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Ex-vivo Microtubule Stability Assay Using *Drosophila* Wing Disc

Jung-Wan Mok and Kwang-Wook Choi*

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 34141, Korea

*For correspondence: kchoi100@kaist.ac.kr

[Abstract] Regulation of microtubule stability is crucial for diverse biological processes, including cell division, morphogenesis, and signaling. Various *in vitro* assays for microtubule stability have been developed to identify and characterize proteins involved in controlling microtubule stability. Here, we introduce a simple *ex-vivo* assay for identifying potential microtubule regulators in the wing imaginal disc of *Drosophila melanogaster*. This assay utilizes silicon rhodamine-tubulin (SiR-Tub) as a cell-permeable fluorogenic dye for labeling microtubules. In an attempt to increase the sensitivity of the screen, we designed an assay using a sensitized microtubule condition. Wing discs are treated with SiR-Tub followed by demecolcine, a microtubule inhibitor, to partially label impaired microtubules. Under this sensitized condition, we can test whether overexpression or downregulation of a gene can enhance or suppress the weakened SiR-Tub labeling. This assay allows highly sensitive detection of microtubules in developing larval tissues. Hence, it provides a useful tool for identifying new microtubule regulators in both unfixed and fixed imaginal discs in *Drosophila*. This strategy may also be applied to characterize microtubule regulators in tissues from other model organisms.

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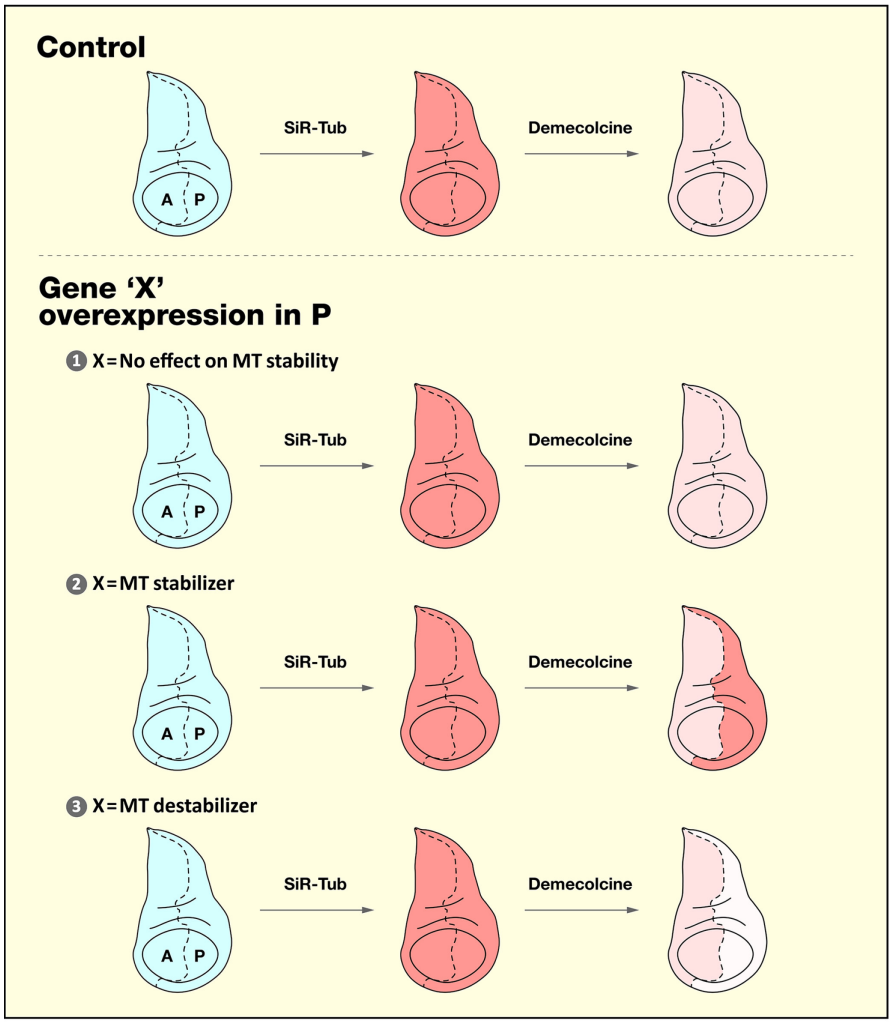
Graphic abstract:



Graphical summary of *Ex-vivo* microtubule stability assay using *Drosophila* wing disc.

Keywords: *Drosophila melanogaster*, Microtubule stability, Wing disc, SiR-Tub, *Ex vivo*, Demecolcine

[Background] Microtubules are dynamically controlled by various modulatory proteins that act as stabilizers or destabilizers. Although some of these regulatory factors have been identified, additional microtubule regulators are yet to be found. Thus, it is pivotal to have a reliable method to detect microtubules and their stability.

Among antibodies or chemical reagents for labeling tubulin, SiR-Tub is an excellent tool for detecting microtubules due to its high specificity, cell permeability, and strong fluorescent signal. Since SiR-Tub was first introduced in 2014 (Lukinavičius *et al.*, 2014), it has been utilized for visualizing microtubules in different organisms (Derivery *et al.*, 2015). SiR-Tub has also been used to label microtubules in *Drosophila* larval tissues, including brain, gut, and body wall muscle (Lukinavičius *et al.*, 2018). However, most of these applications of SiR-Tub labeling were limited to imaging of microtubules in various cells and tissues.

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Recently, we have combined the SiR-Tub labeling method with genetic application for testing the role of a novel *Drosophila* protein named microtubule-associated N-terminal acetyltransferase 9 (Mnat9) (Mok and Choi, 2021). In this assay, we used the larval wing imaginal disc, the primordium for the adult wing, as an *ex-vivo* system. Because a wing disc consists of distinct anterior and posterior compartments, it is possible to drive Mnat9 overexpression specifically in one of the compartments by the *Gal4-UAS* system (Brand and Perrimon, 1993) while the other compartment is used as an internal control. Interestingly, although Mnat9 overexpression is insufficient to induce a noticeable change in normal microtubules, it strongly suppresses microtubule defects caused by a demecolcine treatment. This provides evidence for the role of Mnat9 in promoting microtubule stability. This result also suggests that SiR-Tub labeling under a sensitized microtubule condition can be an efficient way of testing the function of microtubule regulators that might not be identified by simple loss- or gain-of-function approaches.

Microtubules have been extensively studied, but genetic regulation of their function and stability is far from complete, and a number of microtubule regulators remain to be found. Hence, we introduce SiR-Tub labeling as an *ex-vivo* assay for identifying and testing microtubule regulatory proteins. This assay system has four experimental elements: (i) SiR-Tub is used for labeling microtubules in either unfixed or fixed tissues. (ii) Wing discs are used as *ex-vivo* organs (but other imaginal discs may also be used). (iii) Demecolcine is used to generate a sensitized background by partially impairing microtubules. However, such sensitized microtubule conditions can also be induced by silencing a known microtubule stabilizer, such as Tau (Bowne-Anderson *et al.*, 2015; Barbier *et al.*, 2019). (iv) An experimental gene of interest is down- or up-regulated in specific tissues by targeted expression. Compared with existing protocols for studying microtubule stability, our assay has technical advantages for sensitive detection of microtubules in developing organs. An additional merit of this protocol is its applicability to *ex vivo* assays and genetic screens for identifying regulators of microtubule stability. In the protocol below, step-by-step procedures from dissection to SiR-Tub labeling and imaging will be described in detail. Although this protocol is mainly designed for testing microtubule regulators in the wing disc of *Drosophila*, it may also be adapted for identifying or testing regulatory factors of microtubules in other invertebrates and vertebrate genetic model systems.

Materials and Reagents

1. 9-well glass plate (Merck, Corning, catalog number: 7220)
2. Syringe filter 0.22 μm (Merck, catalog number: SLGV033R)
3. Parafilm (Merck, Sigma-Aldrich, catalog number: P7793)
4. 150 mm Petri dish (Thermo Fisher, Nunc, catalog number: 150468)
5. Micro-tubes (Sarstedt, catalog number: 72.706)
6. Vectashield (Vector, catalog numbers: H-1000, H-1200)
7. Slide glass (Superior Marienfeld, catalog number: HSU-1000612)
8. 22 × 22 mm Cover glass (Superior Marienfeld, catalog number: 0102052)

9. Coverslip Sealant (Fisher Scientific, Biotium, catalog number: NC0154994)
10. Nail polish
11. Fly stock: *en-Gal4* (#25752 or #30564 from Bloomington stock center)
12. Fly stock: UAS-X (X: gene of interest. Obtain it from stock centers or make transgenic flies using injection services.)
13. Fly food (vial/bottle): A standard recipe from Bloomington stock center, all the ingredients and plastic vial/bottles from Hansol Tech in Korea.
14. SiR-tubulin kit (Containing SiR700 tubulin & verapamil. Spirochrome, catalog number: SC002)
15. Demecolcine (Merch, Sigma-Aldrich, catalog number: D7385)
16. Express Five SFM (Thermo Fisher, Gibco, catalog number: 10486025)
17. Insulin (Merck, Sigma-Aldrich, catalog number: I6634)
18. EGS (Thermo Fisher, Thermo Scientific, catalog number: 21565)
19. Sodium azide (Sigma-Aldrich, catalog number: S2002)
20. PBS tablet (Thermo Fisher, Gibco, catalog number: 18912014)
21. Triton X-100 (Samchun chemicals, catalog number: 00T0818)
22. Normal goat serum (Jackson Immunoresearch Laboratory, catalog number: 005-000-001)
23. Ethanol (Merch, Sigma-Aldrich, catalog number: 1.00983)
24. Acetic acid (Merck, catalog number: 695092)
25. Dissection medium (see Recipes)
26. 0.5% PBT solution (see Recipes)
27. Blocking buffer (see Recipes)
28. Washing buffer (see Recipes)
29. Fly food (see Recipes)
30. Stock solutions (see Recipes)

Equipment

1. Pipettes (Thermo Fisher, Thermo Scientific, catalog numbers: 4641010N, 4641050N, 4641080N, 4641100N)
2. LSM 710 Confocal Microscope (Zeiss, model: LSM 710)
3. Shaker (FinePCR, model: SH30)
4. Incubator (JSR, model: JSGI-150T)
5. Forceps (FST, Dumont, catalog number: 11255-20)

Software

1. Zen 2009 light Edition (Zeiss, https://www.zeiss.com/microscopy/int/products/microscope-software/zen-lite.html)

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Procedure

A. Genetic cross

1. Collect *en-Gal4* virgin female flies from bottles.
2. Collect *UAS-X* male flies from bottles or vials.
   Collect virgins and males under anesthesia using a carbon dioxide pad.
   Note: Do not leave flies on carbon dioxide pad for over 5 min, as this could decrease the viability and fecundity of flies.
3. Place anesthetized flies together in a vial (for a small scale experiment, 5 virgin females + 3-5 males) or bottle (for large scale, 30 virgin females + 10-20 males) for mating as shown in step 1 of Figure 1.

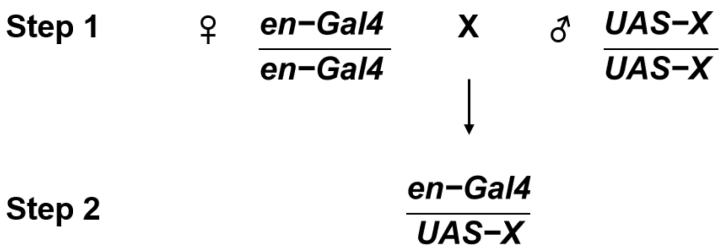

Figure 1. Fly cross scheme.

4. Incubate mating vial/bottle at room temperature (RT) for a day.
5. Move vial/bottle to an incubator set at 29°C (Gal4 activity increases in correlation with temperature. A temperature of 29°C provides a maximal Gal4 activity and robust overexpression of target gene 'X,' but could be harmful to fertility and viability depending on the Gal4 driver and UAS-X strains. Hence, temperature may be adjusted to achieve optimal expression of gene X).
6. Wait until 3rd instar larval progeny carrying en-Gal4 and UAS-X are found (step 2 in Figure 1) (It normally takes 5 to 6 days).

B. Demecolcine stock solution preparation

1. To a demecolcine glass vial (5 mg), add 1.35 ml of pure ethanol to make a 10 mM stock solution.
   Note: You can also make a 1 mM stock solution to minimize pipetting error. But, if your target cells/tissues are sensitive to ethanol, you can use a more concentrated form (100 mM stock) or use DMSO as a solvent.
2. Aliquot and store the solution at -20°C.

C. Tissue dissection

1. Acclimate aliquoted Express Five SFM medium to RT if it was stored in the fridge.
2. Add the insulin supplement to Express Five SFM medium for a concentration of 6.2 µg/ml to make the dissection medium.

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Note: To prevent contamination, it is recommended to add insulin in the lab hood.

3. Collect wandering 3rd instar larvae with forceps.
   Note: Do not grab larvae strongly with the forceps. It could tear or crush tissues.
   a. Collect at least 5-10 larvae per genotype.
   b. Put larvae in a 9 well glass dish and wash with 200 µl of PBS at least two times to remove any residual substances from the larvae surface.
   c. After thorough washing, transfer larvae to 200 µl of dissection medium made at Step C2.
      Note: Do not leave larvae in dissection medium for more than 30 min.
4. Put a single larva in each well of a new 9 well glass dish that contains 200 µl of dissection medium (Do not put the dish on ice).
5. (Critical step) Dissect larva with forceps [For a detailed video protocol, please see Purves and Brachmann (2007)].
   a. Gently grab the mouth hook of the larva with one forceps and use the other forceps to hold the larva gently at 1/3 body length from the mouth hook (Figure 2B).
   b. Gently pull the mouth hook while holding the body (You can also pull the body instead of the mouth hook).
   c. When the larva is torn apart, get rid of the body part and keep the imaginal discs attached to the mouth hook. Imaginal discs can be identified by their transparent sac-like morphology. Eye-antenna discs are attached to the larval brain. Wing discs are the largest among the imaginal discs and have a characteristic shape, as shown in the graphic summary
   d. Revert the remaining part inside-out using both forceps.
      Note: This step needs to be done with caution. Sharp tips of forceps may tear or harm the imaginal discs.
   e. Dissection speed should be fast enough to minimize tissue damage or over-incubation, especially for labeling unfixed samples. If it is hard to speed up, try to keep the incubation time the same for all samples.

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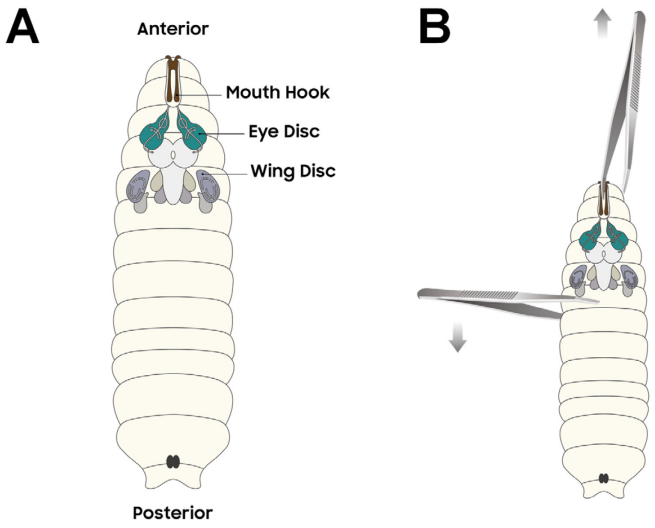

Figure 2. Graphical scheme of larval dissection.

(A) Schematic diagram of larval organs. (B) Graphical explanation of the imaginal disc dissection. Two forceps are used to grab a larva. The body is torn apart by pulling the forceps. The imaginal discs and the brain are attached to the mouth hook in the anterior portion of the larva. The imaginal discs can be exposed by reverting the anterior part inside-out.

6. When the dissection step is finished, transfer the dissected tissues to a new well containing 200 µl of fresh dissection medium.

D. Sample preparation and imaging for live unfixed tissues (Protocol 1)
   1. Add SiR500-tubulin (1:500) and verapamil (final concentration: 10 µM) to the dissected tissues.
      Note: Dilution of SiR-tubulin and verapamil may vary depending on tissues. Optimal dilution should be determined experimentally for different tissue types.
   2. Incubate tissues with agitation (100 rpm) for 30 min at RT.
      Note: Minimize light exposure during incubation by using aluminum foil, as shown in Figure 3.

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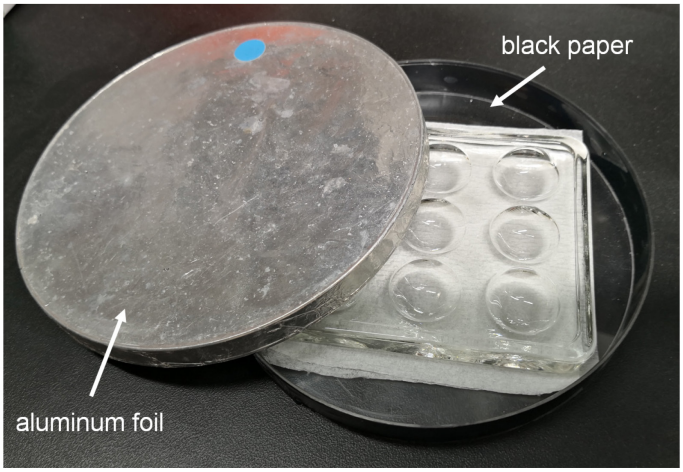

Figure 3. Light shielding chamber with 9 well glass plate.
The 150 mm Petri dish is wrapped with aluminum foil and black paper to make a light shielding chamber.

3. Briefly wash the tissues at least twice with 200 µl of dissection medium.
4. (Critical step) Further incubate tissues in 200 µl of dissection medium containing 50 µM demecolcine for 15 min at RT with agitation (100 rpm).
   Note: For a control without demecolcine, dissected tissues are incubated in the same way but without demecolcine. Precise timing of demecolcine treatment is critical to get consistent and meaningful results. The length of incubation can be varied according to the target gene or application, so some preliminary tests are needed to determine the optimal incubation time.
5. After incubation, mount the wing discs as follows.
   a. Transfer the tissues to 200 µl of dissection medium containing 50 µM demecolcine without any washing step.
      Note: The following dissection steps should be completed within 15 min for best results.
   b. Place 10 µl of dissection medium on a glass slide.
   c. (Critical step) Separate the wing discs using forceps and mount them on the glass slide.
   d. After mounting 3-5 discs, cover the samples using a coverslip.
   e. Gently wipe excess media with Kimwipes and seal with coverslip sealant.
      Note: Nail polish can be used instead of commercial sealant.
6. Perform live imaging using a confocal microscope.
   a. Carefully select wing discs prior to imaging.
      Damaged or folded imaginal discs should be excluded to avoid misinterpretation.
      Check whether microtubules are evenly labeled with SiR-Tub, as shown in Figure 4.
   b. Use a Cy5 filter set, as the optical property of SiR-Tub is as follows: absorption wavelength (λabs) - 652 nm, and fluorescence wavelength (λfl) - 674 nm.
   c. To find optimal imaging conditions (laser intensity, imaging speed, averaging numbers), considering that slow imaging or high laser intensity should be avoided to minimize photobleaching. With the LSM710, set the laser to less than 10% of the maximum intensity

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with imaging speed 1.

d. (Optional) For more detailed analysis, a time-series image acquisition may be used. In this case, the total imaging time should not exceed 30 min per wing disc.

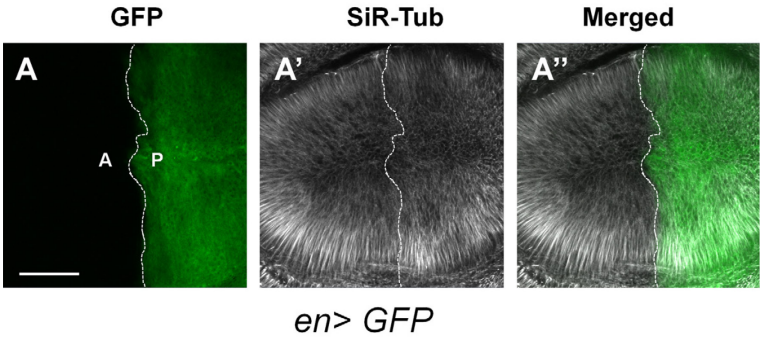

Figure 4. Optimal example of SiR-Tub wing imaginal disc staining.

(A-A'') Undamaged and unfolded wing disc with even SiR-Tub labeling. A: Anterior. P: Posterior. Scale bar: 50 µm. GFP: green. SiR-Tub: white.

E. Sample preparation and imaging for fixed tissues (Protocol 2)

1. (Critical step) Incubate dissected tissues (from Procedure C) in dissection media with or without 50 µM demecolcine for 75 min at RT with agitation (100 rpm).

   Note: For control without demecolcine, dissected tissues are incubated in the same way but without demecolcine. The precise timing of demecolcine treatment is critical to get consistent and meaningful results. The length of incubation can be varied according to the target gene or application, so some preliminary tests are needed to determine the optimal incubation time.

2. After incubation, wash out residual demecolcine from tissues with gentle pipetting at least twice using 200 µl of dissection media.

   Note: Each wash should not exceed 1 min.

3. After removing the wash buffer, fix tissues with 100 µl of fixative (EGS 10 mM in PBS) for 10 min with agitation (100 rpm) at RT.

   Note: EGS fixative is strongly recommended. Other fixatives like 4% paraformaldehyde (PFA) or periodate-lysine-paraformaldehyde (PLP) work poorly in this application. Fixing time and temperature should be optimized prior to the experiment.

4. Remove the fixative.

   Note: EGS incubation at RT may cause precipitation of white aggregates. However, such precipitation has no significant negative effect and can be cleared by several washing steps.

5. Wash briefly with 200 µl of PBS twice for 5 min total with agitation (100 rpm).

6. After removing the PBS, incubate tissues with 200 µl 0.5% PBT solution (see Recipes) for 30 min at RT with agitation (100 rpm).

   Note: This step is for tissue permeabilization. The concentration of Triton X-100 can be modified for different applications.

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7. After removing the 0.5% PBT solution, incubate the tissues with 200 µl of blocking buffer (see Recipes) for 30 min at RT with agitation (100 rpm).
   Note: If you are not going to use antibodies for immunostaining, skip steps from E8 to E11.
8. During the blocking step, prepare the primary antibody sets in 100 µl of blocking buffer at the desired concentration.
9. After blocking, remove the blocking buffer and add the primary antibody to the tissues. Incubate for 1 h at RT with agitation (100 rpm).
   Note: You can also incubate overnight at 4°C.
   a. After incubation, remove the primary antibody solution and rinse with 200 µl of washing buffer (see Recipes) for 1 min. Then, remove the washing buffer.
   b. Add 200 µl of washing buffer for 10 min at RT with agitation (100 rpm).
   c. Remove the washing buffer. Repeat this washing step at least three times.
10. During the washing step, prepare the secondary antibody sets in 100 µl of blocking buffer at the desired dilutions.
11. After the washing step, remove the washing buffer and add the prepared secondary antibody sets to the tissues.
12. Add SiR-Tub (1:500) to the tissues and incubate for 1 h at RT with agitation (100 rpm).
    Note: Minimize light exposure during incubation using aluminum foil, as shown in Figure 3.
13. Add 200 µl of washing buffer and incubate for 10 min at RT with agitation.
14. Remove the washing buffer. Repeat this washing step at least three times.
15. After the washing step, replace the washing buffer with 200 µl of PBS.
16. (Critical step) After incubation, perform further dissection and mounting.
    a. Drop 10 µl of mounting solution (Vectashield) on the glass slide.
    b. Dissect wing discs using forceps and mount them on the glass slide.
    c. After mounting 3-5 discs, cover the samples using a coverslip.
    d. Gently wipe excess media with Kimwipes and seal with a coverslip sealant.
       Note: Nail polish can be used instead of commercial sealant.
17. Perform imaging using a confocal microscope.
    a. Carefully select imaginal discs prior to confocal imaging.
       i. Damaged or folded imaginal discs should be excluded to avoid potential misinterpretation.
       ii. Check whether microtubules are evenly stained with SiR-Tub as shown in Figure 4.
    b. Use a Cy5 filter-set as per SiR-Tub's optical property: λabs, 652 nm, and λfl, 674 nm.
    c. Find optimal imaging conditions (laser intensity, averaging number, and imaging speed) considering that slow imaging or high laser intensity should be avoided to minimize photobleaching. With the LSM710, set the laser to less than 10% of the maximum intensity.
    d. (Optional) Use the Z-stack image acquiring function to get serial plane images along the z-axis.

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Data analysis

Microtubule regulators can play diverse functions in stabilizing microtubules. Loss or overexpression of some critical factors may lead to severe changes in the pattern of microtubules. In contrast, alterations of some modulatory factors such as Mnat9 may not result in obvious defects, although they have important functions in regulating microtubules. Hence, more sensitive assays are needed to identify such factors. The assay described here uses a sensitized condition and provides a powerful tool for identifying important regulatory genes involved in microtubule stability.

For analysis of SiR-Tub labeling, an assay for overexpression of gene X will serve as a simplified example. The purpose of this assay is to determine whether overexpression of the gene X in the posterior domain by *en-Gal4* can alter the pattern of SiR-Tub labeling in the targeted region, in the absence or presence of demecolcine in the wing disc. Below, a few expected results outlined in the graphical abstract and Table 1 will be discussed.

Table 1. Example of experimental sets and possible analysis

Sample Type	Genotype	Without Demecolcine	With Demecolcine	Analysis
Control	*en> GFP*	A = P	A = P	No effect on MT stability.
Experimental 'X' overexpression	*en> X, GFP*	A = P	A = P	No effect on MT stability.
〃	〃	A = P	A < P	'X' stabilizes MT in MT sensitized condition.
〃	〃	A = P	A > P	'X' destabilizes MT in MT sensitized condition.
〃	〃	A < P	-	'X' stabilizes MT and/or increases MT formation.
〃	〃	A > P	-	'X' destabilizes MT and/or decreases MT formation.

1. Control: It is essential to confirm that the pattern of Sir-Tub labeling in the posterior domain of the *en>GFP* control wing disc should be similar to that of the anterior domain (Figure 4A', Figure 4A). Then, the effects of gene X overexpression (*en > gene X*) in the posterior domain can be compared with the SiR-Tub pattern in the anterior control domain. Note that SiR-Tub labeling is significantly reduced by demecolcine treatment (Figure 5B).

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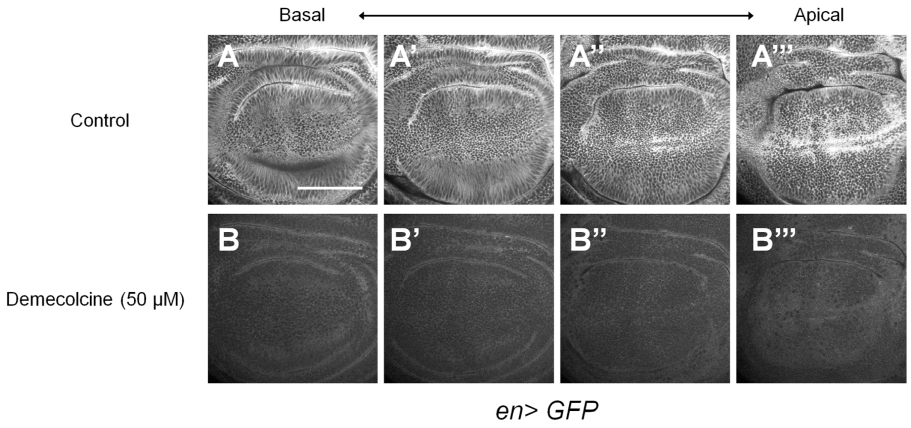

Figure 5. SiR-Tub labeling patterns in control wing disc and demecolcine-treated wing disc. (A-A''') Consecutive z-stack plane images of a control wing disc labeled with SiR-Tub in the absence of demecolcine. (B-B''') SiR-Tub labeling is weakened in consecutive images of a demecolcine-treated wing disc. Scale bar: 100 μm.

2. No change in SiR-Tub labeling: (i) Assay without demecolcine: If the intensity of SiR-Tub labeling in the posterior domain is similar to that of the anterior domain, gene X may not be sufficient to promote microtubule formation or its stability (Figure 6). However, gene X may have a permissive function in maintaining microtubule stability. Thus, it is important to test whether gene X is required for microtubule stability by testing the effects of loss-of-function for gene X. (ii) Assay with demecolcine: If gene X has no function in promoting microtubule stability, gene X overexpression will not alter the pattern of SiR-Tub labeling in the posterior domain even in the demecolcine-treated sensitized condition.

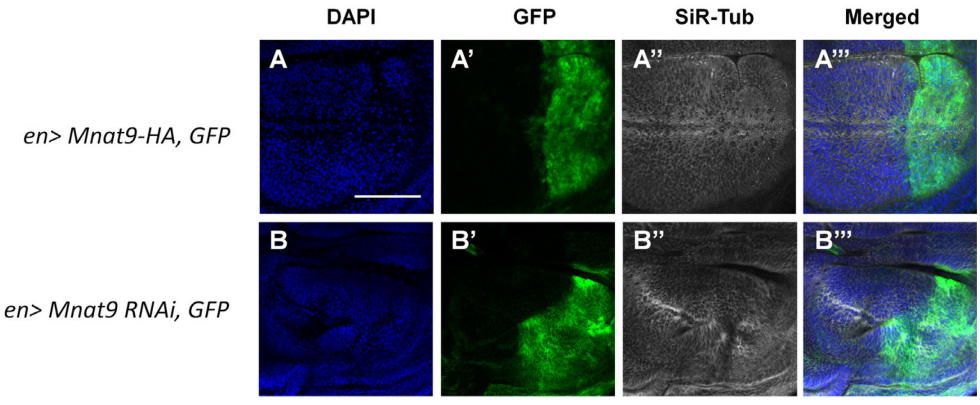

Figure 6. SiR-Tub labeling of wing imaginal discs without demecolcine treatment (Fixed samples).
(A-A''') Effects of Mnat9 overexpression in the posterior part of the wing disc using *en-Gal4*. (B-B''') Effects of Mnat9 knockdown in the posterior part of the wing disc using *en-Gal4*. Anterior: A. Posterior: B. Scale bar: 100 μm. DAPI: blue. GFP: green. SiR-Tube: white.

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3. Enhanced SiR-Tub labeling by gene X overexpression: (i) Assay without demecolcine: If SiR-Tub labeling is enhanced in the posterior region compared with the anterior control region, gene X is likely to be a positive regulator of microtubules. (ii) Assay with demecolcine. If gene X overexpression can enhance SiR-Tub labeling in the absence of demecolcine, it may also show a strong enhancement in SiR-Tub labeling for demecolcine-treated wing discs as well. However, there might be cases where gene X overexpression can increase SiR-Tub labeling only in the sensitized condition. In this case, gene X overexpression may be critically important when microtubules are destabilized, although it may not be essential under normal microtubule conditions (Figure 7). Note that SiR-Tub labeling can also be enhanced by knocking down microtubule destabilizing factors.

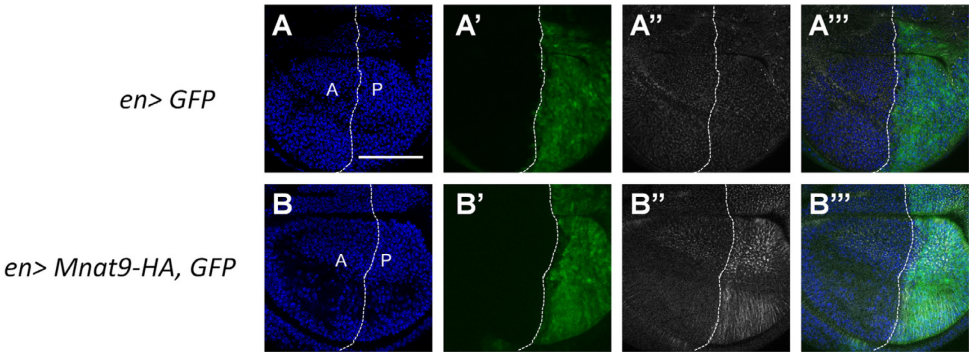

Figure 7. Demecolcine-treated wing imaginal discs (Fixed samples).
(A-A''') Control. (B-B''') Mnat9 overexpression suppresses microtubule dissociation caused by demecolcine treatment. Anterior: A, Posterior: B. Scale bar: 100 μm. DAPI: blue. GFP: green. SiR-Tube: white.

4. Reduced SiR-Tub labeling: (i) Assay without demecolcine: If gene X is a potent factor for destabilizing microtubules, its overexpression may lead to reduced SiR-Tub labeling in the posterior region. (ii) Assay with demecolcine. Such microtubule destabilizer may facilitate the effects of demecolcine, resulting in a further decrease in SiR-Tub labeling. When SiR-Tub labeling is strongly reduced by gene X overexpression, the targeted posterior region of the wing disc may also show morphological abnormalities due to destabilized microtubules. In severe cases where posterior cells undergo cell death, it may be difficult to interpret the results of reduced SiR-Tub labeling. In such cases, it would be worth checking SiR-Tub labeling in the presence of cell death inhibitors such as p35 or Diap1, to minimize the effects of cell death. Although SiR-Tub labeling can be reduced by overexpressing a microtubule destabilizer, a similar reduction may also be found by silencing microtubule-stabilizing factors. Hence, it is ideal to test both gain- and loss-of-function conditions when analyzing a gene's function.

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Recipes

1. Dissection medium
   a. Add insulin supplement to Express Five SFM medium at a concentration of 6.2 µg/ml.
   b. Mix well using an orbital shaker and sterilize using a 0.22 µm pore size syringe filter (You don't have to adjust the pH of the medium).
   c. Aliquot the medium in small volumes (~10 ml) and seal the tube with parafilm.
   d. The above steps should be performed within a lab hood to avoid contamination.
   e. Store the medium at 4°C and use within a day.
   f. Place the dissection medium at RT for at least 15 min prior to dissection.
2. 0.5% PBT solution
   a. Add 1 ml of Triton X-100 to 9 ml of sterilized distilled water to make a 10% Triton X-100 solution. (As Triton X-100 is very viscous, cut the bottom 1cm of the 1 ml tip with sterilized scissors before pipetting.)
   b. Add 1 ml of 10% Triton X-100 solution to 19 ml of filtered 1x PBS solution and mix well (Working pH range of the solution is between 6.0 and 8.0).
   c. Store at RT before use. This solution is stable for several months.
3. Blocking buffer
   a. Thaw a 1 ml aliquot of frozen normal goat serum on ice.
   b. Add 0.6 ml of 10% Triton X-100 solution and 1ml of normal goat serum to 18.4 ml of 1× PBS.
   c. Add sodium azide to the mixture at a final concentration of 0.02% (working pH range of the solution is between 6.0 and 8.0).
   d. Mix well and store at 4°C (the blocking buffer can be stored for a month without significant loss in activity).
4. Fly food (BDSC Cornmeal Food, for 10 L)
   Water 9.2 L
   Yeast 160 g
   Soy flour 92 g
   Yellow cornmeal 670 g
   Agar 53 g
   Light corn syrup 0.7 L
   Propionic acid (>99% pure, 13.4 M) 44.2 ml
   Information for the fly food is described at the Bloomington Drosophila Stock Center website (https://bdsc.indiana.edu/information/recipes/bloomfood.html).
5. Stock solutions
   a. Demecolcine: Demecolcine powder was solubilized with pure ethanol to make 1 mM stock solution.
   b. Insulin: As insulin shows low solubility at neutral pH, acetic acid (pH 2-3) was used to make

a 2 mg/ml stock solution. The solution was aliquoted, snap frozen, and stored at -20°C.

c. Sodium azide: To make a 10% stock solution, 10 g of sodium azide was dissolved in 100ml of distilled water.

6. Washing buffer
   a. Add 1.5 ml of 10% Triton X-100 solution to 48.5 ml of 1× PBS.
   b. Mix well and store at RT (You don't have to adjust the pH of this buffer. The washing buffer can be stored for months without significant loss of activity).

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Competing interests

The authors declare no competing interests.

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