Published in final edited form as:

Methods Cell Biol. 2015 ; 128: 243–269. doi:10.1016/bs.mcb.2015.01.021.

Reconstituting ParA/ParB-mediated transport of DNA cargo

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Abstract

Protein gradients play key roles in subcellular spatial organization. In bacteria, ParA adenosine triphosphatases, or ATPases, form dynamic gradients on the nucleoid surface, which imparts positional information for the segregation, transport, and positioning of chromosomes, plasmids, and large protein assemblies. Despite the apparent simplicity of these minimal and self-organizing systems, the mechanism remains unclear. The small size of bacteria along with the number of physical and biochemical processes involved in subcellular organization makes it difficult to study these systems under controlled conditions in vivo. We developed a cell-free reconstitution technique that allows for the visualization of ParA-mediated cargo transport on a DNA carpet, which acts as a biomimetic of the nucleoid surface. Here, we present methods to express, purify, and visualize the dynamic properties of the SopABC system from F plasmid, considered a paradigm for the study of ParA-type systems. We hope similar cell-free studies will be used to address the biochemical and biophysical underpinnings of this ubiquitous transport scheme in bacteria.

INTRODUCTION

Classical motor proteins, such as myosin or kinesin, and cytoskeletal elements, such as actin filaments or microtubules, have long been thought to be the main drivers of intracellular transport and positioning (Vale, 2003). But improved cell biology techniques have unveiled the ubiquity of protein gradients on biological surfaces, as a primary mode of spatially organizing a wide variety of large cargoes in bacteria such as chromosomes, plasmids, and proteinaceous organelles (Kieckbusch & Thanbichler, 2014; Vecchiarelli, Mizuuchi, & Funnell, 2012). For both chromosome and plasmid segregation, or “partition,” ParA-type systems are the most common microbial transport scheme (Baxter & Funnell, 2014). Par systems are minimal, encoding only two proteins: ParA, a deviant Walker-type ATPase, that forms dynamic protein gradients on the nucleoid upon interacting with its stimulator, ParB, which binds to a centromere site on the plasmid or chromosome and forms a “partition complex” that demarcates the DNA as cargo (Figure 1). How ParA gradients are generated on the nucleoid and provide the driving force for ParB-bound cargo segregation, transport, and positioning over the bacterial nucleoid remains unclear.

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AUTHOR CONTRIBUTIONS

A.G.V. developed, performed, and wrote all methods, except for the SopA–GFP–his₆ purification method, which was improved upon and written by J.A.T. K.M. developed the methods and edited the chapter.

Most low-copy plasmids use ParA-type partition systems as their principle method to ensure inheritance and stability in a cell population, making them excellent models in studying the mechanism of bacterial DNA segregation (Baxter & Funnell, 2014). ParA, along with its cognate ParB stimulator, uniformly distributes plasmid copies over the long axis of the bacterial nucleoid so that at least a single plasmid copy is inherited by each daughter cell following cell division (Figure 1). ParAs have weak ATPase activity that is synergistically stimulated by ParB and nonspecific DNA (Ah-Seng, Lopez, Pasta, Lane, & Bouet, 2009; Barilla, Carmelo, & Hayes, 2007; Davis, Martin, & Austin, 1992; Ebersbach et al., 2006; Pratto et al., 2008; Watanabe, Wachi, Yamasaki, & Nagai, 1992). ParB also significantly increases the rate of ParA release from nonspecific DNA (Hwang et al., 2013; Vecchiarelli, Hwang, & Mizuuchi, 2013), suggesting that adenosine triphosphate (ATP) hydrolysis by ParA is coupled to ParA release from the nucleoid. Therefore, we proposed that ParA dynamically binds the nucleoid until contact with plasmid-bound ParB locally depletes ParA in the vicinity of the cargo (Hwang et al., 2013; Vecchiarelli et al., 2010, 2013; Vecchiarelli, Neuman, & Mizuuchi, 2014). In our diffusion-ratchet model, the ParA depletion zone and the associated gradient generated by plasmid-bound ParB are utilized for the directed transport of cargo over the nucleoid.

One of the first Par systems to be identified and considered a paradigm for the study of ParA-mediated DNA segregation is the SopABC system of the *Escherichia coli* F plasmid (Ogura & Hiraga, 1983). In the F Sop system, the ParA-type ATPase is called SopA and the ParB-type stimulator is called SopB, which binds to the plasmid centromere site, *sopC*. We have recently reconstituted the F Sop system from purified components, and the system dynamics were visualized in a DNA-carpeted flow cell, which acted as an artificial nucleoid surface. In vivo the partition complex appears to chase and redistribute SopA on the nucleoid (Castaing, Bouet, & Lane, 2008; Hatano, Yamaichi, & Niki, 2007). When using a plasmid substrate bearing the *sopC* centromere site as cargo, we were successful in reproducing several aspects of the system dynamics observed in vivo except for persistent and directed plasmid motion (Hwang et al., 2013; Vecchiarelli et al., 2013). We proposed that our flow cell did not provide the surface confinement needed for a persistent interaction between the plasmid and the DNA carpet. When using a magnet above the flow cell to artificially confine *sopC*-coated magnetic beads on the DNA carpet, we found that SopB-bound *sopC*-beads locally released SopA to form a SopA depletion zone on the DNA carpet (Vecchiarelli et al., 2014). Spatial confinement of the bead was required to maintain the SopA depletion zone and the directed transport of the bead. Our cell-free reconstitution of this fascinating positioning system has provided direct evidence toward the proposal that, under spatial confinement, ParA gradients on the nucleoid surface are used to transport large bacterial cargos. Using the methods detailed in this chapter, we hope more research can be conducted in a similar manner to further build on the underlying mechanism.

1. METHODS

Here, we present an itemized description of our procedure to reconstitute and visualize ParA-mediated DNA transport using purified and fluorescent-labeled components from the F plasmid SopABC system. These methods were employed in our recent publications (Hwang et al., 2013; Vecchiarelli et al., 2013, 2014).

1.1 SOPA AND SOPB PROTEIN PURIFICATION

1.1.1 Expression and purification of SopA fused to Green Fluorescent Protein (SopA-GFP)—Like many ParAs, fluorescent fusions of SopA have been shown to be functional in vivo (Ah-Seng, Rech, Lane, & Bouet, 2013; Castaing et al., 2008; Hatano et al., 2007), and we have shown that the biochemical activities of SopA-GFP are similar to that of wild-type SopA (Vecchiarelli et al., 2013).

1.1.1.1 Buffers, reagents, and equipment

1.1.1.1.1 Buffers

- Lysis Buffer: 50 mM HEPES-KOH (pH 7.6), 1 M KCl, 10% Glycerol, 20 mM Imidazole (pH 7.4), 2 mM β -mercaptoethanol
- His Buffer: 50 mM HEPES-KOH (pH 7.6), 1 M KCl, 10% Glycerol, 1 M Imidazole (pH 7.4), 2 mM β -mercaptoethanol
- Q-Buffer A: 50 mM MES-KOH (pH 6), 200 mM KCl, 10% Glycerol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM β -mercaptoethanol
- Q-Buffer B: 50 mM MES-KOH (pH 6), 1 M KCl, 10% Glycerol, 0.1 mM EDTA, 2 mM β -mercaptoethanol
- SopA Concentration Buffer: 50 mM HEPES-KOH (pH 7.5), 2 M KCl, 10% Glycerol, 0.1 mM EDTA, 2 mM dithiothreitol (DTT)
- SopA Buffer: 50 mM HEPES-KOH (pH 7.5), 600 mM KCl, 10% Glycerol, 0.1 mM EDTA, 2 mM DTT.

1.1.1.1.2 Reagents

- BL21(AI) Competent Cells (Life Technologies, Cat. # C6070-03)
- pET15b protein expression vector (EMD Millipore, Cat. # 69661)
- Luria Bertani liquid and solid media (KD Medical)
- Carbinecillin (Invitrogen, Cat. # 10177-012)
- Antifoam Y-30 Emulsion (Sigma-Aldrich, Cat. # A5758)
- DTT (Sigma-Aldrich, Cat. # 43815)
- Isopropyl β -D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, Cat. # I6758)
- L-(+)-Arabinose (Sigma-Aldrich, Cat. # A3256)
- Lysozyme from chicken egg white (Sigma-Aldrich, Cat. # L6876)
- Protease Inhibitor Cocktail Tablets, EDTA-free (Sigma-Aldrich, Cat. # S8830)
- Whatman[®] GD/X 0.45 μ m syringe filters (GE Healthcare, Cat. # 6876-2504)
- A 5-mL HisTRAP HP cassette (GE Healthcare, Cat. # 17-5248-02)
- HiPrep 26/10 Desalting Column (GE Healthcare, Cat. # 17-5087-01)

- Mono Q 5/50 GL Column (GE Healthcare, Cat. # 17–5168-01)
- HiLoad 16/600 Superdex 200 pg Column (GE Healthcare, Cat. # 28–9893-35)
- Amicon Ultra Centrifugal Filters, 10K MWCO (EMD Millipore, Cat. # UFC501024)
- Liquid Nitrogen.

1.1.1.1.3 Equipment

- Innova 44 Shaking incubator (New Brunswick Scientific)
- Notched Fernbach flasks (2.5 L)
- Flasks (125 mL)
- Ultracentrifuge
- 45Ti fixed angle rotor and tubes (Beckman Coulter)
- HarvestLine System (Beckman Coulter, Cat. # 369256)
- Beckman JLA 8.1 rotor
- Centrifuge
- Cell Homogenizer
- Microfluidizer (Microfluidics Corp.)
- Peristaltic Pump
- AKTA Protein Purification System (GE Healthcare)
- NanoDrop 2000 Spectrophotometer.

1.1.1.2 Detailed procedures

1. The gene *sopA-GFP-his₆* is cloned into the multiple cloning site of the vector pET15b to create the pX2 plasmid, used for inducible expression under the control of a bacteriophage T7 promoter. pX2 is transformed into BL21 (AI) cells and a 100 mL overnight culture containing 100 µg/mL of carbenicillin is grown at 20 °C with shaking at 225 rpm. LB supplemented with 100 µg/mL of carbenicillin and a drop of Antifoam Emulsion (1 L per 2.5 L Fernbach flask × 4) is prewarmed to 37 °C and inoculated with 10 mL of overnight culture per flask. The cells are grown at 37 °C with shaking at 225 rpm to an optical density of 0.1. The incubation temperature is decreased to 20 °C, and the cells are grown to an optical density of 0.5. The incubation temperature is decreased once more to 16 °C, and the cells are grown to an optical density of 0.6. Protein expression is then induced by the addition of 10 mL of a 0.1 M IPTG/20% Arabinose solution to each flask. Cells are then grown overnight with shaking (~16 h induction). The cells are transferred to 1 L Beckmann bags and bottles, which are spun in a JLA 8.1 rotor at 4500 rpm for 1 h. The supernatant is poured out, and

the cell pellets are frozen in the bags with liquid N₂ and stored at –80 °C till ready for purification.

2. The frozen cell pellets are combined in a beaker with 10 mL of cold Lysis Buffer per gram of cell pellet (~150 mL), three Protease Inhibitor Mixture Tablets and 1 mg/mL lysozyme. A homogenizer is used to ensure that the cell pellets are thoroughly dispersed, and two passes through a Microfluidizer lyses the cells. The lysate is cleared with a 30 min ultracentrifugation at 35,000 rpm and 4 °C using a 45Ti rotor and Beckmann tubes. The lysate is then passed through a 0.45 mm syringe filter. Using a peristaltic pump, the cleared lysate (~200 mL) is loaded at a flow rate of 2 mL/min onto two 5 mL HisTRAP HP cassettes connected in series and equilibrated with Lysis Buffer. The loaded columns can be stored at 4 °C overnight. Using an AKTA purifier, the protein is eluted with a 20 mM to 1 M imidazole gradient (total volume = 60 mL). When using a system with multiple ultraviolet (UV) detectors, the absorbance at 395 nm should be tracked in addition to A280 nm to detect the GFP signal. Peak protein fractions (5 × 5 mL fractions ≈ 25 mL) for these absorbances are pooled and concentrated to approximately 15 mL using an Amicon Ultra Centrifugal Device (10,000 MWCO) spun at 4500 × g for 15 min at 17 °C (repeated as necessary). To remove Imidazole and reduce the KCl concentration, the sample is run at a rate of 5 mL/min over a 26/10 salt-exchange column equilibrated in Q-Buffer A. The sample is then loaded at a rate of 1 mL/min onto a 1 mL Mono Q column equilibrated in Q-Buffer A. The protein is then eluted with a 200 mM to 1 M KCl gradient. The peak fractions detected by A280 and A395 absorbance are pooled, diluted twofold with SopA Concentration Buffer, and concentrated to approximately 3 mL. The sample is then passed over a HiLoad 16/600 Superdex gel-filtration column equilibrated in SopA Buffer. If the protein precipitates during the preparation, it will elute as a mixture of a troublesome aggregated species (~288 kDa) and active monomer/dimer equilibrium (~143–72 kDa). The peak fractions corresponding to a SopA–GFP–his₆ dimer are pooled, concentrated to 1–2 mg/mL, frozen with liquid nitrogen, and stored at 80 °C.

1.1.1.3 Notes

- This protocol typically yields approximately 50 mg of SopA–GFP–his₆ from 4 L of cells. Purity can be assessed throughout the purification by running a 4–12% Bis-Tris sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel.
- Concentrated SopA–GFP–his₆ (>100 μM) begins to precipitate when in buffers containing <0.5 M KCl. This precipitation results in the formation of a higher molecular weight species. The presence of this species can cause complications during experiments, and should therefore be avoided. This is particularly problematic during Mono Q chromatography. As such, the time SopA–GFP–his₆ spends in Buffer Q should be minimized and the SopA–GFP–his₆-containing fractions stored at room temperature. Further, the protein should not be concentrated to >2 mg/mL after the Superdex 200 column.

1.1.2 SopB expression, purification, and fluorescent labeling—Many ParBs are not fully functional when fused to a fluorescent tag. Indeed this is the case in our hands when attempting to fuse SopB with a variety of fluorescent proteins (data not shown). Therefore, to visualize SopB, we perform dye labeling of SopB-his₆ after its purification. Labeled SopB was functional for stimulating SopA ATPase activity and binding specifically to *sopC* DNA as determined by gel shifts (Vecchiarelli et al., 2013).

1.1.2.1 Buffers, reagents, and equipment

1.1.2.1.1 Buffers

- Lysis Buffer: 50 mM HEPES–KOH (pH 7.6), 1 M KCl, 10% Glycerol, 20 mM Imidazole (pH 7.4), 2 mM β -mercaptoethanol
- S-Buffer: 50 mM MES–KOH (pH 6), 80 mM KCl, 10% Glycerol, 0.1 mM EDTA, 2 mM DTT
- SopB Buffer: 50 mM HEPES–KOH (pH 7.5), 150 mM KCl, 10% Glycerol, 0.1 mM EDTA, 2 mM DTT.

1.1.2.1.2 Reagents

- BL21(AI) Competent Cells (Life Technologies, Cat. # C6070–03)
- pET15b protein expression vector (EMD Millipore, Cat. # 69661)
- Luria Bertani liquid and solid media (KD Medical)
- Carbinecillin (Invitrogen, Cat. # 10177–012)
- Antifoam Y-30 Emulsion (Sigma–Aldrich, Cat. # A5758)
- DTT (Sigma–Aldrich, Cat. # 43815)
- IPTG (Sigma–Aldrich, Cat. # I6758)
- L-(p)-Arabinose (Sigma–Aldrich, Cat. # A3256)
- Protease Inhibitor Cocktail Tablets, EDTA-free (Sigma–Aldrich, Cat. # S8830)
- Whatman GD/X 0.45-mm syringe filters (GE Healthcare, Cat. # 6876–2504)
- 5 mL HisTRAP HP cassette (GE Healthcare, Cat. # 17–5248-02)
- HiPrep 26/10 Desalting Column (GE Healthcare, Cat. # 17–5087-01)
- MonoS 5/50 GL Column (GE Healthcare, Cat. # 17–5168-01)
- Superdex 200 10/300 GL Column (GE Healthcare, Cat. # 17–5175-01)
- Amicon Ultra Centrifugal Filters, 10K MWCO (EMD Millipore, Cat. # UFC501024)
- Alexa Fluor 647 C2-maleimide (Life Technologies, Cat. # A-20347)
- Liquid Nitrogen.

1.1.2.1.3 Equipment: Same as that used for SopA–GFP expression and purification (see Section 1.1.1.1.3).

1.1.2.2 Detailed procedures

1. The gene *sopB-his₆* is cloned into the multiple cloning site of the vector pET15b to create the pX8 plasmid, used for inducible expression under the control of a bacteriophage T7 promoter. pX8 is transformed into BL21 (AI) cells, and a 50 mL overnight culture is grown at 37 °C with shaking at 225 rpm. LB supplemented with 100 µg/mL of carbenicillin and a drop of Antifoam Emulsion (1 L per 2.5 L Fernbach flask × 4) is inoculated with 10 mL of overnight culture per flask. The cells are grown at 30 °C with shaking at 225 rpm to an optical density of 0.4. The incubation temperature is dropped to 16 °C, and to induce protein expression, 10 mL of a 0.1 M IPTG/20% Arabinose solution is added to each flask. Cells are then grown overnight with shaking (~16 h induction). The cells are transferred to 1 L Beckmann bags and bottles, which are spun in a JLA 8.1 rotor at 4500 rpm for 1 h. The supernatant is poured out, and the cell pellets are frozen in the bags with liquid N₂ and stored at –80 °C till ready for purification.
2. The frozen cell pellets are combined in a beaker with 150 mL of cold Lysis Buffer and three Protease Inhibitor Tablets. A homogenizer is used to ensure that the cell pellets are thoroughly dispersed, and two passes through a Microfluidizer lyses the cells. The lysate is cleared with a 30-min ultracentrifugation at 35,000 rpm and 4 °C using a 45Ti rotor and Beckmann tubes. The lysate is then passed through a 0.45-µm syringe filter. Using a peristaltic pump, the cleared lysate (~200 mL) is loaded at a flow rate of 2 mL/min onto a 5 mL HisTRAP HP cassette equilibrated in Lysis Buffer. Using an AKTA purifier, the protein is eluted with a 20 mM to 1 M imidazole gradient. Peak protein fractions (6 × 5 mL fractions ≈ 30 mL) are tracked by A280 absorbance, pooled, and concentrated 3.5-fold using an Amicon Ultra Centrifugal Device (10,000 MWCO) spun at 4500 × g for 1.5 h at 4 °C. Imidazole is removed by running the sample at a rate of 5 mL/min over a 26/10 salt-exchange column equilibrated in S-Buffer. The sample is then loaded at a rate of 1 mL/min onto a 1 mL MonoS column equilibrated in S-Buffer, and the protein is eluted with an 80 mM to 1 M KCl gradient (total volume = 30 mL). The peak fractions detected by A280 absorbance are pooled, concentrated, and passed over a Superdex 200 gel-filtration column equilibrated in Sop Buffer. The peak fractions corresponding to a SopB–his₆ dimer (72.4 KDa) are pooled, concentrated to 2–5 mg/mL, frozen with liquid nitrogen, and stored at –80 °C.
3. To fluorescently label SopB, 10 mM DTT is added to a 0.5 mL fraction of SopB–his₆, and the sample is exposed to N₂ gas for 5 min while on ice. The sample is then passed through a Superdex 200 column equilibrated in Sop Buffer without DTT. The peak fractions (~6 mL) are pooled and concentrated threefold. Alexa Fluor 647 C2 maleimide (10 mM), dissolved in water, is added to the sample at a 2:1 dye to protein ratio, and the reaction mixture is incubated in the

dark at 23 °C for 30 min. The labeling reaction mixture is quenched with 10 mM DTT, and the free label is removed using a Superdex 200 column equilibrated in Sop Buffer with 2 mM DTT. The labeled protein is once again concentrated, and the average labeling efficiency is determined with a Nano-Drop 2000 spectrophotometer using the Beer–Lambert law by comparing the protein and dye absorbencies at 280 and 647 nm, respectively (SopB MW = 36.2 KDa, $\epsilon = 12,200 \text{ M}^{-1} \text{ cm}^{-1}$; Alexa Fluor 647 $\epsilon = 265,000 \text{ M}^{-1} \text{ cm}^{-1}$). The average SopB monomer:Dye ratio is typically 90–100%.

1.1.2.3 Notes

- This protocol typically yields 50 mg of SopB–his₆ from 4 L of cells. Purity can be assessed throughout the purification and labeling protocol by running a 4–12% Bis-Tris SDS-PAGE gel.
- SopB contains three cysteine residues that can potentially be modified: C51, C196, and C307. We constructed and purified SopB mutants with a single cysteine remaining at position 51, 196, or 307 to determine the labeling efficiency at these sites and whether Cys to Ser mutation at the other two sites compromise SopB function. Labeling at C51 and C196 was very poor (<10%), whereas labeling efficiency at C307 was typically between 90% and 100%. However, C51S or C196S mutation altered SopB function in vitro. We therefore label wild-type SopB–his₆ with the assumption that the vast majority is labeled at C307.

1.2 DNA-CARPETED FLOW CELL

We perform all cell-free reconstitutions in a flow cell coated with nonspecific DNA, which acts as a biomimetic of the nucleoid surface. This description is similar to that found in Vecchiarelli et al. (2013).

1.2.1 Flow cell assembly

1.2.1.1 Reagents and equipment

1.2.1.1.1 Reagents

- NOCHROMIX Glass Cleaning Reagent (Sigma–Aldrich, Cat. # 328693)
- Sulfuric Acid (Sigma–Aldrich, Cat. # 339741)
- Optical Adhesive 61 (Norland Products, Cat. # 6101)
- Silica Slide with two holes 30 mm apart (ESCO Products, Job # 93538, Part # 2R1300402H)
- Acrylic double-sided Transfer Tape (25 μm thick; 3M)
- Cover Glass (24 $\times$ 50–1 mm; Fisher Scientific, Cat. # 12–548–5M)
- Nanopore 10–32 Coned inlets (IDEX Health & Science, Cat. # N-333–01)
- Hypodermic Needle Aluminum Hub (Tyco Healthcare, Gauge 23 $\times$ 1 A)

- Disposable plastic syringe (1 mL)
- Kim Wipes
- Slide storage box
- Dry N₂ gas.

1.2.1.1.2 Equipment

- Slide glass washing chamber with magnetic stirrer
- PE-2000 Plasma Etcher with O₂ gas (South Bay Technology)
- Laser Cutter (Epilog, Mini-Model 8000)
- Flow cell Press (Custom build)
- Forced Air Oven (Salvis Lab, Thermocenter)
- Blak-Ray UV lamp (Model UVL-56 – Long Wave UV-366 nm, 115 V; UVP Inc.)
- Needle-nose forceps.

1.2.1.2 Detailed procedures

1. In a beveled slide glass washing chamber with a magnet stirrer, slides are treated overnight in NOCHROMIX glass cleaning reagent dissolved in sulfuric acid according to the manufacture's instruction. The slides are thoroughly rinsed with deionized H₂O, blow dried with N₂ gas, and plasma cleaned with O₂ gas for 20 min.
2. A 4-mm-wide by 34-mm-long flow channel is cut with a laser etcher on a piece of 25-mm-thick acrylic transfer tape. One side of the tape is aligned on a 24- × 50-mm cover slip. This assembly is then stuck to a cleaned slide with the drilled inlet and outlet holes aligned with the ends of the flow channel.
3. With a 1-mL syringe and needle, optical adhesive is piped around the holes of the two nanoports. The holes of the nanoports are then aligned with the drilled holes of the glass slide and placed gently so that glue does not ooze into the holes. The flow cell is then placed on a UV lamp for 45 min to cure the glue and adhere the nanoports.
4. The assembled flow cell is placed in a custom-made flow cell press, which lightly presses the nanoports, cover slip, and slide together. The flow cell is then baked at 100 °C for 45 min. After cooling, the flow cell is removed from the press and placed in a slide box for storage.

1.2.1.3 Notes

- Other glass cleaning reagents, such as Piranha or Hellmanex, can be used in place of NOCHROMIX with similar results.

1.2.2 DNA carpet—A high-density DNA-carpeted flow cell was made by a three-way conjugation starting with a supported lipid bilayer with biotinylated lipid head groups on the flow cell surface, followed by a coating of neutravidin, which then tethers linear DNA fragments biotinylated at both ends.

1.2.2.1 Buffers, reagents, and equipment

1.2.2.1.1 Buffers

- Terminal Transferase Buffer: 20 mM Tris-acetate (pH 7.9), 50 mM Potassium Acetate, 10 mM Magnesium Acetate (New England Biolabs; Cat. # B0315L)
- TE Buffer: 10 mM Tris (pH 8), 1 mM EDTA
- TN100 Buffer: 10 mM Tris (pH 8), 100 mM NaCl
- TN100 Mg Buffer: 10 mM Tris (pH 8), 100 mM NaCl, 5 mM MgCl₂
- Sop Buffer: 50 mM HEPES–KOH (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 10% Glycerol

1.2.2.1.2 Reagents

- CoCl₂ (2 mM; New England Biolabs, Cat. # B0315L)
- Terminal Transferase (20,000 U/mL; New England Biolabs, Cat. # M0315L)
- Sonicated Salmon Sperm DNA (10 mg/mL; Agilent Technologies, Cat. # 201190)
- Biotin deoxycytidine triphosphate (dCTP) (0.4 mM; Life Technologies, Cat. # 19518–018)
- Illustra MicroSpin S-200HR columns (GE Healthcare, Cat # 27–5120-01)
- 18:1 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) (25 mg/mL; Avanti Polar Lipids, Cat. # 850375C)
- 18:1 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-(Cap Biotinyl) (Biotinylated-DOPE; Sodium Salt) (25 mg/mL; Avanti Polar Lipids, Cat. # 870273C)
- Borosilicate glass culture tubes (13 × 100 mm; Kimble Chase, Cat. # 73500 13100)
- Borosilicate glass serological pipettes (1 mL; Kimble Chase, Cat. # 72100–11100)
- Millex-GV Syringe filter units, 0.22 µm, polyvinylidene difluoride (PVDF) (Millipore, SLGV025NB)
- Glass vials with caps (2 mL) and polytetrafluoroethylene (PTFE) septa (National Scientific, Cat. # C4010–21)
- Flow cell (see assembly above)

- BD Luer-Lok disposable syringes (1 mL; Becton, Dickinson and Company, Cat. # 309628)
- 1/16 × 0.02 Teflon tubing (IDEX Heath and Science, Cat. # 1515XL)
- 10–32 Female to Female Luer-Lok Adapter (IDEX Heath and Science, Cat. # P-659)
- FingerTight PEEK Adapter (IDEX Heath and Science, Cat. # F-120X)
- Neutravidin (Thermo Scientific, Cat. # 31000)
- Chloroform
- Ethanol
- Parafilm
- Dry N₂ gas.

1.2.2.1.3 Equipment

- PHD 2000 Programmable Syringe pump (Harvard)
- 37 °C and 70 °C water bath
- Table top centrifuge
- Spectrophotometer
- Speed Vac Lyophilizer
- Glove box filled with N₂ gas
- Sonicator 3000 (Misonix) with Cup Horn & Stand (Model 448; Qsonica).

1.2.2.2 Detailed procedures

1. Lipids are brought to room temperature while four glass tubes are rinsed with 1 mL of water, then ethanol, and then chloroform. With a glass pipette, 1 mL of 25 mg/mL DOPC is spiked with 20 µL of 25 mg/mL of Biotinyl Cap PE. The lipids are gently mixed, and 250 µL is transferred to each of the four rinsed glass tubes. In a fume hood, dry N₂ gas is used to dry the lipid mix. The tube is rotated while drying to make an even lipid cake. The cakes are dried further in a speed vac for 3 h at 45 °C. TN100 buffer (1.25 mL) is added to each of the dry cakes, the tubes are sealed with parafilm, and the lipids are left to hydrate overnight at room temperature in an N₂-filled glove box.
2. The next day, the tubes are vortexed for 1 min and sonicated (power = 70 W) in a water cup at 16 °C for 15 min (2 min pulses with 30 s rest). The solution clears as Small Unilamellar Vesicles (SUVs) are produced. Combine the volumes and pass through a 0.22-µm syringe filter into glass vials. N₂ gas is blown into the vials for 1 min, the vials are sealed with parafilm and stored at 4 °C up to one month. The SUVs are used to make the supported lipid bilayer with biotinylated head groups on the flow cell surface (see below).

3. Salmon sperm DNA fragments are biotinylated at both ends and used as the substrate for making the DNA carpet. The sonicated salmon sperm DNA is first resonicated with 10-s pulses for 5 min at 110 W to an average length of 500 bp in a 4 °C water cup sonicator.
4. A terminal transferase reaction is used to biotinylate both ends of the DNA fragments. The reaction mix consists of 1× Terminal Transferase buffer, 0.25 mM CoCl₂, 0.4 mg of the resonicated salmon sperm DNA, 40 μM Biotine dCTP, and 320 U of the Terminal Transferase enzyme in a reaction volume of 400 μL. The reaction mixture is incubated at 37 °C for 30 min, and then the enzyme is deactivated at 70 °C for 10 min. The DNA is recovered and concentrated by ethanol precipitation, resuspended in 50 μL TE (pH 8), and the concentration is determined by A₂₆₀ measurement.
5. The 5 mg/mL of SUV stock (50 μL) is diluted down to 0.5 mg/mL with TN100 buffer (450 μL) and added to a 1 mL syringe that is attached to the flow cell inlet with a lure lock and plastic tubing. Be sure there are no bubbles in the plumbing. The SUV mix is slowly pushed into the flow cell for 400 μL and then incubated at 37 °C for 30 min to form the supported lipid bilayer. Excess SUVs are washed out with 500 μL of TN100 buffer at a rate of 0.1 mL/min with a syringe pump.
6. Neutravidin (1 mg/mL) in 200 μL of TN100 buffer is infused into the flow cell at a rate of 0.1 mL/min and incubated at room temperature for 30 min. Excess Neutravidin is then washed out with 500 μL of TN100 Mg buffer. The biotinylated DNA is diluted to 1 mg/mL with TN100 Mg buffer (100 μL), which was then infused into the flow cell. The nanoports are sealed with parafilm, and the flow cell is kept at 4 °C. On the experiment day, excess DNA is washed out of the flow cell with 500 μL of TN100 Mg buffer.

1.2.2.3 Notes

- The DNA-carpeted flow cell is good for 3 days if stored at 4 °C.

1.3 CARGO–FLUORESCENT PLASMIDS AND CENTROMERE-COATED BEADS

1.3.1 Plasmid substrate

1.3.1.1 Buffers, reagents, and equipment

1.3.1.1.1 Buffers

- Restriction Digest Buffer #3 (New England Biolabs)
- Restriction Digest Buffer #4 (New England Biolabs)
- DNAPI Buffer: 50 mM Tris–HCl (pH 7.8), 5 mM MgCl₂, 10 mM 2-mercaptoethanol, 10 μg/mL of bovine serum albumin
- T4 Ligase Buffer (New England Biolabs; Cat. # M0202S)
- Tris–acetate–EDTA Buffer: 40 mM Tris, 20 mM Acetate, 1 mM EDTA
- TE Buffer: 10 mM Tris (pH 8), 1 mM EDTA

1.3.1.1.2 Reagents

- pBR322 plasmid (4.4 Kb)
- pBR322::*sopC* plasmid (4.7 Kb)
- Nt BspQI Nicking Enzyme (New England Biolabs, Cat. # R0644S)
- Phenol:CHCl₃:Isoamyl Alcohol (UltraPure 25:24:1 v/v)
- Ethanol
- Deoxyribonucleotide (dNTP) mix (Life Technologies; Cat. # 18427–013)
- dCTP (Life Technologies; Cat. # 18253–013)
- Alexa Fluor 647–12-OBEA-dCTP (Life Technologies; Cat. # C21559)
- DNA Polymerase I (Life Technologies, Cat. # 18010–025)
- SYBR Gold DNA Stain (Life Technologies, Cat. # S-11494)
- Illustra MicroSpin S-400HR columns (GE Healthcare, Cat # 27–5140-01)
- QIAquick polymerase chain reaction (PCR) Purification Kit (Qiagen, Cat. # 28104)
- Ethidium Bromide
- 400 U/μL T4 Ligase (New England Biolabs, Cat. # M0202S)

1.3.1.1.3 Equipment

- Water Bath
- Vortex
- Table top centrifuge
- Nanodrop Spectrophotometer
- Typhoon Imager (GE Healthcare).

1.3.1.2 Detailed procedures

1. To create a fluorescent-labeled and supercoiled plasmid substrate encoding the *sopC* centromere-like site, the *sopC* site from F plasmid is first amplified by PCR with primers encoding the restriction sites SphI and BamHI. The amplified *sopC* fragment and the vector, pBR322, are digested with the restriction enzymes SphI and BamHI. The fragment is then cloned into pBR322 using standard cloning techniques to create pBR322::*sopC*.
2. The pBR322::*sopC* plasmid (10 mg) is nicked with the restriction enzyme Nt BspQI in Buffer #3 at 50 °C for 1 h in a total reaction volume of 400 μL (Figure 2). An equal volume of Phenol:CHCl₃:Isoamyl Alcohol is added to the reaction mixture and mixed by vortex. The aqueous phase containing plasmid DNA is transferred to a new tube, concentrated by ethanol precipitation, and resuspended in 50 μL of TE (pH 8) buffer.

3. The nicked pBR322::*sopC* plasmid (~8 mg) is fluorescently labeled in a nick translation reaction containing 30 mM dNTPs, 10 μ M dCTP, 20 μ M dCTP–Alexa647, and 30 U of DNA Polymerase I in DNAPI Buffer at a final volume of 150 μ L (Figure 2). The reaction mixture is incubated at 16 °C for 30 min and then 80 mM EDTA is added to stop the reaction. Unincorporated nucleotide is removed using a microspin S-400HR column spun at $700 \times g$ for 1 min. The labeled DNA is concentrated by ethanol precipitation, and resuspended in 50 μ L of TE Buffer.
4. The nick in the fluorescent-labeled pBR322::*sopC* plasmid (~6 mg) is then repaired with 1600 U of T4 Ligase in Ligase Buffer with 20 μ g/mL of Ethidium Bromide to negatively supercoil the plasmid (Figure 2). Ligase is added last, and the reaction is incubated at 23 °C overnight in the dark. The reaction mixture is incubated at 65 °C for 20 min to deactivate Ligase. A QIAquick PCR purification kit is used to purify the DNA, which is eluted with 50 μ L TE Buffer. DNA concentration and labeling efficiency are measured with a Nanodrop spectrophotometer. The typical labeling efficiency is 2–5 Alexa-dyes per plasmid. The final sample is run on a 1% Agarose gel and scanned with a Typhoon fluorescence imager to confirm the plasmid is fluorescently labeled. The gel is then stained with SYBR Gold DNA stain to verify the plasmid is supercoiled. The plasmid substrate is stored at –20 °C.

1.3.2 Centromere-coated magnetic beads

1.3.2.1 Buffers, reagents, and equipment

1.3.2.1.1 Buffers

- ThermoPol Reaction Buffer (New England Biolabs, Cat. # M0254S)
- Wash Buffer: 10 mM Tris–HCl (pH 8.2), 1 M NaCl, 1 mM EDTA
- Binding Buffer: 10 mM Tris–HCl (pH 8.2), 1 M NaCl, 1 mM EDTA, 0.2% Tween20
- Elution Buffer: 30 mM Tris–HCl (pH 7), 100 mM KCl, 1 mM EDTA

1.3.2.1.2 Reagents

- pBR322::*sopC* plasmid (PCR template)
- PCR Primers for *sopC* DNA amplification: (Primers synthesized by IDT)

Primer Sequence (5' to 3')	Location on pBR322 (bp)	Notes
Biotin ³ -CTGCTGAAGCCAGTTACCTTCGG	2997–3019	<i>sopC</i> -DNA conjugation to streptavidin-coated beads
Alexa 647-CCGCGTTTCCAGACTTTACG	1636–1619	Fluorescent labeling of the <i>sopC</i> DNA PCR fragment
CCGCGTTTCCAGACTTTACG	1636–1619	For an unlabeled PCR <i>sopC</i> fragment

- 10 mM dNTPs (Life Technologies; Cat. # 18427–013)

- 100 mM MgSO₄ (New England Biolabs, Cat. # M0254S)
- 2000 U/mL VENT Polymerase (New England Biolabs, Cat. # M0254S)
- SYBR Gold DNA Stain (Life Technologies, Cat. # S-11494)
- Illustra MicroSpin S-400HR columns (GE Healthcare, Cat # 27-5140-01)
- MyOne Streptavidin C1 Dynabeads (Life Technologies, Cat. # 65001)
- 20 U/μL PstI Restriction Enzyme (New England Biolabs, Cat. # R0140S)
- Phenol:CHCl₃:Isoamyl Alcohol (UltraPure 25:24:1 v/v)
- Ethanol.

1.3.2.1.3 Equipment

- PCR Thermocycler
- Tabletop centrifuge
- Tube rotator
- BioMag Separator (Advanced Magnetix Inc.)
- Nanodrop Spectrophotometer.

1.3.2.2 Detailed procedures

1. To create *sopC*-coated magnetic beads, an *sopC* DNA fragment (3.36 kb) is first PCR amplified from the plasmid template pBR322::*sopC*. The fragment contains an *sopC* site (12 × 43 bp tandem repeat = 516 bp) flanked by 1.7 and 1.1 kb of pBR322 sequence upstream and downstream of the *sopC* site, respectively. Primers were designed so that the *sopC* fragment was biotinylated at one end for conjugation to streptavidin-coated beads, and Alexa Fluor 647 labeled at the other end for visualization. The 100 μL of PCR reaction mixture contains 1× ThermoPol Buffer, 0.5 μM of each primer (MM1810-BIO and MM1810-A647), 0.6 nM pBR322::*sopC* template, 1 mM dNTPs, 2 mM MgSO₄, and 4 U of VENT polymerase. The PCR reaction conditions are An equal volume (100 μL) of Phenol:CHCl₃:Isoamyl Alcohol is added to the reaction mixture and mixed by vortex. The DNA-containing aqueous phase is transferred to a new tube, concentrated by ethanol precipitation, and resuspended in 90 μL of TE (pH 8) buffer. The DNA is then purified further with a microspin S-400HR column spun at 700 × g for 1 min. Amplification of the *sopC*-DNA fragment is verified by running the sample on a 0.8% Agarose gel. The gel is scanned with a Typhoon Imager to identify the fluorescent-labeled PCR product. The gel is then stained with SYBR Gold to verify there are no nonspecific PCR products that were amplified. The concentration and Alexa Fluor 647-labeling efficiency of Biotin-*sopC*-Alexa647 DNA is measured with a Nanodrop spectrophotometer.

Denature	95 °C	5 min	
Denature	95 °C	30 s	30 Cycles
Anneal	53 °C	30 s	
Elongate	72 °C	1.5 min	
Final elongation	72 °C	10 min	
Store	4 °C		

Table adapted from Vecchiarelli et al. (2014).

2. The Biotin-*sopC*-Alexa647 DNA fragment is then conjugated to streptavidin-coated beads. Fifty microliters of 10 mg/mL of MyOne Streptavidin C1 Dynabeads (0.5 mg) is added to a tube, which is placed in a BioMag magnetic separator that pulls the beads to the side of the tube. The supernatant is aspirated, the tube is removed from the magnet, and the beads are washed with 1.5 mL of Wash Buffer. The washes are repeated twice more. The washed beads are then resuspended in 1.3 mL of Binding Buffer. The Biotin-*sopC*-Alexa647 DNA fragment (~10 pmoles) is added to the beads and incubated with gentle rotation for 1 h at 23 °C in the dark. The beads are washed thrice with 1.5 mL of Binding Buffer, using the BioMag separator to isolate the beads between washes. The beads are finally resuspended in 50 µL of Elution Buffer.
3. The number of DNA fragments conjugated per bead is estimated by a PstI digest, which cleaves the DNA immediately adjacent to the biotinylated end of the *sopC*-DNA fragment; thus releasing the DNA for quantification. The *sopC*-coated beads (5 µL) are digested off the beads in Buffer #3 with 20 U of PstI restriction enzyme for 1 h at 37 °C. The reaction mixture is then placed on the BioMag separator to remove the beads from solution. The supernatant is transferred to a new tube, and 40 µL of Elution Buffer is added. An equal volume of Phenol:CHCl₃:Isoamyl Alcohol is added and the sample is mixed by vortex. DNA in the aqueous phase is transferred to a new tube, concentrated by ethanol precipitation, and resuspended in 50 µL TE (pH 8) buffer. The *sopC*-DNA concentration is then measured with a Nanodrop spectrophotometer.

1.3.2.3. Notes

- DNA-binding capacity on the beads depends on the DNA fragment size. With a 3.36-kb *sopC*-DNA fragment, this protocol yields 1000 ± 200 fragments per 1-mm bead.
- For unlabeled *sopC*-coated beads, using the PCR primer that is not end labeled with Alexa Fluor 647.

1.4 BIOPHYSICAL ASSAYS

We have published results using several biophysical assays to study ParA-mediated transport on a DNA-carpeted flow cell, which acts as a nucleoid biomimetic (Hwang et al., 2013; Vecchiarelli et al., 2013, 2014). In all cases, we use TIRFM imaging to examine this

process (Figure 3). Here, we describe how to perform the experiments in detail, and briefly discuss data analysis.

1.4.1 TIRF imaging Sop-mediated plasmid dynamics on a DNA carpet

1.4.1.1 Buffers, reagents, and equipment

1.4.1.1.1 Buffers

- Sop Buffer: 50 mM Hepes–KOH (pH 7.5), 100 mM KCl, 10% (v/v) glycerol, 5 mM MgCl₂, 2 mM DTT, 0.1 mg/mL α-casein, 0.6 mg/mL ascorbic acid, 2 mM phospho(enol)pyruvate (PEP), and 10 µg/mL of pyruvate kinase.

1.4.1.1.2 Reagents

- α-Casein (Sigma–Aldrich, Cat. # C6780)
- DL-DTT (Sigma–Aldrich, Cat. # D0632)
- Ascorbic acid (Sigma–Aldrich, Cat. # 5960)
- PEP (Sigma–Aldrich, Cat. # 860077)
- Pyruvate Kinase (Sigma–Aldrich, Cat. # P7768)
- Adenosine 5'-triphosphate (disodium salt) (ATP) (Sigma–Aldrich, Cat. # P7768)
- SopA–eGFP–ehis₆
- Sop–Behis₆
- Sop–Behis₆ labeled with Alexa Fluor 647 (SopBeAlexa647)
- pBR322::sopC plasmid labeled with Alexa Fluor 647
- 1-µm Magnetic beads coated with sopC–DNA labeled with Alexa Fluor 647
- DNA-carpeted flow cell
- YOYO-1 DNA Stain
- 1 mL BD Luer-Lok disposable syringes (Becton, Dickinson and Company, Cat. # 309628)
- TFZL 1/16" × 0.02" Plastic tubing (IDEX Heath and Science, Cat. # 1515XL)
- 10–32 Female to Female Lure-Lok Adapter (IDEX Heath and Science, Cat. # P-659)
- FingerTight PEEK Adapter (IDEX Heath and Science, Cat. # F-120X)
- Micrometering Valve (IDEX Heath and Science, Cat. # P-446)
- N42 1.5" × 0.25" cylindrical magnet (K&J Magnetics, Cat. # D4X8)
- Goniometer magnet mount (Custom modified from Charles Supper 4-axis goniometer)
- Immersion Oil–Type DF (Lens/Coverslip interface) (Cargille Labs, Cat. # 16242)

- Immersion Oil–Type FF (Slide/Prism interface) (Cargille Labs, Cat. # 16212)

1.4.1.1.3 Equipment: The reaction mix is loaded into a 1-mL plastic syringe and flowed into the DNA-carpeted flow cell using a neMESYS Low Pressure Syringe Pump (Cetoni) (Figure 3(A)). Fluorescent proteins and/or DNA are visualized using prism-type total internal reflection fluorescence (TIRF) illumination custom-built around a Nikon Eclipse TE2000-E microscope as previously described (Han & Mizuuchi, 2010; Ivanov & Mizuuchi, 2010). A 488-nm diode-pumped solid-state laser beam (Sapphire, Coherent) and a 633 nm HeNe laser beam (Research Electro-Optics) are combined using a custom-built fiber optic system (OZ Optics Ltd), and used for TIRF excitation of GFP and Alexa Fluor 647, respectively (Figure 3(B)). Fluorescence emission is collected through a 60× Plan Apo, NA 1.4 oil-immersion objective (Nikon) with an additional 1.5× magnification placed before the camera to give a 180-nm/pixel resolution. The laser excitation lines are blocked with notch filters (NF03–488E and NF03–633E, Semrock), and images are acquired with an electron multiplier CCD (Andor IXON+ 897) through a Dual-View Module (630DCXR cube, Photometrics; short/long pass filters, SP01–633RS, LP02–633RE, Semrock) for simultaneous imaging the GFP and Alexa Fluor 647 fluorescence.

1.4.1.2 Experimental procedures

1. Casein (1 mg/mL) in Sop Buffer (0.5 mL) is infused into the DNA-carpeted flow cell at a rate of 0.1 mL/min and incubated at room temperature for 30 min. This step blocks nonspecific protein binding to the flow cell surface. Excess casein is then washed out with 500 μ L Sop Buffer. The flow cell is then mounted onto the microscope stage with immersion oil (Type DF) between the objective lens and flow cell, while the reaction sample is prepared.
2. To assemble the reaction, 5 μ M SopA–GFP–his₆ is preincubated with 2 mM ATP, while 10 μ M SopBehis₆ is preincubated with 1 nM pBR322::sopC plasmid DNA. Both 30 μ L samples are incubated for 20 min at 23 C. Preincubation allows SopA to bind ATP, and SopB to bind its *sopC* site on the plasmid substrate thereby preforming the partition complex. The two samples are then mixed and diluted to the final concentrations of 0.5 μ M SopA–GFPEhis₆, 1 μ M SopBehis₆, 0.1 nM pBR322::sopC plasmid DNA and 2 mM ATP in a reaction volume of 300 μ L. To covisualize both Sop proteins, SopBehis₆ is mixed 9:1 with SopBehis₆ labeled with Alexa Fluor 647 and the unlabeled *sopC*–plasmid. To covisualize SopA–GFP with the plasmid substrate, pBR322::sopC plasmid labeled with Alexa Fluor 647 is used along with dark SopB–his₆.
3. The sample is then loaded into a 1-mL plastic syringe, and plastic tubing is used to connect to a micrometering stop valve, which is connected to the flow cell inlet nanoport. The same valve type is also placed at the flow cell outlet to prevent residual flow after flow stoppage. The syringe is mounted on a pump, and the sample is infused into the flow cell at 20 μ L/min for 1–2 min. Flow is stopped for data acquisition.
4. The excitation lasers (488 and 633 nm) are focused through a fused silica prism onto the mounted flow cell. Use immersion oil (Type FF) to mount the prism

onto the flow cell. The typical laser power values of 488 and 633 nm illumination are 15 and 500 μ W, respectively. Microscopy experiments are performed at 24 °C. The camera settings for acquisition are digitization 14-bit at 3 MHz, preamplifier gain 5.2, vertical shift speed 2 MHz, vertical clock range: normal, EM gain 40, camera temperature set at –98 °C, baseline clamp ON.

1.4.1.3 Analysis

1. Establish the best exposure time for visualizing the system dynamics on the DNA carpet. We typically acquire images with an exposure time of 100 ms at a frame rate of 0.2 Hz.
2. Metamorph 7 software (Molecular Devices) is used for camera control and image acquisition. The two-color data are simultaneously recorded as 14-bit stacks (.stk). The two colors are split and aligned using the “split view” display function, and the files are then transferred to ImageJ software (NIH) for image processing and analysis.
3. To quantify Sop system dynamics on and around a plasmid that tethers to the DNA carpet, a circular region of interest (ROI) is drawn around a plasmid focus in the red channel (Figure 4(A)). The plasmid focus can be identified either by pBR322::*sopC*-Alexa647 intensity or by SopB-Alexa647, which loads onto and around *sopC* to form a large partition complex. Use the same ROI in the green channel to monitor changes in SopA–GFP intensity as a result of plasmid tethering and release over time. Draw an additional ROI in the green channel on the DNA carpet away from tethered plasmids, which serves as a negative control for changes in SopA–GFP intensity.
4. The movie should encompass both the plasmid-tethering and -release events (Figure 4(B)). A typical intensity trace as a function of time would show a constant SopA–GFP intensity on the DNA carpet (ROI 1). A SopB-bound plasmid focus would tether to the DNA carpet with a high level of SopA–GFP. SopB stimulates the release of SopA–GFP from the complex as well as from the surrounding DNA carpet. Once all SopA–GFP anchor points are removed, the plasmid complex detaches from the DNA-carpet and the SopA–GFP depletion zone refills.
5. By performing single-molecule intensity measurements of SopA–GFP, SopB–Alexa647, and pBR322::*sopC*-Alexa647 as described previously (Hwang et al., 2013; Vecchiarelli et al., 2013), the fluorescence intensities can be converted to an estimation of protein density and plasmid copy number.

1.4.1.4 Notes

- Our TIRF illumination has a Gaussian shape in the field of view with measured horizontal and vertical half max widths of approximately 65×172 nm at 488 nm. Therefore, intensity quantification of fluorescent species is taken from the center of the illumination profile.

- Before an experiment, YOYO-1 DNA stain can be used to image the DNA carpet and verify a high DNA density on the flow cell surface, and ensure that the surface is free from scratches or bubbles in the chamber, which would destroy the DNA carpet.

1.4.2 TIRF imaging of Sop-mediated transport of surface-confined beads—

We proposed that the narrow cytosolic space between the nucleoid and the inner membrane *in vivo* is critical to ParA-mediated transport as it promotes frequent associations between plasmid-bound ParB and nucleoid-bound ParA—a requirement for sustained plasmid motion (Vecchiarelli et al., 2014). Our flow cell depth (25 μm) does not provide the surface confinement needed to maintain contact between the plasmid substrate and the DNA carpet. Therefore, to mimic surface confinement on the nucleoid, we recapitulated the F Sop system using magnetic beads coated with *sopC* centromere DNA, which were artificially confined to the DNA carpet by a magnet placed above the flow cell (Vecchiarelli et al., 2014).

1.4.2.1 Experimental procedures

1. Experimental procedures using the magnetic beads are essentially the same as when using the plasmid substrate as described above (see Section 1.4.1.2). The main difference highlighted here is the use of beads in the sample and a magnet aligned 12 mm above the flow cell, which pulls the magnetic beads and artificially confines them on DNA-carpeted flow cell surface.
2. Before performing an experiment with Sop system components, 1 mg/mL of the *sopC*-coated magnetic beads labeled with Alexa Fluor 647 in Sop Buffer is flowed into the flow cell. After flow stoppage, the valves at the flow cell inlet and outlet are closed to stop residual flow. The magnet is then aligned using a goniometer mount so that bead motion is freely diffusive over the time course of an experiment (Figure 5(A)). Beads are then washed out of the flow cell with Sop buffer and the same flow cell is used for the experiment.
3. To assemble the reaction equipment, 5 μM SopA–GFP–his₆ is preincubated with 2 mM ATP, while 10 mM SopBehis₆ is preincubated with 1.6 mg/mL of the *sopC*-coated beads. Both 30 μL samples are incubated for 20 min at 23 °C. The samples are then mixed together and diluted to the final concentrations of 0.5 μM SopA–GFP–his₆, 1 μM SopBehis₆, 1.6 mg/mL of the *sopC*-beads and 2 mM ATP in a reaction volume of 300 μL . To covisualize SopA–GFP with the beads, use the *sopC*-beads labeled with Alexa Fluor 647 along with dark SopB–his₆. To covisualize both Sop proteins, SopB–his₆ is mixed 9:1 with SopB–his₆ labeled with Alexa Fluor 647. In this case, use dark *sopC*-coated beads that have not been fluorescently labeled during the PCR of the *sopC* fragment (see Section 1.3.2).

1.4.2.2 Analysis

1. Image acquisition and analysis are performed as when using a plasmid substrate (see Section 1.4.1.3).

2. Unlike plasmid cargo that is no longer visible by TIRFM after release from the DNA carpet, the *sopC*-coated beads exhibit directed movement while being artificially confined to the DNA carpet by the magnet (Figure 5(B) and (C)). Sop-mediated directed bead motion is quantified by first taking the trajectories of moving beads. To obtain bead trajectories, the background from the red channel, which images the *sopC*-Alexa647 beads, is subtracted using a sliding window length of 40 pixels with the Mosaic Plug-in for ImageJ. The Octane plug-in is then used to track bead trajectories.
3. Trajectories are then used to calculate the MSD over time as described previously (Vecchiarelli et al., 2014) (Figure 5(D)). Freely diffusing beads have MSDs that fit a linear curve, whereas directed beads fit a quadratic function that is characteristic of directed motion.

1.4.2.3 Notes

- Establish the best exposure time for visualizing bead dynamics on the DNA carpet. We typically take time-lapse movies at a frame rate of 0.2 Hz.

DISCUSSION AND SUMMARY

With conventional microscopy methods the bacterial cell is just a handful of pixels, which makes it difficult to elucidate the mechanistic principles governing subcellular spatial organization in vivo. Our cell-free reconstitution technique allows for the visualization of ParA-mediated transport systems on a DNA carpet, and has provided direct evidence toward a novel mode of transport that does not use a classical cytoskeletal element or motor protein. Rather, the data suggest that these systems establish a moving ParA ATPase gradient on the nucleoid, which generates the force required for the directed movement of spatially confined cargos (Vecchiarelli et al., 2014), such as plasmids, chromosomes, or even large protein machineries.

Although this work has provided a mechanistic framework, many critical features are still unknown and can be addressed with our cell-free technique, albeit with several technical improvements. First, instead of using external forces to confine cargo, microconfinement chambers that passively confine multiple copies of cargo would allow for the study of cargo segregation and equidistant positioning, which are key functions of ParA-type systems that have yet to be reconstituted in vitro. Second, magnetic beads are very different from plasmid cargo. However, the cargos shown to use ParA-type transport to date are composed of plasmid or chromosomal DNA of varying size, as well as proteinaceous organelles, suggesting cargo composition is not a critical factor (Vecchiarelli et al., 2012). Regardless, more physiologically relevant cargos will certainly be useful in the chambers discussed above. Third, nucleoid-associated proteins as well as other DNA-binding proteins likely impact the system dynamics in vivo, and their addition to the cell-free setup may unveil further details. Finally, one of the most fascinating features of this mode of transport is the exploitation of the nucleoid surface as a matrix for cargo transport. This feature raises a number of questions with respect to the nature of the nucleoid surface and how its mechanical properties may influence the transport mechanism (Lim et al., 2014). By

changing the length and/or topology of the DNA making up the carpet in the flow cell, we can start to address these biophysical aspects. Along with cell biology, genetics, and biochemical approaches, we anticipate that in vitro reconstitution and imaging will soon unveil the mechanism governing ParA-mediated segregation, transport, and positioning of large bodies in bacteria.

ACKNOWLEDGMENTS

This work was supported by the intramural research fund for the National Institute of Diabetes and Digestive and Kidney Diseases (to K.M.) and by the Nancy Nossal Fellowship (to A.G.V.).

REFERENCES

- Ah-Seng Y, Lopez F, Pasta F, Lane D, & Bouet J-Y (2009). Dual role of DNA in regulating ATP hydrolysis by the SopA partition protein. *Journal of Biological Chemistry*, 284(44),30067–30075. 10.1074/jbc.M109.044800. [PubMed: 19740757]
- Ah-Seng Y, Rech J, Lane D, & Bouet J-Y (2013). Defining the role of ATP hydrolysis in mitotic segregation of bacterial plasmids. *PLoS Genetics*, 9(12), e1003956 10.1371/journal.pgen.1003956. [PubMed: 24367270]
- Barilla D, Carmelo E, & Hayes F (2007). The tail of the ParG DNA segregation protein remodels ParF polymers and enhances ATP hydrolysis via an arginine finger-like motif. *Proceedings of the National Academy of Sciences of the USA*, 104(6), 1811–1816. 10.1073/pnas.0607216104. [PubMed: 17261809]
- Baxter JC, & Funnell BE (2014). Plasmid partition mechanisms. *Microbiology Spectrum*, 2(6). 10.1128/microbiolspec.PLAS-0023e2014.
- Castaing J-P, Bouet JY, & Lane D (2008). F plasmid partition depends on interaction of SopA with non-specific DNA. *Molecular Microbiology*, 70(4), 1000–1011. [PubMed: 18826408]
- Davis MA, Martin KA, & Austin SJ (1992). Biochemical activities of the ParA partition protein of the P1 plasmid. *Molecular Microbiology*, 6, 1141–1147. [PubMed: 1534133]
- Ebersbach G, Ringgaard S, Moller-Jensen J, Wang Q, Sherratt DJ, & Gerdes K (2006). Regular cellular distribution of plasmids by oscillating and filament-forming ParA ATPase of plasmid pB171. *Molecular Microbiology*, 61(6), 1428–1442. 10.1111/j.1365-2958.2006.05322.x. [PubMed: 16899080]
- Han Y-W, & Mizuuchi K (2010). Phage Mu transposition immunity: protein pattern formation along DNA by a diffusion-ratchet mechanism. *Molecular Cell*, 39(1), 48–58. 10.1016/j.molcel.2010.06.013. [PubMed: 20603074]
- Hatano T, Yamaichi Y, & Niki H (2007). Oscillating focus of SopA associated with filamentous structure guides partitioning of F plasmid. *Molecular Microbiology*, 64(5), 1198–1213. [PubMed: 17542915]
- Hwang LC, Vecchiarelli AG, Han Y-W, Mizuuchi M, Harada Y, Funnell BE, et al. (2013). ParA-mediated plasmid partition driven by protein pattern self-organization. *EMBO Journal*, 32(9), 1238–1249. [PubMed: 23443047]
- Ivanov V, & Mizuuchi K (2010). Multiple modes of interconverting dynamic pattern formation by bacterial cell division proteins. *Proceedings of the National Academy of Sciences of the USA*, 107(18), 8071–8078. 10.1073/pnas.0911036107. [PubMed: 20212106]
- Kieckbusch D, & Thanbichler M (2014). Spatiotemporal organization of microbial cells by protein concentration gradients. *Trends in Microbiology*, 22(2), 65–73. 10.1016/j.tim.2013.11.005. [PubMed: 24342487]
- Lim HC, Surovtsev IV, Beltran BG, Huang F, Bewersdorf J, & Jacobs-Wagner C (2014). Evidence for a DNA-relay mechanism in ParABS-mediated chromosome segregation. *eLife*, 3, e02758 10.7554/eLife.02758. [PubMed: 24859756]
- Ogura T, & Hiraga S (1983). Partition mechanism of F plasmid: two plasmid gene-encoded products and a cis-acting region are involved in partition. *Cell*, 32, 351–360. [PubMed: 6297791]

- Pratto F, Cicek A, Weihofen WA, Lurz R, Saenger W, & Alonso JC (2008). Streptococcus pyogenes pSM19035 requires dynamic assembly of ATP-bound ParA and ParB on parS DNA during plasmid segregation. *Nucleic Acids Research*, 36(11), 3676–3689. 10.1093/nar/gkn170. [PubMed: 18477635]
- Vale RD (2003). The molecular motor toolbox for intracellular transport. *Cell*, 112(4), 467–480. 10.1016/S0092-8674(03)00111-9. [PubMed: 12600311]
- Vecchiarelli AG, Han Y-W, Tan X, Mizuuchi M, Ghirlando R, Biertümpfel C, & Mizuuchi K (2010). ATP control of dynamic P1 ParAeDNA interactions: a key role for the nucleoid in plasmid partition. *Molecular Microbiology*, 78–91(1), 78.
- Vecchiarelli AG, Hwang LC, & Mizuuchi K (2013). Cell-free study of F plasmid partition provides evidence for cargo transport by a diffusion-ratchet mechanism. *Proceedings of the National Academy of Sciences of the USA*, 110(15), E1390–E1397. 10.1073/pnas.1302745110. [PubMed: 23479605]
- Vecchiarelli AG, Mizuuchi K, & Funnell BE (2012). Surfing biological surfaces: exploiting the nucleoid for partition and transport in bacteria. *Molecular Microbiology*, 86(3), 513. [PubMed: 22934804]
- Vecchiarelli AG, Neuman KC, & Mizuuchi K (2014). A propagating ATPase gradient drives transport of surface-confined cellular cargo. *Proceedings of the National Academy of Sciences of the USA*, 111(13), 4880–4885. 10.1073/pnas.1401025111. [PubMed: 24567408]
- Watanabe E, Wachi M, Yamasaki M, & Nagai K (1992). ATPase activity of SopA, a protein essential for active partitioning of F plasmid. *Molecular and General Genetics*, 234(3), 346–352. [PubMed: 1406581]

FIGURE 1. The ParA-type plasmid partition system.

Three plasmid-encoded components are essential for plasmid stability—an ATPase, its stimulator and a centromere-like site on the plasmid. The ParA ATPase (or SopA from F plasmid) binds DNA nonspecifically and colocalizes with the nucleoid, while its stimulator, ParB (or SopB), binds to the centromere-like site (*sopC*) to form a “partition complex” on the plasmid cargo. The partition complex locally removes ParA from the nucleoid, forming dynamic ParA gradients. How ParA gradients produce a driving force for cargo movement over the nucleoid is a subject of intense study and remains controversial.

FIGURE 2. Creating a supercoiled and fluorescently-labeled *sopC*-plasmid.

The plasmid pBR322::*sopC* is fluorescently labeled to visualize its movement over the DNA-carpeted flow cell. We have developed an efficient labeling protocol that does not require intercalating dyes and produces a negatively supercoiled plasmid. The restriction enzyme Nt.BspQ1 nicks the pBR322 backbone at a site located approximately 180° from *sopC*. DNA polymerase I is used with dNTPs and Alexa647-labeled dCTP to label the DNA. Ethidium Bromide promotes negative supercoiling before a final ligation reaction that covalently closes the nick. The final product is a negatively supercoiled and fluorescently labeled plasmid bearing the *sopC* centromere site. This protocol can be used to incorporate a variety of dyes without significant perturbation to plasmid topology.

FIGURE 3. Cell-free setup for visualizing SopAB-mediated dynamics of plasmids or beads on a DNA carpet.

(A) SopA and SopB proteins are preincubated with plasmid or bead cargo bearing the *sopC*-centromere site in Sop buffer containing ATP. The sample is then flowed into the DNA-carpeted flow cell. Flow is stopped to visualize how SopA and SopB mediate cargo dynamics on the DNA carpet. (B) Excitation lasers are directed onto the flow cell at an angle that allows total internal reflection (TIRF). The evanescent wave (blue haze) produced by the incident and reflected light propagates into the flow cell ~100 nm. SopA-GFP (green) is covisualized with either SopB-Alexa647, or one of two possible cargos labeled with Alexa647, the pBR322::*sopC* plasmid or *sopC*-coated beads. (i) ATP-bound SopA binds the DNA carpet and tethers the plasmid by SopA–SopB interaction, (ii) until SopB locally stimulates the removal of SopA. (iii) Once all SopA anchor points are removed, the plasmid is released and is no longer observable via TIRFM. A magnet above the flow cell surface confines *sopC*-coated magnetic beads on the DNA carpet, which supports persistent SopA removal and directed bead movement. (See color plate)

FIGURE 4. Analyzing Sop system effects on *sopC*-plasmid dynamics on the DNA carpet. (A) Time-lapse images show *sopC*-plasmid tethering by SopA-GFP (green) and SopB-Alexa647 (red) on a DNA-carpeted flow cell. ATP-bound SopA-GFP binds the DNA carpet and tethers SopB-bound plasmid. SopB stimulates SopA-GFP release around the plasmid focus forming a SopA depletion zone. Once all SopA anchor points are removed, the plasmid substrate is released and the depletion zone refills. (B) Time course of SopA-GFP intensity associated with the DNA carpet (black, ROI 1 from A) or the plasmid partition complex (green, ROI 2 from A). The dashed lines indicate when the *sopC*-plasmid (red, ROI 3 from A) was tethered with high SopA-GFP content, and released from the DNA carpet when all SopA-GFP anchor points are removed. Following plasmid release, SopA-GFP refills the depletion zone on the DNA carpet. Fluorescence intensities were normalized to the values prior to plasmid tethering ($t = 0$). Scale bar = 2 μ m. (See color plate)

FIGURE 5. Analyzing Sop system effects on *sopC*-coated bead dynamics on the DNA carpet.

(A) Still image of a freely diffusing *sopC*-coated bead (red). Beads are first flowed onto the DNA carpet without Sop proteins to align the magnet and ensure the beads are confined on the DNA carpet while still able to freely diffuse in the x- and y-axes. The yellow line is the bead trajectory over 20 min. (B) Still image of a directed *sopC*-coated bead in the presence of SopA-GFP (green) and SopB-Alexa647 (red). Like the plasmid substrate, SopB stimulates the removal of SopA in the vicinity of the bead cargo. The white line is the bead trajectory over 5 min. (C) Time lapse of SopA-GFP and SopB-Alexa647 on and around a bead traveling from the right to the left. The surface-confined bead moves directionally toward higher concentrations of the SopA gradient on the perimeter of the depletion zone. (D) The Mean Square Displacement (MSD) of directed (red) and freely diffusing beads (gray) are plotted against time. The black line is the average MSD for freely diffusing beads. (See color plate)