



Chapter 2

Using Budding Yeast as a Model to Understand Dynein-Mediated Cargo Transport

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Abstract

Cytoplasmic dynein-1 is a minus end-directed microtubule motor that transports numerous cargoes in cell types throughout the evolutionary spectrum. Dynein is regulated by various motor-intrinsic and motor-extrinsic factors that enhance its processivity, recruit it to various cellular sites, or otherwise promote or restrict its activity. Studying dynein activity in higher eukaryotes is complicated by various factors, including the myriad functions in which this motor participates, and the consequential pleiotropic effects associated with disrupting its activity. Budding yeast has long been a powerful model system for understanding this enormous motor protein complex, which is highly conserved between yeast and humans at the primary sequence and structural levels. Studies in budding yeast are simplified by the fact that dynein only performs one known function in this organism: to position the mitotic spindle at the site of cell division. Monitoring dynein-mediated spindle movements in budding yeast provides a powerful tool for the quantitative measurements of various motility parameters, and a system with which to assess the consequence of mutations in dynein or its regulators. Here, we provide detailed protocols to perform quantitative measurements of dynein activity in live cells using a combination of fluorescence microscopy and computational methods to track and quantitate dynein-mediated spindle movements. These methods are broadly applicable to anyone that wishes to perform fluorescence microscopy on budding yeast.

Key words Dynein, Live-cell imaging, Yeast transformation, Nuclear migration, Spindle positioning

1 Introduction

Dynein is a highly conserved microtubule-dependent motor complex that is required for the transport of a wide variety of cargoes in numerous organisms and cell types. Examples of dynein cargoes include membrane-bound organelles (e.g., lysosomes, early endosomes, nuclei), proteins, RNAs, and even the mitotic spindle. The large variety of cargoes for this motor complex means that dynein must be highly regulated such that individual cargoes are

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transported with a high degree of spatial and temporal precision. This is likely achieved in large part by the growing number of adaptor molecules (e.g., BicD2, Hook3), which link dynein to its various cargoes and also activate its motility. In addition to the cargo adaptor molecules, dynein is regulated by a number of extrinsic regulators, including dynactin (dynein activator), the lissencephaly protein LIS1, and Ndel1.

A number of recent advances in recombinant protein production as well as recent progress in understanding this large motor complex have yielded invaluable insight into the biochemical basis for cargo specificity and transport [1–3]. However, a clear understanding of how dynein and its regulators ensure appropriate transport in live cells is still somewhat lacking. This is due in large part to the complications associated with studying dynein activity in cells. Such complications are due to the myriad roles that dynein plays in higher eukaryotes, the pleiotropic consequences of disrupting dynein function in these cells, and the fact that the dynein accessory genes (e.g., light-intermediate chain, intermediate chain) are encoded by multiple genes.

To overcome such difficulties, we and others have employed the simple eukaryotic organism budding yeast (*Saccharomyces cerevisiae*) as a model system for studying dynein and its regulators. In addition to the ease of manipulating its genome, this organism possesses only a single gene that encodes each of the dynein accessories—all of which are highly conserved with those found in higher eukaryotes—and is highly amenable to live-cell microscopy. Moreover, dynein function in this organism is unambiguous and easily measured and quantified, enabling direct assessment of the role of dynein regulators (or mutations therein) in various aspects of cellular dynein function and regulation. Specifically, the only known function for dynein in budding yeast is to position the mitotic spindle at the future site of cell division—the bud neck—prior to anaphase onset. Deletion of genes encoding dynein (or dynactin) subunits leads to situations in which the spindle remains within the mother cell, ultimately leading to a binuclear phenotype in which the mother inherits both copies of the duplicated genome, and the daughter gets none. Besides this severe consequence of dynein dysfunction, more nuanced and subtle defects in dynein function can also be directly assessed and quantitated by imaging spindle movements in real time in live cells [4–6]. This assay provides quantitative metrics of dynein activity, including velocity, displacement, as well as several “activity” metrics (e.g., frequency or extent of dynein-mediated spindle movements). For example, we have recently employed this assay to determine the consequences of disease-correlated mutations on dynein function [6]. In addition to assessing motility parameters, this assay can also reveal the manner

by which dynein is directionally tuned such that the spindle is moved toward—and not away from—the bud neck [4].

Here, we describe our methods for performing this live-cell microscopy-based assay, and our pipeline for quantitatively analyzing these movies. In addition, we also provide a generalized protocol for performing genetic manipulations of budding yeast (e.g., knocking out or mutating genes of interest). Our intention is to aid researchers who are unfamiliar with handling budding yeast and to guide those who are interested in studying dynein in this simple model organism. Our methods are also broadly applicable to anyone that is interested in genetically manipulating and/or imaging budding yeast.

2 Materials

2.1 Generation of PCR Cassettes for Yeast Genome Manipulation

1. Plasmids with selectable markers. There are many such plasmids with selectable marker “cassettes” (which include the open reading frame along with a suitable promoter and terminator). Several of these are available from Addgene, with many of them being based on the original family of plasmids developed by the Pringle (see <https://www.addgene.org/browse/article/6064/>) or Hieter labs (e.g., pRS303, pRS304, pRS305, pRS306) (*see Note 1*).
2. DNA polymerase kit for PCR, such as Accuprime Pfx (from Thermo Fisher) or Q5 polymerase (from NEB).
3. Thermal cycler.
4. Desalted, custom-designed oligonucleotides (custom ordered) (*see Fig. 1 and Note 2*).
5. Agarose gel running apparatus.
6. Tris–acetate–EDTA (TAE) buffer. 40 mM Tris, 20 mM acetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA).
7. Molecular biology grade agarose. We typically prepare 0.9–1.0% agarose gels by melting agarose in TAE buffer.
8. Dye to visualize DNA, such as SYBR Safe (Thermo Fisher).
9. Light box with appropriate wavelength light (e.g., UV) to visualize and excise DNA fragment.
10. Gel DNA recovery kit.
11. Heat block maintained at 50–60 °C.

2.2 Yeast Growth and Transformation

1. A laboratory yeast strain, such as W303, S288C, or YEF473. Our laboratory routinely uses the haploid YEF473 given its consistent and reproducible behavior with respect to dynein function/dysfunction.
2. Sterile toothpicks.

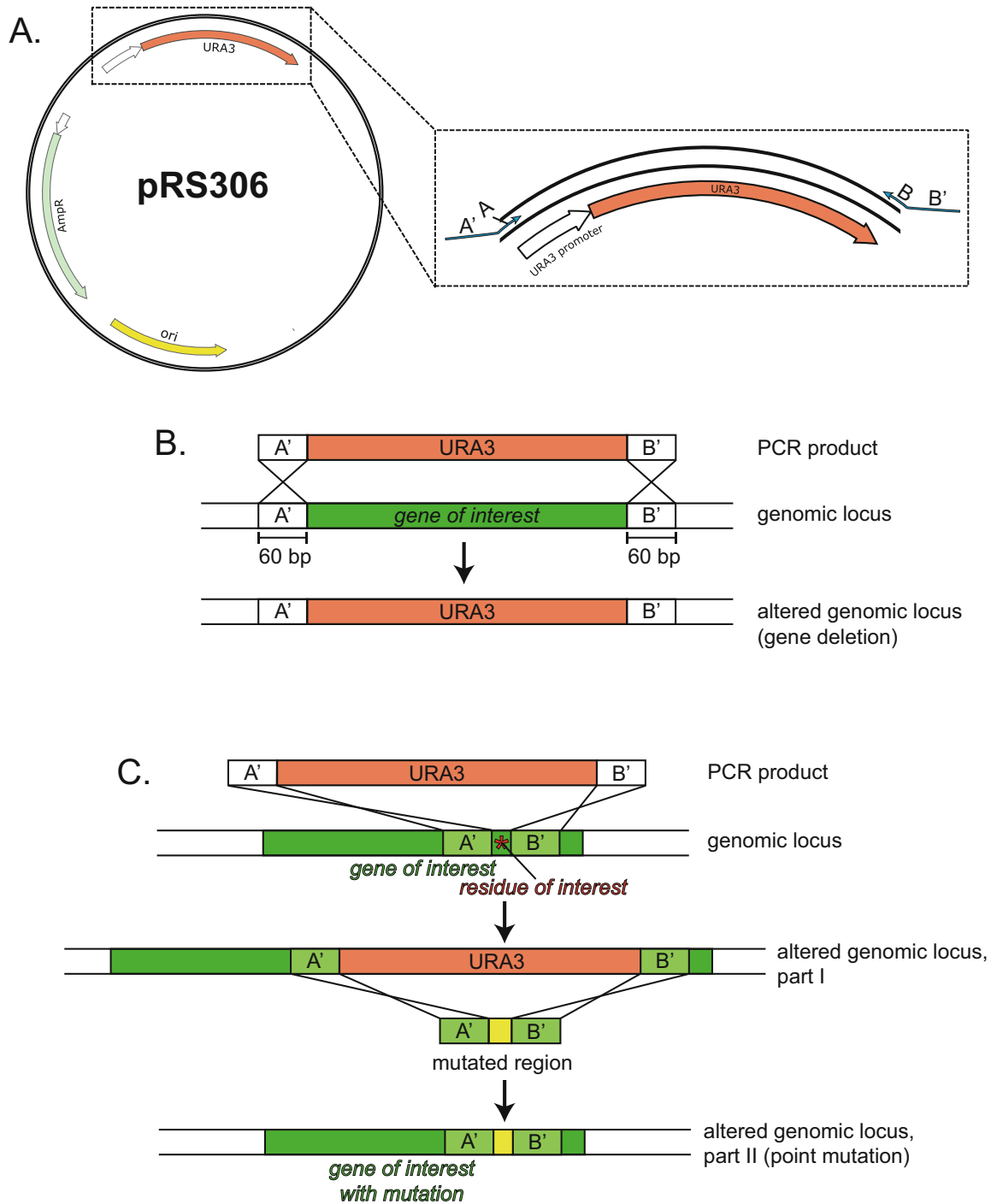


Fig. 1 PCR-based genomic manipulation strategy. **(a)** Map of pRS306 [10], a plasmid containing the *URA3* cassette that is useful for gene knockouts or generating mutations. Sequences A and B indicate forward and reverse priming sequences, respectively, while A' and B' indicate regions with sequences used for homologous recombination (generally 60 bp). **(b)** and **(c)** Schematics depicting recombination strategies for knocking out a gene of interest **(b)** or generating a point mutation/small deletion/insertion **(c)**. Mutations are generated in two distinct steps: (1) the *URA3* cassette is integrated into the genome at the site of interest, and (2) a PCR product with the desired mutation replaces the *URA3* cassette. Transformants in the 2nd step are selected on media containing 5-FOA

3. Sterile culture tubes with cap. We use 16 mm × 100 mm glass tubes with plastic caps.
4. YPAD medium. 10 g/L bacto-yeast extract, 20 g/L bacto-tryptone, 20 g/L glucose, 40 mg/L adenine hemisulfate. Autoclave to sterilize.
5. Shaking incubator and baffled flasks for yeast growth.
6. Centrifuge with swinging bucket rotor capable of accommodating sterile culture tubes and 1500× *g*.
7. Sterile water.
8. Lithium sorbitol solution: 100 mM lithium acetate, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 1 M sorbitol. Sterilize in an autoclave.
9. Sterile 1.7 mL tubes.
10. Salmon sperm DNA (10 mg/mL), sheared and denatured (sheared by sonication, 10 × 1 min, and then denatured by boiling at 100 °C for 10 min).
11. PEG solution: 100 mM lithium acetate, 10 mM Tris-HCl pH 8, 1 mM EDTA, 40% PEG 3350. Sterilize in an autoclave.
12. Sterile dimethyl sulfoxide (DMSO).
13. 42 °C water bath.
14. 30 °C plate incubator.
15. Microcentrifuge.
16. Selective dropout base agar (DOBA; Sunrise Science; San Diego, California, USA). Supplement with appropriate complete supplement mixture (e.g., complete minus uracil; Sunrise Science; San Diego, California, USA), autoclave, and pour into sterile Petri dishes to generate appropriate solid selective media (e.g., DOBA minus uracil).
17. Sterile glass beads (2.5 mm in size).

2.3 Yeast Imaging

1. Sterile culture tubes.
2. Imaging media (also known as Synthetic defined, or SD media). Dissolve the following in 900 mL of water: 3.4 g yeast nitrogen base (Sunrise Science), 5 g (NH₄)₂SO₄, 0.79 g complete supplement mixture (Sunrise Science). After autoclaving to sterilize, add 100 mL of a sterilized (by autoclaving) 20% glucose solution (2% final). Note that we typically prepare bottles with 100 mL of media.
3. SD media supplemented with 200 mM hydroxyurea. Dissolve 0.152 g of HU into 10 mL of sterile SD media. This should be prepared immediately prior to use.
4. Shaking incubator.

5. Centrifuge with swinging bucket rotor capable of accommodating culture tubes.
6. Molecular biology grade agarose.
7. Glass slides.
8. Glass coverslips (number 1.0 or 1.5, 22 mm squares).
9. Cotton swab.
10. Wax heated to melting temperature in a small beaker ($\sim 100^\circ\text{C}$).
11. Confocal or wide-field fluorescence microscope with a high numerical aperture (e.g., 1.49) 60x or 100x objective.

2.4 Image Analysis Using MATLAB and MS Excel

1. Computer with ImageJ/FIJI, Matlab (version R2016b; MathWorks), and MS Excel.
2. The following Matlab codes can be found in the supplemental materials to this article: (A) *Spindle_Tracker.m*, (B) *Stack_Threshold.m*, and (C) *Relative_coordinate_mapper.m*.
3. The *make_combined_XY_YZ_movie_in_Fiji_19Zs* FIJI macro is included in the supplemental materials.
4. The MS Excel file *SOA_TEMPLATE.xlsx* is also included in the supplemental materials. Table 1 includes example output data generated by analyzing the example time-lapse TIFF stack listed as item 5.
5. We have also included a crop of a single-budded yeast cell for practice analysis (*crop.tif*, fluorescence TIFF stack; and *DIC.tif*, both of which are needed). The TIFF stack is a time (15 min, images acquired every 10 s) and Z series (19 Z slices spaced $0.2\ \mu\text{m}$ apart) of a wild-type yeast cell expressing GFP-Tub1. The mother cell is on the left, and the daughter cell is on the right in this example.

3 Methods

Here, we describe a general overview for manipulating the yeast genome (e.g., deleting genes and/or adding a fluorescent tag to a gene of interest). These generalized methods can be applied to deleting, overexpressing, or mutating a gene of interest, to determine how it might affect dynein function. We describe the steps required for (1) generating the PCR products for chromosomal tagging and/or deletion of yeast genes, (2) transformation of yeast strains with the PCR products, (3) slide preparation for microscopy, and (4) imaging of fluorescent proteins in living yeast cells. Note that assessing dynein activity in budding yeast requires imaging the mitotic spindle and microtubules. To visualize microtubules, we rely on the integration of a fluorescent protein (FP)-Tub1

expression cassette, of which there are several options, including those we described previously [7].

3.1 Generation of PCR Tagging/Knock-Out Cassettes

We describe generalized methods here for the integration of a selectable marker into the yeast genome such that a gene of interest is either deleted or mutated. Homologous recombination of a selectable marker (e.g., *URA3*) into the genome using DNA sequences that are homologous to the 5' and 3' regions flanking a gene of interest (see Fig. 1; A' and B' sequences; note we typically use 60 base pairs of complementarity) will result in the deletion of the gene (Fig. 1a). Alternatively, point mutations or small/targeted deletions can be made using a two-step strategy in which the *URA3* gene is first integrated in such a way that the region of interest is replaced with the *URA3* gene. In a subsequent step, the *URA3* gene is replaced with a double-stranded DNA donor strand (e.g., generated by PCR, or using a custom-designed gene block) with the mutation or deletion of interest (see Fig. 1b). Employing the *URA3* gene is especially useful for mutagenesis schemes given the availability of a drug that can be used for counter-selection against *URA3* integrants: 5-fluoroorotic acid (5-FOA).

1. Using a commercial DNA polymerase kit, amplify the selectable marker of interest from the chosen plasmid (see Fig. 1 and **Note 1**). As shown in Fig. 1, *URA3* is commonly used due to its use as a selectable marker, but also for counter-selection against *URA3*⁺ transformants in a subsequent step (using 5-FOA; see Fig. 1b). We routinely obtain the *URA3* cassette from pRS306 (see Fig. 1). We aim for 2–10 µg of PCR product for a typical yeast transformation (described below in Subheading 3.2).
2. Run the PCR on an agarose gel, and excise the band that corresponds to the desired product (i.e., the selectable marker with homologous sequences on 5' and 3' ends).
3. Use a commercial gel DNA recovery kit to isolate the PCR product from the agarose, and to concentrate the product to a small volume (~10–15 µL).

3.2 Prepare and Transform PCR Product into Yeast

1. The afternoon/evening before the transformation, use a sterile toothpick to inoculate a sterile culture tube containing YPAD with a colony of appropriate yeast cells. Incubate the cells in a 30 °C shaking incubator set to 220 rpm. We typically grow cells in 3 mL of YPAD media.
2. The next morning, freshly dilute the cells into a sterile tube of YPAD media. We typically dilute 0.5–1.0 mL of cells into 3 mL of fresh YPAD media.
3. Incubate the cells in a shaking incubator set to 30 °C for 2–6 h.
4. Pellet the cells in a centrifuge at 1500× *g* for 5 min.

5. After decanting the media, resuspend the cells in 3 mL of sterile water.
6. Pellet the cells as in step 4.
7. After decanting water, resuspend the cells in 3 mL of sterile lithium sorbitol solution.
8. Pellet the cells as in step 4.
9. After decanting the lithium sorbitol solution, resuspend the cells in a small volume of the lithium sorbitol solution. We typically resuspend the cell pellet in 300–400 μL , which provides 3–4 aliquots of competent cells.
10. Aliquot 90 μL of the lithium sorbitol solution/cell suspension into sterile 1.7 mL tubes, and add 10 μL of the boiled salmon sperm DNA to each. These 100 μL aliquots can be used immediately for transformation, or can be stored at $-80\text{ }^{\circ}\text{C}$ for future use (*see* **Note 3**).
11. Add purified PCR product (from Subheading 3.1) or Tub1 tagging plasmid (*see* **Note 4**) to competent cells (from step 10) and immediately add 600 μL of PEG solution. Invert 5–6 times to mix.
12. Incubate cells at $30\text{ }^{\circ}\text{C}$ for ≥ 30 min in a shaking incubator set at ~ 180 rpm. Alternatively, invert the mixture every 10–15 min if incubation is done without shaking.
13. Add 38 μL sterile DMSO and immediately invert 5–10 times to mix well.
14. Heat-shock the cells in a $42\text{ }^{\circ}\text{C}$ water bath for 15 min.
15. Pellet transformed cells in a microcentrifuge ($1500\times g$, 1 min), aspirate the supernatant, and resuspend the cells in 1 mL of YPAD.
16. Incubate cells in $30\text{ }^{\circ}\text{C}$ shaking incubator set to 220 rpm (*see* **Note 5**) for 30–90 min. Especially when extended incubations are used, it is important that the tube cap is secured (using a small piece of parafilm, or a cap lock) to prevent the cap popping open due to CO_2 buildup.
17. Pellet the cells in a microcentrifuge ($1500\times g$, 1 min), and aspirate all but $\sim 100\text{ }\mu\text{L}$.
18. Pour ~ 20 – 30 sterile 2.5 mm glass beads onto a selection plate. Resuspend the cell pellet in the remaining YPAD by pipetting up and down, pipet the cell suspension onto the plate (e.g., use DOBA minus uracil plate in the case of the URA3 marker), and spread cells evenly by shaking. Discard the glass beads (*see* **Note 6**).
19. Incubate the plate in a $30\text{ }^{\circ}\text{C}$ incubator until colonies appear (typically 2–3 days).

20. Use sterile toothpicks to pick single colonies from the transformation plate and streak onto a new selection plate (we typically streak at least 8 colonies). Colonies are streaked to single colonies in order to clonally purify. Incubate plate at 30 °C until well isolated single colonies appear (2–3 days).
21. Repeat step 20 to ensure sufficient clonal purification of the transformed cells.
22. Genotype clonal isolates using PCR (*see Note 7*). If a point mutation or small deletion was introduced, we strongly suggest sequencing the entire region that was engineered to ensure the region is as expected.

3.3 Yeast Imaging

Here, we describe a generalized method to mount yeast cells for fluorescent imaging, along with a means to image cells arrested with the DNA-synthesis inhibitor hydroxyurea (HU), which prevents anaphase onset, and permits observations of many dynein-mediated spindle movements. Although HU can be easily applied to any yeast strain, arresting cells using genetic-based techniques are another option. For example, conditional depletion of CDC20—an activating subunit of the anaphase-promoting complex/cyclosome (APC/C)—using an inducible promoter (e.g., *GAL1p*, *MET3p*) provides an alternate means to enrich for cells with many dynein-mediated spindle movements.

1. Inoculate 3 mL imaging/SD media liquid culture from a single colony of a desired strain of interest. Note that after genome manipulation and validation (as described above), maintenance of selective pressure is no longer necessary.
2. Grow cells overnight in a 30 °C shaking incubator set to 220 rpm, after which they typically reach an optical density (OD_{600 nm}) of 1–3.
3. The next morning, dilute the cells into fresh media (typically 300–500 μ L of cells into 2.5–3.0 mL of fresh SD media), and incubate in a shaking incubator as above.
4. After 1–2 h, pellet cells in a swinging bucket rotor (500 $\times g$, 2–3 min), discard the media, and resuspend the cells in 3 mL of freshly prepared SD media supplemented with 200 mM hydroxyurea.
5. Return cells to shaking incubator for 2.5 h.
6. After 2.5 h, transfer 1–1.5 mL of arrested cells to a 1.7 mL centrifuge tube, pellet the cells (500 $\times g$, 1–2 min), discard the supernatant leaving a small volume of media behind (~30–40 μ L), and resuspend the pelleted cells in the remaining media. Cells are now ready for imaging, and may be used for up to 1 h. If imaging several different yeast strains, stagger your

HU treatment so that cells will be imaged precisely after a 2.5 h arrest duration.

7. Place two strips of clear adhesive tape on a slide, making sure the area between allows another slide to sit on the edges of the tape, perpendicular to the first (see Fig. 2; Step 1). Avoid introducing air bubbles between the slide and tape, as this may interfere with removal of the slide, and may lead to unevenness in the agarose pad.
8. In a 50 mL conical tube, prepare a 1.7% agarose gel by dissolving 0.17 g of molecular biology grade agarose into 10 mL SD media. If you wish to maintain HU arrest throughout the imaging period, 200 mM HU should be included in this mixture. Place the tube in a beaker of water (to temper the heat such that the media/agarose mixture does not burn) and microwave until agarose is completely melted. Be sure to loosen the cap on the conical tube so that the pressure does not cause the tube to explode.
9. Use a P1000 to pipet ~200 μ L of agarose mixture onto the slide into the space between the 2 pieces of tape, being careful to minimize bubbles (Fig. 2; Step 1). Working quickly, arrange the top slide above and perpendicular to the bottom slide at an angle ($\sim 30^\circ - 45^\circ$) to the bench top, and allow it to gently drop on to the hot agarose mixture so that the slide pushes any air bubbles away from the center. Let the agarose mixture cool for ~1–5 min.
10. Working carefully, slide the top slide off laterally (Fig. 2; Step 2); use a razor blade to cut any agarose that may be sticking to the top slide during this movement. Cut next to both pieces of tape with a razor blade (Fig. 2; Step 3) and then carefully peel off the tape while holding the slide down on the bench top. Cut the agarose pad into a neat square approximately 1 cm on all sides, removing any excess agar from the slide.
11. Pipet 1 μ L of resuspended cell slurry (from Step 6 above) into one corner of the agarose pad (Fig. 2; Step 4). Tilt a coverslip at an angle next to the agarose pad and let it gently fall on the cells such that the coverslip spreads the cells across the agarose pad (Fig. 2; Step 5). Do not apply pressure on the cover glass after it has been placed on the agarose. If cells do not spread evenly across the agarose pad, the cell slurry may be too dense. If so, add additional HU-containing media to the cell pellet in Step 6.
12. Use a cotton swab to apply small drops of melted wax onto each corner of the cover glass to anchor it, and then seal all the edges around the cover glass (Fig. 2; Step 6). The cells are now ready for imaging (*see* **Note 8**).

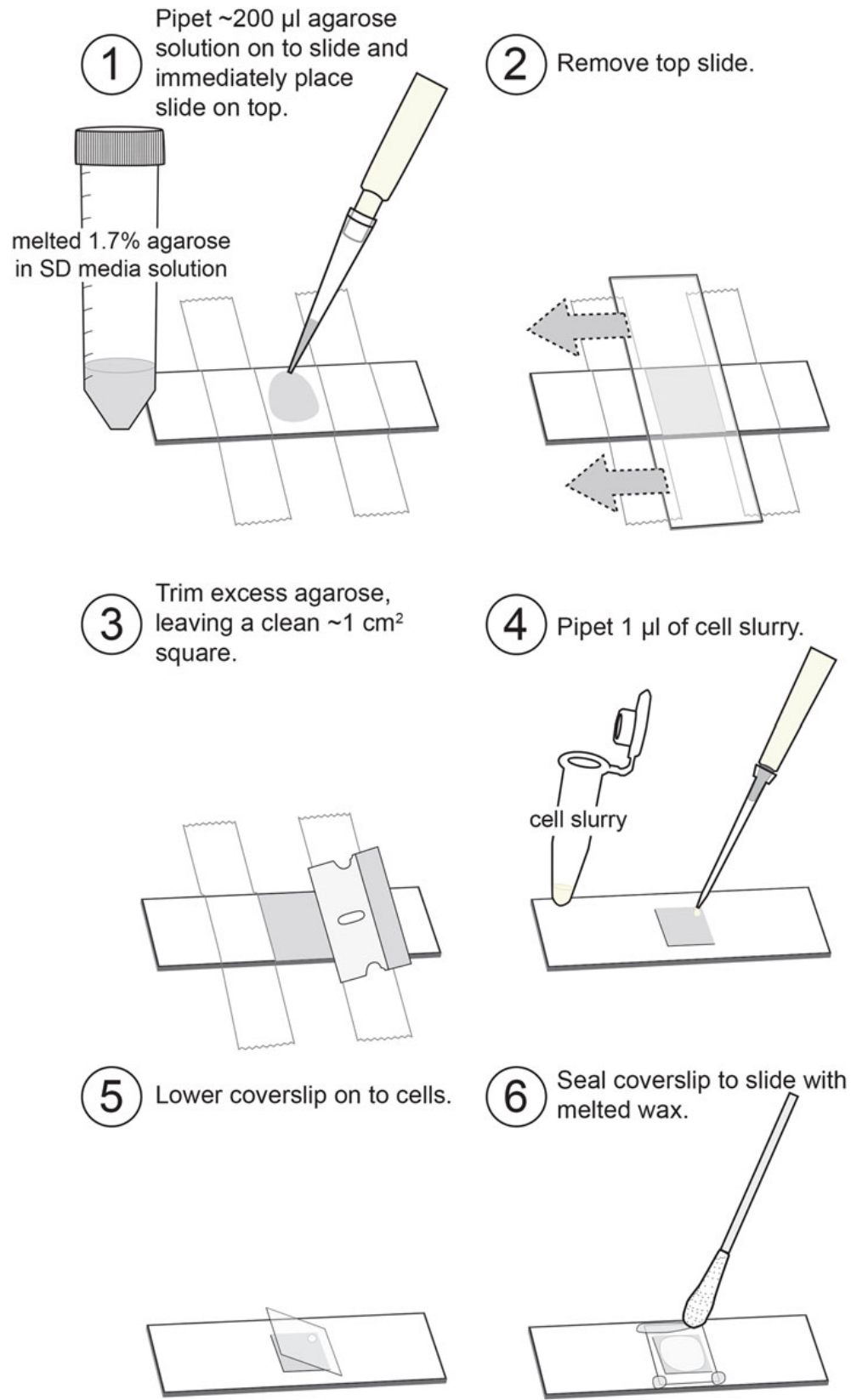


Fig. 2 Preparation of yeast cells for live-cell microscopy. See text (Subheading 3.3) for details

13. Place slide on microscope stage and collect time-lapse and Z-stack images. We generally acquire a single brightfield or DIC image (will be useful for analysis, described below), as well as 19–23 Z slices spaced 0.2 μm apart (over a 4–5 μm range) every 10 s for 10–15 min using a spinning disk confocal microscope (alternatively, 5–7 Z slices spaced 0.4–0.5 μm apart for a wide-field microscope setup will suffice). A given agarose pad can be used for 2–3 such movies, providing the cells remain in place. Such movies of HU-arrested cells generally provide numerous instances of dynein-mediated spindle movements.

3.4 Image Analysis Using Matlab and MS Excel

Here, we describe our pipeline for analyzing dynein-mediated spindle movements in yeast. Although the movies of dynein-mediated spindle movements can be analyzed manually, we have developed a tracking routine/pipeline that significantly improves the throughput. Although the pipeline provides a powerful tool to analyze spindle movements in yeast cells, it is indeed imperfect, as it is unable to parse dynein-mediated movements from those that are due to diffusion or otherwise (e.g., a microtubule pushing against the cell cortex can also displace the spindle). Thus, it is important that the output from the tracking routine is manually cross-checked against the movie such that dynein-mediated events are manually selected. For those that are new to this analysis, it is very helpful to compare spindle movements in wild-type cells to those from *dyn1 Δ* cells, in which no dynein-mediated events are apparent. This will provide a helpful point of comparison.

We generally score various aspects of the dynein-mediated spindle movements [6, 8], including the following: (1) velocity, (2) displacement per event, (3) frequency of events, (4) total displacement of events per minute. In addition to the extent of the movements, we have also scored events based on direction (with respect to the mother–daughter cell axis) and relative residence of the spindle with respect to cellular landmarks (e.g., cell cortex and bud neck) [4].

1. Copy *Spindle_Tracker.m*, *Stack_Threshold.m*, and *Relative_coordinate_mapper.m* to [Documents > Matlab] folder. In the Matlab file explorer, double-click the file names of these two files to bring them up in the Matlab editor.
2. Using ImageJ/FIJI, generate crops of brightfield/DIC image and corresponding fluorescence time lapse (using the “Restore selection” option in ImageJ/FIJI will ensure the images correspond perfectly). Note that *Spindle_Tracker.m* routine will work with both maximum intensity projections (to track spindle movements in 2-dimensions), or unprojected TIFF stacks (to track spindle in 3-dimensions) (see **Notes 9 and 10**).

3. Add folder with cropped TIFF stack(s) and brightfield/DIC images to Matlab path (find in file explorer, and right-click to add to path).
4. Double-click the *Spindle_Tracker.m* file in the explorer to open it in the Matlab Editor window. Edit the imaging parameters (*z_depth*, spacing between Z slices; *x_um* and *y_um*, X and Y pixel dimensions; *total_slices*, number of Z slices per time point; *total_frames*, total number of time points) such that they are correct.
5. Run *Spindle_Tracker.m* by clicking in the corresponding Editor window, and then hitting “Run.” This will make the Stack Thresholder window open, which is where you will set the threshold for image analysis.
6. Use the Adjust Threshold slider to designate the fluorescence signal that will be used for analysis (delineated with a magenta overlay). Be sure to adjust the threshold such that only a bright region within the spindle is selected. If a Z stack is being used, the Z-slice slider will permit you to choose the Z slice with the brightest signal. Note that only one time point is shown at a time. If more than 20 time points were acquired, the threshold window will permit you define a new threshold every 20 frames (to account for photobleaching).
7. After thresholding is complete, a “Save Workspace Variables” dialog box will direct you to choose a folder into which you will save the output of the tracking routine. If tracking is successful, a plot will appear that displays a 2D version of the output (irrespective of whether a 3D dataset was used).
8. In the Workspace, double-click the *Master_ds* data array to visualize raw data output. Select all data, and copy/paste it into cell A2 (“PASTE MATLAB OUTPUT HERE”) of the *SOA_TEMPLATE.xls* MS Excel file provided. Each tab (#1 through 18) is designed to accommodate data from a single cell (the “1” tab is pre-filled with a test data set for illustrative purposes), with the final tab (“ALL DATA”) providing a summary and compilation of select data.
9. Manually curate dynein-dependent events, and calculate velocity and displacement values for each independent event. We recommend manually inspecting tracking data output by comparing it to the respective movie for side-by-side comparison of tracking data to the movie. We typically generate a combined XY and XY maximum intensity projection so events can be observed from above (via the XY projection) and the side (from the XZ projection). We have provided a FIJI macro to easily convert a Z stack into a combined XZ/XZ projection (*make_combined_XY_YZ_movie_in_Fiji_19Zs*; see **Note 11**).

10. To manually curate tracking data, highlight those column values that correspond to a dynein event (e.g., see green shaded columns in Table 1 of the provided *SOA_template.xlsx*). Be wary that some frames might appear as missing in tracking data (see blue cells in Table 1). This is generally due to thresholding issues and the tracking routine “losing” the spindle for one or more frames, which can usually be remedied by repeating *Spindle_Tracker.m* with different thresholding.
11. Determining whether an event is a direct consequence of dynein is impossible in a wild-type yeast strain since other forces (i.e., those arising from the *KAR9* pathway) can also move the spindle (*see Note 12*). However, we use three criteria that are fairly good indicators: (A) an astral microtubule appears to be “plastered” against the cell cortex (in either the XY or XZ projection) during the spindle movement, (B) the microtubule appears to slide along the cortex, and (C) the spindle moves in the direction of the sliding/plastered microtubule. See example time-lapse movie, and corresponding analysis in *SOA_template.xlsx* (Table 1).
12. Data from velocity and displacement means (per event) that are input in columns S and T in *SOA_TEMPLATE.xlsx* file are automatically pooled in columns X and Y, and in the ALL DATA tab.
13. If the relative coordinates for the spindle are desired (e.g., with respect to the neck, or the mother/daughter cells), the “*Relative_coordinate_mapper.m*” routine can be used. Double-click on this file in the explorer and edit the file path in the Matlab Editor (*matFilename*) such that the output file from Step 7 is chosen (the *.mat* file). Be sure the folder with the data output from Step 7, and the DIC files have been added to the Matlab path (right-click to add if necessary).
14. When prompted, click on the bud neck region of the DIC image. This will define the bud neck as the zero point, with the mother coordinates being -1 to 0 (relative values), and the daughter cell coordinates being 0 to 1 . Note the values are relative, and not absolute; thus, the size of the respective cell compartments is all normalized to 1. This routine will output several images, including (1) the brightfield/DIC image with the overlaid tracking data, (2) the brightfield/DIC image with the overlaid heatmap of spindle centroid positions, (3 and 4) heatmaps of spindle centroid positions in mother and daughter cell, and (5) a histogram of relative spindle positions (see Fig. 3).
15. The main output for the coordinate mapping routine can be found in the “*normalizedDistanceFromNeck*” array (in the Workspace). This contains a 1D projection of positions (i.e.,

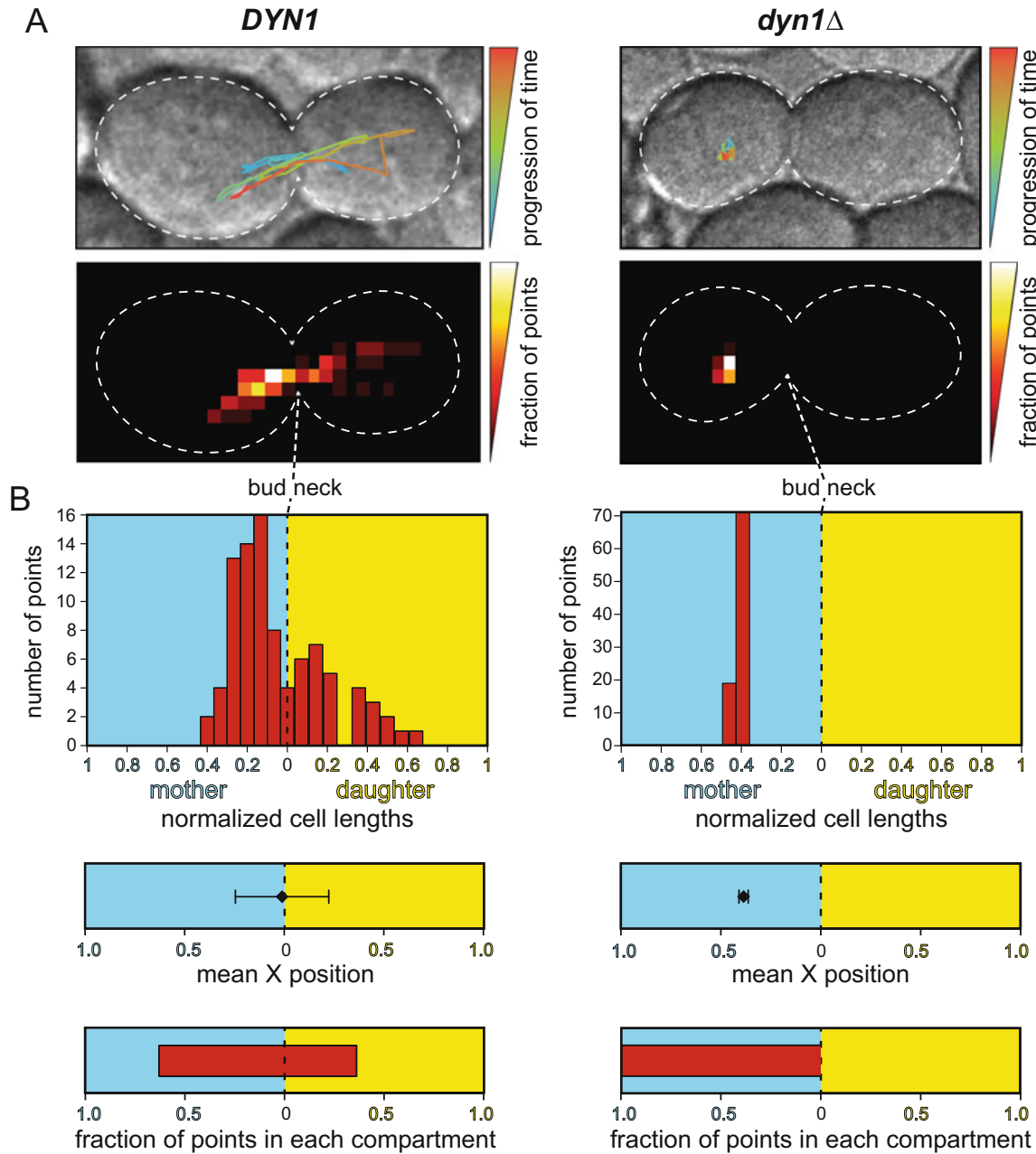


Fig. 3 Example output data from the spindle tracking and coordinate mapper routines. (a) Representative DIC image with spindle tracks overlaid (top; time progression indicated by color gradient from cyan to red), or cell outline with heatmap depicting track density (bottom). Note the lack of spindle motility in the *dyn1Δ* cell on the right. (b) Representative output from the coordinate mapping routine depicting the relative distribution of spindle coordinate data with respect to the bud neck, mother, and daughter cells (top), as well as the mean spindle position (middle), and the relative fraction of spindle coordinates in the mother and daughter cells (bottom)

all positions are projected onto a single X-axis) that can be used to make a histogram of positions, and to calculate mean distances from neck.

4 Notes

1. Note that there are a variety of plasmids offering several selectable markers that can be used for manipulation of the yeast genome. These include auxotrophic markers (e.g., LEU2, URA3, TRP1, HIS3) and antibiotic resistance markers (e.g., Kan^R, Hygro^R, Nat^R). For auxotrophic markers to be an option, it is important that the yeast strain being used is auxotrophic for the chosen marker (e.g., possesses a mutated *URA3* gene, such as *ura3-52*) that can be complemented by the wild-type copy (e.g., *URA3*). The mutant allele will ideally have a low probability of reversion. For a table of commonly used auxotrophic markers, see the *Saccharomyces* genome database (https://wiki.yeastgenome.org/index.php/Commonly_used_auxotrophic_markers).
2. Design the forward and reverse oligonucleotides such that they contain gene-specific sequences for insertion of the PCR product in the appropriate genomic locus. See Fig. 1 for typical strategy for gene deletion. The binding sites and the priming sequences for the forward and reverse oligos are chosen such that the promoter region and terminator (if any) are included in the PCR product. Primers for commonly used plasmids are available in the literature (e.g., [9]).
3. We find that transformation efficiency is higher with freshly prepared/non-frozen cells. If using frozen cells, thaw cell aliquot slowly on ice prior to proceeding with protocol.
4. Tub1-tagging plasmids such as those we described previously [7], generally require an enzyme digest prior to being transformed. We typically digest 500–2000 ng of plasmid (many of which are deposited on Addgene: <https://www.addgene.org/browse/article/18536/>) prior to transformation.
5. This incubation step increases the efficiency of transformation, but is not necessary in cases where an auxotrophic marker is being used. If an antibiotic (e.g., hygromycin) is being used as the selection agent, this incubation becomes more important.
6. Glass beads can be reused by washing and re-sterilizing. To do so, combine all used beads in a wide glass baking dish. Thoroughly rinse beads with water, and then submerge them in a 0.1% SDS solution. Agitate the beads by hand every 10 min for 30 min, and then rinse them thoroughly with water until the detergent appears to be mostly gone (apparent by a lack of

bubbles). Submerge the beads in a solution of 10% hydrochloric acid for ≥ 1 h, and then rinse them ~ 10 – 15 times with water. Pour off as much of the water as possible, and let the beads air dry in the baking dish overnight. The next day, the beads can be aliquoted into glass bottles, and autoclaved to sterilize.

7. Cells can be used directly in a PCR by adding a small portion of a single colony to a reaction mixture. We typically employ Taq polymerase in a 20 – 30 μL reaction with 0.5 μM of a suitable forward and reverse primer. The target amplicon is typically <1500 bp as larger products tend to be more difficult to obtain.
8. The size of the agarose pad, as well as the density of the cell slurry, can impact the quality of the slide. If the cells are too dense, or the agarose pad too small (i.e., if the number of cells is too high for the size of the agarose pad), the cells may have limited room to settle on the agarose pad, which will lead to the cells moving around, which is non-ideal for time-lapse imaging.
9. If relative spindle coordinate information is desired (i.e., with respect to cell periphery and/or mother–daughter cells), it is important to rotate the image crops such that the mother and daughter cell are consistently situated on a specific side of the image. For example, we always position the mother cell on the left, and the daughter cell on the right.
10. Ensure the cropped TIFF stack has only one bright object (i.e., one spindle). More than one will confound the tracking routine. Additional objects should be eliminated using a drawing tool such as the polygon tool (pressing command-F with black selected will fill in the designated region with black, thus obscuring the unwanted fluorescence signal).
11. To use this FIJI macro, ensure that the correct Z-slice number is input prior to running it (the “group” value in line 7: *run (“Grouped Z Project...”, “projection=[Max Intensity] group=19”)*).
12. To more confidently determine that a spindle movement is a consequence of dynein, and not Kar9, we generally delete *KAR9* [4, 6, 8].

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