Dynamic Confocal Imaging of Mitochondria in Swimming Tetrahymena and of Microtubule Poleward Flux in Xenopus Extract Spindles

Paul Maddox1, Arshad Desai2, E. D. Salmon1, T. J. Mitchison3, Karen Oogema2, Tarun Kapoor3, Brian Matsumoto4, and Shinya Inoué (Marine Biological Laboratory, Woods Hole, Massachusetts 02543)

We report here the use of a real-time, spinning-disk confocal scanning unit (Yokogawa Electric CSU-10; 1) mounted on a Leica microscope to investigate the internal dynamics of fluorescently labeled microtubules in mitotic spindles. We aimed, in particular, to test the spatial and temporal resolution and the optical sectioning capability of the microscope setup.

Live Tetrahymena were stained with the fluorescent probe, Mito Tracker Green FM (Molecular Probes, Cat#M7514). Excitation was provided by the 488-nm line from a Krypton-Argon gas laser, which was passed through the spinning disk in the CSU-10. Green fluorescence from the mitochondria was collected back through the microscope to investigate the internal dynamics of fluorescently labeled microtubules in mitotic spindles. We aimed, in particular, to test the spatial and temporal resolution and the optical sectioning capability of the microscope setup.

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1 University of North Carolina, Chapel Hill, North Carolina.
2 EMBL, Heidelberg, Germany.
3 Harvard Medical School, Boston, Massachusetts.
4 University of California, Santa Barbara, California.
back into mitosis by addition of a small amount of CSF-arrested extract. The resulting cytoplasmic extract contained metaphase spindles (sperm nuclei were added for a source of chromatin) that were easily observed by addition of fluorescent labels (DAPI for DNA and x-rhodamine-labeled purified tubulin for microtubules).

In previous studies, photo-uncaging of fluorescently labeled tubulin in spindles was used to show that there is a bulk flow of polymer originating near the spindle mid-zone and progressing toward each pole region. This flow, termed microtubule poleward flux, was found to move at about 2 μm/min in Xenopus extract spindles; this is the same rate as the chromosome-to-pole movement during anaphase in this system. Therefore, it was proposed that poleward microtubule flux could be the force generator for anaphase A chromosome separation (3).

The object of this study was to image the dynamics of individual microtubules within spindles. Because the spindles are about 40-μm long and contain thousands of microtubules, conventional wide-field fluorescence micrographs lack fine structural detail (Fig. 1C). Lowering the ratio of labeled to unlabeled tubulin subunits in the cytoplasmic extract leads to a low density of label along the polymerized microtubule lattice (4, 5). This distribution in turn creates bright speckles of fluorescence that serve nicely as internal fiduciary marks along the lattice. One is then able to visualize either polymerization or depolymerization, as well as the flux of subunits within polymers and the movement of whole polymers (5). A problem with this technique, however, is that the small amounts of labeled tubulin (less than 0.1%) make visualization of gross spindle morphology difficult (Fig. 1D).
Mitotic Xenopus extract spindles with more than 0.1% of the total tubulin pool labeled were observed with the CSU-10 real-time confocal unit, which was coupled to an Orca cooled CCD camera (Hamamatsu Photonics, Bridgewater, New Jersey). With this system, the dynamics of microtubules in the middle of spindles could be seen, as could the fibrous structure of the polymer mass (Fig. 1A). MetaMorph software (Universal Imaging Corp., West Chester, Pennsylvania) was used to control the CSU-10 shutter as well as the camera. This allowed time-lapse imaging for up to 10 min, exposing the sample for 0.75 s to the 568-nm line of the laser every 10 s, with little photobleaching. A Leica 100×/1.3 NA objective lens was used to increase light-gathering efficiency, as well as to match magnification to the resolution limit imposed by the pixel size of the CCD chip.

Analysis of the time-lapse images revealed that microtubules add subunits to their plus ends while losing subunits from their minus ends near the spindle poles. Plus ends were located throughout the half spindle, and the movement of fluorescent speckles was often seen traversing the entire spindle (Fig. 1B). Preliminary measurements of the poleward movement of fluorescent speckles indicated a rate of about 2 μm/min, corresponding nicely to previous values (4, 5). The confocal images revealed that microtubules were clustered into bundles within the spindle (Fig. 1A). Also clearly visible were fluorescent-speckled microtubules extending out and away from the main spindle (Fig. 1A, arrows), often for great lengths (up to 50 μm). Poleward speckle movement was detected in this population of microtubules, indicating that plus ends do not have to be within the spindle to facilitate poleward microtubule flux and disassembly near the poles.

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Effects of Vanadate on Actin-dependent Vesicle Motility in Extracts of Clam Oocytes

Torsten Wöllert¹, Ana S. DePina, and George M. Langford
(Dartmouth College, Hanover, New Hampshire 03755)

The effect of sodium orthovanadate on actin-based vesicle transport (1) was examined in extracts obtained from oocytes of the surf clam Spisula solidissima. Vanadate, an analog of inorganic phosphate (Pι), inhibits microtubule-based motors at low concentrations (5–50 μM) without affecting actin-based motors, and inhibits tyrosine phosphatases selectively at higher concentrations (0.5–1.0 mM) (2). The higher concentrations of vanadate were used to determine whether myosin-dependent vesicle transport in clam oocyte extracts is regulated by tyrosine phosphatase activity.

Clam oocyte extracts were incubated at 18°C for 45 min and then treated with 0.5 μM rhodamine-phalloidin to fluorescently label actin filaments, and 0.5 or 1.0 mM vanadate to inhibit tyrosine phosphatases. Vesicle transport on actin filaments was monitored for 60 min by video microscopy, and images of the actin filaments on the coverslip surface were recorded at regular intervals by epi-fluorescence microscopy. Vesicle transport on actin filaments was unaffected by vanadate during these experiments. Both motile activity and the velocity of vesicle transport in the control and the treated extracts were the same. However, the actin filament network in the extracts was altered significantly by vanadate treatment. Vanadate reduced actin filament nucleation and stimulated the formation of actin bundles. In the control samples at 15 and 60 min (C in Fig. 1), single actin filaments formed on the coverslip surface and bundles were rarely seen. In the vanadate-treated samples (V in Fig. 1), a 3-D network of bundles formed, and the density of the network increased over time (60 min). The bundles of actin filaments in the vanadate-treated extracts supported bi-directional movement of vesicles. The actin filament bundles in these extracts were disrupted by treatment with Triton X-100 (0.05 and 0.1%), indicating that actin assembly may be dependent on the presence of membranes in the extracts, as observed in Xenopus oocyte extracts (3, 4). These results suggest that some of the membranes in these extracts have the ability to nucleate actin filament assembly, although vesicle transport was driven by myosin motors.

The inhibitory effect of vanadate on tyrosine phosphatases is well established (2, 5), and the actin filament bundle formation observed in these extracts was most likely due to an increase in actin cross-linking activity upon inhibition of tyrosine phosphatases by vanadate. However, vanadate can influence many different activities in cells when used at high concentration. At millimolar concentrations, it can inhibit the myosin motor by forming a stable complex with ADP (6), but under our assay condition, in the presence of ATP, formation of myosin-ADP-Vι is slow, with an inhibition t1/2 of 1 h (7). Therefore, the slow formation of the inhibitory complex explains the failure to inhibit myosin-dependent vesicle transport in clam oocyte extracts. Vanadate has the potential to inhibit other activities requiring ATP, including Na+/K+ ATPase (8), acid and alkaline phosphatases (9, 10), phosphofructokinase (11), and adenylate kinase (12), as well as actin polymerization (13). The complexity of the vanadate effect

¹ University of Rostock, Rostock, Germany.


Literature Cited