CHAPTER TEN

Engineering Defined Motor Ensembles with DNA Origami

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Abstract

Many cytoskeletal motors function in groups to coordinate the spatial and temporal positioning of cellular cargo. While methods to study the biophysical properties of single motors are well established, methods to understand how multiple motors work synergistically or antagonistically are less well developed. Here, we describe a three-dimensional synthetic cargo structure made using DNA origami, which can be used to template defined numbers and types of cytoskeletal motors with programmable geometries and spacing. We describe methods for building the DNA origami structure, covalently attaching motors to DNA, forming the motor–DNA origami structure complex, and single-molecule assays to examine the motile properties of motor ensembles.
1. INTRODUCTION

Cytoskeletal motors and the dynamic filaments they move on generate the organization critical for a cell to maintain homeostasis, grow, divide, and communicate with neighboring cells. Motors carry out these functions either by directly transporting cargos or by generating pulling forces (Hirokawa, Niwa, & Tanaka, 2010; Vale, 2003). In this review, we focus on microtubule-based motors, but the same methods could be applied to the actin-based myosin motors. Cytoplasmic dynein and kinesin are the microtubule-based motors that move cargos toward the minus and plus ends of microtubules, respectively. The ultimate spatial and temporal distribution of cargos depends on the arrangement, number, and type of motors on the cargos (Bryantseva & Zhapparova, 2012; Welte, 2004). While great strides have been made in understanding the mechanisms of single cytoskeletal motor proteins (Block, 2007; Vale, 2003), much less is known about the mechanisms governing motor ensembles, which are important in physiological contexts. Bridging the gap between observations of single motors and motor ensembles is critical to understand the biophysical mechanisms that govern how multiple motors coordinate or interfere to produce the complex motility observed in cells. While some methods to study motor ensembles in vitro have been described (Bieling, Telley, Piehler, & Surrey, 2008; Mallik, Petrov, Lex, King, & Gross, 2005; Vale, Malik, & Brown, 1992; Vershinin, Carter, Razański, King, & Gross, 2007), it has not been possible to control the absolute numbers of motors using these methods. The use of DNA nanotechnology to build synthetic cargos offers a platform for exquisite control over motor number, type, and spacing (Derr et al., 2012; Rogers, Driver, Constantinou, Kenneth Jamison, & Diehl, 2009). DNA origami (Shih & Lin, 2010), building three-dimensional (3D) shapes out of DNA, is modular and allows for sophisticated structures to be designed, built, and modified rapidly and easily. Alternatively, a duplex DNA scaffold can be used to template one or two motors as described in Chapter 11 by Rogers and colleagues.

In this chapter, we describe techniques to template motor proteins on a DNA origami structure. These methods can be used to understand the biophysical mechanisms of motor proteins in vitro with single-molecule motility assays (Fig. 10.1). We provide methods for folding and purifying the 3D DNA origami structure, attaching oligonucleotides (oligos) to motor proteins, forming the motor–DNA origami structure complex, and single-molecule
assays to determine the motile properties of motor ensembles. Throughout we discuss practical and theoretical considerations and methods for validation and quality control. Updates to these techniques can be found online (https://reck-peterson.med.harvard.edu/protocols).

2. BUILDING THE DNA ORIGAMI STRUCTURE

There is an expanding list of nanoscale devices that can be built using 2D and 3D DNA origami (Douglas, Bachelet, & Church, 2012;
Software, tutorials, and other resources for sharing and building DNA origami structures exist, creating a lower barrier for adapting this technology (Douglas et al., 2009). While DNA origami can be used to build structures of varying geometries (Dietz, Douglas, & Shih, 2009; Douglas et al., 2009; Ke, Voigt, Gothelf, & Shih, 2012; Shih & Lin, 2010), the 12-helix bundle (12hb) described here is a simple, straightforward design (Fig. 10.2A) (Derr et al., 2012). It is composed of six inner helixes surrounded by six outer helixes. All origami structures are constructed by folding a long, single-stranded (ss) piece of scaffold DNA by the annealing of short (~20–60 nucleotides) ss oligos, called staples, which bind to noncontiguous sequences on the scaffolding strand. In our design, ss oligos protruding from the structure, called handles, offer sites for motor, fluorophore, or biotin attachment. Handles can be designed every 14 nm on any of the six outer faces by including nonhomologous sequence to the 3' end of the staple strands. Staples that are positioned in the structure at handle locations are referred to as either positive handles (those that contain a handle sequence) or negative handles (those lacking a handle sequence). Thus, there are 15 sites on each of the six faces of the outer helixes that allow for up to 90 uniquely addressable motor attachment sites. We avoid placing handles on the ends of the origami structure (positions 0 and 14; Fig. 10.2A), as these staples are the least stably associated with the structure (B. Goodman, N. Derr, W. Shih, & S. Reck-Peterson, unpublished data).

The ssDNA scaffold used to fold the 3D origami structure is purified from M13 phage as previously described (Bellot, McClintock, Chou, & Shih, 2013). 273 short oligo staples are required to fold the DNA origami structure. The sequence of the M13 genome we use (p8064; Bellot et al., 2013) and the complementary oligo staples can be found online (https://reck-peterson.med.harvard.edu/protocols). Any chemical moiety conjugated to antihandle oligos (an oligo that is complementary to the handle sequence) can be added to the structure, provided it can withstand the high temperatures associated with the folding protocol. In our hands, fluorophores, biotin, and photocleavable linkers remain functional after the folding process. New protocols for enhanced folding have been developed and could be considered for optimizing yield and fidelity (Sobczak, Martin, Gerling, & Dietz, 2012).

The methods described here have been optimized for our 12hb structure, although similar methods can be used to fold and purify other DNA
origami structures. Magnesium concentrations for folding different structures must be optimized empirically as described earlier (Douglas, Dietz, et al., 2009). New origami structures can be designed using caDNAno software (cadnano.org) (Douglas, Marblestone, et al., 2009), and additional design principles are discussed in the literature (Castro et al., 2011; Dietz et al., 2009; Ke et al., 2012; Shih & Lin, 2010).

![Figure 10.2](image-url) Design and analysis of the 12hb DNA origami structure. (A) Design of the 12hb DNA origami structure. The 12hb is composed of six inner helixes and six outer helixes. The structure is 14 nm in diameter and 225 nm in length with optional handle sites every 14 nm. (B) 12hb folding is analyzed by glycerol gradients. After folding, the 12hb is sedimented through a 10–45% glycerol gradient as described (Lin, Perrault, Kwak, Graf, & Shih, 2013) and fractions from the gradient are run on a 2% agarose TBE gel. The excess staples remain in the lower percentage glycerol fractions, while the denser folded 12hb is found in the higher percentage glycerol fractions (box). (C) Handle strand incorporation into the 12hb is assessed by a DNA PAINT experiment (Derr et al., 2012; Jungmann et al., 2010). In this technique, short (10 bp), fluorescent antihandles base pair with the handles of surface-immobilized 12hb structures (Jungmann et al., 2010). Due to the short stretch of homology, stochastic binding of the labeled antihandles generates the ability to perform high-precision localization over many frames of a movie. (D) A DNA PAINT experiment of a 12hb bearing seven handles. A histogram of the number of handle sites observed reveals that the majority of structures have five or more assessable handles. The inset shows a pseudo-image of the handle locations generated from a movie of a single 12hb structure.
2.1. Folding the DNA origami structure

1. Mix the following reagents at room temperature:
   • 2.5 µl of 20 × folding buffer.
   • 5 µl of 1 µM p8064 scaffold (final scaffold concentration is 100 nM). For methods for purifying the p8064 scaffold, see Bellot et al. (2013).
   • Core staples to a final concentration of 600 nM (Table S1 in Derr et al., 2012).
   • Negative handle staples (Table S2 in Derr et al., 2012) to a final concentration of 600 nM.
   • Positive handle staples (Table S3 in Derr et al., 2012) to a final concentration of 10 µM. All staple sequences can be found online (https://reck-peterson.med.harvard.edu/protocols).
   • Fluorophore-conjugated oligos complementary to the handle strands, referred to as antihandles, can be included to label the chassis. If included, they should be added to a final concentration of 10 µM.
   • Add ddH2O to bring the final volume to 50 µl.

2. Using a thermal cycler, run the following protocol:
   • 80 °C for 5 min.
   • 14 additional cycles of 5 min with 1 °C temperature decrease per cycle.
   • 65 °C for 30 min.
   • 35 additional cycles of 30 min with 1 °C temperature decrease per cycle.
   • Hold at 4 °C.

3. Proceed directly to the purification of the folded origami structure to remove excess staples (recommended). Alternatively, the folded structure can be stored at 4 °C (do not freeze the folded structures) for several days.

4. Notes:
   • Either a positive or a negative handle staple (but not both) should be used at each handle site position. This ensures that the 12hb structure folds properly as the positive and negative handle staples also have sequence required for binding the scaffolding strand.
   • Core staples should have a final concentration of 6 × the p8064 scaffold.
   • All positive handle staples should have a final concentration of 100 × the p8064 scaffold.
- Folding and purification can be monitored using 2% agarose gel electrophoresis. Gels and running buffers should be supplemented with 11 mM MgCl₂. Gels may need to be run in water baths to prevent melting of the DNA origami structures. Melted structures appear as a smear rather than a tight band.

Solutions

1. 20 × Folding buffer
   - 100 mM Tris, pH 8.0
   - 20 mM EDTA
   - 320 mM MgCl₂

2.2. Purification of the folded DNA origami structure

1. The folded 12hb origami structure is purified away from excess staple strands using velocity gradient centrifugation as described earlier (Lin et al., 2013).
2. Use Amicon 100K MWCO filters (Millipore) for buffer exchange, glycerol removal, and to concentrate the DNA origami structures.
3. The folded structure should be stored in buffer with at least 11 mM MgCl₂ at 4 °C. Lower MgCl₂ concentrations or temperatures can lead to unfolding.

2.3. Determining the integrity of the folded structure

To determine the integrity of the folded DNA origami structure, several methods can be used. Here we briefly describe agarose gel electrophoresis, transmission electron microscopy (TEM), and DNA PAINT.

1. Agarose gel electrophoresis can be used to visualize the folded chassis compared to the initial input in the folding reaction. The folded chassis will run faster in the gel. Run the folded chassis on a 2% agarose, 1 × TBE gel containing 11 mM MgCl₂ and 0.7 μg/ml ethidium bromide. The MgCl₂ should be added after boiling the agarose in TBE buffer. Run the gel at a constant voltage of 70 V for 90 min (Fig. 10.2B). Running the gel at a voltage >70 V will cause the gel to overheat, causing the DNA origami structure to denature and run as a smeared band. We routinely place our gel apparatus in an icebox to dissipate heat and exchange the running buffer if the gel is run for longer than 2 h.
2. TEM techniques can be used to assess the structure using previously described methods (Douglas, Marblestone, et al., 2009).
3. DNA PAINT techniques can be used to observe handle incorporation (Fig. 10.2C) as described previously (Derr et al., 2012; Jungmann et al., 2010). To immobilize the DNA origami on a cover slip, biotin-labeled handles are included during the folding reaction. The biotin-labeled origami structures can then be adhered to avidin-coated cover slips. Previously, using the DNA PAINT method we found that handles are incorporated with 80% efficiency. Modifying staple length and other design principles may improve the efficiency of handle incorporation (Sobczak et al., 2012).

Solutions

1. 10× TBE buffer
   - 450 mM Tris, pH 8.1
   - 450 mM Boric acid
   - 10 mM EDTA

2. Agarose gel
   - 2% Agarose
   - 1× TBE buffer
   - Boil to dissolve agarose
   - Add MgCl₂ to 11 mM

3. Gradient buffers
   - 1× TBE buffer
   - 11 mM MgCl₂
   - 15–45% glycerol

4. Agarose gel running buffer
   - 1× TBE buffer
   - 11 mM MgCl₂

3. COVALENT ATTACHMENT OF OLIGONUCLEOTIDES TO MOTOR PROTEINS

We use the SNAP-tag technology (NEB) to attach oligos to motor proteins (Keppler et al., 2003). The motor proteins we express and purify are fusion proteins with the SNAP-tag (Derr et al., 2012; Qiu et al., 2012). The SNAP-tag is appended to the cargo-binding region (or “tail”) of the motor. The first step in linking a motor to DNA is to link antihandle oligos (oligos that will base pair with positive handle staples on the origami structure) to the SNAP ligand, benzylguanine (BG). The BG-oligo conjugate is then linked to the motor-SNAP-tag fusion protein to produce an
oligo-linked motor. The oligo-linked motors are then attached to the 12hb origami structure via base pairing.

3.1. Conjugating BG-NHS to an amine-oligonucleotide

1. Using a needle, add nonaqueous DMSO to a vial containing dry BG-NHS (NEB) to a final concentration of 20 mM. All reagents should be at room temperature to reduce condensation. Unused BG-NHS can be stored in a tube sealed with parafilm for up to 4 months at –20 °C.
2. Resuspend the amine-functionalized oligos in water to a final concentration of 2 mM. Take a small sample (~1 µl) and dilute 1:3000 for a gel sample (pre).
3. Mix and incubate the following at room temperature for 30 min:
   - 4 µl of 2 mM amine-oligo in aqueous solution
   - 8 µl of 200 mM HEPES, pH 8.5
   - 12 µl of 20 mM BG-NHS in DMSO
4. Run the reacted mixture through a 0.1-µm filter (Ultrafree-MC with Durapore membrane; Millipore) to remove any precipitate from the solution. Take a small sample (~1 µl) and dilute 1:500 for a gel sample (post).
5. Run 5 µl of “pre” and “post” oligos on a 20% polyacrylamide TBE gel (Life Sciences) at 200 V for 65 min as specified (http://www.lifetechnologies.com/order/catalog/product/EC6315BOX).
6. Stain the gel with Sybr Gold (Invitrogen) following the standard protocol (Fig. 10.3A; http://probes.invitrogen.com/media/pis/mp11494.pdf).
7. Use appropriate software (e.g., ImageJ) to quantify the percentage of oligo that was successfully linked to the BG ligand.

3.2. Purification of BG-oligonucleotides

Purification of BG-oligos away from unreacted BG-NHS is critical because unreacted BG-NHS can competitively inhibit downstream labeling of the protein through reaction with the SNAP-tag.

Figure 10.3 Oligo labeling of motors and formation of the 12hb–motor complex. (A) Labeling of oligos with BG is assessed by 10% agarose TBE gel electrophoresis. A mobility shift of the oligo after addition of BG is observed. Oligo dimers from cross-linking during oligo synthesis are also visible at the top of the gel. “Pre” and “post” refer to samples that were taken before or after the reaction, respectively. (B) Labeling of purified SNAP-tagged kinesin motors with BG-oligos is assessed by 4–12% PAGE. SNAP-tagged kinesin motors (pre) are mixed with BG-oligos and microtubules in the presence of AMPPNP and centrifuged. Most of the BG-oligo-SNAP-motor complex is found in the pellet (P1) fraction. ATP is added to the pellet fraction to release the motor from microtubules and the mixture is centrifuged a second time. Most of the motor is now found in the supernatant (S2) fraction. A small gel shift of the BG-oligo-labeled SNAP-tagged kinesin is observed. (C) Attachment of oligo-labeled motors to the 12hb structure is assessed by 2% agarose TBE gel electrophoresis. 12hb origami structures with 1, 2, 3, or 4 oligo-dynein attachment sites reveal an upward mobility shift that corresponds to the programmed handle number when mixed with antihandle oligo-labeled dynein. TAMRA-labeled 12hb structures are visualized. (D) Attachment of oligo-labeled motors to the 12hb origami structure can also be analyzed by transmission electron microscopy (Derr et al., 2012). Negatively stained dynein motors can be seen bound to a 12hb with four dynein attachment sites. Scale bars: 40 nm.
2. Remove excess packing buffer with the following protocol:
   * Prepare and assemble the column as directed by the product manual.
   * Centrifuge for 2 min at $1000 \times g$ at $4 \, ^\circ C$. Discard the packaging buffer.
   * Exchange the column buffer with the desired buffer for protein labeling by adding 500 μl of buffer to the gel and centrifuging for 1 min at $1000 \times g$ at $4 \, ^\circ C$. Discard the buffer and repeat the column washing procedure three additional times.

3. To purify the BG-oligos from excess free BG-NHS, apply 24 μl of the sample to the first column. Centrifuge for 1 min at $1000 \times g$ at $4 \, ^\circ C$ and collect the eluate containing the BG-oligos. Repeat this procedure three additional times.

4. Use a UV spectrophotometer to measure the final oligo concentration.

5. Store the BG-oligos at $-20 \, ^\circ C$.

3.3. Labeling SNAP-tagged motor proteins with BG-oligonucleotides

We use two different strategies for labeling motors with oligos. For SNAP-ZZ-tagged dynein purified from yeast, we label the protein while it is attached to IgG sepharose beads during the purification (Qiu et al., 2012). For SNAP-6×HiS-tagged kinesin (Case, Pierce, Hom-Booher, Hart, & Vale, 1997), we use a microtubule affinity step after the nickel column purification because the positive charge on the nickel column binds negatively charged oligos (Derr et al., 2012). It is critical to remove the unreacted BG-oligo from the motor-oligo product to ensure that the unreacted oligo does not compete with the motor-oligo for binding to the 12hb origami structure.

1. Protocols for polymerizing tubulin to form microtubules can be found online (http://mitchison.med.harvard.edu/protocols.html).

2. Mix the following components. The final concentrations are listed in parentheses.
   * 50 μl of 3.5 μM kinesin (2 μm)
   * 22 μl of ~240 μM BG-oligo (60 μM)

3. Incubate the mixture at room temperature for 15 min. Add the following in order. The final molar concentration of each component is listed in parentheses.
   * 0.85 μl of 0.1 M AMPPNP (1 mM)
   * 0.17 μl of 10 mM taxol prepared in DMSO (20 μM)
   * 0.85 μl of 660 units/ml Apyrase (6.6 units/ml)
11 µl of 50 µM microtubules (6.5 µM). It is important that taxol is in the buffer before the microtubules are added.

ddH₂O to a final volume of 85 µl

4. Mix by gently flicking the tube (to avoid microtubule shearing) and incubate at room temperature for 15 min.

5. Centrifuge the mixture through 110 µl of a 60% glycerol cushion in a TLA100 (Beckman) or equivalent rotor at 108,000 × g at 25 ºC for 15 min.

6. Remove the top layer and a small amount (~10 µl) of the top of the cushion. Save 4 µl for SDS-PAGE analysis (S1).

7. Gently wash the sides of the tube and glycerol cushion by adding 110 µl of BRB12 + taxol above the glycerol cushion and removing the wash and some of the cushion. Repeat three times until the entire cushion is removed without disturbing the pellet.

8. Wash the pellet three times with BRB12 + taxol without disturbing the pellet.

9. Resuspend the pellet containing kinesins bound to microtubules in 50 µl of release buffer. Save 3 µl for SDS-PAGE analysis (P1).

10. Incubate the mixture for 5 min at room temperature.

11. Pellet the microtubules by spinning at 278,000 × g for 6 min at 25 ºC in a TLA 100 rotor (Beckman) or equivalent.

12. Remove the supernatant and add 10 µl of BRB12 + taxol containing 60% sucrose to a final concentration of 10% sucrose. Save 3.6 µl (post-sucrose addition) of the supernatant for SDS-PAGE analysis (S2).

13. The final kinesin-oligo-containing supernatant should be aliquoted, flash-frozen in liquid nitrogen, and stored at −80 ºC. There will be a mixture of labeled and unlabeled kinesin if the reaction has not gone to completion, but only the labeled kinesin will bind to the DNA origami structure.

14. Resuspend the pellet in 50 µl of BRB12. Save 3 µl for a gel sample (P2).

15. Analyze the fractions by SDS-PAGE and SYPRO Red (Life Sciences) staining (Fig. 10.3B) alongside a protein concentration standard to measure the labeling efficiency (the percent of kinesin that shifts to a higher molecular weight due to covalent linkage to the ~8 kDa oligo) and yield (the final concentration of S2 sample by densitometry compared to a protein standard).

Solutions

1. BRB12
   - 12 mM PIPES, pH 6.8
1. Mix the following on ice for 30 min:
   * 5 µl of 10 nM 12hb origami structure
   * 5 µl of >800 nM oligo-labeled motor
   * (Optional) 5 µl of >800 nM of a second oligo-labeled motor (e.g., opposite polarity motor or mutant motor).

2. To assess complex formation, run the 12hb alone, the motor alone, and the motor–12hb complex on a 2% agarose gel in 1× TBE supplemented with 11 mM MgCl₂ and 0.5% lithium dodecyl sulfate (LDS). Gel
samples are mixed with 4 × NuPAGE LDS sample buffer (Life Sciences) and incubated on ice for 5 min.

3. Run the gel at 70 V for 90 min at room temperature (Fig. 10.3C).

4. Complexes can also be observed on an EM grid with standard negative stain techniques (Fig. 10.3D) as previously described (Derr et al., 2012).

5. If excess motors will interfere with downstream procedures, a gel filtration step can be performed using Sephacryl S-500 HR resin (GE Healthcare) in a microchromatography spin column (Bio-Rad):
   * Dilute the 12hb–motor complex in Dynein assay buffer (DAB) to a final volume of 50 µl and apply to a 450 µl Sephacryl S-500 HR column.
   * Centrifuge for 10 s at 1000 × g and collect the supernatant.

Solutions

1. DAB
   * 30 mM HEPES, pH 7.4
   * 50 mM K-acetate
   * 2 mM Mg-acetate
   * 1 mM EGTA, pH 7.5
   * 10% Glycerol

5. SINGLE-MOLECULE MOTILITY ANALYSIS OF MOTOR–DNA ORIGAMI COMPLEXES

To determine the motile properties of motor–DNA origami complexes, we use single-molecule motility assays, which can provide the velocity, run length, and stepping behavior of individual motor-driven cargos. For analysis of microtubule-based motors, taxol-stabilized microtubules are immobilized to a cover slip via biotin–streptavidin (described in detail in Gennerich & Reck-Peterson, 2011). Fluorescently labeled motor–cargo complexes are then observed moving along microtubules in the presence of ATP using time-lapse, total internal reflection fluorescence (TIRF) microscopy (described in detail in Derr et al., 2012).

One important consideration is the positioning of the fluorescent dyes on the 12hb structure and interpretation of the data based on these positions. In our experiments, we have positioned 5 oligo-linked dyes at the end of the 225-nm-long DNA origami structure. Because the structure can rotate around a single microtubule-motor attachment, if a motor at the end opposite the fluorophore attachment site is the only motor making microtubule contact, jumps of 450 nm in the fluorescent signal can occur. This becomes
particularly important when interpreting bidirectional movements or high-precision stepping experiments.

A variation of the standard motility assay involves attaching photocleavable positive handle oligos to the 12hb structure such that the role of individual motor types can be assessed dynamically. We used this experimental design to determine whether a tug-of-war was occurring between dynein and kinesin motors attached to the same DNA cargo structure (Derr et al., 2012). In this experiment, either dyneins or kinesins are attached to the 12hb structure by photocleavable handles. These handles have a nitrobenzyl spacer group (Integrated DNA Technologies) inserted just prior to the portion of the handle that will hybridize to the motor antihandle, providing a means to sever any designated motor from the origami structure with 405-nm laser light. In our experiments, we used photocleavage and release of one motor type to determine the role of the remaining motor type (Fig. 10.4A) (Derr et al., 2012). In this work, cargos containing photocleavable kinesins were labeled with TAMRA, and cargos with photocleavable dyneins were labeled with Cy5. The following protocol outlines a photocleavage experiment.

1. Prepare a flow cell with biotinylated and fluorophore-labeled microtubules immobilized on the surface via a biotin–streptavidin sandwich as previously described (Gennerich & Reck-Peterson, 2011).

2. Dilute the 12hb–motor complex to single-molecule conditions (∼10–100 pM) in motility buffer containing 2.5 mg/ml casein and 1/2 oxygen scavenger mix in a final volume of 20 μl.

3. Flow the above mixture into the motility chamber and seal the chamber with vacuum grease.

4. Image the chamber immediately as the oxygen scavenger system will acidify the buffer after ∼1 h. Buffer with low buffer capacity such as BRB12 can only be used for ∼20 min.

5. We use an Olympus IX-81 inverted objective TIRF microscope (Qiu et al., 2012) equipped with 405-, 561-, and 640-nm lasers (Coherent). A dual-band sputtered emission filter (z561/635rpc and etCy3/Cy5m, Chroma) in the main optical path is used to image rapidly between the 561- and 640-nm channels. The 561-nm laser is controlled by an acousto–optical tunable filter (NEOS; 10 ms response time) and the 640-nm laser is controlled by a fast mechanical shutter (SmartShutter, Sutter; 25 ms response time).

6. For experiments with yeast dynein and human kinesin (Derr et al., 2012), we have imaged the 561-nm (cargo bearing photocleavable kinesins) and 640-nm channels (cargo bearing photocleavable dyneins)
at 1 image/s, with 100 ms exposure times in each channel with 1.5 mW of power measured at the objective. To induce photocleavage, after 1 min of imaging the 405-nm laser (0.5 mW at the objective) is pulsed at 1 image/s, with 400 ms exposure times. Imaging is continued for a total of 4 min. As a control, we image motor–cargo complexes lacking the photocleavage handles.

7. To measure the kinetics of photocleavage, experiments with immobile motor–cargo complexes are performed in motility chambers lacking ATP (Derr et al., 2012).

Figure 10.4 Photocleavable oligos allow release of motors from the 12hb. (A) To program the removal of specific motors from the 12hb structure, photocleavable linkers are added to select handle strands (circles). Illumination with 405-nm light induces photocleavage of the motors bearing the photocleavable linker (inset). (B) Two types of 12hb structures with different fluorophores were designed for simultaneous imaging. Kinesin cleavable, mixed-motor 12hb structures (left) were labeled with Cy5 and had photocleavable linkers on the kinesin handle strands. Dynein cleavable, mixed-motor 12hb structures (center) were labeled with TAMRA and contained dynein handle strands with photocleavable linkers. After 1 min of imaging both the TAMRA and Cy5 channels, a 405-nm laser was pulsed in between frames to induce cleavage (dotted line) showing that 12hb structures move in the expected direction when the opposing motor is cleaved. Different colored fluorophores allow the simultaneous observation of the two different motor ensembles during the same experiment (right). Vertical scale bar: 1 min; horizontal scale bar: 10 μm.
Solutions

1. DAB + casein
   - Supplement DAB with casein to a final concentration of 2.5 mg/ml.

2. Oxygen scavenger mix (Yildiz et al., 2003)
   - 50 µl of 1 × DAB
   - 15 µl of 20 mg/ml glucose catalase (Roche)
   - 7.5 mg glucose oxidase (*Aspergillus niger*; Sigma)
   - Vortex the mixture and then centrifuge it at 16,100 × g for 5 min at 4 °C.
   - Remove the supernatant and aliquot, freeze in liquid nitrogen, and store at −80 °C.

6. DATA ANALYSIS CONSIDERATIONS

   Single-molecule behavior of the motor–cargo complexes is analyzed by generating kymographs (position vs. time plots, Fig. 10.4B) using ImageJ. Automated particle tracking software can also be used (Jaqaman et al., 2008; Ruhnow, Zwicker, & Diez, 2011). Analysis of motor ensembles, as opposed to individual motors, raises some challenges, including very long run lengths and bidirectional behavior. Motor–cargo complexes containing multiple dynein or kinesin motors that we have studied typically generate run lengths >10 μm (Derr et al., 2012). Thus, to achieve accurate run lengths, we analyze long microtubules (typically >65 μm) for sufficient amounts of time (Derr et al., 2012). To generate long microtubules, we avoid pipetting microtubules through small-gauge pipette tips and mix microtubule solutions by gently flicking the tubes. Raising the ionic strength of the buffer can also be used to shorten the run lengths of dynein and kinesin ensembles as both motors’ microtubule binding is based on ionic interactions (Redwine et al., 2012). To analyze potential bidirectional motility of motor–cargo complexes containing both dyneins and kinesins, it is important to consider the position of the fluorescent labels on the DNA origami cargo as described in Section 5.

7. SUMMARY AND FUTURE DIRECTIONS

   It is likely that motor ensembles drive most cargo transport *in vivo* (Gross, 2004). Thus, the ability to control the number, type, and spatial arrangement of motors on a synthetic cargo structure is an important tool
for determining the biophysical properties governing movements by motor ensembles. For example, by using DNA origami to program motor ensembles of one to seven identical polarity motors (either dyneins or kinesins), we determined that motor number had very little affect on directional velocity (Derr et al., 2012). We also programmed DNA origami structures to bind varying ratios of dynein and kinesin. Many of these mixed-motor ensembles were immotile; however, by using a photocleavage experiment, we found that this apparent tug-of-war could be resolved by removing one motor species (Derr et al., 2012).

DNA origami is an ideal method for these types of experiments because it offers precise engineering control of shape at scales that are biologically relevant (Douglas et al., 2012; Douglas, Dietz, et al., 2009; Douglas, Marblestone, et al., 2009; Langecker et al., 2012; Shih & Lin, 2010). Spherical objects can now be designed, which may more accurately resemble organelar cargos (Erickson, Jia, Gross, & Yu, 2011). DNA origami also allows the incorporation of a variety of chemistries to gain dynamic control. In addition to photocleavable groups, \textit{cis–trans} isomerization groups, disulfide bridges, DNA aptamers, and other controllable chemistries could be applied in the future to create dynamic systems. The rapidly decreasing costs of oligos and freely available software to design DNA origami structures should make this method feasible and approachable for many laboratories.

DNA origami methods are a valuable addition to the biophysicist’s toolbox for the study of groups of actin- or microtubule-based molecular motors. We envision future experiments that will determine, in greater detail, the biophysical properties of motor ensembles. For example, linking origami structures to objects that can be trapped by optical tweezers will allow force production to be probed in the context of varying numbers or types of motors. Understanding how regulatory proteins affect motor ensembles will also be an important area where synthetic DNA technology can aid in modeling the complexity found in physiological contexts. Finally, while methods to build DNA or RNA structures \textit{in vivo} or introduce folded nucleic acid structures into cells are in their infancy (Guo, 2010; Pinheiro, Han, Shih, & Yan, 2011), breakthroughs in these areas will open doors for using DNA origami to probe motor function in cells.

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