Video Article

Live Imaging to Study Microtubule Dynamic Instability in Taxane-resistant Breast Cancers

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Abstract

Taxanes such as docetaxel belong to a group of microtubule-targeting agents (MTAs) that are commonly relied upon to treat cancer. However, taxane resistance in cancerous cells drastically reduces the effectiveness of the drugs' long-term usage. Accumulated evidence suggests that the mechanisms underlying taxane resistance include both general mechanisms, such as the development of multidrug resistance due to the overexpression of drug-efflux proteins, and taxane-specific mechanisms, such as those that involve microtubule dynamics.

Because taxanes target cell microtubules, measuring microtubule dynamic instability is an important step in determining the mechanisms of taxane resistance and provides insight into how to overcome this resistance. In the experiment, an *in vivo* method was used to measure microtubule dynamic instability. GFP-tagged α-tubulin was expressed and incorporated into microtubules in MCF-7 cells, allowing for the recording of the microtubule dynamics by time lapse using a sensitive camera. The results showed that, as opposed to the non-resistant parental MCF-7_{CC} cells, the microtubule dynamics of docetaxel-resistant MCF-7_{TXT} cells are insensitive to docetaxel treatment, which causes the resistance to docetaxel-induced mitotic arrest and apoptosis. This paper will outline this *in vivo* method of measuring microtubule dynamic instability.

Video Link

The video component of this article can be found at https://www.jove.com/video/55027/

Introduction

The leading cause of breast cancer mortality is through metastasis[1,2]. Taxanes, such as docetaxel and paclitaxel, are currently used as first-line regimens in the treatment of metastatic breast cancer[2,3,4,5,6]. They are part of a group of microtubule-targeting agents (MTAs) that disrupt microtubule dynamics. However, one of the greatest challenges to using taxanes in curative therapy is the development of taxane resistance in cancer cells, which leads to disease recurrence[7]. Drug resistance accounts for more than 90% of all deaths among patients with metastatic breast cancer[7].

Microtubules are formed by the polymerization of α- and β-tubulin heterodimers[8,9]. The precise regulation of microtubule dynamics is important for many cellular functions, including cell polarization, cell cycle progression, intracellular transport, and cell signaling. Dysregulation of microtubules and their dynamics will disrupt cell function and result in cell death[10,11]. Depending on how they cause this dysregulation, MTA drugs can be classified as either microtubule stabilizing agents (*i.e.,* taxanes) or microtubule-destabalizing agents (*i.e.,* vinca alkaloids or colchicine-site binding agents)[20]. Despite their opposite effects on microtubule mass, at a sufficient dosage, both classes can kill cancer cells through their effects on microtubule dynamics[21].

Taxanes function primarily by stabilizing the microtubule spindle[12], leading to chromosomal misalignment. The subsequent perpetual activation of the spindle assembly checkpoint (SAC) arrests the cell in mitosis. Prolonged mitotic arrest then causes apoptosis[13,14]. Taxane interacts with microtubules through the taxane binding site on β-tubulin[8,15], which is only present in assembled tubulin[16].

Multiple mechanisms for taxane resistance have been proposed[9,17]. These mechanisms include both general multidrug resistance due to the overexpression of drug-efflux proteins and taxane-specific resistance[5,9,18,19]. For example, taxane-resistant cancer cells may have altered expression and function of certain β-tubulin isotypes[5,9,19,20,21,22,23]. By using an *in vivo* method to measure microtubule dynamic instability, we show that, when compared to non-resistant, parental MCF-7_{CC} cells[17], the microtubule dynamics of docetaxel-resistant MCF-7_{TXT} cells are insensitive to docetaxel treatment.

To better understand the function of MTAs and the exact mechanism of taxane-resistance in cancerous cells, it is essential to measure microtubule dynamics. Here, we report an *in vivo* method of doing so. By using live imaging in combination with the expression of GFP-tagged

tubulin in cells, we can measure the microtubule dynamics of MCF-7_{TXT} and MCF-7_{CC} cells with and without docetaxel treatment. The results can help us design more effective drugs that can overcome taxane resistance.

Protocol

1. Preparing the Cells for Live Imaging

1. **Cell culture and seeding**
   1. Use MCF-7 breast cancer cells selected for resistance to docetaxel (MCF-7_{TXT}) and their non-resistant parental cell line (MCF-7_{CC}). The detailed selection process and the characterization of these selected cell lines were described previously[24].
   2. Grow all cells in 10 cm culture dishes at 37 °C in a medium composed 90% of Dulbecco's modified Eagle's medium (DMEM) and 10% of fetal bovine serum (FBS) and supplemented with non-essential amino acids. Maintain the medium in a 5% CO_2 atmosphere. Maintain MCF-7_{TXT} cells at 5 nM docetaxel.
   3. Seed cells on coverslips for live imaging. Sterilize 24 mm poly-L-lysine-coated glass coverslips by rinsing them with 70% alcohol and then exposing them to UV overnight. Place the coverslips into a 6-well culture plate of 35 mm. Place one coverslip in each well.
   4. Remove the medium from the 10-cm culture dish and wash the cells with PBS. Add 0.5 mL of trypsin-EDTA to the dish to detach the cells from the dish. Add 5 mL of culture medium to the dish to neutralize the trypsin following the detachment of the cells.
   5. Count the cells with a hemocytometer to determine the number of cell per unit volume.
   6. Add approximately 10^5 cells to each well based on the cell number per unit volume. After shaking it gently, put the plate back into the incubator to allow the cells to attach to the coverslips.

2. **Expression of GFP-tagged α-tubulin**
   1. To assay microtubule dynamics, transduce the cells with GFP-tagged α-tubulin with the commercial GFP transduction control (*e.g.,* BacMam) according to the manufacturer's protocol.
   2. Culture the cells in the well for 36 h in a 37 °C cell culture incubator to allow complete adhesion and 60% confluence.
   3. Calculate the appropriate volume of GFP-tagged α-tubulin to add to each well, according to the following equation:

$$\text{Volume of GFP-tubulin} = \frac{\text{number of cells x desired PPC}}{1 \times 10^8 \text{ GFP-tubulin particles/mL}}$$

      The number of cells is the estimated total number of cells in the well at the time of labeling and the desired PPC is the number of particles per cell.
      NOTE: Seeding the MCF-7 cells should have doubled the cell count from 10^5 to 2 x 10^5 with a desired PPC of 20, the volume needed for each coverslip is therefore 40 µL. For different cells, the volume calculated based on the above formula may not be the best. The exact volume should be adjusted according to the actual expression level of GFP-tubulin.
   4. Mix the GFP-tagged α-tubulin several times by inversion to ensure a homogenous solution. Do not vortex.
      NOTE: For the control, the Null (control) reagent lacks any mammalian genetic elements and can be used to help determine potential baculovirus-mediated effects and background fluorescence. A non-targeted GFP transduction control may also be used, which will light up the entire cell.
   5. Replace the culture medium with fresh medium. Add 2 mL of the new medium and 40 µL of GFP-tagged α-tubulin to each well.
   6. Shake the plate gently to allow complete mixing of GFP-tagged α-tubulin with the culture medium. Return the plate to the culture incubator and incubate for 24 h to allow the expression of GFP-tubulin.

3. **Treat cells with docetaxel**
   NOTE: This experiment is to test the effect of docetaxel on microtubule dynamic instability. Thus, the cells are treated with the desired concentration of docetaxel.
   1. Prepare a medium composed 90% of DMEM and 10% of FBS and supplemented with non-essential amino acids. Use DMEM without phenol red because phenol red may interfere with the fluorescence of GFP-tubulin. Add the desired concentration of docetaxel (ranging from 10 nM to 10 µM). The stock solution of docetaxel is 10 mM in dimethyl sulfoxide (DMSO).
   2. Pre-warm the docetaxel-containing medium to 37 °C in the tissue culture incubator.
   3. Replace the normal culture medium with the docetaxel-containing medium and incubate for 30 min in the tissue culture incubator. There should be at least 3 coverslips per well for each docetaxel concentration.

2. Live Imaging to Examine Microtubule Dynamic Instability

1. **Setting up the system**
   1. Perform experiments in a chamber maintained at 37 °C and 5% CO_2. Acquire fluorescence images by time lapse with a microscopic system equipped with a 60X, 1.42 NA oil objective lens on an inverted fluorescence microscope and with a CCD camera.
   2. Set up the cells for live imaging. Pre-warm both the sample holder and the medium to 37 °C. Choose a coverslip to examine. Mount the coverslip of interest on a sample holder and incubate it with 1 mL of the docetaxel-containing medium prepared in step 1.3.1.

2. **Live imaging of microtubule dynamic instability**
   1. Identify the cells that will be used for imaging. They should be flat, have a high fluorescence intensity of expressed GFP-tubulin, and have clear microtubule structures.
   2. Choose one cell from the group of identified cells from step 2.2.1 to examine first. Focus on the peripheral area of the identified cell. Set up the program for exposure time and the frequency of image acquisition. As exposure time is usually 0.5 s, the images will be acquired every two s.

3. Allow an additional 20 min for the steps from 1.33 to 2.2.3. Thus,exactly 50 min after the addition of docetaxel, as described in step 1.3.3, start imaging (this is for standardization).
4. Record the microtubule dynamics for 2 min, taking a photo every 2 s. In total, acquire 60 images for the identified cell.
5. Move to the next identified cell and repeat steps 2.2.2 to 2.2.4. Repeat the procedure until 5 cells have been imaged. It is imperative to do this before the time reaches 70 min after the addition of docetaxel, as described in step 1.3.3.
6. For each treatment condition, repeat steps 2.2.1 to 2.2.5 for 3 different coverslips. In total, image 15 cells, which is enough data to measure microtubule dynamic instability.

3. Image Processing and Data Analysis

1. **Deconvolution of images**
   1. Deconvolve the acquired images to achieve a high quality. Open the deconvolution program equipped with the microscope system.
   2. Click "Process" and select "Deconvolve." Drag the image file to the "Input" window and click "Do." The image file will be deconvolved, and the deconvolved file will be saved as a new file automatically.

2. **Generation of videos**
   1. Click the deconvolved image file to open with equipped software.
   2. Click "File" and select "Save as movie." Select the "Movie format" as "AV" and the "Animation style" as "Forward." Set the "Compression quality" to "100%" and the "Frame rate" to "5/second."
   3. Check the box of "Animate through time" and click "Do it." The video will be generated.

3. **Measurement of microtubule shortening and growth rate**
   1. Identify the microtubules that will be used for measurement. For each cell, only a few microtubules will be able to have their shortening or growth followed clearly and completely.
   2. Examine each frame of the video to identify the frame that shows the starting length of the microtubule and the frame with the most extended or shortened microtubule. Measure the change in length between the two microtubules and the time elapsed between the two frames.
   3. Calculate the rate of growth or shortening by dividing the change in length by time (the unit is μm/s). For each treatment condition, follow at least 10 cells and measure at least 20 microtubules.
   4. Calculate the average and the standard error for each treatment condition. Analyze the statistical difference between the conditions using the Student test. Report the data in a table and/or in a chart.

Representative Results

Using the protocol presented here, we studied the effects of docetaxel on the microtubule dynamics of normal (MCF-7_{CC}) and docetaxel-resistant (MCF-7_{TXT}) breast cancer cells. Two sets of images show the effects of docetaxel (0.5 μM) on microtubule growth and shortening in MCF-7_{CC} and MCF-7_{TXT} cells (**Figure 1A**).

We also calculated the rate of microtubule growth or shortening under these conditions for the two cell lines and showed that at 0.5 μM, docetaxel strongly inhibits the tubulin dynamics of MCF-7_{CC} cells but not MCF-7_{TXT} cells (**Figure 1B**).

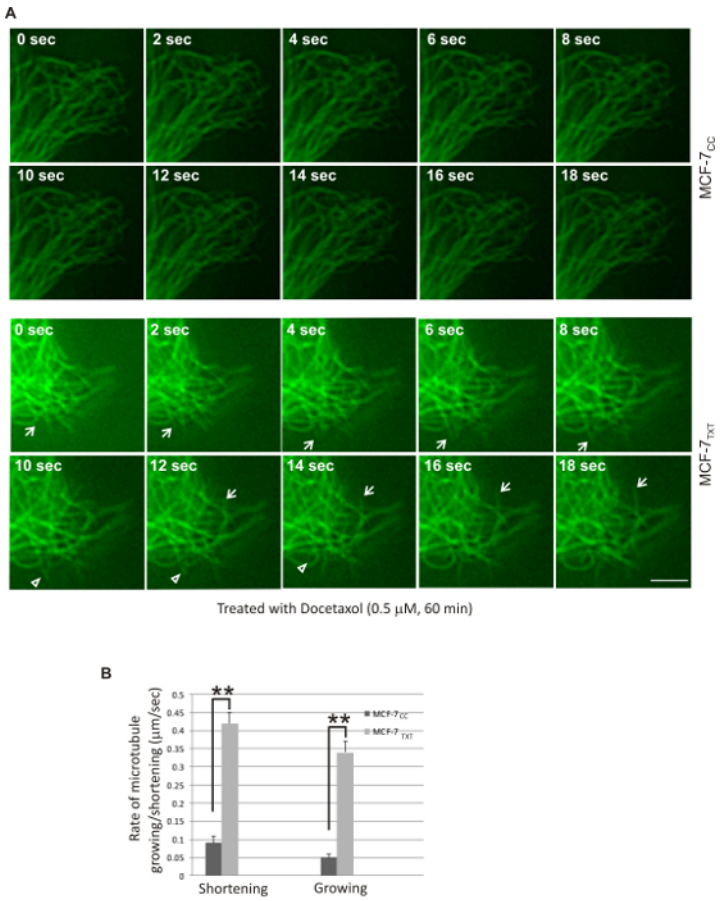

Figure 1: The Effects of Docetaxel on the Microtubule Dynamics of MCF-7_{TXT} and MCF-7_{CC} Cells. The live imaging was performed as described. (**A**) Selected images from the live imaging of microtubule dynamics in MCF-7_{CC} and MCF-7_{TXT} cells following treatment with 0.5 μM docetaxel for 1 h. The arrow indicates the extending microtubules. The arrowhead indicates the shortening microtubules. Size bar = 5 μm. (**B**) The extension rate and shortening rate of microtubules were measured from the recorded images. Each datum is the average of 20 measurements from at least 8 different cells. The error bar is the standard error. ** indicates that the difference is statistically significant, with $p < 0.01$. Please click here to view a larger version of this figure.

Discussion

There are two major methods to measure microtubule dynamic instability: *in vitro* and *in vivo*. In the *in vitro* method, purified tubulin is used to measure microtubule dynamic instability with computer-enhanced time-lapse differential interference-contrast microscopy. In the *in vivo* method, microinjected fluorescent tubulin, or expressed GFP-tubulin, is incorporated into microtubules. The dynamics (growth and shortening) of the microtubules is then recorded by time-lapse using a sensitive camera in the peripheral region of interphase cells[10,20,25]. While various types of microscopes have been used for this purpose, the protocol we reported here uses a deconvolution microscope.

In vivo, dynamic instability occurs only at microtubule plus ends (the ends with the β-tubulin facing outward), whereas the minus ends (the ends with the α-tubulin facing outward) remain the same length[26]. On the other hand, for microtubules assembled *in vitro*, dynamic instability occurs at both ends of the microtubules, with the plus ends being more robust than the minus ends[27]. The main advantage of analyzing dynamic instability *in vitro* is that it provides mechanistic information about the specific role of different agents on microtubule dynamics in the absence of cellular MAPs. Moreover, under *in vitro* conditions, dynamic instability can be studied at both ends. This provides insight into the mechanisms by which drugs or regulatory proteins affect microtubule stability at the opposite ends. Historically, the minus ends are tracked much less than the plus ends[10]. Much of our understanding of microtubule behavior comes from studies on microtubules assembled from purified tubulin *in vitro*.

However, while many of the basic principles of microtubule behavior gained from these studies can also be applied to microtubules in cells, there are certain differences in microtubule dynamics between *in vitro* and *in vivo*. In cells, microtubules often grow at a five- to ten-fold faster rate, and transitions between growth and/or shortening occur ~10 times more frequently than with microtubules assembled from purified tubulin *in vitro*. There are also dramatic changes in microtubule organization and dynamics throughout the cell cycle, which reflect a high degree of spatial and temporal regulation of cellular microtubule behavior. This behavior cannot be studied by *in vitro* research. Furthermore, the regulation of microtubule dynamics is achieved by tubulin posttranslational modification, tubulin isotype expression, and a large group of MAPs and other microtubule-interacting proteins that either stabilize or destabilize microtubules[28,29]. Therefore, studying microtubule instability in living cells is much more physiologically applicable.

The method described here is a simple and very reliable protocol to study microtubule dynamics in living cells. The steps that require more attention are the selection of the cell for observation and the determination of the exposure time. It is critical to select cells that are flat and have

high fluorescence intensity of GFP-tubulin. It is also desirable to reduce the intensity of excitation and the exposure time to avoid the fading of the GFP fluorescence. Certainly, like all of the other *in vivo* methods, this protocol is not suitable to study microtubule dynamic instability under various manipulated conditions. For example, different isoforms of α- and β-tubulin may behave differently in terms of microtubule dynamics. Only *in vitro* methods can be used to study the effects of individual purified tubulin isoforms on microtubule dynamic instability. Moreover, compared with the microinjection of fluorescence-labelled tubulin, the expression of GFP-tagged tubulin by transfection or transduction of plasmids may exhibit a relatively lower signal-to-noise ratio. However, expression of GFP-tagged tubulin in cells by transfection or transduction of plasmids allows more freedom to experimentally alter other cellular characteristics.

The protocol reported here could be used for various studies. On one hand, it can provide novel and critical insights regarding the function of microtubules during various phases of the cell cycle, especially in mitosis. It can also be used to study the effects of various cellular processes, such as migration, on microtubule dynamics. On the other hand, it can be used to study the effects of various microtubule inhibitors (many of them are cancer drugs) on microtubule dynamics in normal and cancerous cells under more physiological conditions. We have used this method to study the effects of various anti-mitotic cancer drugs on microtubule dynamic instability in both docetaxel resistant and non-docetaxel resistant breast cancer cells.

Disclosures

The authors declare that they have no competing financial interests.

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