FRAP of microtubules reassembled in standard in vitro buffers appears to result from the mobility of whole microtubules, and not from the rapid turnover of tubulin throughout the microtubules as appears to occur for labile microtubules in vivo.

CONTROLS AND CORRELATIONS

The major assumption in our experimental approach is that FRAP measures the steady-state dynamics of tubulin within microtubules in the living cells. This assumption appears justified for several reasons. The rate and extent of FRAP are similar when a small region of the spindle is bleached, when a bar is bleached across the half-spindle, and when the whole half-spindle is bleached. Thus the movement of whole microtubules is not responsible for the FRAP. Photobleaching does not alter spindle birefringent retardation (Fig. 6) and does not change microtubule assembly in interphase CMTC as viewed by high-voltage electron microscopy or antitubulin immunofluorescence methods [Saxton et al., 1984]. This evidence supports our assumption that FRAP measures steady-state tubulin incorporation into microtubules, not a reassembly response to irradiation damage such as that produced by UV microbeam irradiation [Forer, 1965; Leslie and Pickett-Heaps, 1983]. Consistent with this conclusion is the observation that photobleaching half-spindle fluorescence several times during mitosis does not noticeably alter the rate of mitosis or cytokinesis [Salmon et al., 1984c, Saxton et al., 1984].
Leslie et al. [1984] have extensively studied the effects of photobleaching on microtubule assembly in vitro. Extensive photobleaching of DTAF-tubulin that has been polymerized into microtubules in vitro did not cause microtubules to depolymerize or fragment. No effects on microtubule polymerization and little photoactivated crosslinking of proteins were observed under conditions typical for laser photobleaching experiments with living cells: 0.1–1-sec bleach periods, less than 70% bleach, in a reducing environment containing 2 mM glutathione, and a ratio of DTAF-tubulin to unlabeled tubulin in the microtubules of 0.1 or less. However, photobleaching was found to kill polymerization activity and induce crosslinking of tubulin and microtubule-associated proteins for longer bleaching periods in nonreduced buffers at higher ratios of DTAF-tubulin to unlabeled tubulin.

The in vivo FRAP results correspond nicely to other data on spindle microtubule dynamics. First, the incorporation of DTAF-tubulin in microtubules to steady-state levels should occur at rates and extents corresponding to the FRAP characteristics following microinjection of DTAF-tubulin into cells. There is a direct correspondence between the rate of FRAP and the rate of “forward” incorporation [Hamaguchi et al., 1985; McIntosh et al., 1986; Saxton et al., 1984; Wadsworth and Salmon, 1986a]. Visually, the magnitude of fluorescence in the spindle appears to achieve steady-state levels within 1–2 min following injection of DTAF-tubulin into metaphase BSC-1 cells. Within 10 min following injection, the magnitude of fluorescence is nearly identical to the fluorescence obtained after spindles are initially disassembled by cooling and then reassembled by rewarming to 37°C (Fig. 11). Given available evidence, there is a quantitative correlation between the rate of FRAP and the rate of uptake of tubulin into the spindle and CMTC, and a qualitative correlation in the extent.

The second correlation is that the non-steady-state rate and extent of microtubule disassembly, which result when steady-state assembly is abruptly blocked, should occur at rates and extents equal to or greater than the kinetics of FRAP. We have used colchicine or other drugs, such as nocodazole, which bind to the same site on tubulin as does colchicine, to abruptly block microtubule assembly in living cells [Salmon et al., 1984a; Cassimeris et al., 1985], and the approximate half-times for depolymerization are given in Table 2 for spindles, CMTC, and mid-bodies in BSC-1 cells. There is a direct correlation with the rate of FRAP for each type of microtubule array. For both spindles and the CMTC there is a minor fraction of microtubules which are differentially stable as predicted by the incomplete recovery of fluorescence after photobleaching, but the percentage of the total initial population of microtubules is not known yet.

**ANALYSIS OF MICROTUBULE ASSEMBLY MODELS**

The FRAP results have demonstrated that a minor population of microtubules in the mitotic spindle (probably the kinetochore microtubules) and in the CMTC is differentially stable, similar to the midbody microtubules. This

![Diagram](image)

**Fig. 11.** A demonstration that tubulin turnover is rapid throughout the nonkinetochore spindle and astral microtubules. A prometaphase BSC-1 cell was injected with DTAF-tubulin. After 10 min at 37°C the fluorescence was 9220 pps, measured by using an attenuated 2.5-μm-diameter Gaussian laser beam passing through the central region of a half-spindle. The cell was cooled to 4°C for 10 min to depolymerize completely the nonkinetochore and astral microtubules and then rewarmed to 37°C to induce reassembly. When fluorescence in the half-spindle was measured 10 min later, it was slightly higher than it had been before cooling.
TABLE 2. Comparison for Different Microtubule Arrays of the Rate of FRAP and the Rate of Depolymerization Induced by Nocodazole or Cooling

<table>
<thead>
<tr>
<th>Microtubule array</th>
<th>Rate or Half-time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FRAP (32°C)1</td>
</tr>
<tr>
<td>BSC-1 spindle (non-k)</td>
<td>&lt; 40</td>
</tr>
<tr>
<td>BSC-1 CMTC</td>
<td>200</td>
</tr>
<tr>
<td>BSC-1 Midbody</td>
<td>Very slow</td>
</tr>
<tr>
<td>In vitro centrosome aster</td>
<td>Very slow⁴</td>
</tr>
<tr>
<td>In vitro gelled microtubules</td>
<td>Very slow⁴</td>
</tr>
</tbody>
</table>

¹This report; Saxton et al. [1984].
²Salmon et al. [1984a].
³Cassimeris et al. [1985].
⁴(37°C), Leslie et al. [1984].

differential stability was predictable by their stability to other microtubule depolymerization agents. Why these microtubules are stable is of considerable interest, but the underlying mechanism is unknown. There is evidence that microtubules can be stabilized by "capping" both ends with a complex that blocks tubulin association-dissociation [Salmon et al., 1976; Rieder, 1982; Leslie and Pickett-Heaps, 1983; Mitchison and Kirschner, 1984c]. The binding of microtubule-associated proteins along the length of microtubules may promote stability, and stabilization may also depend on ATP-dependent reactions [Debrabander et al., 1981; Bershadsky and Gelfand, 1981]. Preliminary evidence shows that the percentage of FRAP of both the spindle and the CMTC is substantially reduced when BSC-1 cells are treated with inhibitors of ATP production [Wadsworth and Salmon, 1986c].

What is the dominant steady-state pathway of microtubule assembly for the dynamic majority of microtubules in the spindle and CMTC? We will consider the spindle first and then the CMTC, because the conclusions are similar but the details are slightly different. The FRAP results support two major conclusions about the steady-state assembly of the labile nonkinetochore spindle microtubules: (1) tubulin throughout these microtubules is exchanged with tubulin in the cytoplasmic pool with a half-time of about 20 sec at physiological temperature, and (2) tubulin incorporates into microtubules at sites throughout the half-spindle.

The rapid and extensive FRAP of the nonkinetochore spindle microtubules clearly demonstrates that simple equilibrium exchange of tubulin subunits at microtubule ends is not the dominant pathway of steady-state microtubule assembly [Salmon, 1984; Salmon et al., 1984c]. Figure 12 diagrams the incorporation of new tubulin subunits at the (+) end of a microtubule after photobleaching. The mean length of incorporation, $\bar{x}$, is given by

$$\bar{x} = \frac{2k_d}{\pi} / 1.634 ~ \mu m$$

where $k_d$ is the tubulin dissociation, $t$ is time after photobleaching, and 1.634 is the approximate number of tubulin dimers/μm of microtubule [Hill and Kirschner, 1982; Zeeburg et al., 1980]. For the sea urchin spindle, the $k_d$ has been estimated to be about 1,000 dimers/sec for an average steady-state microtubule length of 5.5 μm [Salmon et al., 1984a]. From Eq. 3, the time required to exchange all tubulin dimers in a microtubule that is 1 μm long is 1,051 sec, and for a microtubule 5.5 μm long it is 31,833 sec. The exact distribution of microtubule lengths over the 10–12-μm-long half-spindle is not known, but these times are at least two orders of magnitude too long compared to the 16-sec half-time of spindle FRAP observed. The same argument can be made for the BSC-1 spindle.

Poleward treadmilling of microtubules as proposed by Margolis and Wilson (Fig. 2B) predicts initial recovery of fluorescence proximal to the metaphase plate region, translocation of the bleached region toward the pole during fluorescence recovery, and continuous poleward flow of tubulin poleward due to the "head to tail" steady-state assembly of the microtubules. In actuality, when the whole half-spindle is photobleached, fluorescence recovery appears uniform throughout the half-spindle (Fig. 6) [Salmon et al., 1984c; Saxton et al., 1984], and no poleward translation of a narrow bleached bar pattern occurs during fluorescence recovery (Fig. 7) [Wadsworth and Salmon, 1986a,c, 1986d]. We have tested for poleward flow of tubulin by comparing the rate of FRAP for circular beams of different diameters. If FRAP is due to flow, then the half-time of fluorescence recovery should be proportional to the beam diameter. We found that the rate and extent of FRAP are independent of beam diameter (Table 1) [Wadsworth and Salmon, 1986a,c, 1986d]. The FRAP of nonkinetochore spindle microtubules is not

Fig. 12. Sketch of the incorporation of tubulin subunits into a free end of microtubule at steady-state by simple equilibrium association-dissociation events. $\bar{x}$ is the mean distance from the end of a microtubule in which tubulin dimers are replaced as a function of time.
of the distribution of a rhodamine-labeled antibody to tubulin, which stains all microtubules, to corresponding photographs of the distribution of insoluble DTAF-tubulin. Only DTAF-tubulin incorporation at the ends of microtubules distal from the centrosome and growth of new microtubules from the centrosome were reported to occur over several minutes following injection of the DTAF-tubulin into the cell. Because injection of DTAF-tubulin into the cell raises the concentration of total tubulin within the cell, these results do not strictly represent incorporation at steady state. Nevertheless, these initial findings do indicate that tubulin incorporation does not occur at sites along the length of cytoplasmic microtubules at steady state. Similar studies for microtubules within the mitotic spindle are not yet available.

We have recently analyzed the dynamics of cytoplasmic microtubule depolymerization in human monocytes when assembly is abruptly blocked with the tubulin-binding drug nocodazole [Cassimeris et al., 1985]. Cells on coverslips were fixed at various times after treatment with nocodazole to block assembly abruptly and then extracted and stained for immunofluorescence by using tubulin antibodies. All microtubules extend from the centrosome within a cell and they were traced from images on a video monitor generated by using an SIT video camera and focusing up and down in the microscope. Individual microtubules were found to depolymerize rapidly once disassembly was initiated, but initiation occurred very asynchronously among the population of microtubules. This behavior is directly predictable from the dynamic instability model.

An interesting correlation occurs between the differences in the rate of FRAP of cold-labile microtubule arrays and their sensitivity to colchicine or colchicine-like drugs (Table 2). Nonkinetochore spindle and astral microtubules and the CMTC are rapidly depolymerized by cooling, as are reassembled microtubules in vitro [Purich and Kristofferson, 1984]. However, these microtubules have significantly different responses to colchicine which correspond to the large differences in FRAP in vivo and in vitro (Table 2). Colchicine binds to the tubulin dimer at a site not exposed when the dimer is assembled into the wall of a microtubule [reviewed in Salmon et al., 1984a]. The colchicine-tubulin dimer complex (TC) is the active agent which promotes microtubule depolymerization in vivo [Wadsworth and Salmon, 1986a; McIntosh et al., 1986], apparently by interfering with microtubule elongation. In vitro, TC blocks microtubule elongation [Dustin, 1984], but rapid and extensive depolymerization of microtubules does not occur as it does in vivo. An interesting explanation for the difference in response to colchicine in vitro and in vivo is that in the living cell conditions favor strongly the dynamic instability mode of steady-state microtubule assembly, whereas in the in vitro reassembly buffers the simple equilibrium mode is strongly favored. In vivo, TC complex blocks microtubule elongation, but normal,

accounted for by the treadmilling model, which means that treadmilling is not likely to be the dominant pathway of steady-state microtubule assembly in the spindle.

Either the dynamic equilibrium model, initially proposed by Inoué [Inoué and Sató, 1967; Inoué and Ritter, 1975] or the dynamic instability model proposed by Mitchison and Kirschner [1984a-c] (Fig. 2b) could account for the rapid, uniform, and extensive FRAP of spindles [Salmon et al., 1984c; Wadsworth and Salmon, 1986a, 1986d]. The dynamic equilibrium model proposes that tubulin enters and leaves the microtubule polymer at sites along the length of the microtubule, for which there is some evidence from cryo-electron microscopy [Mandelkow and Mandelkow, 1985]. Microtubules could also be continuously breaking and reannealing along their length [Salmon et al., 1984c]. The dynamic instability model proposes that tubulin exchanges only at the end of a microtubule, the (+) end of microtubules grown from the centrosomes and spindle poles. Rapid and extensive exchange of tubulin within the nonkinetochore spindle microtubules could occur if these microtubules were continuously, but asynchronously, going through alternating, relatively persistent phases of rapid growth and rapid shortening as Mitchison and Kirschner propose. They suggest that the transition between growth and shortening is regulated by the hydrolysis of GTP ligated to tubulin after incorporation into the microtubule lattice. When GTP hydrolysis "catches up" with elongation (Fig. 2B), subunit association is inhibited, and rapid depolymerization ensues [Carlier et al., 1984; Mitchison and Kirschner, 1984a-c]. Experiments to determine sites of tubulin incorporation at the resolution of single microtubules are required to distinguish between the dynamic equilibrium and dynamic instability models.

The average length of microtubules in the interphase CMTC of BSC-1 cells appears, from immunofluorescence micrographs of cells stained with antibodies to tubulin, to be greater than 20 μm [Vandre et al., 1984]. The same arguments can be made for the pathways of tubulin incorporation into the microtubules of the interphase CMTC as have been made above for the nonkinetochore spindle microtubules. FRAP is too fast to be the result of simple equilibrium exchange of tubulin subunits at microtubule ends, and there is no evidence for translation of the bleached region during FRAP [Saxton et al., 1984]. The same conclusion was reached from FRAP studies of the CMTC with a fluorescent analog to a microtubule-associated protein, MAP2, purified from mammalian brain [Scherson et al., 1984].

Recently, Soltys and Borisy [1985] have provided evidence that DTAF-tubulin does not incorporate at sites along the length of microtubules of the CMTC. Interphase tissue culture cells were injected with DTAF-tubulin and then fixed and extracted at various times after injection. To locate regions of DTAF-tubulin incorporation into microtubules they compared photographs
rapid depolymerization continues, while TC also blocks new microtubule growth. Consequently, there is a net loss of microtubule polymer. In vitro, the conditions which produce the rapid, catastrophic depolymerization of microtubules in vivo are absent, and simple equilibrium exchange of tubulin subunits dominates the dynamics of the microtubules.

FUTURE CONSIDERATIONS

Fluorescent analog cytochemistry and FRAP have shown that most microtubules in the spindle and CMTD are much more dynamic than indicated from previous studies of microtubule assembly in vitro. Attention is now focused on the dynamic instability model of microtubule assembly, which does provide simple explanations for the complex behavior of microtubules within the living cell [Mitchison and Kirschner, 1984c]. Approaches similar to the recent studies of Sollys and Boris [1985] should provide clear information on sites of tubulin incorporation into the various types of spindle microtubules at the different stages of mitosis by using secondary labels for DTAF-tubulin, or other tubulin analogs, for electron microscopy. Of particular interest are the sites of tubulin incorporation and dissociation for the differentially stable kinetochore fiber microtubules during chromosome movements. If the dynamic instability model correctly explains the dynamics of the spindle microtubules, then microtubule growth is much faster than expected from microtubule growth rates measured in vitro for tubulin concentrations comparable to the concentration of tubulin in cells [Salmon et al., 1984c]. It would be useful to know what oligomer is the polymerizing subunit of tubulin in vivo. Once the dynamics of microtubules are established, then valuable information on the dynamics of microtubule-associated proteins can be achieved by using FRAP approaches [McIntosh et al., 1986; Scherson et al., 1984]. These approaches will be valuable, in particular, for determining the molecular and physiological basis of regulation of microtubule assembly in living cells.

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