Published in final edited form as:

Methods Cell Biol. 2010 ; 97: 81–90. doi:10.1016/S0091-679X(10)97005-2.

Photoactivatable Green Fluorescent protein-Tubulin

U. Serdar Tulu* and Nick P. Ferenz†

*Department of Biology, Duke University, Durham, North Carolina

†Precept Medical Communications, Berkeley Heights, New Jersey 07922 and Department of Biology, University of Massachusetts, Amherst, Massachusetts 01003

Abstract

Direct observations of live cells expressing fluorescently tagged tubulin have led to important advances in our understanding of mitosis. A limitation of this approach is that all of the cells' microtubules are fluorescent and thus observation of the behavior of specific subsets of microtubules is precluded. To address this problem, we have tagged tubulin with a photoactivatable variant of green fluorescent protein (PA-GFP), thereby allowing one to follow the behavior of a subset of tagged molecules in the cell. Here, we describe methods to tag and express proteins with PA-GFP, locally photoactivate the recombinant protein and record the dynamic behavior of the photoactivated molecules in live cells. Use of photoactivatable proteins is a powerful approach to examine dynamic processes, including spindle formation, in diverse cells.

I. Introduction

Understanding the behaviors of cells in an organism and molecules in a cell has been an essential motivation of many scientists in order to understand how organisms function and survive and also to find out possible ways to prevent malfunctions observed in various disease states. Live imaging in this context provides an effective and useful tool to elucidate both cellular and molecular behaviors in their natural environment. With the cloning of green fluorescent protein (GFP) from jellyfish and its expression in other organisms and cells, live imaging technology had a tremendous renaissance which allowed scientists to ask deeper and broader questions.

Vast usage of GFP in biological sciences over the last 2 decades has demonstrated that GFP tagging is a powerful technique that provides a new way to discover the workings of the microuniverse. In fact, it is widely agreed that one movie of a cell can tell more than a picture. GFP tagging provides us with a means to record cellular/subcellular dynamics in real time. With this approach we can observe subcellular architecture as well as the localization of the protein, and since molecules often localize where they function, following proteins tagged with GFP give clues about their function while the cell/organism is still alive.

In our lab, we have used GFP-tagged tubulin to study microtubule behavior in live cells. We demonstrated that cell lines permanently expressing GFP-tubulin can be generated and that the presence of the GFP-tag does not deleteriously alter microtubule behavior (Rusan *et al.*, 2001). In the cell line that we generated, 17% of the total tubulin was GFP-tagged, and the

level of unlabeled tubulin was reduced to 82% of that in the parental cell line. To determine if the GFP-tag on tubulin altered the parameters of microtubule dynamic instability, we compared dynamics in interphase cells expressing GFP-tubulin with parental cells injected with fluorescent tubulin. The results showed that the parameters of dynamic instability were not significantly different in the two cases, demonstrating that this cell line is a useful tool for measuring microtubule dynamic behavior. Moreover, the mitotic index and doubling time for these cells were not different from the parental cells. Using these cells, we showed for the first time in mammalian cells that microtubule dynamicity is increased as cells progress from interphase to mitosis and that the time microtubules spend in an attenuated state, or pause, is dramatically reduced (Rusan *et al.*, 2001).

Although these (and other similar cell lines) have been extremely useful for analysis of microtubule distribution and dynamics, the behavior of individual microtubules is difficult to follow because the microtubules are uniformly fluorescent [as viewed by wide-field or confocal fluorescence microscopy; but see (Bicek *et al.*, 2009)] and the density of microtubules can be high in many cell regions. One way to overcome these limitations is to microinject cells with lower levels of fluorescent tubulin, thereby creating microtubules with only a few fluorescent subunits, called “speckles,” at any location along the microtubule (Waterman-Storer and Salmon, 1998). The speckles can then be followed over time to reveal additional features of microtubule dynamics. Although powerful, fluorescent speckle microscopy (FSM) usually requires that cells are individually microinjected with low levels of tubulin, a time-consuming procedure. Similarly, FRAP can be used to locally reduce the fluorescence and record the kinetics of fluorescence recovery and reveal motion of the bleached regions (Bancaud *et al.*, 2010).

A. Rationale

An alternative to FSM and FRAP is the use of photoactivatable GFP. This variant of GFP has a threonine to histidine mutation at position 203 of the wild-type EGFP protein and is not fluorescent until it is exposed to ultraviolet light (Patterson and Lippincott-Schwartz, 2002). Because the UV illumination can be spatially controlled, a user-selected subset of photoactivatable variant (PA)-GFP-tagged proteins can be activated, while the remainder is left inactive and thus nonfluorescent. In the following sections, we describe the methods that we use to generate cells expressing PA-GFP-tubulin and to analyze the behavior of microtubules in these cells.

B. Expressing PA-GFP-Tubulin in Mammalian Cells

To generate PA-GFP tubulin, we replaced the EGFP sequence with the sequence encoding PA-GFP using either a pCMV or an IRES vector. We have had particularly good success when using the IRES vectors because the tagged gene, here PA-GFP-tubulin, and the selection marker are expressed from a single promoter, thus improving the selection step (Ferenz *et al.*, 2010). This is particularly important when generating PA-GFP cell lines because the tag is nonfluorescent until activated and so cloning is performed “blind.” Using a pCMV vector expressing PA-GFP-tubulin, we screened ~72 colonies, each a potential cell line. These colonies were either nonphotoactivatable or not suitable for imaging. In contrast,

using an IRES vector, we screened ~24 colonies and obtained one useful cell line expressing PA-GFP-tubulin.

Our cell line of choice for analysis of microtubule dynamics is LLC-Pk1 cells. These cells are derived from kidney epithelium and remain flat and spread throughout mitosis (Wadsworth, 2010). They are easy to culture, transfect, and image for long periods. To examine microtubule behavior in live cells we prepare clonal cell lines. Although generating such cell lines is time-consuming, it has several advantages. First, and most important, all the cells in a clonal line are identical. This is in contrast to transiently transfected cells, which show tremendous variation in expression level. Second, if transiently transfected cells are used for photoactivation, it is not possible to gauge the expression level until after the activation is performed, and thus it is difficult to perform experiments on cells with similar levels of expression. Third, several different clonal cell lines can be characterized (for example, by measuring doubling time, mitotic index, and the percentage of abnormal spindles) prior to performing experiments and the line most similar to the parental cells chosen for experimentation. Finally, stable lines are useful for live imaging of transient events. With transient transfection, catching these short moments in cells with appropriate expression levels is very difficult, if not impossible, and can always be questionable since different cells will have different amounts of expression. With stable cell lines, experiments can also be done much more efficiently because all the cells in the population are fluorescent.

To make a clonal cell line, we first transfect parental cells with PA-GFP-tubulin. For this step we find that nucleofection offers a comparatively higher level of transfection efficiency and requires a smaller amount of purified plasmid. Alternatively, cells can be transfected with any of a number of commercially available lipid-based transfection reagents.

Following transfection cells are grown for ~48 h to allow expression of the antibiotic resistance gene, and then subjected to ~2 weeks of positive selection in the presence of the appropriate antibiotic. During this period, the medium is changed frequently to remove dead and dying cells; surviving cells are subcloned as needed. Following the selection period, cells are trypsinized and seeded into 100 mm dishes at a very low density, so that individual cells generate colonies well separated from other colonies on the dish. Within 1–2 weeks of growth, these individual colonies will be visible by eye; each colony is a potential cell line. At the same time, aliquots of the antibiotic-selected cells are also frozen as a back-up in case a suitable cell line is not obtained. Cloning rings are used to harvest individual colonies, which are first transferred to individual wells of a 24 well plate, and then (after sufficient growth) transferred to two wells of a 6-well plate, one of which has a coverslip. As an alternative approach, cloning discs can be used to harvest colonies from the 100 mm plate. Cells on the coverslip can be examined for expression of the PA-GFP tubulin by photoactivation of full fields of view. Multiwell plates with a coverslip bottom are also convenient for screening large numbers of clones.

LLC-Pk1 cells are epithelial cells, which grow as colonies. For cells that do not form colonies, i.e., fibroblasts, the selected cells are counted and diluted so that approximately one cell is placed into each well of a 96-well plate. When growth of cells in each well

becomes visible, they are transferred to each well of 24-well plates for further growth. Here, cells in each well can be treated as a potential cell line. They can be analyzed thereafter as we describe above.

For the most of the lines we generated, we were more comfortable using the first method with cloning rings or cloning discs partially because epithelial cells form colonies and do not crawl as much as other cells. In addition, when diluted and plated on a 100 mm dish, cells in a colony can be seen under a simple microscope and can be visually selected in terms of their morphological appearance, an indication of whether they are a clone. Finally, if more than one cell type is detected in a stable cell line (i.e., cells with different levels of fluorescence, an indication that the line is not actually clonal), these procedures above can be performed again to obtain a clonal cell line.

Western blotting of cell extracts is used to identify potentially useful clones and to determine the expression level of the tagged protein relative to the endogenous protein. In the cell lines that we have generated ~10–20% of the total tubulin is tagged with GFP or PA-GFP (Fig. 1). At this expression level, the protein is easily detected by fluorescence microscopy and the cells show no mitotic defects due to the expression of the tagged protein (Rusan *et al.*, 2001; Tulu *et al.*, 2003).

Once one or more cell lines are obtained that expresses a useful level of the tagged protein, additional assays are performed (measurement of doubling time, mitotic index, and % of abnormal spindles) to confirm that there is no detectable effect on cell division (Fig. 1). Depending on the particular cellular processes that are under investigation other assays can be performed to show that the presence of the tagged protein has no, or minimal, effect on these events.

C. Photoactivation

We perform photoactivation experiments on a Nikon Eclipse TE300 inverted microscope. To photoactivate, light from an X-cite 120 (EXFO America, Plano, TX) or 100W mercury arc lamp in the epi-illumination pathway is passed through a D405/20 filter cube (Chroma Technology, Rockingham, VT) to obtain light of the appropriate wavelength for activation (~413 nm) (Fig. 2). A laser of 405 nm can also be used. The activation time is determined by exposing cells to 413 nm light for various intervals, and quantifying the resulting fluorescence from images acquired using identical acquisition settings (Fig. 3). The minimal exposure that results in full fluorescence activation is then used for all subsequent experiments.

The region to be photoactivated is controlled by using a slit or a pinhole in a filter wheel placed in an image plane conjugate with the specimen image plane (Fig. 2). When photoactivation of a larger area is desired, the field diaphragm in the epi-illumination pathway can also be closed to a suitable diameter and used for this purpose. For activation of spindles, we find that a slit of dimensions 25 $\mu\text{m} \times 3 \text{ mm}$ (Lennox Laser, Glen Arm, MD) generates an area of activation of ~2 μm width in the image plane. With this setup, the slit is well defined and sharp, facilitating tracking of the photoactivated area in time-lapse images. Because the slits and pinholes are in a fixed position, we rotate the sample (using a circular

rotating stage) so that an appropriate cellular area is activated. For reference, the location of the slit can be noted on an eyepiece reticle or within the image acquisition software. We use a 100 × objective lens for imaging.

We also need to take into account the fact that imaging wavelength and photoactivation wavelength are not the same; and they may not focus on the same plane (presumably due to chromatic aberration). We figured out in our system that laser light at 488 nm and epi-illumination at 405 nm have different focal distances, which we determined as ~3 μm. To be able to correct for this difference, we wrote a simple journal in MetaMorph that changes the position of objective using a piezo Z-motor [Physik Instrumente (PI), Auburn, MA] at the time of activation. Following activation, the objective is brought back to its original position and imaging is continued by the software. This procedure is essential since it is possible to image one plane and photoactivate another plane if enough care is not exercised.

Cells are imaged, prior to and following activation, using a Perkin Elmer spinning disk confocal scan head (Perkin Elmer, Waltham, MA) and a Hamamatsu Orca ER cooled charge-coupled device camera. Several models of spinning disc systems are now commercially available with lasers appropriate for activation (405 nm) and software controlled positioning of the activation light.

D. Analysis of PA-GFP Tagged Tubulin

We have used PA-GFP-tubulin to examine the dynamics of microtubules in live cells. In our experiments, cells were photoactivated using a rectangular slit or pinholes, and time-lapse images were acquired. For mitotic cells, the area of activation was perpendicular to the spindle long axis. Using this approach, we showed that a fraction of microtubules in prophase cells exhibit behavior consistent with flux, but other microtubules move much more rapidly, both toward and away from the spindle poles (Ferenz and Wadsworth, 2007). In prometaphase cells, microtubules are transported inward, toward the forming spindle, in a dynein-dependent manner (Tulu *et al.*, 2003). Microtubules in mitotic cells have also been activated in the centrosomal region, using a pinhole of appropriate diameter in the image plane. This experimental approach documented the release of microtubules from the centrosome in anaphase cells (Rusan and Wadsworth, 2005). Photoactivation of microtubules in interphase cells, using slits positioned perpendicular to the cell's long axis, has also been used to examine microtubule turnover (Fig. 4). Earlier work, using cells microinjected with tubulin chemically modified with caged fluorescein, showed that microtubules are transported in motile cells (Yvon and Wadsworth, 2000) and that antagonistic forces generated by dynein and myosin regulate microtubule turnover, organization, and movement (Yvon *et al.*, 2001). The use of cells expressing PA-GFP-tubulin to study microtubule behavior in interphase cells will greatly facilitate experimentation.

Following photoactivation, appropriate software (e.g., MetaMorph and Image J) can be used to select the photoactivated area and examine its behavior. To quantify the motion of microtubules, rectangular boxes, typically 1–2 μm in height, were positioned parallel to the spindle's long axis were placed around a photoactivated mark of interest and a montage created. Rates of motion can be obtained from the slope within the montage. The turnover of

microtubules can also be determined by measuring the dissipation of photoactivated fluorescence. Similar procedures can be used to analyze microtubule behavior in interphase cells.

II. Conclusions

In conclusion, we find that photoactivation of PA-GFP-tubulin is a powerful method to examine microtubule dynamics in live cells. Our work and that of others shows that the approach is suitable for diverse cell types and can be used to examine microtubule dynamics at all stages of the cell cycle. Moreover, photoactivation can be performed using standard laboratory equipment and data analysis is easily performed using image analysis software. Finally, a large number of proteins have already been shown to retain their native function after tagging with GFP, so substitution of PA-GFP, or other suitable photoactivatable FP, and performing photoactivation are experimentally straightforward and are likely to provide new information about protein dynamics in the physiological context of the live cell.

References

- Bancaud, A., Huet, S., Rabut, G., Ellenberg, J. Fluorescence perturbation techniques to study mobility and molecular dynamics of proteins in live cells: FRAP, photoactivation, photoconversion, and FLIP. In: Goldman, RD.Swedlow, JR., Spector, DL., editors. *Live Cell Imaging: A laboratory manual*. Cold Spring Harbor Press; Cold Spring Harbor, NY: 2010.
- Bicek AD, Tuzel E, Demtchouk A, Uppalapati M, Hancock WO, Kroll DM, Odde DJ. Anterograde microtubule transport drives microtubule bending in LLC-PK1 epithelial cells. *Mol Bio Cell*. 2009; 20:2943–2953. [PubMed: 19403700]
- Ferenz N, Ma N, Lee WL, Wadsworth P. Imaging protein dynamics in live mitotic cells. *Methods*. 2010; 51(2):193–196. In press. [PubMed: 20085816]
- Ferenz NP, Wadsworth P. Prophase microtubule arrays undergo flux-like behavior in mammalian cells. *Molec Biol Cell*. 2007; 18:3993–4002. [PubMed: 17671163]
- Patterson GH, Lippincott-Schwartz J. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science*. 2002; 297:1873–1877. [PubMed: 12228718]
- Rusan NM, Fagerstrom C, Yvon AC, Wadsworth P. Cell cycle dependent changes in microtubule dynamics in living cells expressing GFP-alpha tubulin. *Mol Biol Cell*. 2001; 12:971–980. [PubMed: 11294900]
- Rusan NM, Wadsworth P. Centrosome fragments and microtubules are released and transported asymmetrically Away From division plane in anaphase. *J Cell Biol*. 2005; 168:21–28. [PubMed: 15631988]
- Tulu US, Rusan N, Wadsworth P. Peripheral, non-centrosome-associated microtubules contribute to spindle formation in centrosome containing cells. *Curr Biol*. 2003; 13:1894–1899. [PubMed: 14588246]
- Wadsworth, P. Studying mitosis in cultured mammalian cells. In: Goldman, RD.Swedlow, JR., Spector, DL., editors. *Live Cell Imaging: A laboratory manual*. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 2010. p. 571–582.
- Waterman-Storer C, Salmon ED. How microtubules get fluorescent speckles. *Biophys J*. 1998; 75:2059–2069. [PubMed: 9746548]
- Yvon AC, Gross DJ, Wadsworth P. Antagonistic forces generated by myosin II and cytoplasmic dynein regulate microtubule turnover, movement, and organization in interphase cells. *Proc Natl Acad Sci USA*. 2001; 15:8656–8661.
- Yvon AC, Wadsworth P. Region specific microtubule transport in motile cells. *J Cell Biol*. 2000; 151:1003–1012. [PubMed: 11086002]

Fig. 2.
Schematic diagram of the microscope used for photoactivation.

Fig. 3. Determination of the optimum time for activation of PA-GFP-tubulin. Phase contrast and fluorescence images of an interphase LLC-Pk1 cell expressing PA-GFP-tubulin. Exposure to 405 nm light is calculated cumulatively (i.e., exposure times on each panel show the total time that the cell was exposed to UV light). Images are acquired with the same settings after each exposure. Time of exposure (seconds) to activation light is shown in the upper left. Bar = 10 μ m.

Fig. 4. Microtubule dynamics in an interphase LLC-Pk1 cell expressing PA-GFP-tubulin. The area of activation is shown by the magenta overlay in the phase image (left). Images from a time series acquired following activation; time following activation is shown at the top of each image (mins: seconds). Bars = 10 μ m.